



D3.15 Third periodic report on JRP

WP3 Joint Research Projects

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GENERAL INFORMATION

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1. Introduction

This report shows the progress of the ongoing Joint Research Projects (JRP) and the overall outcome of the finalized JRP, and is based on the 12 month and final reports respectively. Project Leaders submitted their individual reports at the end of the third year of the One Health EJP, in December 2020.

The actual document is complementary to what is described elsewhere. Details on tasks and activities touching on ethical matters are the subject of the specific deliverable D1.25, the Ethical review report for Y3, and are therefore not copied in the actual document. Also, the parts of the 12 months and final reports that deal with the data management plans and the dissemination activities are available in chapter 4 (Update on the Data Management Plan) and in the section 2.3.3.3.1 (Summary of the progress made by the ongoing and finalized JRP in 2020) of the Periodic Technical Report, respectively.

All publications and deliverables are available through the specific project pages on the One Health EJP website: <https://onehealthjep.eu/projects/>.

2. Summary of the Joint Research Projects performance and progress by the end of year 3

Project deliverables and milestones

PROJET	DUE DELIVERABLES	DELIVERED	% DELIVERED	DELAYED
JRP01-AMR1-IMPART	23	21	91%	2
JRP02-AMR2-ARDIG	14	7	50%	7
JRP03-AMR3-RADAR	27	26	96%	1
JRP04-ET1-MADVIR	10	10	100%	0
JRP05-ET1-TOXDETECT	17	7	41%	10
JRP06-FBZ1-NOVA	31	21	68%	10
JRP07-FBZ2-LISTADAPT	23	17	74%	6
JRP08-FBZ2-METASTAVA	22	20	91%	2
JRP09-FBZ3-AIRSAMPLE	6	5	83%	1
JRP10-FBZ3-MOMIR-PPC	49	18	37%	31
JRP11-FBZ4-MEDVETKLEBS	14	13	93%	1
JRP12-AMRSH5-FARMED	2	1	50%	1
JRP13-AMRSH5-WORLDCOM	12	4	33%	8
JRP14-AMR2.1-FULLFORCE	21	12	57%	9
JRP15-AMR2.1-FEDAMR	20	7	35%	13
JRP16-ET2.2-TELEVIR	6	3	50%	3
JRP17-ET2.2-IDEMBRU	10	2	20%	8
JRP18-ET1.1-MEME	6	3	50%	3
JRP19-ET1.1-PARADISE	6	3	50%	3
JRP20-FBZSH3-DISCOVER	3	2	67%	1
JRP21-FBZ3.1-BIOPIGEE	8	4	50%	4
JRP22-FBZ4.1-TOXOSOURCES	3	3	100%	0
JRP23-FBZSH5-ADONIS	5	1	20%	4
JRP24-FBZSH9-BEONE	6	2	33%	4
TOTAL	344	212	62%	132

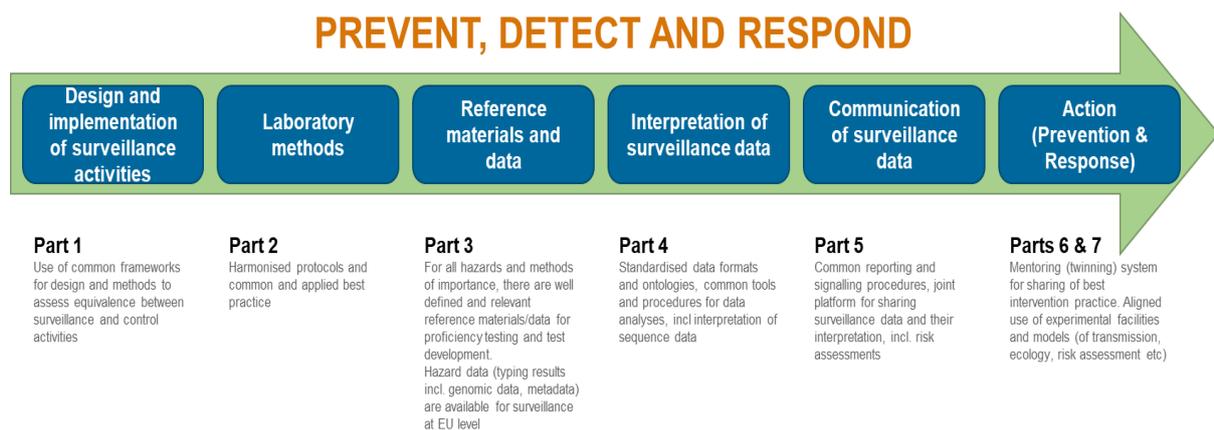


The monitoring of the deliverables gives an indication of the progress that projects make. However, figures should be looked at in detail.

The large majority of the deliverables have been achieved and are uploaded to the One Health EJP website and on Zenodo. Although seven of the first call project have come to an end, yet not all deliverables are marked as available. This is mainly due to publications that are still on-going, either the desk analysis of the results or the writing process itself (10, i.e. IMPART: 2, RADAR: 1, ListAdapt: 6, AirSample: 1). Some deliverables could not be achieved due to the COVID-19 crisis, for instance sampling at farms or the organisation of a final (physical) meeting (IMPART, RADAR, METASTAVA and MedVetKlebs, each 1). Finally, during the course of the project, the outcome justified the modification (sometimes grouping) of deliverables, without any harmful impact on the overall project results.

As for the second call projects, COVID-19 unmistakably interfered with the planning and achievement of the tasks, even more than was the case for the first call projects, since these are affected since the very start of the work.

It is worth noting that many of the JRP deliverables can be categorised according to fields that have been identified in the Integrative Strategy Matrix, as described in the One Health EJP [Strategic Research Agenda](#). This matrix illustrates the successive steps in setting up and implementing surveillance programmes, which serve the prevent-detect-respond approach.



This is a multi-disciplinary process where (reference) laboratories, risk assessors and risk managers from across sectors act together to strengthen the preparedness of the entire system. In such a strategy, cooperation is essential, but collaboration and coordination are highly desirable.

The following examples of JRP and some of their deliverables illustrate this:

Part 1. The design and implementation of surveillance activities:

- NOVA: D3.3 Univariate syndromic surveillance development for foodborne diseases
- IMPART: D1.8 & 2.8 Proposal(s) for epidemiological study to monitor resistance to colistin and to carbapenems
- MAD-VIR: D3.1 Implementation of MAD-VIR’s microarray to INIA and APHA partners

Parts 2 & 3. Harmonised protocols and applied best practice (Laboratory methods) and Databases of reference materials and data, incl. metadata:

- METASTAVA: D1.4 Guidelines for the description of scope and analytical properties of a metagenomic method in a diagnostic context
- TOX-Detect: D1.2 Libraries of MALDI-ToF reference spectra
- PARADISE: D2.1 Protocol for 18S rDNA-based amplicon sequencing for detection of relevant FBPs



- MedVetKlebs: D2.4 Genome sequence data from Klebsiella spp

Part 4. Standardised data formats, aligned data analysis for interpretation of surveillance data:

- ARDIG: D1.1 A report of AMR and AMU data (and data collection activities) in livestock and humans in the seven participating countries, and with indication to its quality, comparability and purpose
- METASTAVA: D1.1 and D1.2 Dataset: reference data metagenomics data generation, and analysis
- NOVA: D3.4 Recommendations about the quality standardization of data produced across the food chain for their use in syndromic surveillance
- All projects: the data management plans (standardized data formats)

Part 5. Sharing and communication of surveillance data, including risk assessments:

- RADAR: D6.2 Policy-targeted report
- ARDIG: D4.2 Annual communication to stakeholders
- IDEMBRU: D2.1 Set-up of a human brucellosis network
- MOMIR-PPC: D4.5 Dissemination to lay-public communities, to policy-makers and regulators, farmers and companies
- FED-AMR: D1.4 Webinars (T1.2.)

Parts 6 & 7. Sharing of best intervention activities (response) and Prevention, aligned use of facilities and models:

- MOMIR-PPC: D2.5 Determine the influence of defined and undefined probiotics on the microbiome signature, the immune response, gut physiology and welfare of pig and/ or chicken
- MOMIR-PPC: D3.5. Definition of intervention measures to target super-shedders
- BIOPIGEE: D2.1, Biosecurity protocol (addressing Salmonella and HEV) designed for data collection in the field

PROJET	DUE MILESTONES	ACHIEVED	% ACHIEVED
JRP01-AMR1-IMPART	11	10	91%
JRP02-AMR2-ARDIG	10	8	80%
JRP03-AMR3-RADAR	35	34	97%
JRP04-ET1-MADVIR	1	1	100%
JRP05-ET1-TOXDETECT	21	14	67%
JRP06-FBZ1-NOVA	24	22	92%
JRP07-FBZ2-LISTADAPT	24	24	100%
JRP08-FBZ2-METASTAVA	8	7	88%
JRP09-FBZ3-AIRSAMPLE	4	4	100%
JRP10-FBZ3-MOMIR-PPC	39	13	33%
JRP11-FBZ4-MEDVETKLEBS	15	6	40%
JRP12-AMRSH5-FARMED	1	1	100%
JRP13-AMRSH5-WORLDCOM	5	1	20%
JRP14-AMR2.1-FULLFORCE	17	8	47%



JRP15-AMR2.1-FEDAMR	12	12	100%
JRP16-ET2.2-TELEVIR	1	0	0%
JRP17-ET2.2-IDEMBRU	16	8	50%
JRP18-ET1.1-MEME	14	9	64%
JRP19-ET1.1-PARADISE	13	3	23%
JRP20-FBZSH3-DISCOVER	14	12	86%
JRP21-FBZ3.1-BIOPIGEE	11	8	73%
JRP22-FBZ4.1-TOXOSOURCES	8	8	100%
JRP23-FBZSH5-ADONIS	8	7	88%
JRP24-FBZSH9-BEONE	16	8	50%
TOTAL	328	228	70%

In principle, milestones are intermediate steps to the accomplishment of deliveries. In practice, milestones and deliverables are sometimes linked.

The same impact as described for the deliverables is applicable to the milestones as well. For instance, meetings planned by IMPART, RADAR and METASTAVA and listed as milestones, could not have been organised due to the pandemic. Also for MedVetKlebs, the milestones relate to the deliverables that have been modified based on the outcome of earlier phases in the project, or that have been merged.

Publications

In total, 65 One Health EJP articles have been published by the JRPs since the start of the One Health EJP in 2018.

1. 2018

JRP06-FBZ1-NOVA

Analysis of consumer food purchase data used for outbreak investigations, a review. Møller FT, Mølbak K, Ethelberg S. *Eurosurveillance*. 2018;23(24). doi:10.2807/1560-7917.ES.2018.23.24.1700503.

https://zenodo.org/record/3747633#.XpCQ_sgzZM0

JRP11-FBZ4-MedVetKlebs

Identification of *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, *Klebsiella variicola* and Related Phylogroups by MALDI-TOF Mass Spectrometry. Rodrigues C, Passet V, Rakotondrasoa A, Brisse S. *Front Microbiol*. 2018;9:3000. doi:10.3389/fmicb.2018.03000.

<https://zenodo.org/record/3660887#.XkEm5mhKiUk>

2. 2019

JRP02-AM2-ARDIG

Antimicrobial Usages and Antimicrobial Resistance in Commensal *Escherichia coli* From Veal Calves in France: Evolution During the Fattening Process. Gay E, Bour M, Cazeau G, et al. *Front Microbiol*. 2019;10:792. doi:10.3389/fmicb.2019.00792.

https://zenodo.org/record/4249017#.YAqc_uhKjcc

The shufflon of IncI1 plasmids is rearranged constantly during different growth conditions. Brouwer MSM, Jurburg SD, Harders F, et al. *Plasmid*. 2019;102:51-55. doi:10.1016/j.plasmid.2019.03.003.

<https://zenodo.org/record/3730621#.Xn3MyYhKi70>



JRP03-AMR3-RADAR

Attributable sources of community-acquired carriage of *Escherichia coli* containing β -lactam antibiotic resistance genes: a population-based modelling study. *The Lancet Mughini-Gras L, Dorado-García A, van Duijkeren E, et al. Planetary Health.* 2019;3(8):e357-e369. doi:10.1016/S2542-5196(19)30130-5
<https://zenodo.org/record/3621285#.XjI8UGhKhPY>

JRP04-ET1-MADVIR

Field samplings of *Ixodes ricinus* ticks from a tick-borne encephalitis virus micro-focus in Northern Zealand, Denmark. Petersen A, Rosenstjerne MW, Rasmussen M, et al. *Ticks and Tick-borne Diseases.* 2019;10(5):1028-1032. doi:10.1016/j.ttbdis.2019.05.005
<https://zenodo.org/record/3634258#.XjI8d2hKhPY>

JRP05-ET1-TOXDETECT

Point-of-Need DNA Testing for Detection of Foodborne Pathogenic Bacteria. Vidic J, Vizzini P, Manzano M, et al. *Sensors.* 2019;19(5):1100. doi:10.3390/s19051100
<https://zenodo.org/record/3935799#.X6Ud-GhKjcc>

JRP06-FBZ1-NOVA

An outbreak of monophasic *Salmonella* Typhimurium associated with raw pork sausage and other pork products, Denmark 2018–19. Helmuth IG, Espenhain L, Ethelberg S, et al. *Epidemiol Infect.* 2019;147:e315. doi:10.1017/S0950268819002073
<https://zenodo.org/record/4249319#.X6UIWmhKjcc>

Non-Typhi, non-Paratyphi *Salmonella*-related hospitalisations in Spain: trends, clinical aspects, risk factors for worse prognosis and hospital costs. Garrido-Esteba M, Latasa P, Ordóñez-León GY, Martínez-Avilés M, de la Torre A, García-Comas L. *Eur J Clin Microbiol Infect Dis.* 2019;38(2):337-346. doi:10.1007/s10096-018-3433-1
<https://zenodo.org/record/4244788#.X6LhGYhKjcc>

Salmonella Surveillance Systems in Swine and Humans in Spain: A Review. Martínez-Avilés M, Garrido-Esteba M, Álvarez J, de la Torre A. *Veterinary Sciences.* 2019;6(1):20. doi:10.3390/vetsci6010020
<https://zenodo.org/record/3635293#.XjIyUGhKhPY>

JRP11-FBZ4-MedVetKlebs

Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. Lepuschitz S, Schill S, Stoeger A, et al. *Science of The Total Environment.* 2019;662:227-235. doi:10.1016/j.scitotenv.2019.01.179
<https://zenodo.org/record/4249232#.X6UhA2hKjcc>

Description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. *tropicalensis* subsp. nov. and *Klebsiella variicola* subsp. *variicola* subsp. nov. Rodrigues C, Passet V, Rakotondrasoa A, Diallo TA, Criscuolo A, Brisse S. *Research in Microbiology.* 2019;170(3):165-170. doi:10.1016/j.resmic.2019.02.003
<https://zenodo.org/record/3660879#.XkEmO2hKiUk>

Description of *Klebsiella spallanzanii* sp. nov. and of *Klebsiella pasteurii* sp. nov. Merla C, Rodrigues C, Passet V, et al. *Front Microbiol.* 2019;10:2360. doi:10.3389/fmicb.2019.02360
<https://zenodo.org/record/3660875#.XkEICGhKiUk>

Outbreak of Yersiniabactin-producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit: Wisgrill L, Lepuschitz S, Blaschitz M, et al. *The Pediatric Infectious Disease Journal.* 2019;38(6):638-642. doi:10.1097/INF.0000000000002258
<https://zenodo.org/record/3661013#.XkFq-OSWwj9>



3. 2020

JRPO1-AMR1-IMPART

Novel IncFII Plasmid Harbours *Bla* NDM-4 in a Carbapenem-Resistant *Escherichia Coli* of Pig Origin, Italy. Diaconu, E. L.; Carfora, V.; Alba, P.; Di Matteo, P.; Stravino, F.; Buccella, C.; Dell'Aira, E.; Onorati, R.; Sorbara, L.; Battisti, A.; Franco, A. *Journal of Antimicrobial Chemotherapy* **2020**, *75* (12), 3475–3479. <https://doi.org/10.1093/jac/dkaa374>.

<https://zenodo.org/record/4451840#.YAfprNhKg2w>

Spill-Over from Public Health? First Detection of an OXA-48-Producing *Escherichia Coli* in a German Pig Farm. Irrgang, A.; Pauly, N.; Tenhagen, B.-A.; Grobbel, M.; Kaesbohrer, A.; Hammerl, J. A. *Microorganisms* **2020**, *8* (6), 855. <https://doi.org/10.3390/microorganisms8060855>.

<https://zenodo.org/record/4447289#.YAWijthKg2w>

First Detection of GES-5-Producing *Escherichia Coli* from Livestock—An Increasing Diversity of Carbapenemases Recognized from German Pig Production. Irrgang, A.; Tausch, S. H.; Pauly, N.; Grobbel, M.; Kaesbohrer, A.; Hammerl, J. A. *Microorganisms* **2020**, *8* (10), 1593. <https://doi.org/10.3390/microorganisms8101593>.

<https://zenodo.org/record/4451836#.YAfndzhKg2w>

ChromID® CARBA Agar Fails to Detect Carbapenem-Resistant Enterobacteriaceae With Slightly Reduced Susceptibility to Carbapenems. Pauly, N.; Hammerl, J. A.; Grobbel, M.; Tenhagen, B.-A.; Käsbohrer, A.; Bisenius, S.; Fuchs, J.; Horlacher, S.; Lingstädt, H.; Mauermann, U.; Mitro, S.; Müller, M.; Rohrmann, S.; Schiffmann, A. P.; Stührenberg, B.; Zimmermann, P.; Schwarz, S.; Meemken, D.; Irrgang, A. *Front. Microbiol.* **2020**, *11*, 1678. <https://doi.org/10.3389/fmicb.2020.01678>.

<https://zenodo.org/record/4447313#.YA7QnehKjcc>

Identification of a *Bla*VIM-1-Carrying *IncA/C2* Multiresistance Plasmid in an *Escherichia Coli* Isolate Recovered from the German Food Chain. Pauly, N.; Hammerl, J. A.; Grobbel, M.; Käsbohrer, A.; Tenhagen, B.-A.; Malorny, B.; Schwarz, S.; Meemken, D.; Irrgang, A. *Microorganisms* **2020**, *9* (1), 29. <https://doi.org/10.3390/microorganisms9010029>.

<https://zenodo.org/record/4451858#.YAfqlNhKg2w>

JRPO2-AMR2-ARDIG

Monitoring Antimicrobial Resistance and Drug Usage in the Human and Livestock Sector and Foodborne Antimicrobial Resistance in Six European Countries. Mesa Varona, O.; Chaintarli, K.; Muller-Pebody, B.; Anjum, M. F.; Eckmanns, T.; Norström, M.; Boone, I.; Tenhagen, B.-A. *IDR* **2020**, *Volume 13*, 957–993. <https://doi.org/10.2147/IDR.S237038>

<https://zenodo.org/record/997236#.XvyQfSgzZMO>

*bla*CTX–M–1/*Inc11-Iy* Plasmids Circulating in *Escherichia coli* From Norwegian Broiler Production Are Related, but Distinguishable. Mo SS, Telke AA, Osei KO, et al. *Front Microbiol.* 2020;11:333. doi:10.3389/fmicb.2020.00333

<https://zenodo.org/record/3701226#.YAqeSehKjcc>

Temporal dynamics of the fecal microbiota in veal calves in a 6-month field trial. Massot M, Haenni M, Nguyen TT, Madec J-Y, Mentré F, Denamur E. *anim microbiome.* 2020;2(1):32. doi:10.1186/s42523-020-00052-6

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The importance of using whole genome sequencing and extended spectrum beta-lactamase selective media when monitoring antimicrobial resistance. Duggett N, AbuOun M, Randall L, et al. *Sci Rep.* 2020;10(1):19880. doi:10.1038/s41598-020-76877-7

<https://zenodo.org/record/4456741#.YAqkH-hKjcc>

Extensive antimicrobial resistance mobilization via multicopy plasmid encapsidation mediated by temperate phages. Rodríguez-Rubio L, Serna C, Ares-Arroyo M, et al. *Journal of Antimicrobial Chemotherapy.* Published online July 28, 2020:dkaa311. doi:10.1093/jac/dkaa311

<https://zenodo.org/record/4244116#.X6KBDjiWxMO>

Stepwise evolution and convergent recombination underlie the global dissemination of carbapenemase-producing *Escherichia coli*. Patiño-Navarrete R, Rosinski-Chupin I, Cabanel N, et al. *Genome Med.* 2020;12(1):10. doi:10.1186/s13073-019-0699-6

<https://zenodo.org/record/3730637#.Xn3Pm4hKi7O>



JRP03-AMR3-RADAR

Analysis of COMPASS, a New Comprehensive Plasmid Database Revealed Prevalence of Multireplicon and Extensive Diversity of IncF Plasmids. Douarre P-E, Mallet L, Radomski N, Felten A, Mistou M-Y. *Front Microbiol.* 2020;11:483. doi:10.3389/fmicb.2020.00483
<https://zenodo.org/record/3968418#.XyQSQTgUIM2>

JRP04-ET1-MADVIR

Novel enteric viruses in fatal enteritis of grey squirrels. Dastjerdi A, Benfield C, Everest D, Stidworthy MF, Zell R. *Journal of General Virology.* 2020;101(7):746-750. doi:10.1099/jgv.0.001431
<https://zenodo.org/record/4249097#.X6kclGhKhM0>

JRP06-FBZ1-NOVA

Modelling spread and surveillance of Mycobacterium avium subsp. paratuberculosis in the Swedish cattle trade network. Rosendal T, Widgren S, Ståhl K, Frössling J. *Preventive Veterinary Medicine.* 2020;183:105152. doi:10.1016/j.prevetmed.2020.105152
<https://zenodo.org/record/4450338>

Using stochastic dynamic modelling to estimate the sensitivity of current and alternative surveillance program of *Salmonella* in conventional broiler production. Apenteng OO, Arnold ME, Vigre H. *Sci Rep.* 2020;10(1):19441. doi:10.1038/s41598-020-76514-3
<https://zenodo.org/record/4271431#.X65ljTiWxPY>

Climatic and topographic tolerance limits of wild boar in Eurasia: implications for their expansion. Bosch J, Iglesias I, Martínez M, De la Torre A. *GES.* 2020;13(1):107-114. doi:10.24057/2071-9388-2019-52
<https://zenodo.org/record/4244729#.X6LYN4hKjcc>

Identifying emerging trends in antimicrobial resistance using *Salmonella* surveillance data in poultry in Spain. Alvarez J, Lopez G, Muellner P, et al. *Transbound Emerg Dis.* 2020;67(1):250-262. doi:10.1111/tbed.13346
<https://zenodo.org/record/3660026#.XvyQnGzZM0>

Spatial Trends in *Salmonella* Infection in Pigs in Spain. Teng KT, Martinez Avilés M, Ugarte-Ruiz M, et al. *Front Vet Sci.* 2020;7:345. doi:10.3389/fvets.2020.00345
<https://zenodo.org/record/4244797#.X6Li lhKjcc>

JRP07-FBZ2-LISTADAPT

First Report on the Finding of *Listeria monocytogenes* ST121 Strain in a Dolphin Brain. Sévellec Y, Torresi M, Félix B, et al. *Pathogens.* 2020;9(10):802. doi:10.3390/pathogens9100802
<https://zenodo.org/record/4244051#.X6J4fziWxM1>

JRP08-FBZ2-METASTAVA

Evaluation of a commercial exogenous internal process control for diagnostic RNA virus metagenomics from different animal clinical samples. Van Borm S, Fu Q, Winand R, et al. *Journal of Virological Methods.* 2020;283:113916. doi:10.1016/j.jviromet.2020.113916
<https://zenodo.org/record/4244771#.X6Lc5ohKjcc>

COVID-19 in health-care workers in three hospitals in the south of the Netherlands: a cross-sectional study. Sikkema RS, Pas SD, Nieuwenhuijse DF, et al. *The Lancet Infectious Diseases.* 2020;20(11):1273-1280. doi:10.1016/S1473-3099(20)30527-2
<https://zenodo.org/record/4457341#.YArXXOhKjcc>

Rapid SARS-CoV-2 whole-genome sequencing and analysis for informed public health decision-making in the Netherlands. The Dutch-Covid-19 response team, Oude Munnink BB, Nieuwenhuijse DF, et al. *Nat Med.* 2020;26(9):1405-1410. doi:10.1038/s41591-020-0997-y
<https://zenodo.org/record/4457379#.YArZ5ehKjcc>

Increased viral read counts and metagenomic full genome characterization of porcine astrovirus 4 and Posavirus 1 in sows in a swine farm with unexplained neonatal piglet diarrhea. Van Borm S, Vanneste K, Fu Q, et al. *Virus Genes.* 2020;56(6):696-704. doi:10.1007/s11262-020-01791-z
<https://zenodo.org/record/4244782#.X6LfzYhKjcc>



JPR09-FBZ3-AIRSAMPLE

Prevalence and antimicrobial resistance of *Campylobacter* isolated from carcasses of chickens slaughtered in Poland – a retrospective study. Wieczorek K, Bocian Ł, Osek J. *Food Control*. 2020;112:107159. doi:10.1016/j.foodcont.2020.107159
<https://zenodo.org/record/3676306>

Campylobacter in chicken – Critical parameters for international, multicentre evaluation of air sampling and detection methods. Johannessen GS, Garofolo G, Di Serafino G, et al. *Food Microbiology*. 2020;90:103455. doi:10.1016/j.fm.2020.103455
<https://zenodo.org/record/3663545#.XkPEvGhKiUk>

Foodborne *Campylobacter*: A multi-center proposal for a fast screening tool in biosecured chicken flocks. Hoorfar J, Koláčková I, Johannessen GS, et al. *Appl Environ Microbiol*. Published online August 7, 2020:AEM.01051-20, aem;AEM.01051-20v1. doi:10.1128/AEM.01051-20
<https://zenodo.org/record/4244138#.X6KEbDiWxM1>

MLST-based genetic relatedness of *Campylobacter jejuni* isolated from chickens and humans in Poland. Wieczorek K, Wołkiewicz T, Osek J, Biggs PJ, et al. *PLoS ONE*. 2020;15(1):e0226238. doi:10.1371/journal.pone.0226238
<https://zenodo.org/record/3628110#.X6FsFjiWxPY>

JRP10-FBZ3-MOMIR-PPC

A multi-scale epidemic model of *Salmonella* infection with heterogeneous shedding. Labarthe S, Laroche B, Nguyen TNT, et al. Calvez V, Grandmont C, Lochërbach E, Poinard C, Ribot M, Vauchelet N, eds. *ESAIM: ProcS*. 2020;67:261-284. doi:10.1051/proc/202067015
<https://zenodo.org/record/4244169#.X6KF4TiWxM0>

Reduction of *Salmonella* Typhimurium Cecal Colonisation and Improvement of Intestinal Health in Broilers Supplemented with Fermented Defatted ‘Alperujo’, an Olive Oil By-Product. Rebollada-Merino A, Ugarte-Ruiz M, Hernández M, et al. *Animals*. 2020;10(10):1931. doi:10.3390/ani10101931
<https://zenodo.org/record/4114070>

Cost-effectiveness analysis of using probiotics, prebiotics, or synbiotics to control *Campylobacter* in broilers. van Wagenberg CPA, van Horne PLM, van Asseldonk MAPM. *Poultry Science*. 2020;99(8):4077-4084. doi:10.1016/j.psj.2020.05.003
<https://zenodo.org/record/4244748#.X6LaalhKjcc>

Dietary supplementation with fermented defatted “alperujo” induces modifications of the intestinal mucosa and cecal microbiota of broiler chickens. Rebollada-Merino A, Ugarte-Ruiz M, Hernández M, et al. *Poultry Science*. Published online August 2020:S0032579120304843. doi:10.1016/j.psj.2020.07.015
<https://zenodo.org/record/4017817#.X6LRjYhKjcc>

Gut microbiota composition before infection determines the *Salmonella* super- and low-shedder phenotypes in chicken. Kempf F, Menanteau P, Rychlik I, et al. *Microb Biotechnol*. 2020;13(5):1611-1630. doi:10.1111/1751-7915.13621
<https://zenodo.org/record/4005830#.X0kCUMgzbcc>

JRP11-FBZ4-MedVetKlebs

Diversity of mucoid to non-mucoid switch among carbapenemase-producing *Klebsiella pneumoniae*. Chiarelli A, Cabanel N, Rosinski-Chupin I, et al. *BMC Microbiol*. 2020;20(1):325. doi:10.1186/s12866-020-02007-y
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Broad-Spectrum Cephalosporin-Resistant *Klebsiella* spp. Isolated from Diseased Horses in Austria. Lončarić I, Cabal Rosel A, Szostak MP, et al. *Animals*. 2020;10(2):332. doi:10.3390/ani10020332
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Klebsiella pneumoniae carriage in low-income countries: antimicrobial resistance, genomic diversity and risk factors. Huynh B-T, Passet V, Rakotondrasoa A, et al. *Gut Microbes*. 2020;11(5):1287-1299. doi:10.1080/19490976.2020.1748257
<https://zenodo.org/record/3929396#.Xv8lrSgzM0>



The ZKIR Assay, a Real-Time PCR Method for the Detection of *Klebsiella pneumoniae* and Closely Related Species in Environmental Samples. Barbier E, Rodrigues C, Depret G, et al. Dudley EG, ed. *Appl Environ Microbiol*. 2020;86(7):e02711-19, /aem/86/7/AEM.02711-19.atom. doi:10.1128/AEM.02711-19
<https://zenodo.org/record/3730608#.Xn3Kk4hKi70>

JRP16-ET2.2-TELEVIR

An alternative workflow for molecular detection of SARS-CoV-2 – escape from the NA extraction kit-shortage, Copenhagen, Denmark, March 2020. Fomsgaard AS, Rosenstjerne MW. *Eurosurveillance*. 2020;25(14). doi:10.2807/1560-7917.ES.2020.25.14.2000398
<https://zenodo.org/record/4247188#.X6QLjWhKjcc>

JRP18-ET1.1-MEME

Cystic Echinococcosis: Clinical, Immunological, and Biomolecular Evaluation of Patients from Sardinia (Italy). Santucci C, Bonelli P, Peruzzo A, et al. *Pathogens*. 2020;9(11):907. doi:10.3390/pathogens9110907
<https://zenodo.org/record/4159681>

Comparison of Two DNA Extraction Methods and Two PCRs for Detection of *Echinococcus multilocularis* in the Stool Samples of Naturally Infected Red Foxes. Skrzypek K, Karamon J, Samorek-Pieróg M, et al. *Animals*. 2020;10(12):2381. doi:10.3390/ani10122381
<https://zenodo.org/record/4384600>

A validated method to identify *Echinococcus granulosus sensu lato* at species level. Santolamazza F, Santoro A, Possenti A, Cacciò SM, Casulli A. *Infection, Genetics and Evolution*. 2020;85:104575. doi:10.1016/j.meegid.2020.104575
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Molecular Characterization of *Giardia duodenalis* in Children and Adults Sampled in Algeria. Belkessa S, Thomas-Lopez D, Houali K, Ghalmi F, Stensvold CR. *Microorganisms*. 2020;9(1):54. doi:10.3390/microorganisms9010054
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Veterinary Students Have a Higher Risk of Contracting Cryptosporidiosis when Calves with High Fecal *Cryptosporidium* Loads Are Used for Fetotomy Exercises. Schaffner DW, ed. Thomas-Lopez D, Müller L, Vestergaard LS, et al. *Appl Environ Microbiol*. 2020;86(19):e01250-20, /aem/86/19/AEM.01250-20.atom. doi:10.1128/AEM.01250-20
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JRP21-FBZSH9-BEONE

Prediction of antimicrobial resistance in clinical *Campylobacter jejuni* isolates from whole-genome sequencing data. Dahl LG, Joensen KG, Østerlund MT, Kiil K, Nielsen EM. *Eur J Clin Microbiol Infect Dis*. Published online September 24, 2020. doi:10.1007/s10096-020-04043-y
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JRP22-FBZ4.1-TOXOSOURCES

Fluorescent Bead-Based Serological Detection of Toxoplasma Gondii Infection in Chickens. Fabian BT, Hedar F, Koethe M, et al. In Review; 2020. Accessed August 20, 2020. <https://www.researchsquare.com/article/rs-34121/v1>

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Impact and relevance of the research projects

In the 12M report, project leaders were explicitly asked to mention on-going and planned collaborations with national or European projects or networks. Most authors mentioned ECDC and/or EFSA as international contacts, but also interactions with relevant EU Reference Laboratories (Brucella, E. coli, parasites, Salmonella, Staphylococci) were referred to. Some of these contacts are already ongoing, but most project leaders plan to reach out to ECDC and EFSA at a later stage, for instance at the occasion of a final workshop or other event. Sporadically, other international organizations such as DG-Health or DG-Agri, OIE and WHO were pointed out. BIOPIGEE mentioned its interaction with HEV-Net, a specialised database on Hepatitis E, maintained by RIVM (NL). Some project leaders also described their contacts with national authorities to keep them updated on project outcomes that may be useful for them. Finally, the reports clearly indicated the collaborations with other One Health EJP projects and with European and national projects. Among those EU projects JPIAMR, EU-JAMRAI, AVANT, EFFORT and IMPACT (EFSA) can be mentioned.

As for the final reports, project leaders were requested to specify any direct or indirect impact the outcome of their project may have for international stakeholders active in the domains of foodborne zoonoses, AMR and/or emerging threats. The authors were also invited to describe how regional or national authorities and stakeholders might implement and/or integrate any of the results from the project; the contacts that have been undertaken should have been listed. The project leaders took various initiatives, each clearly adapted to their domain of interest:

- IMPART reached out to the EU-RL AMR regarding a detailed protocol for the harmonized surveillance of carbapenemase producing Enterobacteriaceae at the European level, but also to EFSA to suggest improvements to existing surveillance programmes. IMPART collaborators linked from the start to EUCAST/VetCAST to setting new ECOFFs.
- RADAR aims at having a supranational stakeholder (EFSA, ECDC) meeting early 2021 in order to disseminate the results and to scout the possibilities for further use of our work. In addition, several consortium partners have been in interaction with their local governments regarding the use of the developed methods and gained knowledge.
- Listadapt underlined the delivery of a large and diverse collection of *Listeria monocytogenes* strains that were isolated from farming/wild animals and their environment. Its importance for future work was stressed, both for the development of new diagnostics as for the improvement of surveillance programmes. Listadapt has reported frequently to the EU-RL *Listeria*.
- METASTAVA stressed the potential use of its newly developed metagenomics guidelines for national and international stakeholders, in order to implement these workflows to improve diagnostic protocols and surveillance programmes.



- AIR-Sample contacted ECDC, EFSA, DG-Health and the EU-RL Campylobacter to present its newly developed tool for on-farm sampling, suited for both bacteriological and genetic analysis.
- MedVetKlebs was in close contact with ECDC to agree on genomic nomenclature of Klebsiella strains for pan-European tracking purposes and built the international network Kleb-NET based on the One Health EJP project.

This overview and the examples clearly demonstrate the impact the JRP has on the work and tasks of national and international stakeholders, be it regarding surveillance programmes, laboratory tasks for diagnosis and characterization, and risk assessors.

Critical risks

Following the Horizon2020 guidelines, the project leaders need to report on any critical risk identified during the management of their project. Not unexpectedly, the COVID-19 crisis was often indicated as the cause of delay in work plan execution and in duties, tasks and reporting. Many leaders expressed their wish to get an extension in time of their project, which would allow to recover some of the valuable time and thus to deliver high-quality outcomes as planned. Also the loss of key personnel was reported, in some cases the project leader her-or himself, or a WP leader. Nonetheless, these issues were appropriately addressed by looking for competent replacements.



3. Reports of the JRP, Year 3

The projects leaders have filled in 12 months reports or final reports for the projects that came to an end in December 2020. The entire contents of these reports have been copied below, except for the paragraphs related to the ethics, the data management and the list of dissemination and communication activities, which can be found elsewhere:

- The third Ethics report, encompassing Joint Research Projects, Joint Integrative Projects and PhD, has been delivered as D1.25 Ethical review report for Y3 (28 Feb 2021) on the Participant Portal.
- All information on the data management plan can be found in chapter 4 of the One Health EJP Periodic Technical Report Y3.
- The list of dissemination activities is available for each project under the section 2.3.3.3.1 “Summary of the progress made by the ongoing and finalized JRP in 2020” of the same Periodic Technical Report.

JRP01-IMPART

1. Summary of the work carried out

For WP1 (Selective isolation, detection and characterization of colistin-resistant *Enterobacteriaceae*), the final ring trial was organised in June 2019. All participants received a final report in November 2020 where a combined use of PCR step and selective commercial media were evaluated. As a result, PCR detection of *mcr*-genes showed 100% specificity and one out of three media tested shows a better sensitivity to selectively isolate colistin-resistant *mcr*-positive strains. Performance of the colistin-resistant screening process is greater for caecal than meat samples in the experimental conditions of this WP1. More trials are needed to test the effect of other matrixes, bacterial concentrations and a broader range of *mcr*-positive strains.

For WP2 (Selective isolation, detection and characterization of carbapenemase-producing *Enterobacteriaceae*), the final ring trial was organised in September 2019. All participants received a report containing their own results compared to what was expected. Preliminary results of the WP2 ring trial were presented at the 2nd OHEJP-ASM. In WP2, the final report was finalized in September 2020. The main outcome indicates that some of the commercially available selective agar media are not sensitive or selective enough to detect bacteria expressing low-level carbapenemase production. Further, there are no commercially available selective agars to detect carbapenem-resistant *Salmonella* spp., as they are non-chromogenic on most media. Some bacteria harbouring specific genes (*bla*_{VIM-1} *E. coli*) that circulate in the livestock population in Europe might be missed using the protocol currently set by the EU decision 2013/652/EU with a pre-enrichment in non-selective BPW-ISO. More studies are needed to test other selective enrichment broths, matrices and bacterial species.

Within WP3 (Establishing epidemiological cut-off values (ECOFFs)), the production and collection of MIC distributions was finished in May 2020. Uploading of the MIC distributions on the EUCAST website was completed in September 2020. The process of calculating epidemiological cut-off values (ECOFFs) based on the uploaded MIC distributions was further delayed because of unexpected problems regarding the acceptance of MIC data by EUCAST due to differences in test method between EUCAST and the IMPART project for the more fastidious growing bacteria. To measure possible media effects, bridging studies have been conducted at WBVR at the end of 2020 and are currently evaluated. The discussion on the difference in test methods does not account for bacteria without supplements in the test media. For this reason the MIC distributions of *Staphylococcus pseudintermedius* are currently analysed and the first accepted ECOFFs for this bacterial species are expected in the beginning of 2021.



Within WP4 (Developing and optimizing a disk diffusion method for antimicrobial susceptibility testing of *Clostridium difficile*), a *Clostridium difficile* strain collection comprising 527 strains was completely characterized regarding PCR-ribotypes, toxin gene and antimicrobial resistance (AMR) profiles (minimum inhibitory concentrations; MICs). An optimized disk diffusion protocol as alternative method for AMR testing was established and inhibition zone diameter (IZD) distributions determined for eight antimicrobials analyzing each strain of the collection. Evaluation of the MICs and IZDs for each strain to determine wild-type and non-wildtype phenotypes correlated well for most but not all antimicrobials. Preliminary results and cut-off values were presented at the 2nd OHEJP ASM. The ring trial to finalize the validation of the proposed disk diffusion method was conducted in June 2020 with seven participating laboratories. Reports were sent out to the participants and uploaded to the OHEJP group website. Overall, the optimized method description proved to be highly reproducible but critical steps could be identified.

Within WP5 (Coordination of the four work packages and knowledge dissemination both internally within and externally beyond the IMPART consortium), emails were sent out by the WP leaders to all consortium members containing general information on the progress of the different WP's. Furthermore, all WP leaders were in contact via Skype every two weeks discussing the organization of IMPART and the progress of the different WPs. Furthermore, three abstracts comprising results from WP2, WP3 and WP4 were presented as poster at the OHEJP ASM online meeting in May 2020. The closing meeting was initially planned in June 2020, but postponed as teleconference to December and later to March 2021 because of COVID-19.

2. Work carried out in the JRP, scientific results

WP1: Selective isolation, detection and characterization of colistin-resistant Enterobacteriaceae (M1-M36)

JRP1-WP1-T1: Describe existing methods to be evaluated in a ring trial (M1-M6)

A literature review had been performed to determine which media were used to isolate colistin-resistant *Enterobacteriaceae*. This review had been complemented by exploring any other commercially available option on the market as off September 2018. We selected both ready-to-use commercial reagents available throughout Europe and in-house prepared reagents. The selected media for the pre-ring trial were: MacConkey Agar + 2mg/L colistin, COLISTIGRAM (SuperPolymyxin™), CHROMagar™ COL-APSE (CHROMagar), CHROMID® Colistin R (BioMerieux). Protocols using selective (1, 2 or 4mg/L of colistin) versus non-selective enrichment broth were also explored.

JRP1-WP1-T2: Preparation of the samples for the pre-ring trial (WP1 and WP2, M7-M8)

In order to avoid false positive detection, our samples to be artificially contaminated (later called “blank” samples) were mostly collected from slaughtered Specific Pathogen Free (SPF) animals from collaborating animal housing facilities (pig and turkey caecal content, turkey meat). Due to unavailability, pig meat had to be purchased at retail shop. Nevertheless, meat and caecal “blank” samples had been checked for absence of colistin resistant determinant by both multiplex PCR (Rebelo et al, 2018, Eurosurveillance) and plating on MacConkey + 2mg/l colistin agar. Non-contaminated sampled had been preserved frozen (-20°C).

Contamination of the “blank” samples of minced meat or caecal content was performed with characterized *mcr*-positive strains originated from animals.

Prior to spiking, a 0.5 McFarland ($\approx 10^8$ CFU/ml) target bacterial suspension in saline (0.9% NaCl) water using fresh colonies had been prepared and diluted to obtain the target concentrations in the pooled samples of minced meat or caecal content (10^2 CFU/g sample). The aliquots were stored at 4°C until shipping on Tuesday, December 11th.



Artificially contaminated aliquots were distributed at 4°C to the three participating labs (NVI, RIVM and WBVR). In order to avoid deviations due to different batches of reagents, ready-to-use media and powders for in-house media were distributed to the participants along with the samples.

JRP1-WP1-T3: Performance of the pre-ring trial and evaluation (M10-M11)

Performance of the pre-ring trial was evaluated all through the first semester 2019. An alternative protocol has been established to improve the detection of positive strains prior to the final ring-trial. The final report of the WP1 pre-ring trial was posted on the IMPART private group on 25 November 2019.

JRP1-WP1-T4: Preparation of samples for the final ring trial (WP1 and WP2, M12-M17)

The protocol for the final ring trial, along with a technical questionnaire, were sent out to the 11 participants at draft stage for consultation on 2 May 2019. The final version of the protocol and the result sheet were distributed to the participants by email on 3 June 2019. A panel of three meat samples and three caecal content samples was prepared at Anses-Fougères laboratory the 13 and 14 June 2019 and stored at 4°C until shipping. Panel of samples and commercially selective agar plates were shipped to the WP1 participants on Monday 17 June 2019.

JRP1-WP1-T5: Performing the final ring trial (M17)

The 11 participants received the parcel on Tuesday 18 June 2019. Ten participants started immediately the analysis, while one was advised to store the samples for a week at -80°C prior to analysis due to bank holiday.

Homogeneity and stability of the samples were assessed at Anses-Fougères laboratory the day of shipment and one day after reception and analysis of the samples by the participating laboratories.

JRP1-WP1-T6: Analysis of the results and reporting (M18-M19)

The analysis of results sheet has been accomplished from September 2019 to May 2020. A final report was draft and sent to the 11 participants for consultation in September 2020. The final report of the ring trial was uploaded to the IMPART EJP website the 4th of November 2020.

JRP1-WP1-T7: Publication in peer-reviewed journal (M25-M30)

This task is delayed and planned for the first half of 2021.

JRP1-WP1-T8: Plan joint implementation (M25-M30)

There will not be a joint implementation plan, because the method for isolation and detection of colistin resistant *Enterobacteriaceae* needs further development.

WP2: Selective isolation, detection and characterization of carbapenemase-producing *Enterobacteriaceae* (M1-M36)

JRP1-WP2-T1: Describe existing methods to be evaluated in a ring trial (M1-M6)

To establish the protocol for the WP2 pre-ring trial, a literature study was performed and a questionnaire was sent to the participants to share experiences between the participants. All information was gathered together with a summary of a questionnaire sent out by the European Union Reference Laboratory on Antimicrobial Resistance (EURL-AR) regarding selective media. Further, the following conditions was discussed: pre-enrichment (which media, selective/non-selective), choice of selective agar plates, incubation temperatures for pre-enrichment and selective agar plates, levels of contamination of the samples, which bacteria/gene-combination to spike with, and what kind of matrix to use (turkey, chicken, pig, cattle).

A list was made of selective agar plates used for detecting carbapenemase producers and the availability and possibility to produce the plates in-house was taken into consideration.



JRP1-WP2-T2: Preparation of the samples for the pre-ring trial (WP2, M7-M8)

All samples were prepared at Anses Fougères as described under

JRP1-WP2-T2: Preparation of the samples for the pre-ring trial (WP1 and WP2, M7-M8)

Meat and caecal “blank” samples was checked for absence of carbapenem resistant determinant by using the EURL-AR method (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/530_esbl-ampc-cpeprotocol-version-caecal-v7-09-12-19.pdf, https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/529_esbl-ampc-cpeprotocol-version-meat-v7-09-12-19.pdf). Non-contaminated sampled had been preserved frozen (-20°C). Contamination of the “blank” samples of minced meat or caecal content was performed with characterized CPE-positive strains originated from animals. The samples were distributed to the three participating laboratories (RIVM, WBVR, and NVI) on Tuesday November 20th at 4°C and processed at arrival within 48 hours.

JRP1-WP2-T3: Performance of the pre-ring trial and evaluation (M10-M11)

The pre-ring trial focused on testing several conditions to detect carbapenemase-producing (CP) *Enterobacteriaceae* to narrow down different possibilities to test in the final ring trial. The focus was to:

1. Test all selective agar plates available in all European countries both in-house and ready-to-use.
2. Test two different temperatures for incubation of the selective agar plates:
 - a) Recommended by the different manufacturers: 35±2°C or 37°C.
 - b) An elevated temperature to try to eliminate unspecific growth: 44°C.
3. Preferably, test DNA extracts from the overnight pre-enrichment broth using a direct PCR protocol.

The performance of the pre-ring trial was evaluated through the first months of 2019 and the final report of the WP2 pre-ring trial was posted on the IMPART group private group in July 2019.

JRP1-WP2-T4: Preparation of samples for the final ring trial (WP1 and WP2, M12-M17)

The samples were prepared at Anses Fougères laboratory on Friday September 6th 2019. The samples were prepared using the same method as for the pre-ring trial (JRP1-WP2-T2).

JRP1-WP2-T5: Performing the final ring trial (M17)

The final ring trial was performed among eleven laboratories participating in IMPART. The samples (n=8) were prepared at Anses Fougères Laboratory on Friday September 6th 2019 and shipped to the eleven participants on Monday September 9th 2019. Analysis started immediately at arrival of the samples. The final protocol was sent as a draft to the participating laboratories for comments on July 5th 2019. Prior to the sample shipment, the ring trial protocol was distributed per email to each participant. The six selective agar plates to be tested, were shipped with the samples.

JRP1-WP2-T6: Analysis of the results and reporting (M18-M19)

Each participating lab received their own report containing what they reported and what was expected in the final ring trial, M27 March 2020. The analysis and drafting of the report from the entire ring trial was delayed due to the COVID-19 situation, but a draft was sent to the participants before the summer holidays (M30) and the report was finalized by M33, September 2020.

JRP1-WP2-T7: Publication in peer-reviewed journal (M25-M30)

The project received a last minute extension from M30 to M36 and due date was December 2020. This task is on-going, but has been delayed. A publication is planned for the first half of 2021. A draft manuscript was ready ultimo December 2020 and distributed to the laboratories participating in this work package.



JRP1-WP2-T8: Plan joint implementation (M25-M30)

There will not be a joint implementation plan, because this project did not result in a new method for isolation and detection of carbapenem-resistant *Enterobacteriaceae*, but ended up doing a multicentre study of an existing method with several selective agar plates, not giving a better harmonized method.

WP3. Establishing epidemiological cut-off values (ECOFFs) (M1-M36)

JRP1-WP3-T1: Inventory, prioritizing and inclusion criteria (M1-M3)

This task was finalized in July 2018 but refined in May 2019. Soon after the physical meeting, an Excel sheet was prepared with a list of bacterial species to be tested. After consultation of the partners by email, it was boiled down to a short list consisting of 17 different bacterial species belonging to either staphylococci, streptococci, *Enterobacteriaceae*, *Pasteurellae* or *Pseudomonas* spp. Based on this short list, a new Excel sheet was sent around by email to all partners on 20 July 2019 in which each partner was asked to fill in exactly which bacterial species they planned to test (or already had tested) including the number of isolates per panel. In this way, an overview was generated of the total number of isolates per bacterial species tested per panel per partner. For most bacterial species selected, at least 5 partners and > 100 strains were to be tested which should be sufficient for setting ECOFFs for these combinations according to EUCAST guidelines.

JRP1-WP3-T2: Production of MIC data (M4-M18)

Task was finalised in May 2020 (collection of MIC distributions completed).

JRP1-WP3-T3: Collection and quality control of MIC data (M4-M18)

Nine partner institutes performed antimicrobial susceptibility testing (AST) of 2,831 bacterial isolates involving 19 different veterinary pathogenic bacteria including staphylococci (*Staphylococcus pseudintermedius*, *S. hyicus*), streptococci (*Streptococcus agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. suis*, *S. canis*, *S. equi* subsp. *zooepidemicus*, *S. equi* subsp. *equi*, *S. equisimilis*), *Pasteurella multocida*, *Mannheimia haemolytica*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Klebsiella variicola*. This resulted in 1,310 MIC-distributions consisting 47,640 MIC-values of 34 different antimicrobials. The collection of MIC results was completed in May 2020. An additional request was sent out in June 2020 to collect raw MIC data. The correctness of the earlier received MIC distributions were reviewed using the raw MIC data files. In case MIC distributions were not in accordance with the raw data, the MIC distribution files were recalculated based on the raw MIC data files. Examples of frequently identified errors in the MIC distributions were incorrect translation of MIC-values at the edges of the test concentrations (\leq or $>$ values) and mistakes in the total number of isolates tested per antibiotic. Finally, all corrected MIC distributions were uploaded in the EUCAST database in September 2020.

JRP1-WP3-T4: Analysis of the data and publication of ECOFFs (M25-M36)

The process of calculating epidemiological cut-off values (ECOFFs) based on the uploaded MIC distributions has been delayed and is currently still ongoing. The first ECOFFs are expected in the first quarter of 2021 for *Staphylococcus pseudintermedius*. On 24 September 2020 a teleconference was held with VetCAST and EUCAST to discuss the outcomes of IMPART WP3. During this meeting it was pointed out that EUCAST implemented a new SOP for determination of ECOFFs and as a result has become stricter on the acceptance of MIC-distributions with regard to the methods used for MIC-testing. The following is stated on the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/): "Using agreed criteria for the acceptance of MIC distributions, the database and ECOFFs are currently being critically evaluated. Official ECOFFs are determined by EUCAST, adhering to protocols generated by the "EUCAST Subcommittee on MIC distributions and the determination of ECOFFs" (SOP10.1) ". As a consequence, WBVR is currently performing bridging studies testing a selection of streptococci and *Pasteurella multocida* isolates to determine the possible effect of using CAMHB + LHB (according to CLSI) instead of MH-F broth (according to EUCAST). Depending on the outcome of these bridging studies MIC distributions of



bacteria tested in CAMHB + LHB will or will not be accepted by EUCAST. Fortunately, this does not include bacterial species tested in CAMHB without supplements. Therefore, the analysis of MIC distributions of *Staphylococcus pseudintermedius* was given priority and the first accepted ECOFFs for this bacterial species is expected in the first quarter of 2021.

WP4: Developing and optimizing a disk diffusion method for antimicrobial susceptibility testing of *Clostridium difficile* (M4-M36)

JRP1-WP4-T1: Establishment of a disk diffusion method for antimicrobial susceptibility testing of *C. difficile* (M4-M14)

To establish a robust protocol for disk diffusion testing of *C. difficile*, we reviewed recent literature regarding this topic and identified critical parameters for reliability and repeatability of inhibition zone diameters (IZD) and growth of *C. difficile*. A first draft protocol was developed that is based on the EUCAST disk diffusion method (v 6.0) and includes recommendations from the literature. For optimization and standardization experiments, ten *C. difficile* strains were selected from the strain collection (see JRP1-WP4-T2) based on different resistance properties. Optimization experiments included the comparison of different media for inoculum preparation (BHI, TPGY, Brucella broth), different turbidity steps (McFarland 0.5 – 4.0) and different solid media (Brucella blood agar, Wilkins-Chalgren-agar, Columbia blood agar) for the disk diffusion itself. Furthermore, different procedures and conditions of anaerobic incubation and pre-treatment were analysed, to be able to propose guidelines later. While the different inoculum and solid media had no significant effect on IZD, the turbidity has to be amended from EUCAST recommendations to reach confluent growth for most of the strains. The biggest variance resulted from different anaerobic conditions and indicates that this factor is the most critical for standardization. The setup which resulted in reliable results and is applicable by most microbiological laboratories, was repeated several times to determine standard deviations and repeatability. Furthermore, the interlaboratory reproducibility will be tested in JRP1-WP4-T4.

Due to problems in recruiting the applied technician and problems in delivery of a gas mixture for working in an anaerobic workstation as well as the availability of culture media, this task including milestone M-JRP1-4 (M8) was delayed.

JRP1-WP4-T2: Assembly and characterization of *C. difficile* strain collection (M4-M12)

This task was finalized in December 2019 with a total number of 527 *C. difficile* strains of human, animal, environmental and food origin from ten partner institutes. All strains were completely characterized according to their PCR-ribotypes, toxin gene profiles and antimicrobial resistance (AMR) phenotypes (minimum inhibitory concentrations; MICs). The results and data will be provided on the OHEJP website.

JRP1-WP4-T3: Performance of a ring trial study (M22-M32)

The ring trial was conducted in June 2020 with seven participating laboratories. The results were reported by 30th June 2020. Reports were sent out to the participants on 25th August 2020 and uploaded to the group on the OHEJP website. The results indicate that the optimized disk diffusion protocol ensures comparable and reproducible results and reveals critical factors, e.g. the equipment for maintaining anaerobic conditions. Standard deviations were overall low but differed depending on the antimicrobial and equipment used.

JRP1-WP4-T4: Producing inhibition zone diameter distributions and proposing cut-off values for *C. difficile* (M17-M33)

Inhibition zone diameter distributions have been determined for eight antimicrobials (clindamycin, erythromycin, metronidazole, moxifloxacin, imipenem, rifampicin, tetracycline, vancomycin) analysing 527 strains of the *C. difficile* strain collection (see JRP1-WP4-T2). Preliminary results and cut-off values were presented at the 2nd OHEJP-ASM and uploaded to the OHEJP website. These results enable to



compare IZDs and MICs for each strain in terms of classifying them to have a wildtype or non-wildtype phenotype. This task was finalized in September 2020 (M33).

WP5: Coordination of the four work packages and knowledge dissemination both internally within and externally beyond the IMPART consortium (M1-M36)

JRP1-WP5-T1: Organization of IMPART (M1-M36)

IMPART consists of five different WP's supervised by WP leaders. The first four WP's have defined scientific goals whereas WP5 is intended for the communication and dissemination of knowledge.

JRP1-WP5-T2: Communication within IMPART (M1-M36)

A kick-off meeting was held on 20th February 2018 at Schiphol airport for all consortium members. All partner institutes were represented by at least one person. During this one-day meeting, the IMPART project was introduced and the tasks and timelines of each work package was discussed with the participants.

During the second year, a physical meeting was held for all consortium members at the OHEJP ASM meeting in Dublin. In addition, the WP leaders sent out emails to all consortium members containing general information on the progress of the different WP's. Furthermore, all WP leaders were in contact via Skype every two weeks discussing the organization of IMPART and the progress of the different WPs.

In the third and final year of IMPART, consortium members were informed by the WP leaders about activities and tasks via emails or teleconferences. In December 2020, a newsletter was sent to all consortium members by email with an update on the status of the different WP's. The closing meeting was initially planned in June 2020, but because of the COVID-19 crisis postponed to December 2020 and recently planned for March 2021 as a teleconference.

JRP1-WP5-T3: Communication beyond IMPART (M1-M36)

In the first year (2018) the IMPART project proposal was presented at the One Health EJP Kick-off meeting in Maisons-Alfort (France) on 30-31 January 2018. Project plans were presented and discussed in more detail during the IMPART kick-off meeting on 20 February 2018 at Schiphol airport. Furthermore, IMPART activities were presented at both Cogwheel meetings organised in 2018 with COMPARE (12 April) and EFFORT (26 October) and shared on the WBVR website as news item in December 2018.

Progress of IMPART research was presented through posters at the OHEJP ASM meeting in Dublin in May 2019. The first results of IMPART was also presented during the annual workshop of the EURL for AMR, which gathers the European NRL for AMR on a yearly basis. The three presentations are available on: <https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx>

In 2020, results of IMPART WP3 were presented during the kick-off meeting of COST action ENOVAT held on 25th of February in Tirana. Information about WP3 was shared in the VetCAST Newsletter 2020 and sent to all VetCAST members by email on 29th of April 2020. Results of WP2, WP3 and WP4 were presented as digital posters on 27th-29th of May 2020 at the online OHEJP ASM meeting. Project outcomes and interactions with stakeholders were presented on 12th of October 2020 at the OHEJP 6th Stakeholders Committee Meeting. A short summary of IMPART activities was presented on 4th of November 2020 at the ECDC food- and waterborne disease steering committee meeting. Finally, a first meeting was held with the Communication Team of the OHEJP on 23th of October 2020. It was agreed to start working on the promotion of four deliverables from the IMPART project (one from each work package). The Communication Team will share a draft proposal with the IMPART management team in the beginning of 2021.



3. Project Self-assessment

Work package 1: Selective isolation, detection and characterization of colistin-resistant *Enterobacteriaceae*

The objectives of WP1 were to evaluate the feasibility and performance of different methods existing for isolation of colistin-resistant *Enterobacteriaceae*. An initial protocol was established and evaluated in a pre-ring trial by few partners. This protocol aggregated the best performing commercially or in house methods where matrix (meat or caecal content), animal species (pig, cattle, poultry), bacterial contaminant (*E. coli*, *Klebsiella* or *Salmonella*) and resistance mechanism (*mcr* genes) to be detected were combined. The detection limit of the method was not evaluated because the number of combinations were too high at this stage of development. Evaluation of the pre-ring trial results led to a final amended protocol for which performance were evaluated in a ring trial with all the partners. All the tasks in WP1 have been met as was described in the initial proposal. Due to a huge combination of alternatives and complex flora in some matrices, it was not possible after evaluation of the final ring trial to propose a harmonized method suitable enough to monitor the current prevalence and spread of *mcr* variants in *Enterobacteriaceae*.

Work package 2: Selective isolation, detection and characterization of carbapenem-resistant *Enterobacteriaceae*

The objectives for WP2 in this project was to find an optimal culture method for the detection of relevant carbapenemase-producing *Enterobacteriaceae* (CPE) in meat and caecal samples from animals. A pre-ring trial among three labs was performed to narrow down the different choices to be tested in the final ring trial among eleven labs. We ended up, after an extensive protocol tested in the pre-ring trial, to use pre-enrichment as outlined in the protocol amended in the EU Decision 2013/652/EU. After pre-enrichment, the broth was streaked onto different selective agar media that were commercially available. A new and better method was not validated through the project period. There is a challenge in developing a harmonized method covering several bacterial species harbouring different carbapenemase producing genes. The results from the ring trials in WP2 underline the need to find more optimal culturing methods to detect low-level carbapenemase producing *Enterobacteriaceae* and carbapenemase producing *Salmonella* spp., for which there is no commercially selective agar available that can detect them using a chromogen.

Work package 3: Establishing epidemiological cut-off values (ECOFFs)

The main goal of WP3 was to establish epidemiological cut-off values (ECOFFs) of antimicrobials for veterinary pathogens. The first step in this work package was to reach consensus on the bacterial species and antibiotics to be included. During the kick-off meeting in February 2018, a priority list with bacterial species (based on the results of a questionnaire) was agreed upon. Furthermore, the concept of designing three different antibiotic panels as Sensititre plates was accepted. The antibiotic panels were designed based on registration data of veterinary antibiotics and prescription guidelines. The design of the antibiotic panels took a bit more time than initially planned and Sensititre plates were ordered in August 2018. Unfortunately, there was a serious delay in the delivery of the plates. As a consequence, partner institutes received the Sensititre plates in February and March of 2019, but most partners started with the MIC testing soon afterwards. In the meanwhile, all consortium partners were consulted and the priority list of the bacterial species to be tested was refined and finalised in May 2019. Despite of the forced delay in testing, most partners managed to complete the production of the MIC data before the end of 2019. The MIC testing was fully completed in May 2020. The process of calculating ECOFFs based on the uploaded MIC distributions was further delayed because of unexpected problems regarding the acceptance of MIC distributions in the EUCAST database (as described in chapter 3, JRP1-WP3-T4) and is currently still ongoing. Finally, the analysis of MIC distributions of *Staphylococcus pseudintermedius* was given priority and the first accepted ECOFFs for this bacterial species are expected in the beginning of 2021. Depending on the outcome of the



discussion with EUCAST, ECOFFs for other bacterial species like streptococci are expected to follow in 2021.

Work package 4: Developing and optimizing a disk diffusion method for antimicrobial susceptibility testing of Clostridium difficile

The aim of WP4 was to optimize a disk diffusion method for antimicrobial susceptibility testing (AST) of *Clostridium difficile*, to validate it by an international ring trial and evaluate this method in comparison with the gold standard, agar dilution, in order to distinguish wild-type from non-wild-type antimicrobial phenotypes. According to the proposal, a draft disk diffusion protocol was established based on the assessment of current literature and further optimized. Even though we could not recruit the entire number of 500 *C. difficile* strains directly from project partners, we were able to finally collect 527 *C. difficile* isolates addressing also external partners. Strains were characterized as planned (PCR-ribotype, toxin-gene profile and antimicrobial phenotype (MICs)) and used to establish IZD distributions for eight antimicrobials. Based on these distributions, we proposed cut-off values to distinguish wild type from non-wild type phenotypes except for vancomycin and imipenem due to a lack of non-wild type strains in the strain collection. Furthermore, phenotypic resistance testing against cefotaxime resulted in very high resistance rates which indicated the definition of cut-off values for this antimicrobial class to be not reasonable. So, we replaced cefotaxime by erythromycin for further analyses. For the final ring trial we had to involve external partners because most of the IMPART partners missed expertise/equipment in this special field or laboratory capacities due to the COVID-19 crisis. Though, the ring trial was overall successful and revealed important aspects for further method standardization but lacked participants for reliable statistics.

Work package 5: Coordination of the four work packages and knowledge dissemination

The main goal of WP5 was to coordinate the knowledge dissemination within and beyond IMPART. In general, this was accomplished satisfactory. However, some of the planned activities (physical project meetings) were hindered by the COVID-19 crisis. Distribution of information within IMPART was well secured by the imbedded organisational structure of the project which included the role of a project leader (PL) responsible for the overall coordination of the project and work package leaders (WPL) responsible for the scientific quality and progress of each of the four scientific work packages. In addition, a separate work package (WP5) was defined to coordinate communication and dissemination activities. Within the consortium, information was shared through physical meetings (kick-off meeting and a project meeting in Dublin at the OHEJP ASM conference), regular teleconferences between the PL and WPL, emails by the PL to WPL's and by the PL to the complete consortium and ad hoc communication of WPLs with WP participants depending on the planning of the different work packages. At the end of 2020 a newsletter with updated information about the different WPs was shared with the consortium and the EJP management. Communication outside IMPART was covered by active participation in workshops (EURL-AR), conferences (OHEJP ASM), Cogwheel meetings and several contacts with stakeholders (EFSA, EURL-AR and EUCAST). The closing meeting of IMPART was initially planned in June 2020, but because of the budget neutral extension postponed to December 2020 still hoping to organise a physical meeting. Recently it was decided to plan the closing meeting for 2nd of March 2021 as a teleconference. Although this meeting is rescheduled after the official closing date of the project, it gives the IMPART management team (PL and WPLs) this final opportunity to present and discuss the completed results with the consortium members. Lastly, it was agreed that the Communication Team of the OHEJP will work on the promotion of four deliverables from the IMPART project (one from each work package). The Communication Team will share a draft proposal with the IMPART management team in the beginning of 2021.



4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
01	D-JRP1-1.1	Protocol of methods ready to be used in the pre-ring trial	M6	M12		https://zenodo.org/record/3676410#.X3xKMHkzY2x	2, 9
01	D-JRP1-1.3	Evaluation of the pre-ring trial	M11	M13		https://zenodo.org/record/3676404#.X3xlvHkzY2w	9
01	D-JRP1-1.4	Protocol for the final ring trial	M15	M21		https://zenodo.org/record/3676425#.X3xMkXkzY2y	2, 9
01	D-JRP1-1.5	Notifications of shipment of the samples for the final ring trial	M16	M17		https://zenodo.org/record/3676431#.X3xLB3kzY2y	8
01	D-JRP1-1.6	Evaluation of the final ring trial	M21	M35		Full report confidential until paper is published (D-JRP1-1.7). Short evaluation uploaded to Zenodo: https://zenodo.org/record/4460952#.YA5amNhKg2w	9
01	D-JRP1-1.7	Publication in an open-access peer-reviewed journal	M30		M39	Public	8



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
01	D-JRP1-1.8	Proposal(s) for epidemiological study to monitor resistance to colistin	M30		No date	The design of an epidemiological study proposal will be very difficult based on this detection method, because the methods need further development.	1
01	D-JRP1-2.1	Protocol of methods ready to be used in the pre-ring trial	M6	M11		https://zenodo.org/record/3676433#.X3xLLnkzY2y	2, 9
01	D-JRP1-2.3	Evaluation of the pre-ring trial	M10	M19		https://zenodo.org/record/3676437#.X3xMtnkzY2y	9
01	D-JRP1-2.4	Protocol for the final ring trial	M15	M21		https://zenodo.org/record/3676439#.X3xLI3kzY2y	2, 9
01	D-JRP1-2.5	Notifications of shipment of the samples for the final ring trial	M16	M17		https://zenodo.org/record/3676431#.X3xLB3kzY2y	8
01	D-JRP1-2.6	Evaluation of the final ring trial	M26	M33		Full report confidential until paper is published (D-JRP1-2.7). Short evaluation uploaded to Zenodo: https://zenodo.org/record/4452580#.YAgxg9hKg2w	9
01	D-JRP1-2.7	Publication in an open-access peer-reviewed journal	M30		M39	Public	8
01	D-JRP1-2.8	Proposal(s) for epidemiological study to monitor resistance to	M30		No date	The design of an epidemiological study proposal will be very difficult based on this detection method. Such a study should be conducted in close	1



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
		carbapenems				cooperation with EURL and EFSA.	
01	D-JRP1-3.1	Priority list	M3	M2		https://zenodo.org/record/4068251#.X3xshXkzY2w	2, 9
01	D-JRP1-3.2	Analysis of the data	M30		M39	All MIC distributions have been uploaded to the EUCAST database and have the status 'Created'. This database is only assessable by a select number of people from VetCAST and EUCAST (data curators). After acceptance of MIC distributions the data will be available for all visitors of the EUCAST website as diagrams with aggregated distributions: https://eucast.org/mic_distributions_and_ecoffs/	2, 9
01	D-JRP1-3.3	Publication of ECOFFs on EUCAST website	M30		M39	Public on EUCAST website; www.eucast.org	2, 9
01	D-JRP1-4.1	Collection of inhibition zone diameter distributions	M21	M36		Confidential until paper is published (D-JRP1-4.2)	9
01	D-JRP1-4.2	Publication in an open-access peer-reviewed journal	M30		M39	Public	8
01	D-JRP1-4.3	Performance of a ring trial study	M27	M33		Full report confidential until paper is published (D-JRP1-4.2). Short evaluation uploaded to Zenodo: https://zenodo.org/record/4457449#.YA5bHhKg2w	2, 9



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
01	D-JRP1-5.1	Invitation to the kick-off meeting sent to participants	M1	M1		Public ; https://zenodo.org/record/3676441#.X3xJcXkzY2y	10
01	D-JRP1-5.2	Kick-off meeting notes sent to participants	M3	M2		https://zenodo.org/record/3678185#.X3xJ9HkzY2x	8
01	D-JRP1-5.3	IMPART News online on One Health EJP website	M30	M36		Public, uploaded to Zenodo : https://zenodo.org/record/4463049#.YBQTn-hKhM0	8
01	D-JRP1-5.4	Protocols and video tutorials online on One Health EJP website	M18	M21		Only protocols	8
01	D-JRP1-5.5	Invitation to the final meeting sent to participants	M21	M36		Date set on 2 nd of March 2021. Invitation uploaded to Zenodo: https://zenodo.org/record/4457438#.YA5cVthKg2w	8
01	D-JRP1-5.6	Final meeting notes sent to participants	M30		M39	Public	10

* Categories of Integrative activities: 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities; 7. Prevention: aligned use of facilities and models; 8. Other (please specify); 9. This is supportive to an integrative activity; 10. This is not an integrative activity



Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
01	M-JRP1-1	Kick-off meeting (notes of meeting)	2	Yes		
01	M-JRP1-2	Ordering of microtiter plates with antimicrobials (WP3)	4	Yes	6	Creating the layout of the microtiter plates was more complicated than expected.
01	M-JRP1-3	IMPART EXTRANET in place	6	No		OH-EJP platform opened in month 9, therefore it was decided not to build our own platform.
01	M-JRP1-4	Established disk diffusion method (WP4)	8	Yes	14	Advertised technician position could not be recruited on time. Delay in gas delivery by manufacturer.
01	M-JRP1-5	Completed strain collection (WP4)	10	Yes	12	
01	M-JRP1-6	Performing pre-ring trial (WP1 and WP2)	11	Yes	11 (WP2) 12 (WP1)	
01	M-JRP1-7	Mid-term video meeting to validate the protocol (WP1, WP2)	13	Yes		The protocols were presented by the WP leaders and discussed during the physical project meeting the OHEJP ASM meeting in Dublin and at the EURL meeting in 2019.
01	M-JRP1-8	Performing final ring trial (WP1 and WP2)	17	WP1: Yes WP2: Yes	WP1: 18 WP2: 21	
01	M-JRP1-9	MIC data collection complete (WP3)	M24	Yes		Achieved in M29



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
01	M-JRP1-10	Proposal of cut-off values based on inhibition zone diameter distributions (WP4)	M26	Yes	M35	
01	M-JRP1-11	Final meeting (notes of the meeting)	M30	No	M39	Due to the COVID-19 issues, the final meeting was postponed and is planned at 2 March 2021 as a teleconference.



5. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Spill-Over from Public Health? First Detection of an OXA-48-Producing <i>Escherichia coli</i> in a German Pig Farm, doi: 10.3390/microorganisms8060855 Zenodo: https://zenodo.org/record/4447289#.YAWijthKg2w	yes		yes, €1496,13
ChromID® CARBA Agar Fails to Detect Carbapenem-Resistant <i>Enterobacteriaceae</i> With Slightly Reduced Susceptibility to Carbapenems, doi: 10.3389/fmicb.2020.01678 Zenodo: https://zenodo.org/record/4447289#.YAfMEthKg2w	yes		yes €2629,35
First Detection of GES-5-Producing <i>Escherichia coli</i> from Livestock-An Increasing Diversity of Carbapenemases Recognized from German Pig Production, DOI: 10.3390/microorganisms8101593 Zenodo: https://zenodo.org/record/4451836#.YAfndhKg2w	yes		yes, €1 389.79
Novel IncFII plasmid harbouring blaNDM-4 in a carbapenem-resistant <i>Escherichia coli</i> of pig origin, Italy, doi:10.1093/jac/dkaa374 Zenodo: https://zenodo.org/record/4451840#.YAfPRNhKg2w	yes		yes, €3,603.60



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Identification of a blaVIM-1-Carrying IncA/C2 Multiresistance Plasmid in an Escherichia coli Isolate Recovered from the German Food Chain, doi.org/10.3390/microorganisms9010029 Zenodo: https://zenodo.org/record/4451858#.YAfqINhKg2w	yes		yes, €1180,27
Co-occurrence of the blaVIM-1 and blaSHV-12 genes on an IncHI2 plasmid of an Escherichia coli isolate recovered from German livestock, doi:10.1093/jac/dkaa436 Zenodo: https://zenodo.org/record/4451874#.YAfs1NhKg2w	yes		yes, €1802,40

Additional output

Poster presentations at One Health EJP Annual Scientific Meeting 2019 (22-24 May 2019):

- WP4: susceptibility testing of *Clostridium difficile*, WP5: COM IMPART: Optimisation of a Disc Diffusion Method for Antimicrobial Susceptibility Testing of *Clostridium difficile* (confidential)
- WP5: COM IMPART: Poster summarizing 1st year of activities within IMPART, displayed during OHEJP annual meeting in Dublin, May 2019, <https://zenodo.org/record/4066734#.X3xwdnkzY2w>

Poster presentation at 6th Joint Conference of the DGHM & VAAM 2020, Leipzig, Germany (8-11 March 2020): WP4: Optimization of a Disc Diffusion Method for Antimicrobial Susceptibility Testing of *Clostridioides difficile* (confidential)

Poster presentations at One Health EJP Annual Scientific Meeting 2020 (27-29 May 2020):

- WP2: A multicentre study examining different culturing methods to detect carbapenemase-producing *Enterobacteriaceae*. <https://zenodo.org/record/4066631#.X3xwsHkzY2w>



- WP3 : Susceptibility testing of veterinary pathogenic bacteria as a first step in setting new epidemiological cut-off values (ECOFFs)
<https://zenodo.org/record/4066667#.X3xw23kzY2w>
- WP4: *Clostridioides difficile* antimicrobial susceptibility testing using disc diffusion (confidential)



6. One Health Impact

WP1: in collaboration with EFSA (Beatrice Guerra) and EURL-AR (Rene Hendriksen), we plan to review together the output of this work package to determine if a future surveillance programme is either feasible or relevant at a European level.

WP2: The draft of the protocol for the multicentre study was done in collaboration with the EURL-AR. In the Commission Implementing Decision 2020/1729, the detection of carbapenemase producing *E. coli* on caecal and meat samples will become mandatory from 2021. Both EURL-AR and EFSA would have great benefit of the outcome of the multicentre study to modify the protocol for the harmonized surveillance of carbapenemase producing *Enterobacteriaceae* at a European level, https://www.eurl-ar.eu/CustomData/Files/Folders/21-protocols/530_esbl-ampc-cpeprotocol-version-caecal-v7-09-12-19.pdf and https://www.eurl-ar.eu/CustomData/Files/Folders/21-protocols/529_esbl-ampc-cpeprotocol-version-meat-v7-09-12-19.pdf. We refer to EFSA “Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food producing animals and food” (DOI: 10.2903/j.efsa.2019.5709), where IMPART is specifically mentioned. Further, the EURL-AR will perform a survey to assess how widely used the pre-enrichment is for other parts of the monitoring if antimicrobial resistant bacteria (personal communication Rene Hendriksen; rshe@food.dtu.dk). This as a starting point to reassess the protocol for isolation of carbapenemase producing *Enterobacteriaceae*.

From the start, the activities defined in WP3 were clearly linked to EUCAST/VetCAST when it comes to setting new ECOFFs (PL is member of the steering committee of VetCAST). This has stimulated closer cooperation between VetCAST and EUCAST on a more technical level. Moreover, IMPART WP3 results have been used as starting point for the collection of more MIC distributions in order to set additional ECOFFs for veterinary antimicrobials within COST action ENOVAT (PL is also member of the management committee of ENOVAT). In other words, the efforts for setting new ECOFFs within IMPART will be continued within ENOVAT. It can be concluded that despite the recent problems with the acceptance of MIC distributions in the EUCAST database, IMPART has generated new opportunities for joined actions to further improve standardisation in veterinary diagnostic laboratories regarding methods for susceptibility testing of veterinary bacteria and setting of ECOFFs as will be supported by VetCAST and ENOVAT.

WP4: OHEJP project FED-AMR aims to use the proposed protocol and cut-off values for the agar disk diffusion in IMPART to characterize and compare *C. difficile* strains with regard to their antimicrobial resistance.



JRP02-ARDIG

1. Summary of the work carried out in year 3

The ARDIG project has continued to progress, and after 36 months there have been substantial achievements made by partners, including peer-reviewed publication of papers which are aligned to ARDIG. Details of progress made by each partner for the three scientific work packages (WPs) are described within the report. Several work package specific meetings have also taken place over the past twelve months by teleconference or videoconference and there have also been regular communications within the consortium by email. Both oral and poster presentations made by ARDIG partners were well received at the OH-EJP Annual Scientific Meeting held virtually in May 2020.

WP1 (Comparison of AMR and antibiotic sales/usage data collected through existing national surveillance and research programs and assessment of risk factors). Antimicrobial resistance (AMR) and antimicrobial usage data has been obtained from healthy and diseased animals from different partners and countries. From preliminary analysis of the data it has become apparent that due to differences in methodology and interpretation criteria, it will be difficult to compare MIC or disc diffusion data obtained for AMR from *Escherichia coli* isolated from diseased animals. Therefore, ARDIG partners have been exploring statistical methods to set epidemiological cut-off (ECOFF) values for each antimicrobial. By using a harmonised method to calculate ECOFF for each antimicrobial, a more detailed analysis of trends in the clinical data set could be made across countries, as well as enabling comparisons between clinical and non-clinical *E. coli* datasets.

WP2 (**Longitudinal studies of AMR persistence**). Both Med and Vet partners have been collecting *E. coli* isolates from both retrospective as well as prospective studies, including *E. coli* isolated from urinary tract infections from local hospitals and General Practitioner over 12 months. Several partners have been characterising *E. coli* isolated through national surveillance. In addition, all partners who participated in a whole genome sequencing (WGS) AMR workshop have submitted ~50 WGS each (~450 in total) for analysis by five pipelines: APHA SeqFinder/Abricate, PHE GeneFinder, WBVR, Ariba, ResFinder/PointFinder. MIC determinations to the EFSA panel of antimicrobials for these isolates have also been performed to help compare the AMR genotypes with the corresponding phenotypes.

WP3 (**AMR characterization, transmission of plasmids and fitness of MDR isolates**). All WP3 partners have continued AMR gene, plasmid and mobile genetic element characterization in isolates collected in WP2 by WGS (short and long reads), as well as other molecular techniques. Phylogenetic analysis performed by partners on their dataset have also helped identify transmission events or epidemiological links between isolates of particular sequence types or clones of *E. coli* collected from different hospital or farm settings. In addition, all WP3 partners have participated in the AMR pipeline comparison work to help harmonise *in silico* AMR gene prediction. The results of running WGS data from ~450 isolates, through five different AMR pipelines, have been compared by partners with their corresponding MIC phenotypes. APHA have further analysed the data to assess the impact of methodology on AMR gene prediction, and the consortium will report the final results in a publication.

2. Work carried out in the JRP, scientific results

WP1 Comparison of antimicrobial resistance (AMR) and antibiotic sales/usage (AMU) data collected through existing surveillance, monitoring and research programs and assessment of risk factors (M1-M42)

JRP2-WP2-T1: Exploration and collection of data available on AMR, AMU and potential risk factors (M1-M12)

Task completed. See second annual report, 2019.



BfR

Additional efforts are now being performed to collect further consumption and resistance data with additional risk factors from Germany (RKI) and the United Kingdom (PHE) in the human sector.

JRP2-WP1-T2: Investigation of trends, associations and risk factors (M9-M42)

BfR

BfR is responsible for comparing data on clinical and non-clinical isolates from livestock across countries. These data were made available from the United Kingdom, Norway, France and Germany in the ARDIG WP1 project. Several limitations were encountered regarding the necessary overlaps of antimicrobials, animal categories and time ranges available in the data. The largest overlap of antimicrobials and animal categories in non-clinical and clinical isolates between 2014 and 2017 was found between Germany and France. Hence, French and German data were selected to carry out the statistical analysis. UK data on clinical and non-clinical isolates were also statistically analysed but results were included separately because of the limited overlap on the antimicrobial panel (i.e. ampicillin and tetracycline, but not nalidixic acid and gentamicin) and animal categories (i.e. broilers, but not turkeys and calves). Resistance data on clinical isolates from broilers and turkeys in Norway were limited in number and therefore not included in the statistical analysis.

Univariate analyses were performed for each animal species and antimicrobial/antimicrobial class, assessing each explanatory variable (data type, country and year). Multivariate analyses assessing only relevant variables were carried out when more than one variable per antimicrobial/antimicrobial class and animal species showed significant associations in the univariate logistic regression. Data type variable was analysed as a binary factor (clinical vs. non-clinical), year as a numeric factor and country as a binary factor (For the analysis between Germany and France). Odds ratios were used to determine which populations showed higher resistance odds. Analyses were carried out on national level and across countries. On the national level, in Germany more antimicrobials could be included and there were fewer limitations with respect to non-harmonized use data.

In relation to antimicrobial use data in livestock, they are being assessed in order to try to overcome the lack of harmonization. In case of successfully solving the harmonization issue, they will be included in the analyses as a risk factor. Otherwise they can only be considered on the national level.

Data on clinical isolates from food producing animals in France were obtained in collaboration with the EU-project JAMRAI that strives to establish a European data collection on AMR data from clinical isolates and is therefore confronted with the same issues of lack of harmonization. With the help of the colleagues from ANSES involved in JAMRAI, ARDIG now has access to quantitative data on AMR in clinical isolates of *E. coli* from pigs and poultry in France in addition to the data on cattle that were previously available.

Lack of AMR harmonization on laboratory methods could be partially overcome by the use of the Normalized Resistance Interpretation, a statistical method to generate cut off values that separated a susceptible wildtype population from a non-susceptible population based on the distribution of inhibition zone diameters or minimum inhibitory concentrations. This method was used in the analysis of the German and French data.

APHA

For this task, APHA has carried out the analysis of trends, associations and risk factors for clinical AMR in livestock (cattle, pigs, and chicken). Data comprising only *E. coli* isolates originating from field cases of clinical disease submitted for diagnostic investigations were included in the analyses. The results of antimicrobial susceptibility testing (AST) of clinical *E. coli* isolates were provided retrospectively for a period of 2014 to 2017 by 3 countries: France, Germany and UK (England and Wales). Due to considerable differences in diagnostic methods for susceptibility testing and interpretation criteria/standards between the three countries, as previously reported, new epidemiological cut offs



for the clinical AMR were calculated using the NRI method. This was performed to allow more robust comparison of resistance levels by different livestock species (cattle, pigs and chicken) and over time between the three countries. Ten antimicrobials overlapped between all three countries and at least one of the three species, with six of those overlapping between all three species. For the risk factors analyses, multiple univariable and multivariable logistic regression models were performed for each of the ten antimicrobials and each country separately, including species (cattle, pigs, and chicken) and year (2014-2017) in the models. Additionally, for each country separate analyses were carried out for each species, including different age categories and year to account for the effect of time on resistance levels. Finally, for the six antimicrobials that overlapped between all three species, logistic regression analyses were carried out including country, species and year as exposure variables in the logistic regression models. A report summarising the results from these analyses is in preparation.

Several teleconferences have been carried out between APHA, BfR and ANSES in order to discuss this work. In addition to the analysis carried out, APHA was also involved in discussions around analyses of clinical vs non-clinical AMR data from livestock led by BfR and contributed to such analyses for two antimicrobials for broilers in the UK.

NVI

A meeting (digital) was held together with the partners of WP1 in April 2020, where it was decided to try to explore and use different statistical methods (NRI Kronvall and Turnidge) to set epidemiological cut-off values for each partners own distributions on both data types (clinical and non-clinical isolates) from each animal species, separately. NVI has carried out analysis on its own data of epidemiological cut offs for the clinical AMR based on the NRI method.. Final results will be compared among the partners to assess the usability of the methods and ECOFFs set. Data from 2015, 2017 and 2019 (cattle and pig) are being further investigated for trends. NVI have also contributed with data from clinical isolates from broiler and turkeys to be compared with the data from France, UK and Germany. However our the amount of data was limited, but still worth to be included for comparison in the discussion in the manuscript under preparation.

RKI

The RKI has transferred the extracted AMR and AMU data (2014-2017) from its surveillance system to the joint database after the transfer/sharing agreement had finally been approved. RKI- activities of joint investigation of trends have been delayed due to the ongoing Coronavirus-outbreak. We are dependent on the data managers, who are all obliged to work for COVID-19. To allow for joint analysis of risk factors using comparable human AMR-data (Germany and UK), we have extended the database with additional variables including county type (rural, city) and MIC values.

To improve animal-human AMU comparisons, human community consumption data (expressed as DDD per 1 000 insured persons per day) will be expanded with a variable containing the quantity of active ingredient (in gram) per antibiotic for the years 2014-2017.

JRP2-WP1-T3: Develop recommendations for improved "One Health" surveillance strategies (M25-M42)

This objective is ongoing. Recommendations for improved surveillance strategies and harmonized data collection systems will be drawn following the results of analysis of clinical, non-clinical and human AMR data and their associations with antimicrobial usage performed in collaboration between APHA, BfR and ANSES.

The final recommendations will be based on challenges encountered when attempting to analyse the data across countries and the potential solutions developed to overcome the challenges.



WP2. Longitudinal studies of persistence ESBL/AmpC/carbapenem/mcr-1 and -2/PMQR producing Enterobacteriaceae on farms or hospitals (M1-M42)

JRP2-WP2-T1: Assessment and selection of longitudinal data from historical studies (M1-M12)

NVI

Isolates from a previous study focusing on cephalosporin resistant Enterobacteriaceae have been characterized. All broiler flocks raised on ten broiler farms were sampled during the period from May to October in 2016 and a total of 42 positive isolates were obtained (one isolate per flock). These isolates have been sequenced with Illumina technology in order to study a possible on-farm persistence/transmission between batches of animals on the same farm or broiler house. In total, 11 different *E. coli* Sequence Types were identified. Data on serotypes will be extracted from WGS data. blaCMY-2/IncK2 plasmids were the most common gene/plasmid combination (present in nine different STs). A possible clonal persistence of ESC-resistant *E. coli* at house level was shown for only a minor proportion of the included houses. Isolates from the same house belonging to the same ST could differ by a considerable number of SNPs, shown for ST38 isolates found in three different houses at one farm from several flocks throughout the sampling period. Similar plasmids were detected in different STs, suggesting possible horizontal transfer and/or persistence of plasmids. It is not possible to determine whether different *E. coli* variants and/or ESC resistance genotypes were present simultaneously in a flock, as only a single isolate was characterized per sample. Further analyses of plasmid data in ongoing and a publication is in planned.

We are performing a retrospective study using the i-bird strain collection in collaboration with Lulla Opatowski and Didier Guillemot (Institut Pasteur). The i-Bird study (Individual-Based Investigation of Resistance Dissemination coordinated by D. Guillemot) is a 4-month study which took place in 2009 in a rehabilitation hospital and followed up more than 600 patients and Health Care Workers with weekly rectal swabs performed in patients and human-human proximities recorded from wireless captors. 604 ESBL-Enterobacteriales, including 334 *E. coli*, were isolated from 84 patients. Our objectives are (i) to characterize strains shared by different patients and to infer transmissions; (ii) to identify cases of ESBL-plasmid transfers and (iii) to identify factors contributing to ESBL-*E. coli* transmission in this hospital environment. In order to characterize the within host diversity we have selected several *E. coli* isolates (up to eleven) in some patients. We have first analyzed 205 *E. coli* and 102 *Klebsiella pneumoniae* isolates. The 205 *E. coli* isolates were from 75 patients (we have selected at least one isolate per colonized patient). All the isolates have been sequenced using the Illumina technology (see WP3).

ANSES

Task completed; see annual report 2019.

JRP2-WP2-T2: Isolation of resistant Enterobacteriaceae on farms (M1-M42)

UoS

Task completed. See second annual report, 2019.

WBVR

In 2019 and 2020, a group of approximately 700 veal calves in the Netherlands was individually followed from birth to slaughter on 5 to 6 sampling moments. The animals were born on 13 dairy farms spread throughout the country and transported between 14 and 28 days of age to 8 veal farms for fattening. Rectal swabs were taken at each sampling moment for selective culturing on cefotaxime containing media to determine the prevalence of ESBL/AmpC producing *E. coli*. At the dairy farms the prevalence of *E. coli* ranged from 0-86% (average 26.4%). At all veal farms the prevalence of



ESBL/AmpC producing *E. coli* amongst the animals went up to >50% at least one sampling moment. In 6 farms, prevalence significantly decreased over time.

Extensive records were recorded on farm management, hygiene, antimicrobial usage and health parameters of the animals and statistical analysis is currently carried out to determine correlation with ESBL carriage. WGS data has been generated and is currently analysed to determine if ESBLs occur on veal farms due to between or within farm transmission of ESBL *E. coli*. Based on these analyses, isolates for long-read sequencing in order to determine plasmid transmission on farms between different *E. coli* lineages has occurred.

NVI

Recent data from monitoring in broilers have demonstrated absence of cephalosporin resistant Enterobacteriaceae in Norwegian broiler production. It was therefore decided that a study in pigs will replace the planned broiler study. A pig study was planned in 2018, but recruiting pig herds for this study has been a great challenge. In the spring of 2020 it was decided not to perform a longitudinal study in pigs, but instead participate in a study with longitudinally sampled human bacterial isolates (task3/WP2).

APHA

We have completed the collection of samples for a longitudinal study which focused on two sites of the same UK pig farm which are separated geographically; a non-clinical farm site that houses five age classes of healthy pigs and has ceased group antimicrobial treatments for at least five years, and a clinical farm site that is comprised of three age classes of pigs sent from healthy sites following disease, that have subsequently undergone group and individual antimicrobial treatment. Faecal samples were obtained from both sites from pigs at four time-points at 6 month intervals over 18 months, alongside seagull faecal samples from two time points. Representative *E. coli* were purified from all time points from non-selective and antibiotic selective agar plates (cefotaxime and ciprofloxacin), followed by Illumina whole-genome sequencing (WGS). The WGS data was analysed by reconstructing phylogeny of the *E. coli* isolates, determining presence of AMR genes, plasmid replicon types, in silico Multilocus Sequence Type and mobile genetic elements (WP3).

MIC determination for the isolates collected during visits 4 and 5 has also been completed. An overall analysis of the MIC data across 5 visits has been carried out. Temporal trends were evaluated, as well as a comparison between antibiotic treated and non-antibiotic treated groups of pigs was carried out. A draft publication is in preparation to report these results.

ANSES

Three studies were set up to investigate impacts of antimicrobial usage (AMU) on AMR and microbiota of fattening veal calves.

In a first study (Gay et al, Frontiers 2019), ten fattening farms were selected and visited twice. A total of 50 animals per farm were sampled for ESC-R carriage and other AMR phenotypes upon arrival and 5-6 months later before slaughter. ESBL-producing *E. coli* were collected from MacConkey and selective agar. As main results, ESBL-producing *E. coli* rates significantly decreased in all 10 farms (arrival: 67.7%; departure: 20.4%). Feeding milk containing antimicrobial residues to veal calves is hypothesized to explain the high ESBL loads in animals at the entrance on farms. In the dominant flora, proportions of resistances to amoxicillin, tetracyclines, streptomycin and sulfonamides were very high (>60%) at arrival of animals in the farm and had significantly increased at departure. Proportions of resistances to other beta-lactams than amoxicillin were overall low and significantly decreased during the fattening process. Resistance to quinolones also significantly decreased from arrival to departure. A total of 11 isolates were resistant to colistin (MICs ranging between 6 and 16 mg/L) of which 9 were



detected in animals upon arrival (originating from 7 different farms), and 2 in animals at departure (both originating from the same farm). The proportion of multi-resistant isolates significantly increased from 60.2% upon arrival to 67.2% at departure of animals. The proportion of isolates susceptible to the seven selected antibiotics was 23.3% upon arrival and 7.3% at departure. Only two isolates displayed co-resistances to all seven antibiotics.

In a second study (Massot et al, Animal microbiome 2020), three farms out of the ten from the first study were visited 11 or 12 times at regular intervals of 15 days. A total of 15 calves per farm were sampled and processed as for the first study. Number and types of treatments during fattening were collected. We characterized the dynamics of the fecal microbiota of those calves from two weeks to six months of age. Calves were mainly fed milk replacers throughout the follow-up and received several collective antibiotic treatments at therapeutic doses. We performed 16S rRNA gene sequencing to study the composition of the microbiota and qPCR of the *Escherichia* genus as a proxy of *E. coli* to quantify its commensal populations. The most striking results of this study are (i) the convergence of the fecal microbiota composition among calves, which began during the first month of life, along with an increase in α -diversity, (ii) a decrease in microbiota diversity and the size of the *E. coli* population during or within the 15 days following an antibiotic treatment relative to non-exposed calves of the same age (reduction of the Shannon index by 0.17 and the number of *E. coli*/g of feces by 0.37 log₁₀ (*E. coli*/g)), and (iii) a significant association between the estimated daily dose of milk powder and the relative abundance of four genera (*Megasphaera*, *Enterococcus*, *Dialister*, *Mitsuokella*) and the predicted farm profiles of the number of *E. coli*/g from our model. From the AMR perspective, our data show that administration of collective antibiotic treatment results in a reduction of microbial diversity and size of the *E. coli* population.

In a third study (manuscript in prep.), the same calves as in Massot et al, 2020 were investigated with regard to dynamics of ESC-resistant *E. coli* prevalence and ESC-R clones and plasmids. Results show that the three farms differed by the prevalence of ESC-resistant *E. coli* since a total of 84 ESC-R-, 15 ESC-R- and 76 ESC-R-positive *E. coli* were recovered from farm A, B and C, respectively, in accordance with antibiotic selection pressure. Also, not only a clonal but also a plasmid dissemination has likely occurred in the studied farms, as investigated using S1 PFGE, Southern blot and short read sequencing.

JRP2-WP2-T3: Isolation of resistant Enterobacteriaceae in hospitals and care facilities (M1-M42)

UoS

The UoS has finalised the collection of *E. coli* isolates from human urinary tract infections in collaboration with a local hospital. A total of 272 isolates were collected during a longitudinal study that was undertaken from January to December 2019. Out of 272 isolates, 134 were collected from GP patients (representing community-acquired infections) and 138 from patients admitted to hospital (representing healthcare-acquired infections). The phenotypic resistance profile of the collection showed a higher level of resistance in isolates from hospitals when compared to GPs. DNA from 256 isolates (an average of 10 from the GP clinic and 10 from the hospital per month) has been extracted and sent to PHE for whole genome sequencing.

A second local hospital has collected an additional set of uropathogenic *E. coli* isolates (June 2019 - May 2020). The new set of isolates collected in this longitudinal study will shortly be sent to the UoS and genome sequenced. The antibiotic resistance profile of the isolates and any relevant clinical data from the corresponding patients will also be gathered to evaluate the risks of antimicrobial resistance.

NVI

E. coli isolates from humans with UVI in a large centrally located hospital in Norway, and from GP in the same area were collected (the 20 first isolates of each category, from each month in 2019). The isolates were sent us monthly and they are stored at NVI, together with relevant/requested data. The isolates have been sequenced at NVI and MIC determination have been carried out.



PHE

PHE are collaborating with UoS (Roberto LaRagione, Maria Getino) to short-read WGS sequence a collection of *E. coli* isolated from urinary tract infections occurring in the community and a hospital setting (see above from UoS). The isolates are sequenced and quality checked ready for collaborative analysis.

IP

We performed a longitudinal sampling of *E. coli* responsible for urinary tract infections (UTI). The objective of this study is to compare the diversity and the ARG profiles of *E. coli* strains responsible for UTI in four ARDIG participating countries and using a similar protocol. The work program was to collect each month during 12 months 10 isolates from community infections and 10 isolates from hospital patients. This work was performed in collaboration with Thierry Naas at the Bicêtre Hospital. Due to difficulties to have access to a private microbiology laboratory, we have selected each month 10 isolates from outpatients (from the emergency of the Bicêtre Hospital) and 10 isolates from hospital patients. Isolates were picked randomly from the isolate collection from the Bicêtre Hospital Clinical Microbiology laboratory. In total 250 isolates have been obtained and all isolates have been sequenced by using the Illumina technology (WP3).

JRP2-WP2-T4: Data analysis of collected resistant Enterobacteriaceae on national levels (M22-M42)

BfR

Since the beginning of the ARDIG project, we have characterized *Escherichia coli* isolates recovered from livestock and food in 2017 for their phenotypic and genotypic resistance profile. In one part of the ARDIG project we had focused on (fluoro-)quinolone-resistant isolates, while the second part deals with mobilizable colistin resistances (*mcr*-genes).

WBVR

Between 2014-2019 in the Netherlands, blaCTX-M-14 and blaCTX-M15 harbouring isolates have increased in prevalence in both veal calves and dairy cattle. In-depth molecular characterisation is being performed and currently ongoing but preliminary results show that these are often encoded on phage-like plasmids which cannot be detected by the widely used PBRT method.

IP

In collaboration with Thierry Naas, we are analysing the genomic diversity of carbapenemase producing *E. coli* received by the National Reference Centre laboratory until 2015 (manuscript is in preparation).

NVI

Analysis of data collected in WP2/task1 is ongoing.

APHA

Whole genome sequencing of ESBL/AmpC *E. coli* that have been collected by APHA from national surveillance of livestock (pig and poultry) since 2013 have been analysed for their AMR gene content as well as plasmid diversity. A paper has been published in Science Reports on AMR analysis in *E. coli* from pigs collected between 2013-2017.



PHE

PHE have collected carbapenemase producing Enterobacterales, including *E. coli*, isolated from colonisation and infections by hospital laboratories and referred to the PHE reference national reference lab. The isolates were received by the PHE National Reference laboratory in 2015 and 2016 and have been analysed for their genomic and resistance gene diversity. (collaborative manuscript with Katie Hopkins, in preparation).

JRP2-WP2-T5: Comparative analysis of collected isolates on a Europe-wide level (M30-M42)

APHA, ANSES, BfR, IP, NVI, PHE, UCM, UoS, WBVR

ARDIG partners have submitted WGS of up to 50 *Escherichia coli* isolates per institute to a repository and five partners (APHA, WBVR, PHE, UCM, and NVI), representing the diversity of pipelines (APHA SeqFinder/Abricate, PHE GeneFinder, WBVR, Ariba, ResFinder/PointFinder), have analysed ~450 WGS data through the pipelines.

All partners have performed MICs on the EFSA panel of antimicrobials on their isolates from this panel of 450 isolates, so phenotypic data could be obtained for comparison with the AMR genotypes resulting from each of the 5 bioinformatic pipelines.

Data from human and animal longitudinal studies will be compared between partners in different countries using the chosen bioinformatic pipeline once all sequencing data is available.

UoS

The UoS has agreed to carry out a comparative analysis of isolates harbouring *bla*CMY-2 gene. Short and long read sequences have been requested from the partners involved in WP3, representing 6 different European countries (UK, France, Norway, Germany, Spain and The Netherlands).

APHA

APHA has collected WGS from *E. coli* ST744 isolates from partners in the consortium for further characterisation. The aim is to compare the WGS data by reconstructing phylogeny of the *E. coli* isolates, determine presence of AMR genes and mobile genetic elements present in the isolates.

PHE

PHE has identified short read data, for sharing with partners, from *E. coli* belonging to the ST744, ST1196 and ST38 lineages for sharing with partners to facilitate the comparisons of isolates from specific strains, along with *E. coli* harbouring CTX-M-1 and IncI1 sequences, CMY-2 & IncK/I sequences, and CTX-M-14/15 with no specific plasmid incompatibility sequence.

BfR

In order to strength the collaboration of the ARDIG partners, individual projects of the partners on specific *E. coli* lineages, plasmid types or resistance genes were supported by the exchange of WGS data. Based on the prevailing genome data of the NRL-AR (BfR), we had chosen to support several projects (i.e. ST-744-, ST-1196-, ST-38- or mcr-1-project) by providing short- and/or long-read sequencing data).

WBVR

A collection of over 150 colistin resistant *E. coli* from the Netherlands encoding MCR-1 and MCR-4 have been sequenced using Illumina sequencing. Nanopore long-read sequencing was performed on a sub-



selection of these isolates. The genomes and MCR-encoding plasmids of this collection will be compared with previously published data from APHA and data stored on public repositories in order to perform a global plasmid comparison of MCR plasmids.

WP3. AMR characterization, transmission of plasmids and fitness of MDR isolates (M6-M42)

JRP2-WP3-T1: Detailed molecular characterisation of AMR genes present in human, animal, food and environment isolates from WP1 and WP2 (M6-M18)

APHA, ANSES, BfR, IP, NVI, PHE, UCM, UoS, WBVR

Five partners (APHA/PHE/WVBR/NVI/UCM) have run WGS data from 450 *E. coli* isolates submitted by ARDIG partners (APHA, ANSES, BfR, IP, NVI, PHE, UCM, UoS, WBVR) through their pipelines (APHA SeqFinder/Abriicate, PHE GeneFinder, WBVR, Ariba, ResFinder/PointFinder) to assess the impact of similarities and differences in methodologies commonly used for AMR genotyping within European Institutes on AMR gene predictions. Results from the pipelines analysis have been shared with the consortium, and partners have compared the AMR genotype prediction for each isolate with the corresponding antimicrobial tested by MIC (EFSA panel), using the database/gene catalogue available to them through their Institute. The results of the genotype/ phenotype comparison have been compiled by APHA for further analysis and identified agreements and discrepancies between each approach. A manuscript is currently being prepared from the analysis and will include recommendations for future use of WGS for surveillance activities. Such comparisons are of extreme importance to EFSA and ECDC as they are moving to reporting of AMR data by genotyping, and we expect our study to make a valuable contribution to harmonisation of current approaches between animal and human sectors in this context of One Health.

UoS

The UoS has completed the molecular characterisation (using whole genome sequencing) of 337 *E. coli* isolates from different sources. The set includes 94 isolates from human urinary tract infections, 111 from human blood bacteraemia, 20 from healthy human faeces, 60 from healthy pig faeces, 10 from healthy chicken faeces, 28 from healthy cattle faeces and 27 from avian colibacillosis. Another set of 256 *E. coli* isolates from human longitudinal studies and 85 additional isolates from pig and chicken longitudinal studies are currently being sequenced.

Bioinformatic analysis of the sequenced isolates has been performed, including phylogenetic analysis, pangenome analysis and database searches to create a profile of plasmids, AMR genes, serotype, sequence type, phylogroup and virulence genes for each isolate. The integration of this information together with available phenotypic and clinical data is currently underway to understanding AMR prevalence in humans and animals.

A manuscript comprising the molecular characterisation of *E. coli* isolates corresponding clinical data collected from the bloodstream infection longitudinal study has been drafted in collaboration with a local hospital and will be submitted for peer review in the coming months.

NVI

A collection of more than 260 cephalosporin resistant Enterobacteriaceae has been sequenced using short-read NGS (archived isolates). The strains were isolated from broilers between 2012 and 2016 and can be available for the project. WGS data is also available from a selection of quinolone resistant *E. coli* from Norwegian animals (pigs, broilers, red fox, wild bird). Isolates from WP2 (task 2.1) have been characterized and main findings are described above.

A request for long and short read sequence data for strains with blaCTX-M-1/Incl plasmids have been sent to the consortium members. The outcome will be a comparative study on Incl/blaCTX-M-1 plasmids with data from several European counties (coordinated by NVI).



Sequence data for ST744 isolates have been sent to APHA for inclusion on a study focusing on this ST.

For a more complete analysis of circulating ESBL plasmids/strains in broilers in Norway a selection of isolates have been sequenced using both short and long-read sequencing (isolates with blaCTX-M-1 located on IncI1-ly plasmids). The blaCTX-M-1/ IncI1-ly plasmids studied grouped into two main plasmid lineages ;clonal complex (CC)-3 and CC-7. Our data showed that dissemination of blaCTX-M-1 in Norwegian broiler production is due to both clonal expansion and horizontal transfer of plasmids carrying blaCTX-M-1. The genetic diversity at both strain and plasmid level indicates multiple introductions to Norwegian broiler production. The study was published in *Frontiers in Microbiology*.

WBVR

In the Netherlands, over 350 caecal samples from broilers were collected over a 10 month period on 5 farms in 2018-2019. Using selective culturing on cefotaxim containing media, 23.8% of these samples were positive for ESBL/AmpC producing *E. coli*, very similar to the 23% prevalence of ESBL/AmpC producing *E. coli* found by the national surveillance in 2018. Molecular analysis indicated that of these isolates, 85% produced blaCTX-M-1, blaCMY-2 or blaSHV-12, similar to results of the national surveillance.

As only 1 farm contained ESBL/AmpC producing *E. coli* on more than 3 consecutive sampling moments, 120 *E. coli* isolates from this farm were selected for whole genome sequencing. Phylogenetic analysis indicates that several lineages of *E. coli* are present in the farm which were found in all three barns that were monitored over several production rounds, while each production round was mostly dominated by a specific lineage. On the farm, blaSHV-12 encoded on an IncI1 plasmid was most prevalent, followed by blaCTX-M15 on an IncFIB plasmid and blaCMY-2 on an IncI1 plasmid. While blaCMY-2 was contained to a specific lineage, blaSHV-12 and blaCTX-M-15 were present in more diverse backgrounds and identical *E. coli* that do not contain the plasmids were also present indicating that the resistance genes are disseminated through the farm by horizontal gene transfer.

APHA

The farm isolates were characterised further by analysis of the WGS data. Levels of AMR genes present within indicator *E. coli* from non-selective media varied significantly between sites, with 84% identified as multi-drug resistant (3 or more AMR genes) on the clinical site in comparison to 4% on the non-clinical, with a corresponding difference in Sequence Types (ST) identified. In contrast, *E. coli* isolated on both sites from antibiotic selective media were mostly identical STs, with ST744 being the dominant *E. coli* isolated from ciprofloxacin containing media and ST88 the dominant from cefotaxime media. Persistence of ST744 clones with <10 SNP differences were identified across time-points, age classes of pigs and seagull samples in both sites. Both STs have previously been reported from animals and humans globally.

The presence of *E. coli* of the same ST with few SNP differences across time points, pigs and gulls indicates persistence and transmission of *E. coli* subtypes on and between sites. Further work is planned to identify factors that may be selecting these clones on site and maintaining AMR in the absence/low use of antimicrobials. A manuscript is being prepared on this work.

PHE

Relating to the IncL/M resistance plasmid incompatibility group: Analyses of CPE supported the ongoing pandemic of OXA-48 carbapenemase encoding incL plasmids and also highlighted the emergence of another IncL/M group encoding the NDM carbapenemase, occurring in geographically widespread isolates. Analyses of the plasmids has led to the discovery of multiple different occurrences of NDM incorporation into the IncM type plasmids. To undertake a risk assessment for the threat posed



by these emergent IncM-NDM plasmids, examples of the most notable 'type' have been shared with for fitness comparisons vs. the pandemic IncL OXA-48 plasmid, at the UoS (see below).

BfR

Investigations on (fluoro-)quinolone-resistant *E. coli*: Isolates with MIC values of ≥ 8 mg/L and/or ≥ 0.25 mg/L against nalidixic acid and ciprofloxacin, respectively, were chosen for further analysis. In total, 452 *E. coli* of the National Reference Laboratory for Antimicrobial Resistances (NRL-AR) were screened for the presence of qnrA, qnrB, qnrC, qnrD, qnrS and qnrVC. The most frequent qnr-variant was qnrS (16.1%). Despite of qnrS, the occurrence of other tested qnr genes was rather low. For phylogenetic analysis, qnr-positive *E. coli* isolates were subjected to pulsed-field-gel-electrophoresis (XbaI-PFGE). In general, the phylogenetic dendrogram showed a high heterogeneity but a few isolates did show certain relationship. By S1-PFGE, the plasmidal state of the 103 qnr-positive *E. coli* isolates was determined and the plasmidal localization of the qnr-gene was confirmed by DNA-DNA hybridization. For determination of the genetic basis, all 103 *E. coli* were subjected to illumina (NextSeq) sequencing. For all isolates, the ST-type distribution, matrix belongings and occurrence of known resistance genes, distinct to qnr are available. To determine the diversity of prevailing plasmid types carrying qnr-genes, in-silico plasmid finishing was conducted. For this, we had used the refSNPer workflow (https://gitlab.com/bfr_bioinformatics/refsnper/-/tree/master). The pipeline elects the closest reference, by mapping the input sample to a chosen reference set and identifies the coverage by using bedtools. With this, we had classified several qnr-carrying plasmids to known reference plasmids and track putative plasmid paths. Moreover, we discovered novel qnr-plasmids, which are not described so far. In this manner, we detected the most frequently detected plasmid to cluster for the groups of IncY (n=19) and IncX (n=27). It has been emphasized before, that there is a correlation between this IncX plasmids, harboring blaTEM and blaCTX-M-15 genes next to qnrS, resulting in ESBL-producing *E. coli* strains. We could confirm this observation through annotation of our sequences. IncX plasmids are regularly described as a group inhabiting the qnrS1 gene. Therefore, we decided to scrutinize this group of plasmids even further. We wanted to find out more about the *E. coli*, prone to inhabit this plasmid type and we wanted to describe a proper plasmid backbone. Moreover, our aim was to scrutinize their differences. With this, we wanted to understand their potential in disseminating qnrS1 and other potential resistance genes. However, the *E. coli* inheriting the IncX-plasmid carrying the qnrS1 were highly heterogenic. We found diverse ST-types of *E. coli* as well as different matrices, which did hold the respective IncX plasmids (Figure1 or Table 2). From the macrorestriction profile, generated with XbaI-PFGE, we confirmed a high diversity of *E. coli*, possibly transmitting this plasmid. Thus, one can conclude that this plasmid, carrying the qnrS1 gene, is spreading over different *E. coli* types within multiple sources. Further, we screened for their conjugational behavior. First, we determined the conjugational behavior in silico with the mob-suit tool. Thus, we found most of the qnrS carrying IncX plasmids to hold the respective relaxase and orit region on the plasmid, suggesting possible transfer. These observation needs to be validated by further laboratory experiments. Altogether, with those comprehensive investigations of qnr-positiv *E.coli* isolates a thorough and complex picture will be generated for the mobile genetic elements and their dissemination as well as their commonalities in commensal *E. coli* within the isolates from the German zoonoses monitoring program in 2017. Further investigations on the fitness of the isolates carrying different qnr-plasmid prototypes will performed in the next months. Therefore, the stability and transmissibility of the different plasmids prototypes will be compared in an isogenic system under slecitive and unselective conditions. Currently, different publications were prepared. One is focusing on the performance of different sequencing platforms for AMR prediction in *E. coli*, while two other will deal with the diversity of qnr-carrying *E. coli* and the diversity of qnr-plasmids.

IP

Analysis of the i-bird collection. The 205 isolates belong to 32 different STs revealing a broad diversity of ESBL-*Ec* carried by the patient. However, 100 belong to ST131, almost 50%. They are carriage isolates



recovered from systematic screening and not clinical isolates, showing that, in this environment, ST131 is dominant even among colonizing ESBL isolates. In-depth phylogenetic analysis of the 205 isolates revealed cases of transmission or of a common source only for ST131 isolates with four disseminated clones (Fig. 1). These isolates clustered in four groups (Figure 1) with exchanges between patients, as exemplified for group 3 identified in seven patients. Furthermore, we observed a within host diversity we aim to further analyze. For this purpose, additional isolates will be sequenced to better characterize the within-host diversity and to infer transmission and directionality. The genomic data will be confronted with temporal and contact data collected during the i-bird project. We also identified three cases of plasmid transmission from *K. pneumoniae* to *E. coli*. In order to deepen our analysis of between and within patient diversification, a representative isolate of the four disseminated ST131 lineage has been completely sequenced by using the long-read sequencing technology (Pacbio).

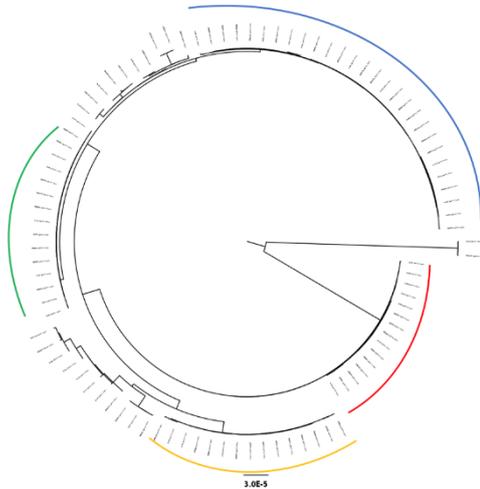


Figure 1 Phylogenetic tree of 100 ST131 isolates from the i-bird project. The majority of isolates clusters in four different clades that have disseminated in the hospital.

Analysis of UTI isolates. The 250 isolates from UTI have been sequenced by the Illumina technology. Preliminary analyses have been performed. The isolates belong to 81 different ST revealing a broad diversity (Fig. 2). The four most frequent STs are also frequently reported in other studies as responsible for UTI (ST73, 31 isolates; ST131, 20 isolates; ST69, 19 isolates and ST95, 18 isolates). Susceptibility to β -lactams has been determined by disc diffusion assays for the first 120 isolates. MICs of 25 isolates for 14 antibiotics were determined by serial microdilution by ANSES for benchmarking pipelines for ARG identification and for genotype phenotype correlation. Antibiotic resistance genes have been detected using Resfinder and candidate mutations have been investigated by using point finder. 21% of the isolates share a mutation in the FQRD region of *gyrA* and/or *parC* and were predicted to show a reduce susceptibility to fluoroquinolons, whereas 11% express a ESBL of the CTX-M family. Only one isolate, of ST410, carries a carbapenemase gene (*bla*_{OXA181}). The next step will be to combine the analysis of UTI *E. coli* from the ARDIG teams participating to the longitudinal study.

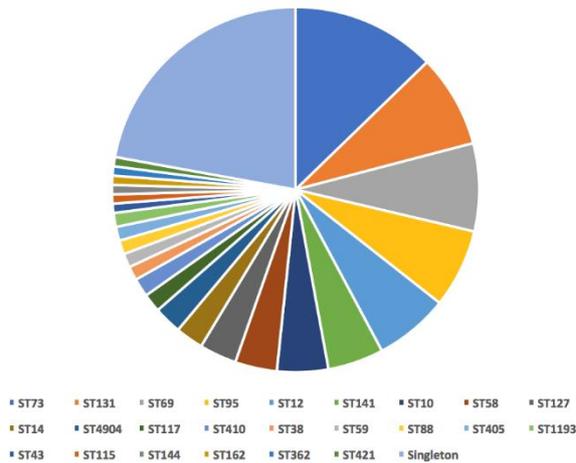


Figure 2 ST distribution among the 250 UTI isolates

In collaboration with Thierry Naas, we are analysing the genomic diversity of carbapenemase producing *E. coli* received by the National Reference Centre laboratory until 2015. CP-Ec are very diverse and belong to 162 different STs. However, 45% of the isolates belong to three dominant lineages: CC10 (including ST167), CC23 (including ST410) and ST38. By an in-depth analysis of ST410 isolates from the NRC and publicly available *E. coli* sequences, we have proposed a model for the dissemination of specific lineages enriched in CP-Ec. These lineages show a reduced susceptibility to β -lactams due to variants in the porin genes *ompC* and *ompF* and to mutation in *ftsI* coding for the penicillin binding protein 3. These lineages are also frequently carrying ESBL genes from the CTX-M family (Patino Navarrete et al. Genome Medicine 2020). A manuscript reporting the analysis of the genome sequences of 714 CP-E. coli isolates taking into account this evolutionary model will soon be submitted for publication. We are currently focusing our analyses on isolates belonging to the ST38 which represents the dominant CP-Ec ST in France but also in the Netherlands. By integrating the analysis of ST38 isolates from animal origin collected by the ARDIG consortium and human isolates we aim to identify additional factors contributing to the acquisition of carbapenemase genes, in particular of those of the *blaOXA48* family and to their dissemination.

JRP2-WP3-T2: Characterisation of prevalent circulating plasmids and their transfer in vitro (M6-M18)

UoS

Transfer of IncL/M plasmids carrying NDM-1 and OXA-48 genes found in local hospitals has been analysed in vitro. The results of these experiments were presented at the OHEJP ASM 2020 in a poster entitled "A broad-host-range plasmid outbreak: dynamics of IncL/M plasmids transferring carbapenemase genes". As a summary, IncL/M plasmids were found to be inter-species vehicles of NDM-1 and OXA-48 and some *E. coli* phylogroups seemed to be less permissive to the acquisition of these plasmids via conjugation. However, additional experiments will be performed to complete the study (e.g. transfer to a larger set of sequenced isolates to find common features impeding plasmid acquisition).

NVI

Comparison studies showed that *bla*CTX-M-1 plasmids circulating in Norwegian broiler production are highly similar to plasmids previously described from broiler production in other countries. Reconstruction of *bla*CTX-M-1/ Inc1-ly plasmids from broilers in Norway showed that a plasmid from an ST57 isolate harboured both Inc1-ly and IncFIB replicons. Further characterization implied that this was an Inc1-ly/IncFIB co-integrated plasmid that consisted of a complete Inc1-ly plasmid and a fraction of an IncFIB plasmid. Several virulence determinants, including *sit*, *iroN* and *hlyF*, were encoded on the IncFIB fraction of the plasmid. The IncFIB specific part was inserted into the accessory module on the Inc1-ly plasmid. Co-integrated Inc1-ly/IncFIB plasmids was found to be present exclusively in ST57 and where detected from a total of five different farms during the six months



sampling periode in 2016, this could indicate a successful plasmid-host combination. Sequence based comparison to ST57 isolates from Dutch and Danish broiler production indicated close genetic relatedness, indicating international dissemination of *E. coli* ST57 with this particular plasmid. The Inc11-Iy/IncFIB co-integrated plasmid and additional Inc11-Iy plasmids with genes encoding cephalosporin resistance have been subjected to conjugation experiments. Recipient strains (n=14) used are laboratory strains as well as wild type strains from broilers. Most plasmids were successfully conjugated, with some exceptions, into various *E. coli* STs, however conjugation into *Klebsiella pneumoniae* was not successful. The hybrid Inc11-Iy/IncFIB plasmid with blaCTX-M-1 (ST57) from broiler was conjugated into only one out of 14 recipient strains. The same conjugation experiments were performed with a non-hybrid Inc11-Iy plasmid (sequence similar to the Inc11-Iy part on the co-integrated Inc11-Iy/IncFIB plasmid). The non-hybrid Inc11-Iy plasmid was successfully transferred into seven of the 14 recipient strains, indicating that the non-hybrid plasmid exhibited a greater promiscuity in terms of acceptance and maintenance by various *E. coli* strains belonging to different ST types. The hybrid and the non-hybrid plasmid transferred into the same host strain (intestinal *E. coli* ST162 from healthy broiler), was subjected to further investigations including effect on growth rates and fitness. In addition, transfer frequencies was investigated. The hybrid plasmid had a lower transfer frequency compared to the non-hybrid plasmid, however, the difference was not significant.

APHA

We have used short and long-read whole-genome sequencing (WGS) techniques to track *Escherichia coli* isolates and identify their associated AMR genes over the duration of 1 year on a UK outdoor pig farm with low antimicrobial usage. Our results showed low levels of AMR *E. coli* presence on this farm but those that were present were likely to be multi-drug resistant (MDR) *E. coli* of particular sequence types (STs), with clones showing epidemiological linkage between pig and wild bird populations. Possible transmission and recycling of the MDR *E. coli* clones within these animal groups drove on-farm persistence for the duration of the study. Most notable was the identification of a large population of sequence type (ST) 744 isolates harbouring up to 14 AMR genes localised on their chromosome within an IS1 flanked transposon region that was variable in AMR gene content but persisted throughout the study within samples obtained from pigs and environmental gull samples. While previous studies have highlighted the importance of horizontal transmission of plasmids as a driver of AMR spread, within our study there was limited evidence of plasmid spread between *E. coli* STs, with plasmids identified instead as remaining associated with individual ST populations, often integrated within the chromosome.

BfR

Investigations on mcr-carrying *E. coli*: Investigations on the occurrence of the mobilizable colistin resistances mcr-4 and mcr-5 among *E. coli* from livestock and food were finished. Overall, more than 900 colistin-resistant *E. coli* isolates from the annual German monitoring programs were screened for the presence of mcr-1 to mcr-9. With the focus on mcr-4- and mcr-5-positive *E. coli*, all information like the MIC, short-/long-read WGS data, XbaI-macrorestriction pattern, plasmid profiles, plasmid transmission were successively determined. We are currently preparing two manuscripts, one the diversity of mcr-4-carrying *E. coli* and one on the description of a novel mcr-5 encoding plasmid. Some initial information on both datasets were recently presented at the ASM EJP conference in Prague. Further in vitro investigations were conducted to determine the influence of different mcr-1 carrying plasmids (>15 plasmid types) on the fitness of *E. coli*. The influence of the different mcr-4 and mcr-5 plasmids were also determined and compared to the results of the mcr-1 plasmids.

WBVR



In the Netherlands, over 350 caecal samples from broilers were collected over a 10 month period on 5 farms in 2018-2019. Using selective culturing on cefotaxim containing media, 23.8% of these samples were positive for ESBL/AmpC producing *E. coli*, very similar to the 23% prevalence of ESBL/AmpC producing *E. coli* found by the national surveillance in 2018. Molecular analysis indicated that of these isolates, 85% produced blaCTX-M-1, blaCMY-2 or blaSHV-12, similar to results of the national surveillance.

As only 1 farm contained ESBL/AmpC producing *E. coli* on more than 3 consecutive sampling moments, 120 *E. coli* isolates from this farm were selected for whole genome sequencing. Phylogenetic analysis indicates that several lineages of *E. coli* are present in the farm which were found in all three barns that were monitored over several production rounds, while each production round was mostly dominated by a specific lineage. On the farm, blaSHV-12 encoded on an IncI1 plasmid was most prevalent, followed by blaCTX-M15 on an IncFIB plasmid and blaCMY-2 on an IncI1 plasmid. While blaCMY-2 was contained to a specific lineage, blaSHV-12 and blaCTX-M-15 were present in more diverse backgrounds and identical *E. coli* that do not contain the plasmids were also present indicating that the resistance genes are disseminated through the farm by horizontal gene transfer.

JRP2-WP3-T3: Fitness cost of AMR and stability of plasmids in different host strain backgrounds (M18-42)

UoS

The UoS has carried out preliminary fitness experiments of an IncL/M plasmid harbouring NDM-1 in different Enterobacteriaceae species. Further studies with a broader range of hosts and *E. coli* phylogroups will be performed in the coming months. Stability experiments in selected hosts will also be undertaken.

NVI

Fitness cost and competitive growth of IncI plasmids with ESBL/pAmpC genes from Norwegian broiler production have been performed. A hybrid IncI1-ly/IncFIB plasmid carrying blaCTX-M-1 (CC3) and a non-hybrid IncI1-ly with blaCTX-M-1 (CC3) was studied. We estimated the maximum growth rates of a recipient *E. coli* (from a healthy broiler) and its respective transconjugants, during 24h of single-strain growth. The IncI1-ly/IncFIB hybrid-plasmid containing culture exhibited a slower maximum growth rate than its plasmid-free counterpart. A similar trend was seen for the non-hybrid plasmid. According to results from competitive growth assays both plasmids caused fitness reduction of the recipient strain, however, fitness cost imposed by the hybrid IncI1-ly/IncFIB plasmid was greater than the non-hybrid plasmid. We also studied shufflon rearrangement in IncI plasmids with ISEcp1-blaCTX-M-1-orf4774 inserted into the shufflon region. Data analysis is currently ongoing.

JRP2-WP3-T4: Measuring AMR plasmid dissemination in mouse and Galleria, and chicken and pig in-vitro models (M24-M42)

UoS

In vivo fitness and transfer experiments with selected hosts and plasmids are being planned using *Galleria mellonella* larvae. Discussions regarding addition studies are ongoing with the APHA, PHE and UCM.

There has been some impact of COVID19 on this Task, delaying laboratory based activities.

WP4: Project coordination and management (M1-M42)

JRP2-WP4-T1: Steering committee quarterly meeting (M1-M42)

Regular teleconference meetings and updates by email have been made to all members in the steering committee within ARDIG.



JRP2-WP4-T2: Consortium members annual meeting (M1-M42)

Due to COVID19 posing restrictions on travel the ARDIG consortium was unable to meet physically for the annual ARDIG meeting, which had been organised to correspond with ECCMID 2020 in Paris, where at least one member from each partner organization was expecting to attend. However, an online Zoom meeting, hosted by UoS, was successfully held between partners. It provided an opportunity for partners from all WPs to interact and discuss the work being performed in ARDIG.

ARDIG partners have used telecommunications and video conferencing facilities, including Zoom, to remain in touch. There have been several meetings held over the past to ensure project is progressing and capture any delay which may have resulted, especially due to the impact of COVID19.

JRP2-WP4-T3: Reporting and communication (M1-M42)

Several work package associated subgroup meetings have been held online to provide time for more in-depth discussion between partners.

For Year 1 ARDIG 9M and 12M reports were submitted in full and in a timely manner. The Year 2 ARDIG 9M report was also submitted in a timely manner. In addition ARDIG submitted their Data Management Plan in full and in a timely manner for Year 1 and Year 2.

There have been a number of publications, presentation (both oral and poster) from ARDIG partners which has included work performed within ARDIG.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
02	D-JRP2-1.2	Description of the specified AMR prevalence/frequency and AMU at population/country/regional level.	M24	M25			Confidential OHEJP: available Zenodo: to be uploaded	Report; 9
02	D-JRP2-1.3	A list of the regions identified for in-depth analysis, and a report including the assessments of parallel trends and estimates of potential associations between AMR and AMU.	M24	M25			Confidential	Report; 9
02	D-JRP2-2.6	Comparative analysis of strains persistence in farms and hospital through longitudinal studies	M30		M42		This is still ongoing as data is still being gathered for comparison across partners. Confidential	
02	D-JRP2-3.3	Predictive modelling of plasmid spread	M30		M42		This is still ongoing as data is still being gathered for comparison across partners.	



							Confidential	
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* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
02	M-JRP2-8	Assessment of ecological and management factors associated with AMR and Antimicrobial usage (from WP1)	M30	Yes	M36	This work has been completed (see Task 1).
02	M-JRP2-10	Collecting of samples and veterinary data, phenotypical testing of resistant isolates from farms and slaughterhouses	M30	Yes	M36	This work has been completed (see Task 1).

4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Monitoring Antimicrobial Resistance and Drug Usage in the Human and Livestock Sector and Foodborne Antimicrobial Resistance in Six European Countries.	Yes	Gold	2590 €



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
10.2147/IDR.S237038 https://zenodo.org/record/997236#.XvyQfSgzZM0			
Stepwise evolution and convergent recombination underlie the global dissemination of carbapenemase-producing Escherichia coli. https://doi.org/10.1186/s13073-019-0699-6 https://zenodo.org/record/3730637#.Xn3Pm4hKi70	Yes	Gold	2656 €
Extensive antimicrobial resistance mobilization via Multicopy Plasmid Encapsidation mediated by temperate phages. https://doi.org/10.1093/jac/dkaa311 https://zenodo.org/record/4244116#.X6KBDjiWxM0	Yes		
The shufflon of IncI1 plasmids is rearranged constantly during different growth conditions 10.1016/j.plasmid.2019.03.003 https://zenodo.org/record/3730621#.Xn3MyYhKi70	Yes	Gold	2590 €
The importance of using whole genome sequencing and extended spectrum beta-lactamase selective media when monitoring antimicrobial resistance. https://www.nature.com/articles/s41598-020-76877-7	Yes	Gold	1844 €



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
https://zenodo.org/record/4456741#.YAgkH-hKjcc			
Antimicrobial Usages and Antimicrobial Resistance in Commensal <i>Escherichia coli</i> From Veal Calves in France: Evolution During the Fattening Process https://doi.org/10.3389/fmicb.2019.00792 https://zenodo.org/record/4249017#.YAgc_uhKjcc	Yes	Gold	2590 €
Temporal dynamics of the fecal microbiota in veal calves in a 6-month field trial https://doi.org/10.1186/s42523-020-00052-6 https://zenodo.org/record/4456718#.YAgfnuhKjcc	Yes	Gold	1390 €
Mobile colistin resistance gene mcr-1 detected on an IncI1 plasmid in <i>Escherichia coli</i> from meat https://doi.org/10.1016/j.jgar.2020.08.018 https://zenodo.org/record/4475507#.YBKNvOhKhM0	Yes	Gold	1660 €
blaCTX-M-1/IncI1-ly Plasmids Circulating in <i>Escherichia coli</i> From Norwegian Broiler Production Are Related, but Distinguishable https://doi.org/10.3389/fmicb.2020.00333	Yes	Gold	



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
https://zenodo.org/record/3701226#.YAgeSehKjcc			

Additional output

The ARDIG partners presented several oral and poster presentations at the 2020 OH-EJP ASM meeting.

Comparison of antibiotic resistance in Escherichia coli from clinical diagnostic submissions and isolates of healthy broilers, turkeys and calves from surveillance and monitoring systems in Germany and France. Octavio Mesa-Varona, Rodolphe Mader, Sophie Granier, Agnes Perrin, Eric Jouy, Jean-Yves Madec, Heike Kaspar, Muna Anjum, Mirjam Grobbel, Martina Velasova, Bernd-Alois Tenhagen. The Tenth International Conference on Antimicrobial Agents in Veterinary Medicine (AAVM), 23-25 Nov. 2020, online



5. On-going and planned collaborations with national or European projects or networks

ARDIG partners are involved in a number of other projects within OHEJP including IMPART, FULL-FORCE, WorldCOM and FARMED. Further, there are a number of OHEJP PhD grants: WILBR, with co-supervision between APHA, SVA and Univ. of Exeter; METAPRO, with co-supervision between UCM, UoS and IP.

UCM are involved in the new H2020 project AVANT, alternatives to antibiotics, started Jan 2020.

APHA are involved in two JPI-AMR projects on AMR in the environment.

There has been interaction between ARDIG colleagues and ECDC and EFSA at the COGWHEEL workshop for WGS, which was organised in September 2020. APHA colleagues Muna Anjum and Manal Abuoun presented both on the AMR pipeline for WGS that is being used within APHA and ARDIG, and also on the WGS AMR workshop that APHA was leading and hosting with ARDIG colleagues, in the UK. We are planning to follow up communication with EFSA in the near future.

ARDIG WP1 has a collaboration with the Joint Action Antimicrobial Resistance and Healthcare-Associated Infections (JAMRAI) project. The main objective of the collaboration is to develop a new method that will allow comparing not harmonized AMR data.

Partner BfR (Dr. B.-A. Tenhagen) is member of the JIACRA working group of EFSA, EMA and ECDC. The group analyses the association of antimicrobial use and antimicrobial resistance on based on data submitted to the European Agencies, i.e. it utilizes the national aggregated data, while in ARDIG concerning AMR there is a focus on individual isolate data. Therefore the two approaches are complementary.

ARDIG WP1 is planning a workshop in early March 2021 in which stakeholders such as EFSA, ECDC, EMA, WHO and OIE will be invited to attend.

Presentation of ARDIG work and progress, has been given to national authorities, including the VMD and Defra in UK, at stake holder meetings.

JRP03-RADAR

1. Summary of the work carried out

Antimicrobial resistance threatens the effective prevention and treatment of an ever increasing range of infections. It is an increasingly serious threat to global public health that requires action across all government sectors and society. Assessment of the importance of different transmission routes and quantifying public health effects (i.e. disease burden) associated with AMR represent major knowledge gaps. The RADAR project contributed significantly to filling these data gaps by producing and harmonizing modelling methodology and frameworks specifically for AMR related problems. A diverse group of international experts in epidemiology, molecular biology, transmission modelling, risk modelling, and disease burden modelling was brought together in the RADAR consortium.

This project achieved its goals in a multi-disciplinary and One Health approach with partners of different expertise (microbiology, epidemiology, risk assessment) and from different domains (public health, veterinary health, food safety). RADAR delivered some interesting and potential useful tools and insights.

1. We delivered a large-scale curated database of (AMR) plasmids from a range of different bacterial species and sources ("COMPASS", Dourarre 2020 *Frontiers in Microbiology*). This novel resource will help researchers understand the genetic plasticity and transmission routes



of plasmids, which are crucial in the fight against the spread of antimicrobial resistant pathogens.. The database is available at <https://github.com/itsmeludo/COMPASS>.

2. We provided an infrastructure for exchanging and annotating risk assessment models in an exchangeable and reproducible file format called FSK and key or desirable features that facilitate access and usability of the inventory (<https://eip-radar.eu/>).
3. We produced state-of-the-art AMR risk assessment models for different food chains in a generic framework. These generic methods may be more crude, but will allow for combining the risks in the different (sub-)categories and may thus help to create a more complete picture of the AMR problems throughout.
4. We produced a framework for the use of machine-learning methods in AMR risk assessment in order to identify risk factors from high-dimensional data with more variables than data points and/or categorical features with many classes.
5. We designed a new burden of disease (BoD) approach suitable for estimating the excess BoD associated with AMR bacterial infection. By 'excess BoD' we mean mortality and morbidity (computed as DALY) associated with resistance, over and above the mortality and morbidity associated with the same – but antimicrobial-susceptible – bacterial infection.
6. We produced a One-Health source attribution model that estimates the relative contribution of reservoirs and transmission routes to AMR (ESBL E. coli) carriership in the population. We adopted established source attribution methods for zoonotic pathogens for specific use for AMR (mainly by introducing humans not only as an endpoint but also as a source).
7. We studied a new paradigm for AMR surveillance based on metagenomics where we showed that metagenomic surveillance is a suitable methodology for population based surveillance of AMR and observed that AMU and AMR are in general correlated both at phenotypic and genotypic levels, but also that other factors play a role in the abundances of AMR.
8. Finally, we developed and applied a Bayesian Evidence Synthesis (BES) approach to integrate all available data and information by combining all relevant data with a priori knowledge and thereby eventually infer estimates on prevalence/incidence of AMR infections / carriership.

2. Work carried out in the JRP, scientific results

WPO: Coordination and communication (M1-M30)

JRP3-WPO-T1: Coordination and project management (M1-M30)

Regular meetings with WP leaders were organized throughout the duration of the project. During these meetings the list of milestones and deliverables (incl. the timelines) were systematically discussed.

JRP3-WPO-T2: Consortium meetings (M1-M30)

The project has seen three physical meetings: the kick-off meeting (Schiphol airport), a half-way meeting (Schiphol airport), and a meeting towards the end of the project (BfR Berlin). During the extension period from Jan to Dec 2020 no physical meetings were organized due to the COVID19 crisis. Regular phonecalls between the project management and WP leaders took place.

JRP3-WPO-T2-ST1: Kick-off meeting

January 2018 Schiphol Airport, The Netherlands. Important first alignments and directions were consolidated.

JRP3-WPO-T2-ST2: Mid-term meeting (M10-M12)

Held January 18th Schiphol Airport, The Netherlands. Scientific progress was presented and discussed. Good agreements for WP alignments were made.



JRP3-WP0-T2-ST3: Final meeting (M30-M30)

Due to the corona crisis it no final meeting took place. Discussions are still ongoing to organize an OH EJP AMR workshop in summer 2021 in combination with other AMR projects.

JRP3-WP0-T3: Annual reports (M1-M30)

JRP3-WP0-T3-ST1: First annual report (M10-M12)

Completed.

JRP3-WP0-T3-ST2: Second annual report (M22-M24)

Completed.

JRP3-WP0-T3-ST3: Third annual report (M28-M30)

This is the final report presented here.

WP1. New genomic information to feed AMR transmission models (M1-M30)

JRP3-WP1-T1: Build collections of high throughput sequencing (HTS) data needed for project-specific milestones and deliverables (M1-M15)

Sub-Task 1.1.1: Plasmids are the keystone of horizontal gene transfer in bacteria and are of major clinical interest due to their contribution to the dissemination of antibiotic resistance genes. As the number of plasmid sequences in public databases is growing exponentially, the creation of a comprehensive and curated complete plasmid database is critical to capitalize on the available data and provide an exhaustive library for the scientist community.

Here, we have compiled and curated a dataset of complete plasmid sequences with associated metadata sourced from the NCBI database. The resultant database contains 12084 plasmid records that have been analysed and summarized using R and online tools such as Gunmap 2 and Krona. Resistance genes acquisitions were identified by BLASTn search against the ResFinder database.

The plasmid database encloses 1564 distinct species, 443 genera, 189 families, 93 orders, 38 classes and 21 phyla. Proteobacteria (66%) and Firmicutes (21%) are the most represented phyla and 38% of the bacterial species belong to the Enterobacteriaceae family. The vast majority of the plasmids (94.5%) are circular and with sizes ranging from 744 bp to 2 555 069 bp. Plasmids were isolated in 126 different countries from 1884 to 2018. In total, 13812 resistance genes were detected among the 12 084 plasmids and they included 503 different resistance variants. From our dataset, we found that 3438 plasmids (28%) carry at least one resistant gene and resistance to beta-lactams, aminoglycosides and sulphonamides are the most frequent. Among these resistant plasmids, 41% are multi-resistant of which 80% were isolated from Enterobacteria.

This curated plasmid database can be easily integrated, as a reference, into pipelines aiming at identifying new plasmids, thus enabling the exploration of the metadata of all complete plasmids in the NCBI database, in light of their predicted antibiotic resistance. This novel resource will help researchers and clinicians understand the genetic plasticity and transmission routes of plasmids, which are crucial in the fight against the spread of antimicrobial resistant pathogens.

This curated plasmid database was integrated, as a reference, into our new developed pipelines (**JRP3-WP1-T2**) aiming at identifying plasmids from sequencing data. This novel resource will help researchers and clinicians understand the genetic plasticity and transmission routes of plasmids, which are crucial in the fight against the spread of antimicrobial resistant pathogens.

The database was named “Compass” and was published in **Frontiers in Microbiology (Dourarre 2020, Frontier in Microb. 24 march 2020)**. See fig.1. for a visual description of the database. The database is available at <https://github.com/itsmeludo/COMPASS>.

Sub-Task 1.1.2: New environmental genomic datasets relevant to various AMR challenges have also been collected and organized in a database for future study. These datasets include: 1)



Salmonella surveillance network (2000-2016) from all food, animal and environmental sectors, a total of **2839 isolates**. This dataset have a particular focus on fluoroquinolone resistance and the plasmidome, 2) Quinolone resistant *E. coli* (QREC) from animals (poultry, pigs, wild birds, foxes) in Norway, 3) Cephalosporin resistant *E. coli* (containing *bla*CMY-2) from poultry in Norway, 4) the pig faecal metagenomics data collection obtained in the frame of the EFFORT H2020 project (<https://academic.oup.com/jac/article/74/4/865/5289505> ; ENA: PRJEB22062), 5) The metagenomics dataset of urban sewages under the H2020 COMPARE project (<https://doi.org/10.1038/s41467-019-08853-3>).

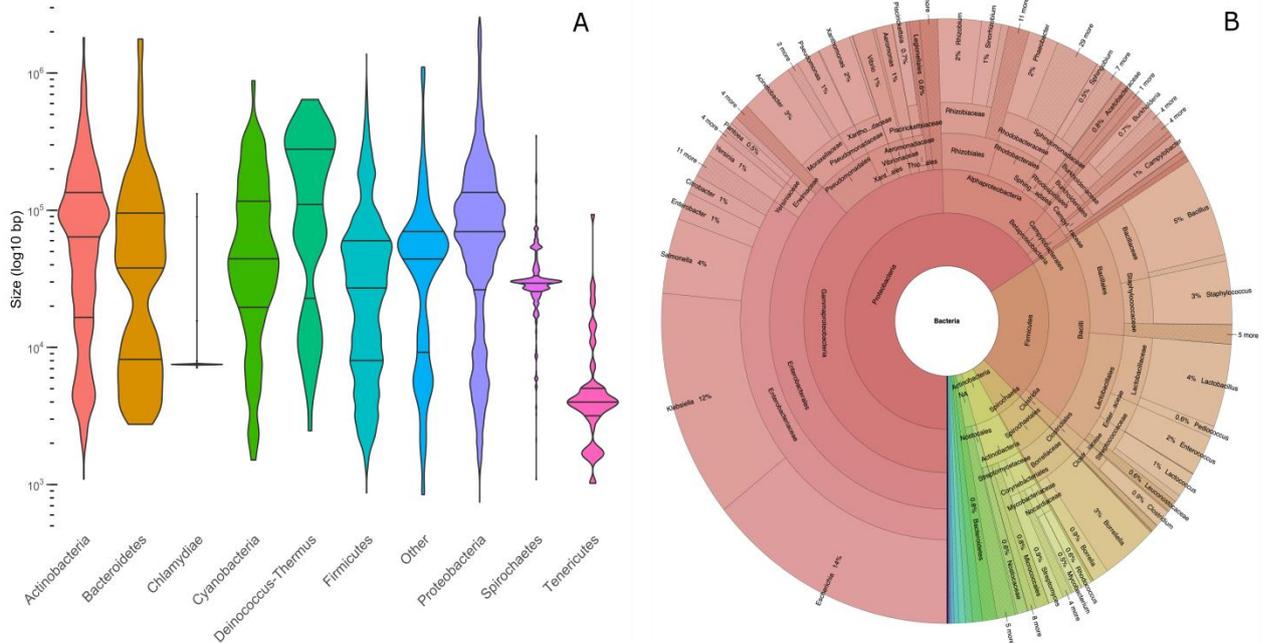


Fig. 1. Description of the COMPASS database. (A) Violin plot displaying plasmid size distribution (log₁₀) among the main phyla (n=12 084). The phylum entitled “Other” is composed of 57 plasmids from 12 minority phyla (n<25) and 129 plasmids missing taxonomy data. (B) Krona plot showing the compositions of taxa and taxonomic ranks (n=12 084).

JRP3-WP1-T2: Develop an innovative automated bioinformatic pipeline integrating de novo plasmid reconstruction and identification (M1-M18)

Identifying and tracking circulating plasmids is heavily relying on the ability to accurately assemble their whole genome sequences and differentiate chromosomal from plasmid contigs. Even though several bioinformatics tools can be applied to reconstruct plasmid sequences from short reads, a contiguous assembly is still difficult to obtain. Plasmid *de novo* assembly based from the coverage and the assembly graph was first performed by SPAdes. In order to assess the performance of different plasmid detection tools, we built a “test dataset” composed of 56 known genomes of *Salmonella enterica* (Chromosome and plasmids) and tested against MOBRecon, PlaScope, Plasflow and HyASP programs. Our COMPASS database was integrated in the detection tools as a reference database to help discerning the plasmids contigs from the chromosomal sequences and to identify the closest plasmid neighbour present in COMPASS. Core plasmid genes necessary for replication (replicon) and mobilization (Relaxase, MPF and *oriT*) were annotated by the MOBtyper program from the MOBsuite package and the plasmid resistome was characterized in silico by detecting resistance genes of the Resfinder database. Finally, we developed an automated plasmid identification pipeline by integrating plasmid assembly (SPADES) and detection programs (PlaScope), annotation tools (MOBtyper and Resfinder) and reference-based identification (Mash program against the COMPASS database) (Fig. 2).

The outputs of the pipeline (plasmid contigs / genes and resistance genes) were designed for handling by microbiologists and modelers to facilitate biological interpretation and integration into transmission



models. Following the validation of our pipeline on the “test dataset”, our novel plasmid tool was used to detect and identify plasmids from the new environmental datasets collected in (T1 subtask 1.2).

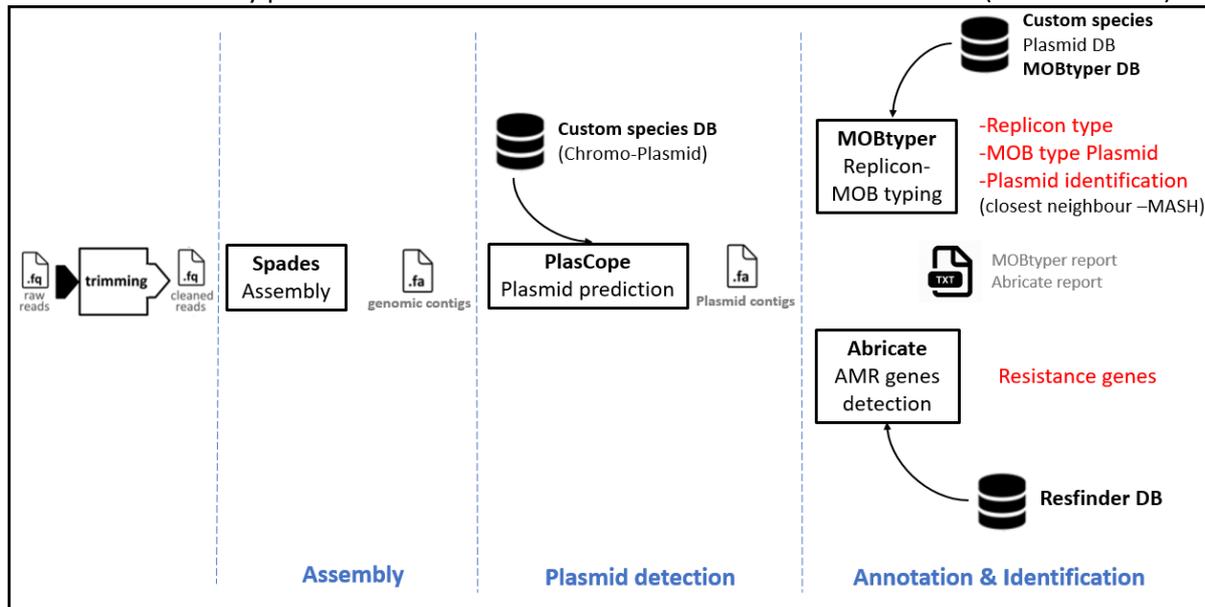


Fig. 2. Scheme of the automated plasmid identification pipeline (described below) by integrating plasmid assembly (SPADES) and detection programs (PlaScope), annotation tools (MOBtyper and Resfinder) and reference-based identification (Mash program against reference database).

JRP3-WP1-T3: Plasmidome: biological annotation and risk assessment (M12-M24)

We used the pipeline we developed in JRP3-WP1-T2 to characterize the plasmidome of 3109 genomes of *Salmonella enterica* strains isolated from all food, animal and environmental sectors (ANSES collection). We successfully identified and reconstructed 2776 plasmids, which were categorized into 242 clusters. Replicon and MOB types identified a diverse population of plasmids. The resistome and the virulome were characterized in all the plasmids and compared within different serovars. Our results suggest that resistant plasmids are shared among different serovars while virulent plasmid (like the pSLT) were restricted to few serovars.

JRP3-WP1-T4: Methods to identify genetic traits associated to AMR (M12-M24)

Genome Wide Association Studies (GWAS) are hypothesis-free methods for identifying genetic variations associated with particular phenotypic traits within a population. Microbial genome-wide association studies (mGWAS) are a new and exciting research field that is adapting human GWAS methods to understand how variations in microbial genomes affect host or pathogen phenotypes. Given the availability of large panels of bacterial genomes combined with phenotypic data in public databases, GWAS have shown promising results for genetic marker discovery and as emerged as a fundamental task in bacterial genomics. To determine best practices for microbial GWAS, it is essential to compare current GWAS methods in terms of their performance across a range of realistic effect sizes, recombination rates and sample sizes. With the growing number of different GWAS softwares available, the choice of tool, methods or workflows presents a major challenge to biologists. Here we present in [Table 1](#) a summary of bioinformatics tools and pipelines available for microbial GWAS and highlight their advantages and limitations. We made an overview of different mGWAS methods (see deliverable 1.4.1 and 1.4.2, combined).

WP2. Pharmacodynamics and transmission models (M1-M24)

JRP3-WP2-T1: On-farm transmission models (M1-M24)

JRP3-WP2-T1-ST1: PK/PD model to assess relationship between animal exposure and change in antimicrobial resistance (M1-M20)

The goal of this work was to refine and develop a “within-host PKPD model” (hereafter simplified as PKPD model) to assess and predict the impact of an AMD treatment (as an input of the model) on the emergence/selection of resistant bacteria within guts and excretion towards faeces (output of the model) for pig, at the individual level and population level (taking into account the inter-individual variability). The PKPD models initially developed for colistin (with a “simple” intestinal Pharmacokinetic) allowed us to identify key points for the development of a PKPD model for amoxicillin and ESBL *E. coli* (**Fig. 3**).

This work highlighted the different tools and methods that exist but also the numerous gaps to develop a mechanistic PKPD model of antimicrobial resistance within guts. Our work outlined the relative importance of the inherent variability of each PK and PD sub-levels for the understanding of the evolution of bacterial populations toward resistance development/selection after a perturbation due to an AMD. The influence of specific bacterial parameters on the plasmid dynamism and its influence on the selection, maintenance or disappearance of resistance could be independent of the initial exposition.

This work outlined that the mechanistic modelling of the digestive tract is still challenging and should be improved but will probably need additional data. Some very mechanistic models of digestion and food transit have already been published but are sometimes theoretical with parameters values not based on experimental measures. The complexity of such model will definitively need inter-disciplinary approach combining mathematicians and pharmacologist/biologists.

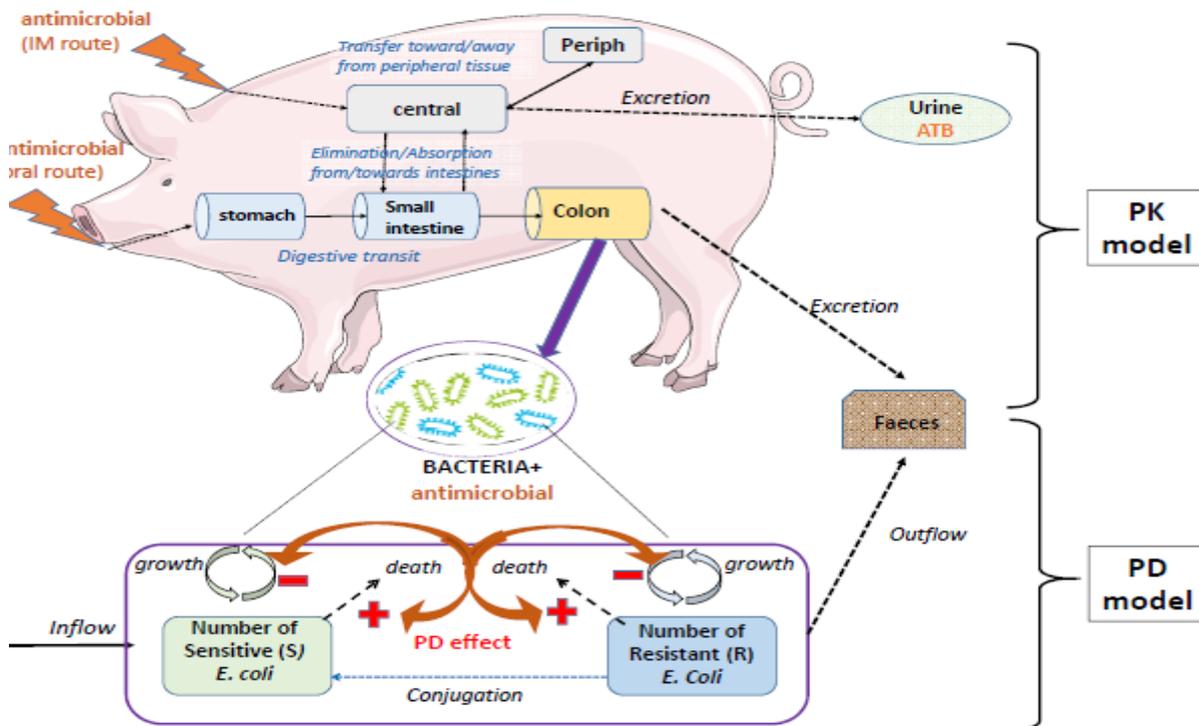


Fig. 3. Schematic diagram for the proposed Pharmacodynamic model.

JRP3-WP2-T1-ST2: Assess relative importance of AMU and clonal dissemination for resistance occurrence (M1-M20).

Simulations were carried-out to study the influence of bacteriological and pharmacodynamic parameters describing 2 bacterial population from a same clone with and without a plasmid coding for



a reduced susceptibility. Pattern analysis and clustering have also been performed and will help to identify key influential parameters. For the PD model, our simulations focused only on one bacterial specie, *E. coli*, and the different case scenario outlined the need to get a better understanding of the bacterial interactions within species. Indeed, these interactions/competitions between strains are likely a major key that affects the dynamics of resistance evolution. However, the inter-species transmission of AMR should also be considered and the development of more complex model including the whole microbiota is currently an ongoing research topic and it will probably be possible within the next years to include ecological models inside the PKPD models.

JRP3-WP2-T1-ST3: Development of on-farm transmission model (M1-M20)

The transmission of antimicrobial resistance (AMR) between animals, their environment, food and humans is a complex issue and more evidence is needed to strengthen current understanding. Models of disease transmission within commercial farm environments can provide further insight to the on-farm transmission dynamics of AMR between animals and their environment, as well as predict the effect of various on-farm interventions. Previous generic models indicate that ESBL resistant bacterial populations may be self-sustaining through horizontal and vertical gene transfer, even in the absence of antimicrobial pressure. However, models focusing purely on the biochemical aspects fail to incorporate the complicated host population dynamics which occur within a farm environment. Here, we present a model framework for incorporating pharmacokinetic-pharmacodynamic (PKPD) models; exploring the complex host/gut bacteria interplay, with on-farm models of disease transmission in order to predict the infection dynamics on pig farms. The model is designed to be adaptable for the simultaneous transmission of multiple bacteria and resistant strains. In addition, the model incorporates the faecal excretion (and subsequent ingestion) of antimicrobial residues from pigs that have been treated with antibiotics. The model is an individual based, stochastic, susceptible-infected-susceptible (SIS) model of the colonisation of pigs with resistant bacteria within commercial pig farms. It is based on a previous model of *Salmonella* transmission on commercial pig farms in the European Union. The model consists of two main components: a pig management component and a bacterial transmission component (Figure 4). The management component simulates the movement of individual pigs throughout their time on the farm. It incorporates different management practices of commercial pig farms, such as when cleaning of the pens occurs, and the simultaneous movement of groups of different aged pigs.

The model output is the average prevalence and concentration of resistant bacteria in the faeces of pigs sent to the abattoir. The model was run with 500 iterations, each iteration representing production from one farm over a 365-day period. The timestep of the model was one day. To account for different farm types, the model subdivides farms into one of three pig production systems: breeder-finisher (where a pig stays on the same farm from birth until it goes to the abattoir), breeder-weaner (where pigs stay on one farm from birth to the weaner stage and then moves to a finisher-only unit) or finisher-only (pigs arrive on the farm after weaning and stay until they leave to go to the abattoir). Farms may run an all-in-all-out (AIAO) production system where batches of pigs are kept in rooms without direct contact between batches, or a continuous production system. AIAO systems were not applicable for the farrowing stage. Farms may also use either an indoor or outdoor production system in the farrowing stage and may use either solid or slatted floor in the pig pens. Different production systems are randomly selected for each iteration.

The model predicted that after introduction of ESBL *E. coli* onto a pig farm, the disease is likely to persist on the farm for more than a year leading to a high level of carriage (13.7%, 5th and 95th percentiles: 0-53.1) and faecal shedding in slaughter-age pigs. The average individual pig prevalence over all iterations in the baseline scenario was 13.7% (5th and 95th percentile: 0%-53.1%). However, the average batch prevalence, i.e. proportion of batches sent to slaughter with at least one positive pig, was 34.6% (5th and 95th percentile: 0%-97.9%) and the average pig prevalence in these batches was 39.6% (5th and 95th percentile: 5.6-56.3).

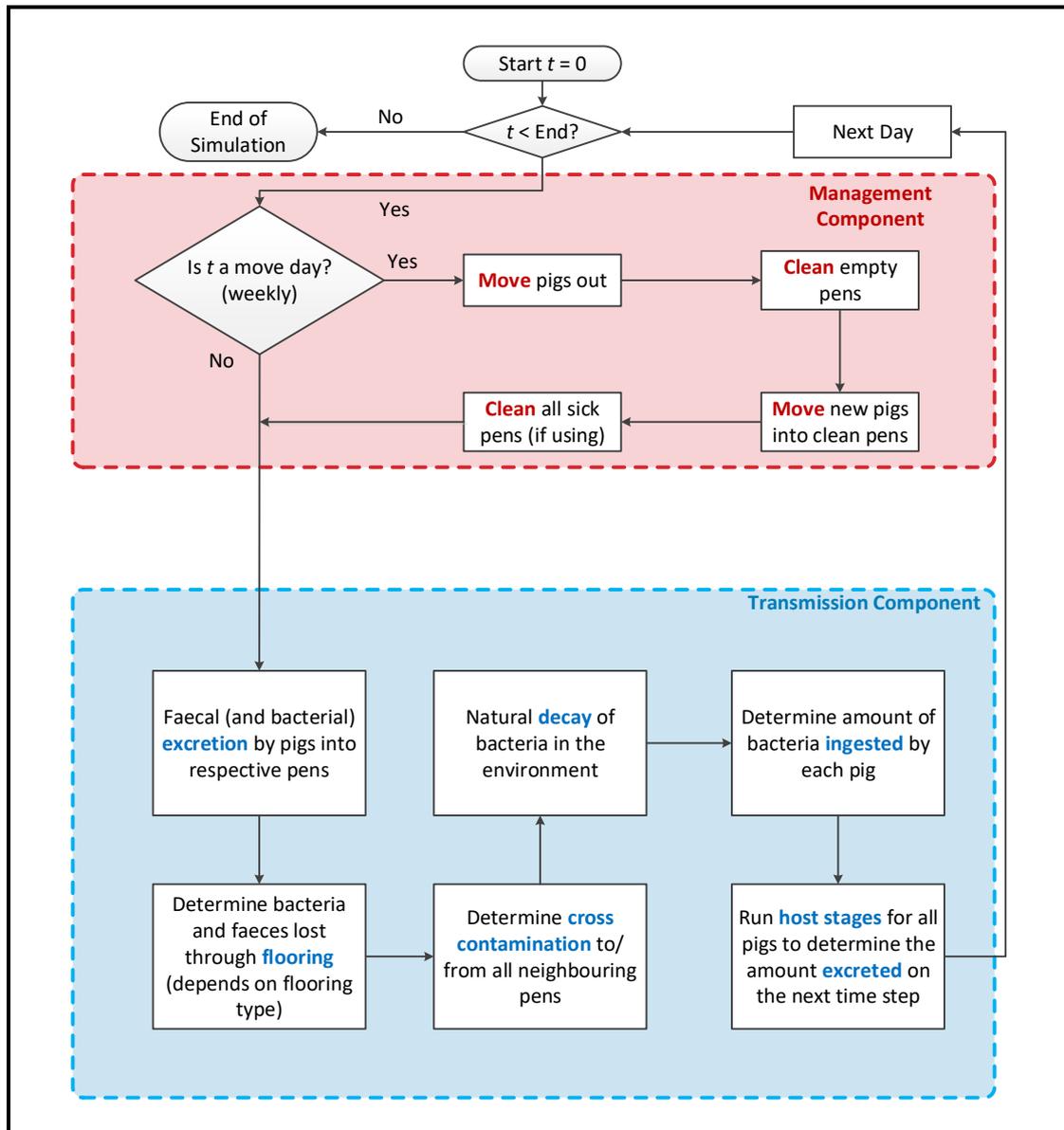


Figure 4: Overview of model framework simulating the colonisation and transmission of resistant bacteria in pigs within a commercial farm environment. The model has both management and transmission components.

JRP3-WP2-T1-ST4: Scenario analysis to assess hypothetical on-farm intervention measures (M6 M20)

Three farm-based interventions were modelled to investigate possible options for reduction of ESBL transmission between pigs. Firstly, sick pigs were simulated to move to a quarantined sick pen during the duration of their antibiotic treatment course. It was hypothesised that this could isolate the pigs which are most likely to be shedding the highest levels of resistant bacteria (due to the antibiotic use), and therefore reduce pen contamination levels. A further intervention was modelled to understand the effect of an enhanced cleaning and disinfection (C&D) protocol.

Isolation of pigs in sick pens for the duration of their antibiotic treatment reduced the number of positive batches whereas an enhanced cleaning and disinfectant (C&D) protocol reduced the within herd prevalence. Both interventions were able to reduce the number of pigs shedding more than 2log₁₀ ESBL *E. coli* from 8.5% (5th and 95th percentiles: 0-29.2) in the baseline scenario, to 0.07% (5th and 95th percentiles: 0-0.3) when sick pens were used and 3.4% (5th and 95th percentiles: 0-16.5) when an enhanced C&D protocol was applied. A combination of the two interventions was most effective at reducing overall prevalence (5.6%, 5th and 95th percentiles: 0-40.6). The results suggest that actions



targeted towards preventing or removing the colonisation of AMR bacteria within the microbiome of pigs are more likely to be effective than steps to remove contamination levels within the pen environment.

JRP3-WP2-T1-ST5: Communication of results (M18-M24)

A first draft of the on-farm model report is currently in internal review, but COVID-19 is delaying progress.

JRP3-WP2-T2: Models for transmission between livestock and human populations

JRP3-WP2-T2-ST1: Development of mathematical models for source-attribution (M1-M22)

A systematic review and meta-analysis was conducted in order to assess relevance of transmission routes of antibiotic resistant bacteria calculated using different methodologies and the relevance of routes per pathogen. This is being transformed in to a scientific paper. PubMed and EMBASE were searched, resulting in 6054 articles published up until January 1st, 2019. Full text screening was performed on 525 articles and 277 are included. This review took considerably more time than anticipated given the huge number of papers that had to be screened. The main conclusion of the review is that, on the one hand, there is a plethora of estimates and studies on risk factors and transmission routes of ABR bacteria, while on the other hand, the relevance of routes on a population scale is missing. As the frequency at which all kind of risk factors and exposure occurs, the relevance of transmission routes on a population level are lacking. For a specific pathogen, i.e., *Escherichia coli* containing β -lactam antibiotic resistance genes, we importance could be obtained 2), but even this study could pinpoint (with some uncertainty) how important different reservoirs are for the acquisition by humans of *Escherichia coli* containing β -lactam antibiotic resistance genes, but could not pinpoint the mechanisms behind the transmission routes. Given the almost complete absence of estimates of the importance of AMR transmission pathways, a mathematical model to calculate reproduction numbers would be highly speculative and any assessment of the effectiveness of intervention measures would lead to high-levels of uncertainty.

JRP3-WP2-T2-ST2: Assessment of intervention measures (M13-M22)

This work was merged with *JRP3-WP2-T2-ST1*

JRP3-WP2-T2-ST3: Communication of results (M18-M24)

Ongoing. A poster was presented at the Epidemics Conference in December 2019 (P1.069) . D2.3 I being transformed into a scientific paper.

WP3. Transmission through the food chain (M1-M30)

This WP focuses on risk assessment models that calculate risks of exposure/infection with ESBL *E. coli* from different sources and through different food chains..

JRP3-WP3-T1: Inventory of available exposure assessment models and related data and transfer to FSK Standard (M1-M24)

JRP3-WP3-T1-ST1: Inventory of available exposure assessment models (M1-M12)

We have completed the development and implementation of the RADAR model inventory. This includes the provision of a proper infrastructure for exchanging and annotating models in an exchangeable and reproducible file format called FSK and key or desirable features that facilitate access and usability of the inventory. The features were defined in the annual report 2018. However, the inventory currently runs on a firebase server hosted by Google (<http://ejp-radar.eu>). It is planned to migrate the web application to a stand-alone BfR server as soon as possible. We have uploaded three models to the inventory, two showcase models and the primary production model from JRP3-WP3-T1-ST2.

JRP3-WP3-T1-ST2: Transfer of available exposure assessment models developed in R (or Matlab) to FSK Standard for at least one type of AMR bacteria and at least one animal (chicken, pig or mussels) (M10-M24)

We have uploaded three models to the inventory, two showcase models and the primary production model from JRP3-WP3-T1-ST2.

JRP3-WP3-T2: Exposure assessment models for different production chains (M1-M24)

JRP3-WP3-T2-ST1: Exposure assessment model for the chicken production chain (M1-M24)

During the project an exposure assessment model for the broiler production chain was developed. This model is a combination of two preexisting models which have been connected with each other (Fig. 4). One of the two preexisting models looks at the primary production and calculates how far ESBL *E. coli* spread within flocks and among flocks. This primary production model is then linked to a model describing how bacterial colonization on the surface of carcasses change over the course of a chicken processing line. The work in this deliverable then focused on the development of a generic model framework and for that the second part, the processing line model, was chosen to create a concrete implementation of how such a generic model might look like. The result is a R package *genpromodel* developed in this project that can help the user to build and customize its own processing model which might not be limited to chicken.

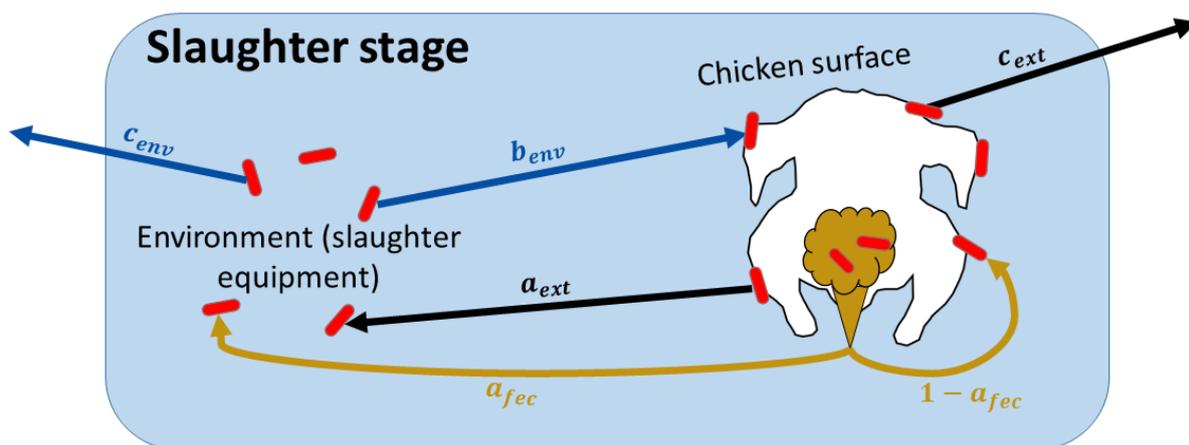


Fig. 4. The Exposure assessment model for the chicken production chain model considers following processes: Bacteria can go from the slaughter equipment to the carcass and the other way around. Bacteria on the carcass and on the slaughter equipment can be inactivated (symbolized by the arrows that lead out of the slaughter stage, indicating that the bacteria which become inactivated no longer take part in the model calculations). And finally bacteria that are in leaked faeces can either go onto the carcass or onto the slaughter equipment. The letters at the arrows describe the proportion of bacteria which transfer from the start point of the arrow to its end point.

JRP3-WP3-T2-ST2: Exposure assessment model for the pork production chain (M13-M24)

The pig processing model is developed in WP2 (primary production) and WP 3.2 (the slaughter process, consumption phase). It is developed to closely link to WP6, where an evidence synthesis approach for combining QMRA and epidemiology in the context of ESBL in the pork chain is developed. The model chain is parametrized for the Netherlands.

With approval of the RaDAR project lead, the dissemination mode of the current deliverable has been changed from scientific report to scientific article, thereby increasing dissemination value. Hence, full coverage of the model development for the slaughter phase, consumer phase, and dose-response will be discussed in detail in a forthcoming publication (Swart et al., in preparation). The exposition below should be considered a brief summary, highlighting the most prominent features.



The full QMRA model consists of a farm model, a slaughter model, a consumption model, and a dose-response model.

JRP3-WP3-T2-ST3: Exposure assessment model for the mussel production chain (M13-M24)

The potential for blue mussels to be a significant source of ESBL - producing *E. coli* to humans is unknown. Blue mussels typically undergo heat treatment before consumption, and commercial produced blue mussels have a food safety regulation limit of 230 *E. coli*/100g for direct human consumption (854/2004/EC, 2004), demanding that shellfish with higher *E. coli* concentrations be moved to cleaner water until the concentration falls below this level. As *E. coli* is an indicator of faecal contamination makes it useful as an indicator of pathogens which spreads this way, but this limit might not be enough to avoid ESBL-producing *E. coli* being transferred to humans from mussels/ shellfish. In addition, consumption of wild-harvested mussels occurs in many coastal areas, particular during vacation times.

The heat treatment performed by the consumer is traditionally kept to a minimum (until the shell opens), so there is a need for public knowledge of the potential for survival of both *E. coli* and ESBL-producing *E. coli* in such a food matrix. There exists little knowledge about the persistence of viable ESBL producing *E. coli* in different food matrixes where only light heat treatment are performed before consumption, but both the maximum obtained temperature within the mussel as well as the duration of certain temperatures will likely have an impact on bacterial survival, determining the amount of remaining viable from the original contamination.

The model for the mussel production chain described here (Fig. 5), is focused on the preparation step which is critical for exposure and disease risk. It addresses the question of how much ESBL *E. coli* bacteria consumers are exposed to when they prepare a batch of blue mussels (*Mytilus edulis*) or similar food item through the common practice of steaming raw shellfish before immediate consumption. Thus, the output of the model is the number of colony forming units of ESBL *E. coli* in a mussel meal depending on the duration and temperature of heat treatment, mussel size and initial contamination levels.

The model for the mussel production chain incorporates two sets of experiments, integrating them through a supervised learning algorithm for a robust output. The first set of experiments involves inoculating live mussels with non-ESBL *E. coli* by allowing them to naturally filter contaminated water in an aquarium, and then batches were steamed in a procedure mimicking common kitchen practices for different time intervals. Samples were taken of raw mussels as well as those subjected to different heat treatments, individual mussels were recorded, and colony-forming units per gram mussel meat (CFU/g) assessed by culturing. This experiment could, however, not utilize ESBL strains due to biohazard procedures, and the steaming does not allow incremental adjustment of temperature. Thus, a second set of experiments addressed this by homogenizing a mix of mussel meat and either non-ESBL or ESBL-producing *E. coli* in a series of in small, heat-resistant plastic bags, and subjecting them to water baths of different temperatures and durations before culturing. Lastly, these approaches were integrated through a simple learning algorithm for a more robust model by linking heat stress captured by “button-type” temperature loggers used in both experiment sets.

Additional work carried out in WP3 concerns a risk assessment for ESBL *E. coli* via sea-food consumption. Extended-Spectrum β -Lactamase (ESBL) producing *Escherichia coli* (EEC) are a significant public health concern. Although previous studies have calculated the exposure through meat consumption, the risk from seafood has not been quantified. A Quantitative Microbiological Risk Assessment (QMRA) model for meat was adapted to incorporate the complexities along the seafood chain. Total exposure of the Dutch population (EEC / year) was highest for raw salmon (48.1%) and smoked eel (43.9%). The top five seafood products (raw salmon, smoked eel, caviar / cod roe, smoked salmon and anchovies) all had a higher annual exposure estimate than any meat product. The high exposure by raw salmon is due partly to the current EU legislation which permits farmed salmon to be sold without prior freezing. With current antimicrobial usage remaining high in aquaculture industries, the results question whether the current legislation is adequate to mitigate against emerging microbiological hazards such as antimicrobial resistance.

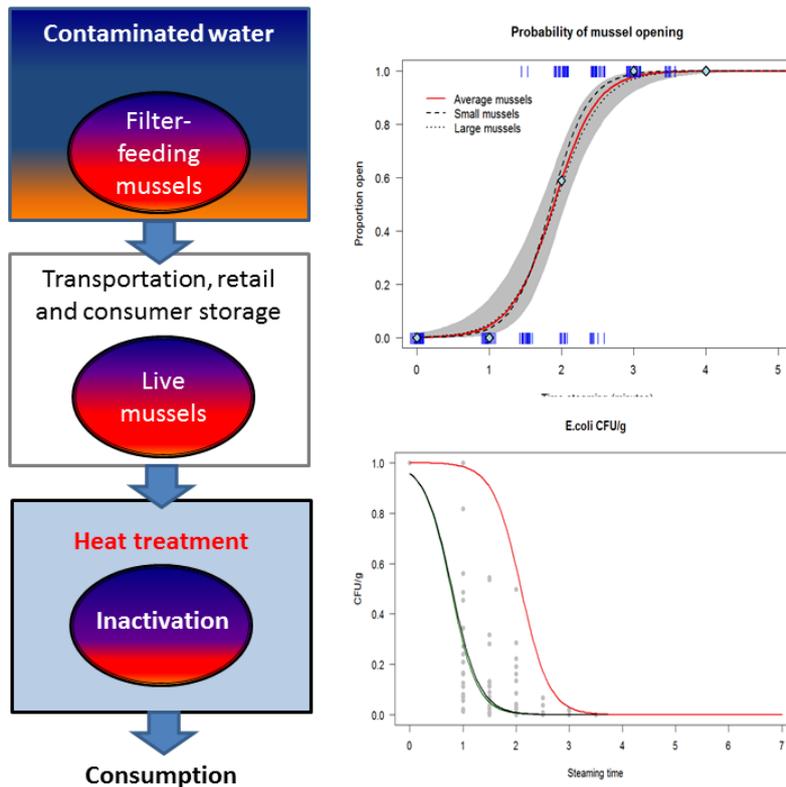


Figure 5. Summary of the production/handling chain from growth and harvest to end consumer for blue mussels. In the growth stage they concentrate any contaminants in their surroundings through filter feeding, and are then kept alive through a more or less unbroken cold chain until they are exposed to a short heat treatment at the point of consumption.

JRP3-WP3-T3: Generic comparative exposure assessment model (M13-M24).

This task focussed on the potential for deriving or creating generic methods for exposure assessments for different production types, different bacteria, different processing etc.. These generic methods may be more crude, but will allow for combining the risks in the different (sub-)categories and may thus help to create a more complete picture of the AMR problems throughout.

In order to study how one could go about developing a generic modelling approach for assessing exposure while taking into account different exposure pathways the following was done. Four in this project independently developed models for three different food chains were compared in a synoptic way identifying conceptual communalities which were then considered central parts of a possible generic exposure modelling approach. The three different food chains were the chicken, pork and seafood chain. Within the seafood chain, there are two models: a comparative exposure assessment model for various types of seafood (fish) and an exposure model focussed on steamed mussels. The models for each food chain have of course their own idiosyncrasies given the different concrete question they try to answer. However, there are still many conceptual communalities, which are useful to highlight in order to identify the generic elements of current state of the art exposure assessment models.

WP4: Machine learning methods for quantification of risk and health effects (M1-M30)

Assessing risk factors from data needs statistical modelling of that data. Here, mostly generalized linear models are applied, including logistic regression, and Poisson regression. The used framework struggles when the data set at hand is not a low-dimensional problem (i.e. only consists of only a few features), the data generating model is unknown and should a bit more ambitious than just a very simple linear one. Very often, we have data sets with almost as many, or even more variables than data points, and categorical features with many classes increase this problem for linear models even



more (i.e. by inflating the design matrix with dummy variables). The classical framework fails here completely, as it has very high requirements on the data in order to successfully do a data analysis. And if the data generation model is unknown and we have to somehow guess a good model, it only gets worse. Here, we investigated the vast possibilities of Machine Learning (also known as statistical Learning, predictive modelling, and algorithmic modelling). On the field of machine learning, we often encounter data sets that are difficult to handle when using the classical statistical framework.

JRP3-WP4-T1: Add state of the art ML models for risk profiling to an inventory of exposure risk assessment models (M1-M2)

JRP3-WP4-T1-ST1, ST2, ST3:

There is a wide variety of different ML algorithm families, and many different packages available in R. Unfortunately, the quality and user guidance of the various packages is very diverse, partly because the writers of the packages are not qualified programmers and there is no peer or code review of R packages. The validation of used packages, especially if they are new, is the responsibility of the user.

Unfortunately, new and interesting packages can be so plagued by programming errors that they become unusable. The same applies to the partially erratic user guidance of the model functions, although a factual standard exists for this in R (see the function *lm*, *nls* and *glm*). Unfortunately, this is not binding, as a peer and code review would then have to be carried out, which is currently practically impossible, as the necessary structures do not exist. Many packages are the result of academic research, and with the end of this research the maintenance and responsibility for the package may cease. Thus these packages disappear again, even if they offer interesting approaches. Or programming errors remain unresolved. This can undermine your own analyses if they are based on the existence of these packages. (Since the underlying code may work, but is often unmaintainable, taking over the old, no longer hosted code would be a task of de-facto rewriting the code. We usually do not have the time or manpower to do this.).

Meta-packages such as *caret* (short for *_C_lassification _A_nd _RE_gression _T_raining*) try to clear up this chaos, and we strongly recommend the newcomer to familiarise himself with this or similar packages. Unfortunately, fixed approaches such as *caret* are only good in machine learning as long as the path is not deviated from. Therefore “only” standard evaluations are possible. But we had to do that here. Furthermore, even *caret* is also not immune to programming errors and poor performance. The R package ‘*caret*’ offers a treatment of supervised machine learning problems from a single source. At the same time it also contains a repository for a very large number of different machine learning models and their variations. The available models in *caret* can be viewed at: <http://topepo.github.io/caret/available-models.html>

We hope that packages such as *caret* will be further developed and maintained so that R remains competitive in the area of machine learning. We have avoided using meta-packages as much as possible.

There are other meta-packages in R for Machine Learning, namely *mlr*, and *mlr3*. But these two packages have the following problems: first of all, they want to do everything very differently from how it is normally done in R. The user guidance reminds more of Python than of R, which is not an advantage. The learning curve is very steep, so that *mlr* and *mlr3* are not worth it for most, relatively simple problems. It is then much more time-saving and efficient to use the underlying ML packages directly, and to program the possibly missing functionalities yourself in R, if these problems exist at all. It also saves the time-consuming research for how to access certain functionalities -- which are easily accessible in the underlying packages -- using *mlr* or *mlr3*. The second problem is that the development of *mlr* was abandoned in favour of *mlr3*. Unfortunately, some special, very advanced possibilities (e.g. multi-label classification) of *mlr*, are currently not available in *mlr3*.



JRP3-WP4-T1-ST4: Repository setup including setup of a Github repository(M7-M12).

Building a separate repository may be deemed superfluous given the existence of the Caret methods library on Github (<http://topepo.github.io/caret/index.html>) for which model evaluation manuals are readily available and a paper evaluating model performance on AMR risk models

JRP3-WP4-T2: Methods for testing model -validity, -sensitivity and –robustness (M13-M21)

In ML, models are developed differently from how we know it from classical statistical modelling. In ML, models with high predictive power are to be created, whereby the concrete form of the model is of little interest. This is called function estimation or approximation, in contrast to the parameter estimations of the assumed (hopefully correct) model in classical statistical modelling.

JRP3-WP4-T2-ST1: Selection of test data set(s) to be used (M13-M14)

For testing of different ML methods we searched for suitable pilot datasets from the AMR research field. We selected suitable ML benchmark data sets, simulated data sets (in silico data sets) with known effect size, variability and interdependence (correlation), and AMR data sets provided by partners within RaDAR and other EJP projects. Some of these data AMR data sets will be provided in the future. Data sets were selected according to our needs: one well behaved data set, one misbehaved data set that exceeds the possibilities of current fitting routines. Both data sets are already analysed and published.

- Fromm et al., Prev. Vet. Med., 117, 2014 (fattening pig herds and MRSA)
- Hille et al., Pred. Vet. Med., (pig herds and ESBL)

JRP3-WP4-T2-ST2: Defining a work bench for assessing model (M15-M17)

Here we present a framework for building an ML model (fig. 6). This consist of different steps.

- a) Splitting data into training and test data.

In ML we have to somehow test or validate the predictive power of our created model. If we had a constant flow of new data at hand, the task would be easy to accomplish. Unfortunately, collecting data is usually complex and expensive, so we generate our own test data from the existing data set. To do this, we retain part of the data set for test purposes and create the model with the remaining data (training data).

- b) Calculating performance measures .

Unlike in the classical stat. modeling, we are not interested in how good the model fits the data. We are interested, how good the model **predicts** the data, especially new unseen data. That is why, we need measures that are able to grasp this information somehow. Most often, the accuracy, and the misclassification rate are used for that.

- c) Hyperparameter optimization.

Many machine learning models have parameters that have to be set before the model is created. These parameters are also called hyperparameter. For example, in the case of random forest models, hyperparameters include the number of trees formed and the number of randomly selected features with which each individual tree is formed. In the elastic net regularisation, the continuous hyperparameters are available for setting the effect strength of the regularisation. These hyperparameters can have a very strong influence on the performance of the model and must therefore be optimised.

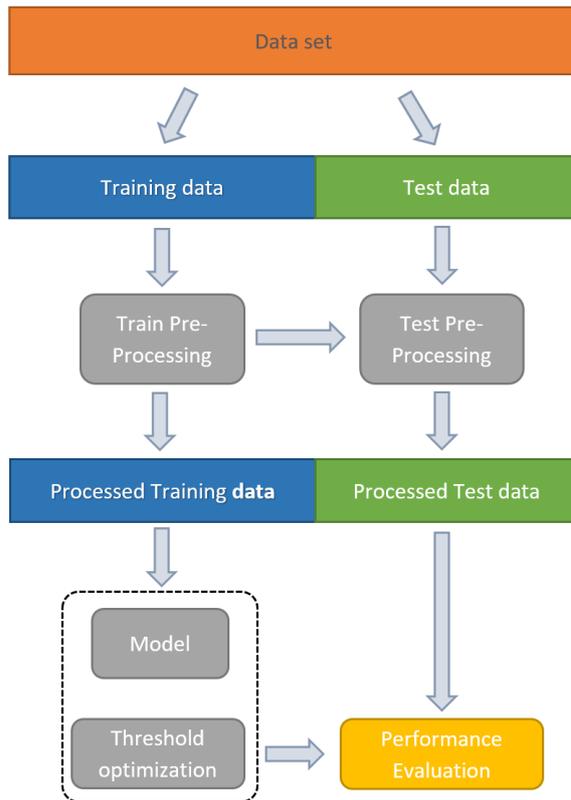


Fig. 6. General flow chart for creating and testing of a machine learning model. Here, only the scheme for one training/test split is shown, but to improve the assessment of the performance many (unique, non-repeated) splits are performed. E.g. for small data sets a leave-one-out cross-validation (or jackknife) is performed, where a single data point is separated from the data set for testing, and the rest of the data is used for training. Then, the training data is pre-processed, if necessary. The pre-processing for training is then used to pre-process the test data. With the pre-processed training data the trained model is built. Many machine learning models for classification do not produce a hard classification, but instead produce a probability, or a probability-like response. With the introduction of a threshold (often 0.5) a hard classification is performed. But it is sometimes an advantage to optimize the threshold, especially if the overall performance of the model is rather weak. Finally, with the model (and the optimized threshold) and the test data the performance measures a computed, and evaluated.

JRP3-WP4-T2-ST3: Model Analysis (M18-M21)

We compared the methods using real data sets (see above) that have already been evaluated by classical means in order to better explore the possibilities of machine learning for our purposes. Both datasets have a very different structure and in the corresponding papers various problems have been encountered during data analysis, which we would like to address with the methods we have chosen.

We concluded that regularized parametric algorithms would satisfy our needs. For the regularization we realized that the elastic net is the most appropriate approach. Three algorithms were selected: logistic regression with elastic net, linear discriminant analysis using the elastic net, and sparse distance weighted discrimination. The last two models need additionally a final probability calibration.

Ongoing: Model analysis has been completed but sensitivity and testing of model robustness ongoing

JRP3-WP4-T3: Literature review of methodologies and compilation of the selected methods (M22-M24)

Combined with T1.



JRP3-WP4-T4: ML and causality. Does it fit together? (M28-M36).

Not finished within the time-span of the RADAR project.

WP5: The burden of disease caused by AMR exposure (M1-M30)

We have modified these goals to take into consideration available personnel resources for WP5 and recent developments in the field. Instead of producing actual, EU-wide, estimates for the disease burden of infection with resistant forms of two bacteria, this task will now concentrate on developing a suitable methodological framework for computing resistance-attributable disease burden, and comparing this methodology against other approaches. We also included source attribution into this WP (redesigned T2).

JRP3-WP5-T1: Methodological framework for AMR burden (previously “Identify data gaps and define target questions for SEJ (Structured Expert Judgment)”)(M1-M12)

We designed a new burden of disease (BoD) approach suitable for estimating the *excess* BoD associated with AMR bacterial infection. By ‘excess BoD’ we mean mortality and morbidity (computed as DALY) associated with resistance, over and above the mortality and morbidity associated with the same – but antimicrobial-susceptible – bacterial infection (Fig. 7).

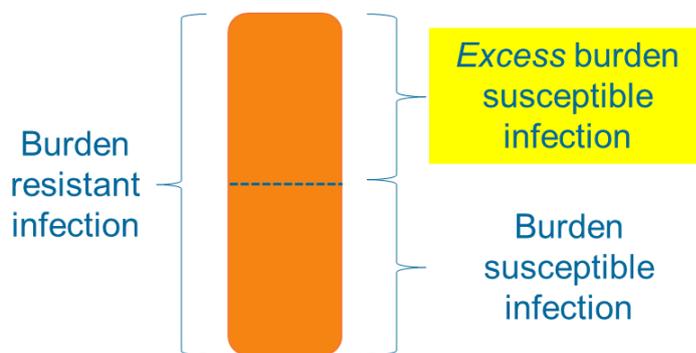


Fig. 7. The concept of the excess burden approach to AMR.

We illustrate this approach for a single infection site (urinary tract) and a single bacterial agent (*E. coli*; extended spectrum beta-lactamase [ESBL]-producing compared with non-ESBL producing *E. coli*). We designed a methodology approach for estimating the BoD of urinary tract infection caused by antibiotic-susceptible and resistant *E. coli*. We modify an existing outcome tree (OT) describing the clinical progression pathway for UTI, and describe the separate transition parameters, disability durations, and disability weights that are needed to fully quantify the health consequences of infection with both susceptible and resistant versions of the same bacterial agent (Fig. 8). We have chosen the DALY as composite measure of departure from ideal health, and adopt the ‘incidence-based’ approach rather than a ‘prevalence-based’ approach to DALY computation. To arrive at a valid estimate of the *excess* BoD of UTI attributable to AMR, we propose to use a counterfactual approach. This involves simply subtracting the total BoD that is expected for antibiotic-susceptible UTI (using the ‘susceptible’ version of the OT), for the same number of incident cases, from the BoD computed using the ‘resistant’ version of the OT. A basic assumption of this approach is that the resistant form of UTI will lead to greater BoD, which may be driven by greater mortality risk, longer disability durations, and higher risks of developing long-term sequelae. Note that an eventual further step is required, for the purposes of RaDAR, to distribute/attribute the estimated excess BoD to various transmission routes, including the food chain.

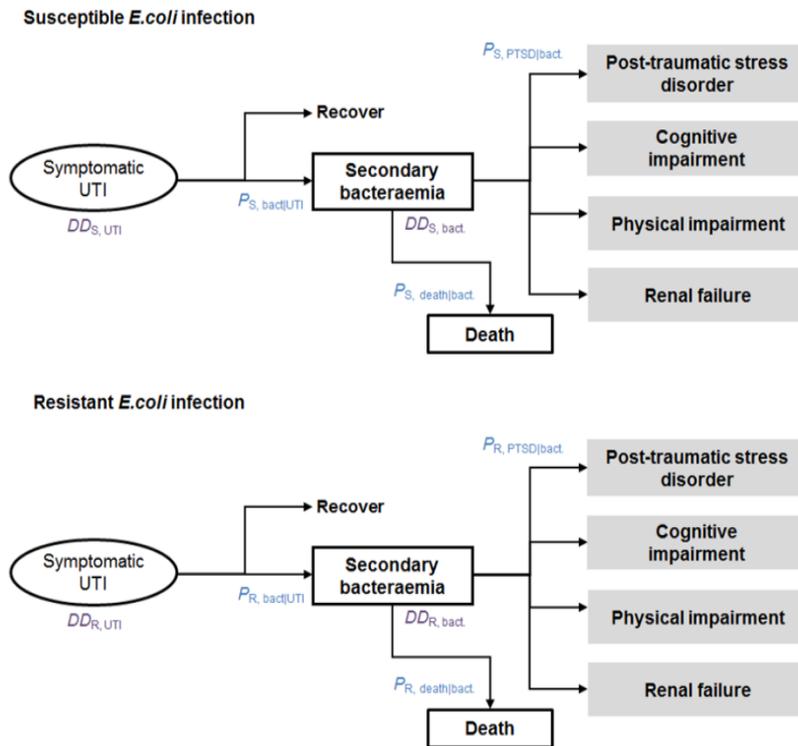


Fig. 8. Proposed outcome trees(s) for UTI, for antimicrobial-susceptible (upper panel) and antimicrobial-resistant (lower panel) infection. Transition probabilities (P) stratified by type of infection ($[S]$ usceptible or $[R]$ esistant) are indicated for several transitions, as are disability durations (DD).

JRP3-WP5-T2: Comparison of AMR burden methods (previously “Defining the seed questions”) (M1-M12)

We compared the proposed method for estimating AMR-attributable BoD with the ‘ECDC method’ (Cassini 2018) in terms of data needs, assumptions required, and limitations/points of concern. The principal difference between methodologies concerns the type of ‘input’ data. The proposed approach requires directly measured incidence; in contrast the ECDC method applies the Rhame-Sudderth formula to convert point prevalence and information on days spent in hospital to incidence. The second main difference between the two approaches concerns the pre-calculation of AMR attributable mortality and duration (i.e., LOS).

For valid estimation of BoD *attributable* to AMR, it seems clear that the relevant steps of the BoD methodology must correctly attribute morbidity and mortality to resistant infection only. Control groups on which the calculation of attributable risk/effect sizes (progression and mortality probabilities, disability durations) depends must represent only patients with susceptible infection, and not uninfected patients. A fundamental limitation of the ECDC method is that incidence is not measured directly; instead the Rhame-Sudderth formula is applied to convert point-prevalence to incidence. This formula can lead to over- or under-estimation of true incidence, and although frequently used, its non-established validity raises questions about conclusions drawn regarding absolute incidence (as opposed to trend analyses or comparisons across countries, for which the formula’s approximation to incidence appears more suitable). All methods suffer from confounding bias in the estimation of AMR-attributable burden, as patient characteristics (e.g., age, presence of co-morbidities, frailty, immunosenescence) can differ between those persons who acquire resistant form of bacterial infection and those who do not, and even if statistical adjustment could be straightforwardly integrated within the DALY computation, it would still be insufficient to correct for such differences due to unmeasured factors. Finally, when calculating attributable risks from longitudinal study data (for transition probabilities between health outcomes) or attributable mortality, it is vital to consider the impact of competing risks, such as discharge from hospital, death,



or development of a complication that precludes observation of the health outcome of interest. The potential for erroneous transition probability calculations (usually over-estimated) is particularly high for patient populations at high risk of such competing events. It is not clear whether the reviewed studies used as basis for the ECDC transitions take competing risks into account, but the proposed BoD approach will apply appropriate methods when inferring the transition probabilities.

JRP3-WP5-T3: Application of AMR disease burden framework to urinary tract infections (previously “Identifying, enrolling and interviewing the experts”) (M4-M30)

We illustrated the approach described above for a single infection site (urinary tract) and a single bacterial agent (*E. coli*; extended spectrum beta-lactamase [ESBL]-producing compared with non-ESBL producing *E. coli*). This has not yet been done for the Netherlands using national-level data and country-specific parameter values. To achieve the goal of producing the most accurate estimate possible, we use systematic review to determine parameter values whenever possible and obtain data on incidence from a nationwide AMR surveillance system.

Urinary tract infections (UTIs) are among the most frequent infections occurring in the community, hospital outpatient and inpatient settings. UTI is a common cause of sepsis in infants and accounts for up to 20–30% of all cases of sepsis, which is a life-threatening complication with a high mortality rate. As UTIs are frequently caused by AMR bacterial agents, a pressing research question is whether or not AMR urinary tract infections are associated with a higher BoD than AMS UTIs.

We performed a systematic literature search to locate parameter values for the risk of progression to bacteraemia, risk of progression to health states following bacteraemia, length of stay, other indicators of duration of illness and mortality risk. a second search was undertaken to locate relevant studies specifically informing the model parameters involving bacteraemia (i.e., $P(\text{Bact}|\text{UTI})$, $DD(\text{Bact})$, $P(\text{Death}|\text{Bact})$). A third systematic literature search was conducted to attempt to find relevant studies specifically to inform $P(\text{Bact}|\text{UTI})$, with restriction to studies of resistant *E. coli* UTIs.

We found an excess burden of 860 DALY, of which 98% was due to premature mortality. The sensitivity analysis, which indicated a 46% higher BoD when the mortality risk parameter was assumed to be age-independent, illustrates that for accurate BoD estimation stratified parameter values are vital. Parameter values obtained from a study population with a certain characteristic (e.g., older age) are not necessarily generalisable to strata of the population that do not match the study participants' profile. We were only able to crudely account for age-dependent mortality risk, and due to lack of suitable data, necessarily had to estimate the reduced risk for younger age-groups from a single, relatively small, national study of mortality due to bacteraemia caused by a different organism (*S. aureus*). The estimated 60 excess deaths in the sensitivity analysis do not appear realistic as an estimate for the annual AMR attributable-mortality among all patients with UTIs caused by ESBL-producing *E. coli* in the Netherlands, and are not consistent with other estimates of annual deaths due to resistant *E. coli* BSIs.

Our estimate of BoD among patients with resistant UTIs computed using the ECDC parameters is not comparable to the BoD for resistant UTIs as reported in Cassini et al. because of the very different approach of attributing BoD due to UTI and infections in other sites that progress to bacteraemia/sepsis to the BoD due to BSI. Namely, the BoD for the health outcome *symptomatic UTI* only was reported at 4.14 DALY (95% UI: 3.22-5.17)[1]. However, by making the assumption that the parameter value for $P(\text{Bact}|\text{UTI})$ for AMS UTIs from the literature source can also be applied to AMR UTIs, we could then calculate the resistant BoD by incorporating this parameter into the rest of the ECDC parameters, which then allows a more direct comparison to our estimates to be made. We found that this approach estimated an 3.3-fold greater BoD among patients with resistant *E. coli* infection compared with the Netherlands-specific model.



JRP3-WP5-T4: Source attribution of AMR for attribution of disease burden to sources (previously “Analysing the data to obtain aggregated responses to the target questions”) (M0-M30)

Although complex dynamics are involved in ESBL-EC and pAmpC-EC transmission, our results provide quantitative links between specific ESBL-EC and pAmpC-EC genes in the open community and their probable human and non-human sources of direct transmission. Approximately two-thirds of community-acquired ESBL-EC and pAmpC-EC carriers are attributable to human-to-human transmission, with the considered non-human sources accounting for the other third (Fig. 9). We also attributed the different ESBL genotypes to reservoirs (Fig. 10). While anthroponotic sources prevail, our findings underpin the need for longitudinal studies and warrant continuous monitoring in both human and non-human populations, because intracommunity ESBL-EC and pAmpC-EC spread alone seems unlikely to be self-maintaining without transmission to and from non-human sources. Transmission routes of antibiotic resistance are complex, with numerous interconnected cycles and subcycles involving different hosts and environments. Because resistant bacteria might pass into humans from animals and food, and via environment-mediated and human-to-human transmission, a One Health approach is needed that values interdisciplinarity and stresses the connections between public, animal, and environmental health, and provides an integrated framework for improving our understanding of the global threat of antimicrobial resistance.

This work was published in Lancet Planetary Health (Mughini-Gras et al. 2019 3:357-369).

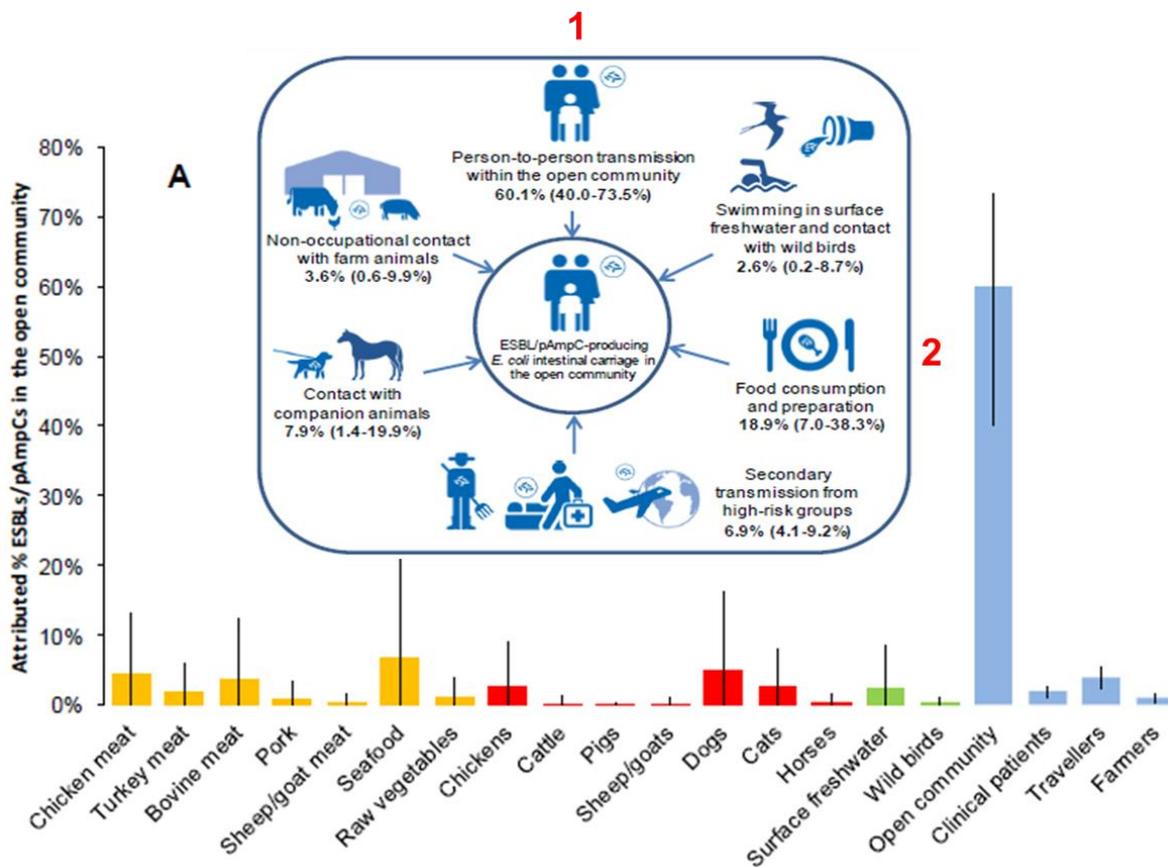


Fig. 9. Summary results of ESBL source-attribution

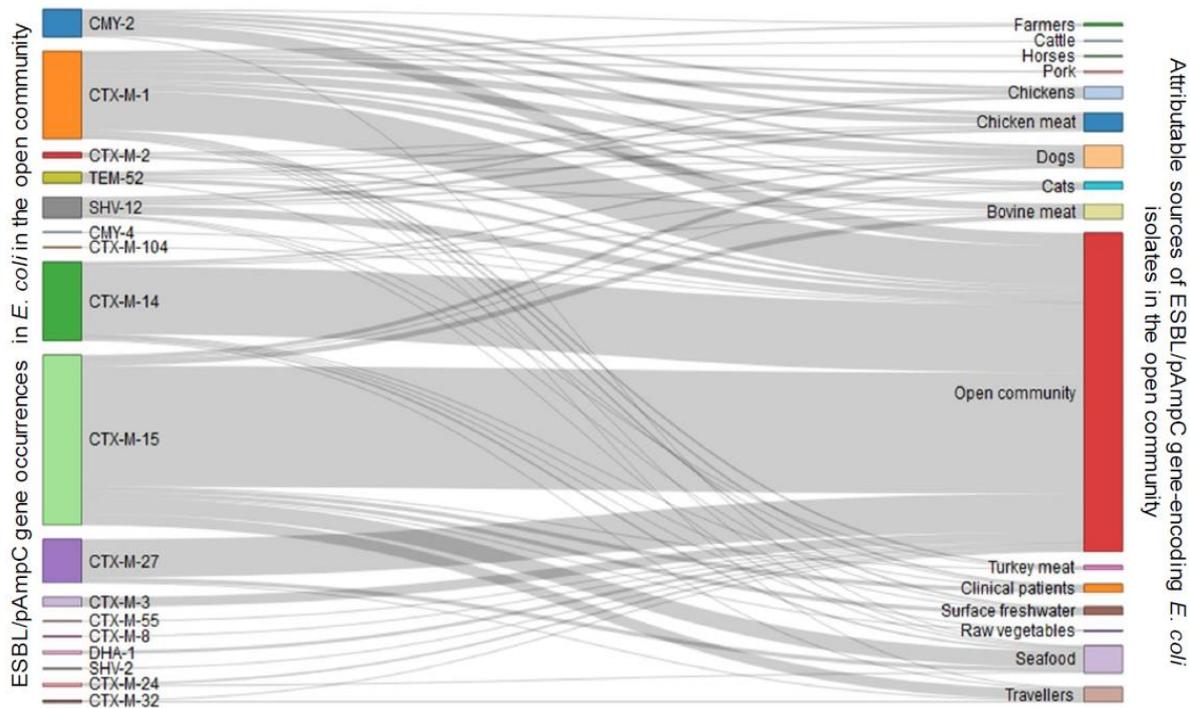


Fig. 10. Attribution of different ESBL genotypes to reservoirs.

EXTRA: JRP3-WP5-T5: A new paradigm for AMR surveillance based on metagenomics (M18-M30)

In this study we recovered and shotgun-sequenced caecal samples collected from pigs under the Danish monitoring of antimicrobial resistance (AMR) over two time periods (1999-2004 and 2015-2018), and compared this to gathered phenotypic results and antimicrobial use (AMU) data for the same time periods. The aim was to compare findings obtained with the present methods employed in AMR surveillance (phenotypic testing of indicator- and pathogenic-bacteria), with a new surveillance paradigm - a metagenomics-based surveillance of AMR determinants. We showed that metagenomic surveillance is a suitable methodology for population based surveillance of AMR and observed that AMU and AMR are in general correlated both at phenotypic and genotypic levels, but also that other factors play a role in the abundances of AMR.

WP6: Integration of information by Bayesian evidence synthesis (M1-M30)

We applied the Bayesian Evidence Synthesis (BES) approach to estimate human carriage of ESBL-producing *E. coli* (ESBL *E. coli*) in the Netherlands that is attributable to pork. BES is a statistical modelling approach that can make use of all available information, by combining all relevant data with a priori knowledge regarding model parameters, and is suitable for estimation of parameters such as prevalence or incidence for which no direct measurements exist, but which may be indirectly informed by other data sources. This approach importantly recognises all sources of uncertainty in the data sources contributing to the uncertainty of parameters of interest. As in meta-analysis, multiple sources of data informing the same parameter are naturally weighted by their accuracy; as a corollary, all data sources contributing information to a given parameter are also essentially weighted by precision. The ultimate aim of implementing a full BES network would be to eventually integrate information from all exposure pathways and all health effects and in multiple countries, but under the lights of the pilot project that RaDAR conforms, to make the initial problem tractable we focused only on ESBL *E. coli* in humans attributable to pork consumption in the Netherlands.

JRP3-WP6-T1: Collect current status data (M1-M6)

We made a descriptive overview of available Dutch data relevant for risk assessment and epidemiological calculations. For risk assessment, this will comprise of ESBL *E. coli* prevalence data but



also data that describe the human exposure intensity to ESBL *E. coli*. For epidemiology, data relate to the distribution of ESBL genes of plasmids in the human population and in the respective reservoirs. Also, risk factors and epidemiological metadata are described.

JRP3-WP6-T2: Build evidence synthesis network for current status database (M1-M12)

The full QMRA pig processing model consists of a primary production farm model (WP2), and a slaughter process model, a consumption phase model, and a dose-response model (WP3), which are described more in detail in their corresponding work package reports. In here we will briefly explain how they are interlaced in the network and then joined together with the EPI model. The purpose from this report is to highlight the most prominent findings after successfully managed to implement the full BSE network. A manuscript describing this study in detail is currently being written (Bonacic Marinovic et al., in preparation) and will be submitted for publication during 2021, together with a manuscript resulting from the task from WP3.2, where the slaughter model and the consumer behaviour model, both part of the QMRA submodel, are also described in detail. We built up a prototype evidence synthesis network for the pork chain as a multi-level bayesian model, where parameters have initial prior distributions whose parameters in turn have prior distributions. This allows to manage the level of desired uncertainty and also to find the most representative values for some priors, e.g., uncertainty in the estimated average values on top of the spread around that estimated value. The code for carrying out the calculations is being written in R for JAGS. As first step we constructed a network inspired on the analysis carried out by Evers & Bouwknecht (2016) and using data there in found. By means of the QMRA analysis there and Markov chain Montecarlo simulations, we calculate posterior distributions for doses to which the population is exposed yearly. Information on the distributions is given as averages and for our code we introduced poisson and normal spreads as first attempt to deal with point estimates. We used a dose-response relation model based on a Beta-Poisson function, where the function parameters are loosely constrained and informed by data from challenge studies. From the Epidemiology side we employed information on duration of carriage (distributions from Teunis et al., 2018) and ESBL carriage attribution to pork (expert opinion) to estimate distributions of incidence per year and prevalence. We joined the two approaches by using the so called zero-crossing method, where the estimated incidence of carriage from both QMRA and Epidemiology approaches is forced to be the same within a given standard deviation. So far, we identified ESBL carriage attribution to pork and the dose-response relation as the culprits of a difference of three orders of magnitude between the posterior distributions for yearly ESBL carriage as calculated independently by the Epidemiology approach and by the QMRA approach.

JRP3-WP6-T3: Update evidence network for information developed in the other work packages (M12-M18)

The results from JRP3-WP3-T2-ST2 were implemented in the evidence synthesis network. The other exposure assessments from WP3 will undoubtedly be valuable for future work. The evidence synthesis framework links with JRP3-WP2-T1-ST3 (development of an on-farm transmission model for ESBL *E. coli* in pigs) and JRP3-WP3-T2-ST2 (development of a model for the pig production chain and consumption phase). Here, the farm models of WP2 was coupled to the pig processing model and consumer phase model is nearly completed, with some final improvements being implemented. Epidemiological inputs to the model were finalized.

JRP3-WP6-T4: Define endpoint for the current project and report results for the evidence synthesis model in its endpoint state (M18-24)

The result of the final model is depicted in Fig. 11, where now the predicted incidences for ESBL *E. coli* for the QMRA and EPI model are very close together. Also, the combined model has a smaller credible interval than both submodels, meaning that precision of the prediction was improved due to the evidence synthesis of the submodels. The final prediction is 5249 (credible interval: 983-24852) ESBL *E. coli* cases due to consumption of pork per year.

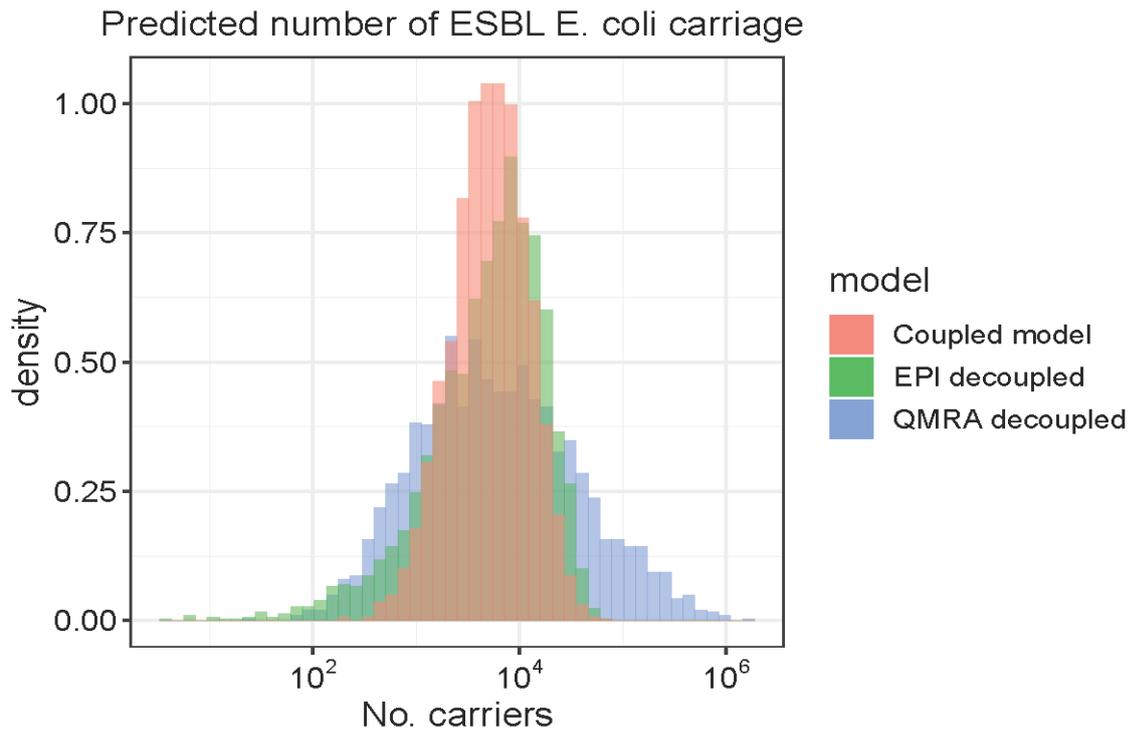


Fig. 11. Predicted number of ESBL E. coli carriers, for the EPI submodel (green), the QMRA submodel (blue) and the coupled model (red).

We presented models for carriage of ESBL producing E. coli in humans. On the one side we have an epidemiological submodel, based on cross-sectional and longitudinal studies. On the other side we have a risk-assessment model, based on a farm-to-fork QMRA approach. Both models are supposed to estimate the same quantity: human carriage. We applied a Bayesian evidence synthesis approach to combine the submodel, integration all data sources in one overarching model.

One major advantage of this approach is that variability and uncertainty are separated, meaning that we get insight in where additional data collection is warranted.

Furthermore, we have shown that it is possible to assess consistency of data sources. In particular, we identified one data source in the cross-sectional data which was inconsistent with the others. Upon closer inspection, this data pertained to farmers, an occupational group for which higher ESBL carriage prevalence is not unexpected as compared to the general population.

Another benefit of the approach is detection of parameters that need a great change to accommodate the joining of the submodels. In other words: the prior (i.e. our knowledge of the parameter value before the model is applied) is very different from the posterior (i.e. our updated estimate of the parameter). We made this explicit by running two scenarios. The first scenario was to deliberately use an incorrect dose-response relation for illness instead of carriage. The second scenario consisted of the correct dose response for carriage only. In the incorrect scenario, we observed that the dose-response parameters shifted far from their initial value to accommodate the model joining. In the second scenario, parameters were hardly affected, and joining of the epidemiological and QMRA models resulted in a more precise consensus estimate than either of the individual models.

So, not only did we obtain more reliable estimates of incidence of carriage, but we also unlocked a toolbox for assessing model and data reliability. A paper detailing the approach is currently being written for submission to a scientific journal.



3. Project self-assessment

Antimicrobial resistance threatens the effective prevention and treatment of an everincreasing range of infections. It is an increasingly serious threat to global public health that

requires action across all government sectors and society. Assessment of the importance of different transmission routes and quantifying public health effects (i.e. disease burden) associated with AMR represent major knowledge gaps. The current knowledge on public health risks and burden of AMR is limited due to the complex nature of the system (humans/food/animal/environment, relation between antimicrobial use and development of resistance, complex multi-directional transmission routes, and divers effects on human health).

The key objective of RADAR was (copied from the project proposal) to systematically integrate top-down epidemiological and bottom-up risk assessment information from diverse modalities to obtain best estimates for attribution, risk factors and health effects as information is collected.

More specifically to objective were:

1. To develop bioinformatics tools to analyses (meta-)genomic data and retrieve information relevant for AMR and its transmission
2. To produce models regarding AMR development, transmission
3. To develop machine learning approaches for risk assessment
4. To fill data gaps by structured expert judgement
5. To produce a Bayesian evidence synthesis of all available data and information in order to come to consensus estimates on source attribution, risks of exposure and health effects.

To that end a diverse group of international experts in epidemiology, molecular biology, transmission modelling, risk modelling, and disease burden modelling was brought together in the RADAR consortium. This was very much a success since corporation went very natural and all members were highly motivated. Little pressure by the project management had to be carried out.

The radar project deviated from the original plan regarding WP5 (burden of disease). Originally we aimed at applying an established method using expert elicitation for burden estimates. This was redesigned to the development of new method where we estimated the excess burden of resistant infection and applied this this to urinary tract infections. In addition we added a new method for AMR source attribution (applied to ESVL E. coli) where we included humans as a possible sources (which is not done in source attributions of zoonotic pathogens). Finally we added a pilot study into the use of metagenomics for AMR surveillance. See fig.12 for an overview of the redesign of WP5.

WP5 old	WP5 new
T-5.1. Identify data gaps and define target questions for SEJ	T5.1 methodological framework for AMR burden
T-5.2. Defining the seed questions	T5.2 Comparison of AMR burden methods
T-5.3. Identifying, enrolling and interviewing the experts	T5.3 Application of burden framework to UTIs
T-5.4. Analysing the data to obtain aggregated responses to the target questions	T5.4 Source attribution of AMR for attribution of disease burden to sources
	T-5.5. Propose and assess a new paradigm for AMR surveillance in pigs.

Fig. 12. Overview of the redesign of WP5 taks.

The RADAR project delivered beyond the state-of-the-art by:

- producing a new curated database of (AMR) plasmids
- development and coupling of models for on-farm development and spread of AMR
- producing new risk assessment models for AMR in different food chains
- defining and testing frameworks for machine learning methods for AMR risk assessment



- developing a new method for specific AMR burden of disease
- developing and applying a new source attribution approach for AMR
- investigating the usefulness of metagenomics for AMR surveillance
- developing and applying methodology to integrate various sources of AMR data in order to come to consensus estimates on exposure and risks of carriage.

In addition the RADAR project published in respected journals like The Lancet Global Health and Frontiers in Microbiology. At least 7 other publications are in preparation.



4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-0.1	First annual report	M12	M18			
03	D-JRP3-0.2	Second annual report	M24	M36			
03	D-JRP3-0.3	Third annual report	M30	M36			9
03	D-JRP3-1.1	Establishment of a database of synthetic, reference and genomic data	M12	M20		https://zenodo.org/record/4476359#.YBLXYfZFw2w	
03	D-JRP3-1.2	Establishment of a database of field (meta-)genomic data	M15	M18		https://zenodo.org/record/4476374#.YBLYzvZFw2w	



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-1.3	Automated assembly pipeline integrating de novo plasmid reconstruction	M12	M24		https://zenodo.org/record/4476381#.YBLZuvZFw2w	
03	D-JRP3-1.4 (AWP2020)	Test and parameterization of the assembly pipeline for metagenomics data	M24	M24		https://zenodo.org/record/4476406#.YBLai_ZFw2w	
03	D-JRP3-1.5 (AWP2020)	Biological annotations of plasmid identified in the pipeline	M24	M24		https://zenodo.org/record/4476436#.YBLbc_ZFw2w	
03	D-JRP3-1.6 (AWP 2020)	WGAS-based method for genomic data analysis	M30	M36		https://zenodo.org/record/4476483#.YBLcQ_ZFw2w	2



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-1.7 (AWP 2020)	Development of regression model for genomic data analysis	M30	M36		https://zenodo.org/record/4476483#.YBLcQ_ZFw2w	9
03	D-JRP3-1.4.3 (AWP2020)	Integration of genetic traits associated to AMR and plasmid content information into models (WP6)	M30		M36	Cancelled (COVID and lack of capacity)	9
03	D-JRP3-2.1	Report on Pharmacodynamics and transmission models	M30	M36		https://zenodo.org/record/4478642#.YBPwkvZFw2w	8
03	D-JRP3-2.2	Report - A risk assessment to predict	M30	M36		https://zenodo.org/record/4478651#.Y	8



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
		the sustainability of ESBL-producing E.Coli carriage within commercial pig farms				BPxH_ZFw2w	
03	D-JRP3-2.3	Report - The relevance of transmission routes of antibiotic resistant bacteria calculated using different methodologies and the relevance of routes	M30	M27		https://zenodo.org/record/4476487#.YBLizfZFw2w	8
03	D-JRP3-2.4	Report -Report on intervention strategies	M30	M36		https://zenodo.org/record/4478291#.YBPDMfZFw2w	8



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-3.1	Inventory with models and related data in FSK standard	M30	M36		https://zenodo.org/record/4476609#.YBL0OvZFw2w	3
03	D-JRP3-3.2	Scientific report on a generic model for the chicken production chain	M30	M36		https://zenodo.org/record/4476623#.YBLpl_ZFw2w	8 or 9
03	D-JRP3-3.3	Scientific report on an adapted model for the pork production chain developed	M30	M36		https://zenodo.org/record/4476637#.YBLrD_ZFw2w	8 or 9
03	D-JRP3-3.4	Scientific report on an model for the exposure assessment of AMR through mussels	M30	M36		https://zenodo.org/record/4476661#.YBLsz_ZFw2w	8 or 9



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-3.5	Scientific report on a generic comparative exposure assessment model	M30	M36		https://zenodo.org/record/4476674#.YBLuTfZFw2w	8 or 9
03	D-JRP3-3.6	Comparative Exposure Assessment of ESBL-Producing Escherichia coli Through Seafood Consumption	NEW	M36		https://zenodo.org/record/4476684#.YBLvYPZFw2w	8 or 9
03	D-JRP3-4.1	Model repository of state of the art ML methods for risk profiling available	M12	M36		https://zenodo.org/record/4476722#.YBLxoPZFw2w	8 or 9
03	D-JRP3-4.2	Recommended methods for risk profiling in investigations on antibiotic resistance	M30	M36		https://zenodo.org/record/4476722#.YBLxoPZFw2w	8 or 9



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
		available					
03	D-JRP3-5.1	The 'excess burden' approach for computing the burden of disease attributable to AMR: Application to urinary tract infection	M30	M27		https://zenodo.org/record/4476732#.YBLyrPZFw2w	9
03	D-JRP3-5.2 (NEW)	A proposal for a new paradigm for AMR surveillance (NEW)	M30	M36		https://zenodo.org/record/4476751#.YBLzY_ZFw2w	9
03	D-JRP3-5.3 (NEW)	Attributable sources of community-acquired carriage of Escherichia coli containing β -lactam antibiotic resistance genes	M36	M36		https://zenodo.org/record/4476761#.YBL1ffZFw2w	9



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories * (1 to 8) <i>(several categories may be applicable)</i>
03	D-JRP3-5.4	Report on structured expert judgement				Cancelled (redesigned WP5; see D D-JRP3-5.1)	
03	D-JRP3-6.1	Publication on final evidence network	M30	M36		https://zenodo.org/record/4476793#.YBL3QvZFw2w	8
03	D-JRP3-6.2	Policy-targeted report	M30		TBC	COVID delay – will be completed first half 2021	9

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M-JRP3-1	Kick-off meeting and report	M1	yes		
03	M-5 .1	Identification data gaps and SEJ (Structured Expert Judgment) target questions	M3	yes		
03	M5.2	Defined seed questions	M6	Yes		
03	M-JRP3-3.1 (See FP)	Complete literature reviews of previous PK/PD and on-farm models (ANSES, APHA)	M5	yes		
03	M AMR3.11 (See FP)	Complete literature reviews of AMR transmission modelling (CVI, NCOH, RIVM)	M5	Yes		
03	M-JRP3-6	Database of available data	M6	yes		
03	M AMR3.2 (See FP)	Develop model frameworks for PK/PD and on-farm model (ANSES, APHA)	M8	yes		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M-JRP3-8	Mid-term meeting and report	M12	yes		
03	M-3.1	Structure for inventory of models and related data developed	M12	Yes		
03	M 4.1	Repository filled with models on Github	M12	Yes		
03	M6.1	Database of available data	M6	yes		
03	M6.2	Functional consensus evidence network	M12	yes		
03	M-AMR3-1.2.1 (see AWP2020)	Test and validation of assembly pipeline on synthetic and reference genomic data	M24	Yes		
03	M-AMR3-1.2.2	Analysis of HTS field data with assembly pipeline	M24	yes		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M-AMR3-1.4.1	Analysis of field genomic data with WGAS-based method	M30	Yes		
03	M-AMR3-1.4.2	Analysis of field genomic data with regression model	M30	Yes		
03	M AMR3.2	Develop model frameworks for PK/PD and on-farm model (ANSES, APHA)	M8	yes		
03	M AMR3.3	Produce case study (collected datasets) baseline results for PK/PD (ANSES)	M15	yes		
03	M AMR3.4	Establish connection between the PK/PD model and the on-farm-model (ANSES, APHA)	M24	yes		
03	M AMR3.5	Establish connection between the PK/PD model and the on-farm-model (ANSES, APHA)	28	YES		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M AMR3.6	Simulations of the PK/PD model for green AMDs (ANSES)	M28	YES		
03	M AMR3.7	Produce case study baseline model results for on-farm model (APHA)	M28	yes		
03	M AMR3.8	Structural adaptations of the PK/PD model according to AMR mechanisms (ANSES)	28	YES		
03	M AMR3.9	Assess hypothetical intervention measures using on-farm model (APHA)	M30	Yes		
03	M JRP3.10	Data gaps in PK/PD modelling and propositions of experimental studies with a defined methodology (ANSES)	M30	YES		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M-AMR3.12	Formulate CM/NGM (CVI, NCOH) and SD (RIVM) transmission models	M24	Yes		
03	M-AMR3.13	Calibrate models using data library (CVI)	M24	Yes		
03	M-AMR3.14	Formulate/investigate interventions (RIVM, NCOH, CVI, BfR43)	28	Yes		COVID delay and granted extension
03	M-AMR3.15	Assimilate project-generated data (NCOH)	28	Yes		COVID delay and granted extension
03	M-AMR3.16	Make recommendations to fill data gaps (RIVM, NCOH, CVI)	M30	Yes		COVID delay and granted extension
03	M-JRP3-34	Final meeting and report	M30	Final report: Yes		COVID delay and granted extension



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
				Final meeting: TBC		
03	M-3.2 (See AWP2020)	Concept for an improved model for the chicken production chain developed	M24	Yes		
03	M-3.3	Concept for an adapted model for the pork production chain developed	M24	Yes		
03	M-3.4	Concept for an model for the exposure assessment of AMR through mussels	M24	Yes		
03	M-3.5	Concept on a generic comparative exposure assessment model	M27	Yes		
03	M4.2	Model development and model assessment completed	M21	yes		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M 0.2.2	Final meeting	M30	NO	M36	COVID delay and granted extension



5. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Analysis of COMPASS, a New Comprehensive Plasmid Database Revealed Prevalence of Multireplicon and Extensive Diversity of IncF Plasmids https://doi.org/10.3389/fmicb.2020.00483 https://zenodo.org/record/3968418#.XyQSQTgUIM2	YES		
Attributable Sources of Community-Acquired Carriage of Escherichia Coli Containing β -Lactam Antibiotic Resistance Genes: A Population-Based Modelling Study https://doi.org/10.1016/S2542-5196(19)30130-5 https://zenodo.org/record/3621285#.Xjl8UGhKhPY	YES		

The following manuscripts are in preparation:

- “Benchmarking of plasmid prediction tools to explore the plasmidome of *Salmonella enterica*” (ANSES)
- “A risk assessment to predict the likelihood of ESBL-producing *E.coli* carriage in slaughter-aged pigs following bacterial introduction onto commercial pig farms: an interdisciplinary approach combining a within-host and a farm model” (APHA/ANSES)



- “The relevance of transmission routes of antibiotic resistant bacteria calculated using different methodologies and the relevance of routes per pathogen: a systematic review and meta-analysis “(NCOH)
- “Machine learning methods for qualification of AMR related risk factors and health effects” (BfR)
- “Defining a methodological framework for estimating AMR related excess disease burden” (RIVM/NCOH)
- “Integration of risk assessment and epidemiology by Bayesian evidence synthesis: a case for ESBL-E. coli attributable to pork” (RIVM)
- “When the heat is on enough: modelling the persistence of ESBL-producing *E. coli* in blue mussels under meal preparation” (NVI)

Additional output:

Due to the corona-crisis little scientific dissemination activities were undertaken. Congresses were cancelled and sparse time available was dedicated to the progress of the project.



6. One Health Impact

This project achieved its goals in a multi-disciplinary and One Health approach with partners of different expertise (microbiology, epidemiology, risk assessment) and from different domains (public health, veterinary health, food safety). RADAR delivered some interesting and potential useful tools and insights.

- We delivered a large-scale curated database of (AMR) plasmids from a range of different bacterial species and sources (“COMPASS”, Dourarre 2020 *Frontiers in Microbiology*), This novel resource will help researchers understand the genetic plasticity and transmission routes of plasmids, which are crucial in the fight against the spread of antimicrobial resistant pathogens. **Moreover this will be potentially of use in future AMR risk assessment model that may integrate genetic data.** The database is available at <https://github.com/itsmeludo/COMPASS>
- We provided an infrastructure for exchanging and annotating risk assessment models in an exchangeable and reproducible file format called FSK and key or desirable features that facilitate access and usability of the inventory (<https://ejp-radar.eu/>). **This will be helpful to the risk assessment community since it makes risk assessment models more transparent and re-useable (with parametrisation based on the local situation/data).**
- We produced state-of-the-art AMR risk assessment models for different food chains in a generic framework. These generic methods may be more crude, but will allow for combining the risks in the different (sub-)categories and may thus help to create a more complete picture of the AMR problems throughout. **This may be helpful to national and supra-national institutes to rank food related AMR (and pathogen) related risks.**
- We produced a framework for the use machine-learning methods in AMR risk assessment in order to identify risk factors from high-dimensional data with more variables than data points and/or categorical features with many classes. **This may be very useful for future risk assessment that will likely integrate more and more diverse data.**
- We designed a new burden of disease (BoD) approach suitable for estimating the excess BoD associated with AMR bacterial infection. By ‘excess BoD’ we mean mortality and morbidity (computed as DALY) associated with resistance, over and above the mortality and morbidity associated with the same – but antimicrobial-susceptible – bacterial infection. **The method will help to add the burden of AMR to a larger set of infectious disease burden estimates used by policy makers to set priorities.**
- We produced a One-Health source attribution model that estimates the relative contribution of reservoirs and transmission routes to AMR (ESBL E. coli) casrriership in the population. We adopted established source attribution methods for zoonotic pathogens for specific use for AMR (mainly by introducing humans not only as an endpoint but also as a source). **This method can be used to help policy makers in ranking and setting priorities regarding intervention strategies.**
- We studied a new paradigm for AMR surveillance based on metagenomics where we showed that metagenomic surveillance is a suitable methodology for population based surveillance of AMR and observed that AMU and AMR are in general correlated both at phenotypic and genotypic levels, but also that other factors play a role in the abundances of AMR. **This will be of interest to public health and veterinary institutes looking for all-in-one surveillance methods.**
- Finally, we developed and applied a Bayesian Evidence Synthesis (BES) approach to integrate all available data and information by combining all relevant data with a priori knowledge and



thereby eventually infer estimates on prevalence/incidence of AMR infections / carriership. **This approach will be very helpful in providing policy makers with consensus estimates based on all available data.**

Now that the scientific work of RADAR is completed we aim at having a **supranational stakeholder (EFSA, ECDC) meeting** early 2021 in order to disseminate our results and scout the possibilities for further use of our work. In addition, several consortium partners have been in interaction with their **local governments** regarding the use of the developed methods and gained knowledge. An example is the interests of the Dutch Ministry of Health in applying the AMR burden methodology more broadly to AMR infections in order to include them in the national burden comparisons aiming at setting intervention priorities.

JRP04-MADVIR

This project came to an end in December 2019, and it was reported in the “D3.11-Second periodic report on ongoing JRPs”, March 2020.

JRP05-TOXDETECT

1. Summary of the work carried out in year 3

The third meeting of the Tox-Detect project has been organized on January 15-16, 2020. After general information dedicated to architecture of the EJP, elements of reporting and budget, a focus on the work package progress has been discussed.

Among hot topics, the consortium decided to launch MALDI-ToF library implementation at NVI (Norway). Moreover, the WP5 and inter-laboratory comparison tests organisation were discussed according to the progress of technical work packages, WP1, WP3 and WP4.

Also, expected times of WP2, WP3 and WP4 deliverables were updated due to technical issues. Consequently, the impact on the expected deadline of WP5 deliverables has been discussed as this WP is directly related to WP3 and WP4 outcomes.

Finally, 6-months extension period for Tox-Detect project was discussed and proposed in order to achieve the deliverables with high quality.

Due to COVID-19 crisis, Tox-Detect consortium studied the impact on WP and especially cases of short contract and technical WP as laboratory activities were strongly limited.

Even if some delays had been observed in the project, the managerial structure remains confident in the positive outcomes of this project for the last year.

2. Work carried out in the JRP. scientific results

WP0: Coordination, management and communication (M0-M42)

JRP5-WP0-T1: General coordination and management of the project (administrative and financial) (M0-M36)

The overall purpose of the management structure is to ensure the timely implementation of the tasks and the smooth running of the project as a whole. Its primary goal is to identify arising opportunities and detect the occurrence of obstacles as early as possible, hence maximise the outcome of the project while preventing delays in its implementation. This will ensure that all tasks and research objectives are performed in due time.

General meetings :



- Third General meeting 15 and 16 January 2020
- Additional general meeting (web conference) on 21,22 and 28 October 2020

Specific meetings

- TC WP1 on 28 January 2020
- Face to Face meeting on WP2 14 February 2020
- TC WP3 on 24 February 2020 about organisation
- TC WP2 on 20 March 2020
- TC WP0 with all partners about the work program impact due to COVID-19 crisis on 10 June 2020
- TC WP4 on 16 June 2020
- TC WP3 on 02 July 2020 : organization and management of WP3
- TC WP1 and WP5 on 17 July 2020 about Maldi-ToF reference spectra library transfer
- TC WP0 on 21 August 2020 with OHEJP WP3 coordination, dedicated to the feedback of REA on the 9M report (year 2020)
- TC WP4 and WP5 on 02 September 2020 about antibody quality and Inter-Laboratory Comparison tests (ILC) dedicated to Immuno Enzymatic methods.
- TC WP2 on 16 September 2020 about supernatant transfer between partners to perform toxicity tests
- TC WP1 and WP5 on 18 September 2020 about MALDI-ToF library transfer
- TC WP3 on 06 October 2020 about Mass spectrometry (MS) method development
- Web meeting WP2 on the preparation and progress of WP2 deliverables

Web meeting WP5 on 1st December 2020 about the organization of ILC (training session)

JRP5-WP0-T2 to JRP5-WP0-T5: Organisation of four face-to-face meetings with all partners (M0-M36)

23 participants representing all Tox-Detect partners were present during the 2020 general meeting (on 15 and 16 January 2020). C Cordevant and A Callegari were invited by the coordination of Tox-Detect Project as SSB member and general coordination, respectively. All participants presented their institutions, activities and involvement in the Tox-Detect project.

The meeting was split into 1.5 days. General discussion dealing with EJP projects took place after the presentation of A Callegari from EJP general coordination. Briefly, he presented Guidelines, specific rules, budget, communication tools and spoke about the possible 6-month extension. A Callegari also highlighted the need to be present in the ASM meeting which would take place in May in Prague (CZ).

Due to the extension of the project until June 2021, ToxDetect coordination implemented an additional general webmeeting (on 21, 22 and 28 October 2020) gathering 20 participants representing all Tox-Detect consortium. R. El Mounaged was invited as EJP general coordination member. All participants presented their WP progresses, future work, schedule and dissemination activities. Methods and strains sharing were discussed and it was decided to share a table summing up the strains requested by each partner and a SOP template. This task was done on December 2020. Also, submission period of deliverables and ILC organisation schedule were updated according to Covid 19 crisis impact and the situation in each partner laboratory on the second semestre of 2020. At the end of the project, it was concluded to organise first a specific meeting for each WP (with only the WP partners) followed by a general meeting to present all the results. Tox-Detect partners were called to register all their dissemination actions on the OH EJP website.



JRP5-WP0-T6: Mandatory reports on network activities: interim activity report, final report (M0-M36)

This task is in progress:

In 2020, 9M report was submitted on september 2020

3rd Year report (this report) is expected on 6 January 2021.

WP1: Constitution of a reference strain collection for *S. aureus*, *B. cereus* and *C. perfringens*

JRP5-WP1-T1: Constitution of *S. aureus* strains collection (M1-M3)

Done. Exchange of strains from the collection between partners is planned. A due Diligence process regarding Nagoya protocol is underway. Focal points of countries, parties of Nagoya protocol, have been contacted to request a PIC and a MAT for the access and use of the strains. Strain transfer is in progress between different partners.

JRP5-WP1-T2: Constitution of *B. cereus* strains collection (M1-M3)

Done. Some *Bacillus cereus* strains proposed by INRAE have been added to the strain collection previously established by partners. Exchange of strains from the collection between partners is planned. A due Diligence process regarding Nagoya protocol is underway. Focal points of countries, parties of Nagoya protocol, have been contacted to request a PIC and a MAT for the access and use of the strains. Strain transfer is in progress between different partners.

JRP5-WP1-T3: Constitution of *C. perfringens* strains collection (M1-M3)

Done. Exchange of strains from the collection between partners is planned. A due Diligence process regarding Nagoya protocol is underway. Focal points of countries, parties of Nagoya protocol, have been contacted to request a PIC and a MAT for the access and use of the strains. Strain transfer is in progress between different partners.

JRP5-WP1-T4: Transfer of libraries of MALDI-ToF reference spectra (M3-M3)

Due time of this task was revised, see report of year 2

The MALDI-ToF reference spectra library has been completed on December 2019, see deliverable D1.2.

Technical and data processing transfer has been discussed with partners for an export planned on March 2020 before organization of a dedicated PT trial (cf WP5). Due to COVID-19, the transfer has been done on October 2020. NVI is implementing the method in order to organise the inter laboratory test on 2021.

WP2 :Characterization of toxins/virulence factors

JRP5-WP2-T1: Characterization of candidate toxin and/or virulence genes using toxicity tests (M4-M24)

The growth conditions were defined and optimized for both *B. cereus* and *C. perfringens*. The corresponding SOP are written. The methods for cytotoxicity testing and High Content Analysis (HCA) tests were developed. The SOPs are also available. The development of these methods an element of the deliverable D2.1 (Report on results from toxicity assays (classical toxicity tests and High Content Analysis), that is currently being prepared.

Bacillus cereus

All *Bacillus cereus* supernatants were tested for toxicity by MTT analysis on Caco2 cells. Due to significant differences in cytotoxicity of certain strains of *B. cereus* compared to results generated from a previous project, it was asked that ANSES and Institut Pasteur prepare new productions of bacterial culture supernatants. These supernatants have been tested for toxin production before shipment to



ANSES-Fougères for cytotoxicity assays. As a result of the COVID-19 crisis, ANSES and Institut Pasteur laboratories were closed from March to June of 2020 delaying the evaluation of toxicity. New supernatants from *B. cereus* strains were received at ANSES in September 2020, and are currently being tested for cytotoxicity at ANSES Fougères. Results from toxicity testing are expected in the beginning of 2021. Based on results from toxicity testing (cytotoxicity and pro-inflammatory effects), a selection of strains will be tested in further detail using a HCA approach. Three to four months will be required to complete these HCA assays.

Clostridium perfringens

Supernatants for the 3 CPE+ and one CPE- selected strains from the collection of ANSES (sequenced for RNA-Seq study) have been sent to Anses Fougères to be tested for cytotoxicity. Three independent productions of supernatants from vegetative and sporulating phases have been tested for cytotoxicity and IL-8 production. In September 2020, Institut Pasteur sent supernatants from their collection to ANSES-Fougères for toxicity testing and HCA assays. Results from toxicity testing (cytotoxicity and pro-inflammatory effects) are expected by mid-january 2021. Based on results from toxicity testing (cytotoxicity and pro-inflammatory effects), a selection of strains will be tested in further detail using a HCA approach. Three to four months will be required to complete these HCA assays.

High Content Analysis assays will investigate apoptosis, DNA Damage, mitochondrial membrane potential and the pro-inflammatory response.

JRP5-WP2-T2: Assessment of virulence and toxin gene expression using RT-PCR and transcriptomic assays (M4-M24)

RNA extraction protocols have been developed and optimized in various culture conditions for *B. cereus* and *C. perfringens* to obtain sufficient RNA to develop qRT-PCR assays and to send for RNA depletion and sequencing. Bioinformatic analysis was performed and differential gene expression analyses have been performed. For both organisms, the methods have been developed and the pipeline for data analysis is functional. The methods and analysis are validated. The development of these methods corresponds to the deliverable D2.2, that is currently been prepared.

The results obtained will allow to study correlation between gene expression and strain patterns.

JRP5-WP2-T3: Correlation of specific toxicity profiles with expression patterns of bacterial toxins/virulence factors (M25-M30)

A first set of analysis allowed to obtain data on comparative gene expression profile in the strain collections. These analysis are currently ongoing and will establish correlation between gene expression and specific strain patterns. Data concerning the correlation of toxicity profiles and expression patterns of bacterial toxins will not be available until all *B. cereus* and *C. perfringens* strains have been completely characterized in terms of cytotoxicity profiles. Results from toxicity testing (cytotoxicity and pro-inflammatory effects) are expected in January 2021.

Correlation analysis can be performed using data from cytotoxicity testing and pro-inflammatory effects. This should be the priority – and can be performed once this data will be available. Data from HCA experiments may (or may not) provide more information concerning correlations between toxin expression profiles and specific toxicity pathways identified by HCA.

WP3: Development of Mass Spectrometry-based proteomics procedures for detection of bacterial toxins and virulence factors

JRP5-WP3-T1: development of Mass Spectrometry-based methods for the detection of new enterotoxins (eg SEG, SEH, SEI) from *S. aureus* (M4-M36)

A scientific collaborator has been hired to carry out analytical development and analyses of Staphylococcal enterotoxins M, N & O. A global method based on an “on filter” digestion was finalized (based on SEB enterotoxin analysis). The selection of peptides for SEM, SEN & SEO has been done



based on the theoretical protein sequence and a LC-MS method has also been implemented. In June 2020 the SEM, SEN & SEO proteins were produced and provided from BfR (P9 – WP4 task 4.1). Only SEN & SEO were pure enough to use for the implementation of the enterotoxin quantification and then the method has been extended for the detection and quantification of SEN and SEO. Due to the COVID-19 crisis and the very limited experimental work during several months, a finalized and optimized method for spiked supernatant analysis will only be available on January 2021. The full validation (specificity, linearity, matrix effect, LOQ, repeatability, reproducibility etc.) of the method (3 days, 3 concentrations & 3 repetitions) is ongoing and a detailed SOP is in preparation in order to allow the transfer of the method to the partners involved in the inter-laboratories comparison tests (WP5). This method will also be applied on strains selected in WP1.

JRP5-WP3-T2: development of Mass Spectrometry-based methods for the detection of toxins and/or virulence factors from *B. cereus* (M4-M36)

A bottom-up LC-MS/MS approach has been set up for the detection of two *B. cereus* toxins: Hemolysin II, and Sphingomyelinase using on samples enriched with recombinant toxins. The selection of proteotypic peptides has been done and the fragmentation conditions optimized to obtain the best signal/noise ratio for all selected transitions. Homogeneity tests for the analysis of bacterial supernatant spiked with toxin standards were performed. We prepared ten samples spiked with two toxins (Sphingomyelinase and Hemolysin II) at high concentration (6 µg) along with three LB alone as control: twenty-six samples (technical duplicate) were analysed by LC-MS/MS on a QExactive Plus coupled to an EasynLC 1200 using a PRM method on selected peptides. Results were analysed using Pinpoint Software.

For every protein at least four peptides are identified with a CV below 20%.

As expected in a PRM experiment average CV is below 20% with 13% and 12% in average per protein.

Homogeneity tests validate the method. Stability tests now remain to be performed followed by Inter-laboratory tests: samples are ready to be sent.

SOP writing is almost completed and will be shared when validated among partners.

JRP5-WP3-T3: Development of Mass Spectrometry-based methods for the detection of toxins and/or virulence factors from *C. perfringens* (M4-M36)

A nanoLC-PRM/MS method has been developed for the detection of *C. perfringens* enterotoxin CPE. It is based on the detection of 6 peptides generated by the tryptic digestion of CPE, achieving a sequence coverage of 19.7%. As a protein standard is not commercially available in Europe, a strain from the Tox-Detect collection was selected for its high CPE production under culture conditions developed by ANSES to use as reference. A homogeneity test was performed on pooled supernatants of this reference strain and 4 out of 6 peptides were found homogenous with a CV of less than 20% and the carryover in the matrix blank was estimated to be less than 0.05%. Serial dilution experiment was performed by diluting the digested reference sample in the digested matrix blank and CPE was detected for dilutions up to 1/128, but limit of detection could not be assessed and requires further work. A first attempt to estimate CPE concentration in the reference strain supernatant pool was carried out by spiking known concentrations of heavy peptides and following the single reference point (SRP) quantification method. However results must be confirmed through new analysis. A preliminary SOP is in preparation in order to allow the transfer of the method to the project partners involved in the inter-laboratories comparison tests (WP5). In addition we contacted other laboratories within our network to inquire about their potential interest in participating in the ILC tests.

JRP5-WP3-T4: Transfer of LC-MS/MS methods (M36-M39)

Transfer of LC-MS/MS methods is postponed due to COVID-19 crisis.



WP4: Development of new immuno-enzymatic assays for detection of *S. aureus* and *B. cereus* toxins and virulence determinants

JRP5-WP4-T1: Development of quantitative immunoassays for five known *S. aureus* and *B. cereus* toxins and virulence factors (M4-M40)

Toxin production procedures for SEM, SEN and SEO have been successful and purified toxins have been sent to the WP3 partners (SEN and SEO, 0.5 mg each, on 25.05.2020; SEM, 0.5 mg on 22.06.2020). Due to the delays in the project, the coordinators have requested that polyclonal antisera for two SEs be generated for the development of the immuno-enzymatic assays (instead of the originally planned monoclonal antibodies). We have complied and purified, recombinant SEN and SEO have been sent (on 22.06.2020) to the antibody company Covalab (arranged for the BfR by Michel Gohar) for immunization of rabbits and generation of the respective polyclonal antisera. The polyclonal antisera for SEN and SEO were received by the BfR on 29 September, 2020. For *B. cereus*, the three toxins have been produced and samples were sent to Institut Pasteur for WP3 task 2. For one of the toxin (sphingomyelinase), the antiserum have already been received and characterized, and an LFA (lateral flow assay) is currently in development. The two other toxins were sent in July 2020 to Covalab for antibodies production, and were expected to be received in mid-September. However, one of the toxins was delivered 5 days after shipment (due to a transporter failure) and was received unfrozen (and was therefore lost). Another batch was prepared and was sent, but because Covalab did not start immunisation procedures in August, the antiserum was received by the end of September.

With receipt of the polyclonal sera at the end of September, development of the corresponding ELISAs was begun at the beginning of October and is ongoing. Concurrently we are preparing fresh SEN and SEO samples for this effort and for the ILT planned for the Spring of 2021. The removal of our affinity tag (the last step in the toxin preparation) remains inefficient, particularly for SEO. We have conducted several fruitful conversations with the Coordinators concerning preparation for the ELISA trial and have been provided by them with the complete scheme for the ILC. We therefore now have a concrete measure for the amount of each toxin needed to carry out all experiments related to this last task once we have determined the detection limit in each case. We therefore expect to be able to deliver the method and all ILC materials by the end of January 2021.

JRP5-WP4-T2: Development of a quantitative immunoassay on a new *B. cereus* toxin or virulence factors (M18-M36)

Antibodies against sphingomyelinase are available and have been characterized. A lateral flow assay is currently in development. For the two other toxins, HlyII and CytK2, the rabbits immunization procedure has been **disrupted** due to the COVID-19 lockdown. A new batch of proteins is in production and will be sent to Covalab to reinitiate rabbits immunization.

JRP5-WP4-ST2.1: Construction of a genetic tool for protein overexpression (M18-M26)

Completed.

JRP5-WP4-ST2.2 : Protein production (M24-M27)

Ongoing for SEN and SEO.

JRP5-WP4-ST2.3 Development of specific Ab (poly/monoclonal) (M25-M28)

Polyclonal antisera against SEN and SEO received by the BfR on 29 September, 2020.

JRP5-WP4-ST2.4 Design of the immunoenzymatic assay (M27-M32)

Task ongoing.

WP5: Inter-laboratory ring trial scheme (M33-M42)

The 5.1 deliverable “Inter-lab tests documents according to ISO/IEC 17043 regulation, adapted to each method” has been uploaded on EJP website on October 2020.



COVID-19 crisis has induced partner laboratories closure, stopping method development during more than 4 months. In consequence, inter-laboratory comparison tests (ILC) organisation has been postponed to 2021.

A training session dedicated to the management of ILC was organized on the 1st December 2020. The objective was to present to the 6 ILC organizers a detailed program and the schedule that could be implemented in the frame of WP5.

JRP5-WP5-T1: Inter-lab test on Maldi-ToF for species identification (M36-M42)

A TC was organised on 28th January 2020, involving Anses and Institut Pasteur. The aim of this TC was to determine strain distribution between WP1 and NVI (Norway) as Maldi ToF ILC organiser. Several TCs were organized on 17 July, 18 September and 9 October 2020 allowing the transfer of MALDI ToF reference spectra library between ANSES (method developer) and NVI (ILC organizer). This transfer was achieved on December 2020, and NVI is currently testing the MALDI-ToF library method in order to organize the Maldi-ToF ILC in 2021.

JRP5-WP5-T2: Inter-lab test on LC-MS/MS (M36-M42)

A TC was organised on 24th February 2020 between Sciensano (Task 5.2 leader) and WP3 partners in order to update deliverables schedule, taking into account the necessary period (10 to 12 months) to organise LC-MS/MS ILC.

LC-MS/MS ILC organizers are currently recruiting potential participants. Clostridium perfringens LC-MS/MS ILC is in progress and should be implemented between January and April 2021.

JRP5-WP5-T3: Inter-lab test on immuno-enzymatic assays (M36-M42)

For Staphylococcal Enterotoxins ELISA method: the method was developed by BfR in the frame of WP4. However, the ILC dedicated to this method will be organised by Anses. For this reason, BfR and Anses are in touch in order to organize the transfer of the method from BfR to Anses. This step is expected in January 2021.

For Bacillus ELISA method: INRAe is in charge of development of the method and also the organisation of ILC.

Both ELISA ILC organizers (Anses and INRAe) are currently recruiting participants, and ELISA ILCs are expected in first semestre of 2021.

WP6: Dissemination, protection and exploitation of results (M0-M45)

JRP4-WP6-T1: dissemination of information within the partners (M0-M45)

A Abdelrahim (WP2) participated to the online OH-EJP TIM “Practical use of NGS”

D Clermont submitted a poster at OHEJP ASM entitled “Establishment of a shared MALDI-ToF reference spectra base, covering three pathogens of interest”

JRP4-WP6-T2: dissemination of information to the outside (M0-M42)

Tox-Detect coordination submitted

- one abstracts at the IAFP symposium “Characterisation of Food Born Outbreaks Due to Emerging Staphylococcal Enterotoxins”,
- one abstract for IAFP round table “Tox-Detect: Development and Harmonization of Innovative Methods for Comprehensive Analysis of Food-Borne Toxigenic Bacteria, Ie. Staphylococci, Bacillus Cereus and Clostridium Perfringens. How can standardization help in validation non-standardized/alternative methods ?”.

The two abstracts were accepted for oral presentations, but the IAFP symposium was cancelled due to COVID-19 crisis.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
05	D-JRP5-1.2	Libraries of MALDI-ToF reference spectra	M24	M33			Confidential, this concern reference strains under MTA and will be published	3
05	D-JRP5-2.1	Report on results from toxicity assays (classical toxicity tests and High Content Analysis)	M32		M38		Confidential, technical data under publication	8 Research and development activities
05	D-JRP5-2.2	Report on TR-PCR and RNAseq data analysis	M32		M38		Confidential, technical data under publication	8 Research and development activities



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
05	D2.3.	Report on correlation between RNAseq data analysis and toxicity assays (including toolbox for toxicity prediction)	M30		M38		Confidential, technical data under publication	8 Research and development activities
05	D3.1	Report on Mass Spectrometry-based methods for the detection of new enterotoxins (eg SEG, SEH, SEI) from S. aureus	M27		M42		Confidential, new developed method, will be published by the end of the project	8 Research and development activities
05	D3.2	Report on Mass Spectrometry-	M27		M42		Confidential, new developed	8 Research and development



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		based methods for the detection of cereulide analogs and enterotoxins from <i>B. cereus</i>					method, will be published by the end of the project	activities
05	D3.3	Report on Mass Spectrometry-based methods for the detection of CPE and virulence factors from <i>C. perfringens</i>	M27		M42		Confidential, new developed method, will be published by the end of the project	8 Research and development activities
05	D3.4	Report on the performance criteria for method harmonisation	M30		M42		Confidential, new developed method, will be published by the end of	2



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
							the project	
05	D4.1.	Report on the immuno-enzymatic assays	M33		M42		Confidential, new developed method, will be published by the end of the project	8 Research and development activities
05	D5.1.	Inter-lab tests documents according to ISO/IEC 17043 regulation, adapted to each method	M25	M33			Public https://zenodo.org/record/4479096#.YBQYXuhKhM0	2
05	D6.1	Dispatch of SOPs	M33		M42		Confidential, technical data under publication	2
05	D6.2	Dissemination of results (publications, conferences...)	M36		M42			



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCod e	Mileston e number	Milestone name	Delivery date from AWP 2020	Achieve d (Yes/No)	If not achieved: Forecast achievem ent date	Comments
05	M-JRP5-04	Exchange of libraries of MALDI-ToF reference spectra	M27	Yes		
05	MS2.2	RT-PCR assays developed	M32	Yes		
05	MS2.3	High content analysis methods developed	M32	Yes		
05	MS2.4	RNAseq data analysed	M32	No	M38	



JRP/JIPC Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
05	MS2.5	Correlation between toxicity profiles and expression patterns assessed.	M30	Yes		
05	MS3.2	Methods developed and assessed	M24	Yes		
05	MS3.3	Methods transferred to partners	M30	No	M39	
05	MS4.3	Immuno-enzymatic assays designed for known S. aureus and B. cereus toxins	M32	Yes		
05	MS4.4	Genetic tools constructed for over-expression of a new B. cereus toxin/virulence factor	M26	Yes		
05	MS4.5	Antibodies produced against new B. cereus toxin/virulence factor overproduced	M32	Yes		
05	MS4.6	Immuno-enzymatic assays designed for a new B. cereus toxin/virulence factor	M33	Yes		
05	MS4.7	Immuno-enzymatic assays transferred to partners	M33	No	M39	



JRP/JIPC Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
05	MS5.1	Dispatch of inter-lab tests documents (according to ISO/IEC 17043 regulation) adapted to each method	M25	Yes		
05	MS5.2	Dispatch of samples and evaluation report to be filled by partners	M27	No	M42	
05	MS5.3	Samples analysed by partners	M34	No	M42	
05	MS5.4	Dispatch of final report	M34	No	M45	
05	MS6.1	Final report dispatched	M36	No	M45	

4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Point-of-Need DNA Testing for Detection of Foodborne Pathogenic 10.3390/s19051100 https://zenodo.org/record/3935799#.YBQaJ-hKhM0	YES		



5. *On-going and planned collaborations with national or European projects or networks*

Progress of ToxDetect Project was presented during the EURL staphylococci annual workshop (April 2020). In fact, LC-MS (WP3) and ELISA (WP4) methods, under development, dedicated to staphylococcal enterotoxins should be used for the Foodborne Outbreak characterization at EURL network level. This need was expressed in (i) the working program of the EURL for CPS 2019-2020 (WORK PROGRAMME of EURL for COAGULASE POSITIVE STAPHYLOCOCCI (INCLUDING STAPHYLOCOCCUS AUREUS, 14/12/2018), and (ii) and in CBRN n°H20.5 program dedicated to development of new tools for characterization of bacterial toxins.



JRP06-NOVA

1. Summary of the work carried out in year 3

On the project management level, several tasks have been influenced by the COVID-19 pandemic. The annual assembly was cancelled just a month before the planned date and there have been needs and requests to investigate how the outbreak affects work in NOVA. Several project members have had to turn their attention to surveillance and management of the outbreak, both on professional and private level. However, based on our inventories, we conclude that with the extension of NOVA into 2021, we will be able to finish our remaining tasks and deliverables.

In WP1 work has been focussed on the studies of potential barriers or opportunities for surveillance across the food chain. After careful preparations, including considering advantages and disadvantages of different methodological approaches, interviews of disease surveillance experts are now ongoing.

The studies in WP2 are led and performed mainly by public health institutes and these have been heavily affected by the COVID-19 pandemic. However, there has still been some progress. For example, a large dataset with food purchase data has been obtained, which will enable analyses of simulated outbreaks. An electronic web module in which consumers can give consent for their purchase data to be used has also been prepared. The work related to electronic food purchase data at the institutional level for investigation of nosocomial foodborne outbreaks is on-going, including submission of a literature review manuscript and a questionnaire study. Work to develop improved tools for food risk mapping and to integrate them into the state-of-the-art tracing tool software FoodChain-Lab also continues.

In WP3, we are working on the methods to integrate signals from different univariate syndromic surveillance systems to improve outbreak detection in humans. The Norwegian veterinary and public health institutes are working on adding veterinary data (*Campylobacter* in poultry) and environmental data (rainfall, temperature) to the current syndromic surveillance system for human gastro-intestinal outbreaks. SVA is developing an explanatory multivariate syndromic surveillance system that processes multiple time series - human *Campylobacter* cases, positive *Campylobacter* slaughter batches in broilers, and weather data (rainfall, temperature) – to calculate the value of evidence for an outbreak for each week. After univariate analysis of seven animal, food and human databases in relation with human gastro-enteritis outbreaks, ANSES is working on the development of detection algorithms that can process temporal signals from multiple time series simultaneously.

WP4 continues the spatial and temporal analyses with a one health perspective, mainly focussing on *Salmonella*, and also AMR. In studies of the role of the environment on disease occurrence, a machine learning algorithm has been developed to correlate infection data with environmental drivers. The plan is to apply this on different scenarios to identify environmental risk factors.

In WP5, the work with different transmission models to investigate potential disease spread and compare surveillance strategies is in the final stage. Two papers based on the work conducted have been published in peer-viewed journals. In order to obtain a better measure of the occurrence of AMR in animal production at national level, the coding of a simulation model for how to utilize metagenomics on samples from very large pools has also been developed. A model for estimating cost-effectiveness of retail sampling (cost) and prevention of cases of human disease (effectiveness) has also been produced.



2. Work carried out in the JRP, scientific results

WP0: Coordination and project management (M1-M42)

JRP6-WP0-T1: Project management (M1-M42)

Monthly meetings with WP leaders have been held. The project leader and the deputy project leader also joined the Project Leaders' Forum at the OHEJP Annual Scientific Meeting online.

A request to extend the project was sent to OHEJP management in May. Due to slow recruiting processes in several partner institutes, we decided already in 2019 that we would ask for an extension. This year, the COVID-19 pandemic has also influenced the work in several partner institutes/countries. The request was endorsed and accepted by the PMT and the members of the SSB in June.

JRP6-WP0-T2: Organise annual assemblies (M1-M42)

Organisation of a third annual assembly was started at the end of 2019 and was planned to be held in Madrid, 23-24 April 2020, hosted by UCM. The meeting was cancelled in March due to the COVID-19 pandemic. Instead, a shorter online meeting was held on the 23rd of April, with short scientific presentations from the WPs and general information.

We have concluded that it will probably not be possible to have a face-to-face meeting during Spring 2021. Instead we will have our annual assembly online. Also, we will investigate the possibility to have a NOVA group face-to-face meeting after the official end of the project, e.g. in connection to other OHEJP activities.

JRP6-WP0-T3: Economic reporting and financial management (M1-M42)

In response to a request by the OHEJP coordination in April, the project partners were asked to report any costs that had been wasted due to the COVID-19 pandemic. Only two partner institutes reported such costs.

Later, the OHEJP coordination also requested that the budget for the third year of the project were updated by each partner and specific budget files were filled in and sent to OHEJP centrally in June. The conclusion from this update was that partners plan to not underspend but to use potentially remaining resources during the extension period in the fourth year.

WP1: Food chain surveillance mapping (M1-M42)

JRP6-WP1-T1: Definition of a joint food borne zoonosis surveillance terminology (M1-M24)

Given the work on a Med-Vet glossary of the participants of the integrative ORION project in which members of NOVA project WP1 also participated in the first year, in order to complete the deliverable of this WP, a collaboration has been established with the respective ORION team, as well as COHESIVE.

WP1-T1 has collected 274 terms from partners leading all WPs. The NOVA Glossary has been delivered to ORION project leader, BfR, in order to incorporate the NOVA Glossary into the common OH Glossary. After this there has been several exchanges with BfR in order to adjust the NOVA Glossary into the common version. The final result can be found at:

<https://ckan-aginfra.d4science.org/organization/about/orionknowledgehub>

The OHEJP Glossary has 3 main functionalities: First the collection of One Health (OH) related terms and definitions in the sectors public health, animal health and food safety. Second, highlighting similarities and differences of terms and definitions between the sectors. Finally, an infrastructure to reference, search and filter terms and definitions.

As an outcome of this cooperation (of all three projects) is a common scientific paper that is currently in final editing before submission to a peer-review journal (June 2020). This publication work is coordinated by BfR.



JRP6-WP1-T2: Mapping of surveillance: data, regulatory framework, key stakeholders, opportunities and barriers (M1-M42)

A methodology has been developed and put in practice to identify barriers and opportunities in food-borne disease surveillance from a One Health perspective. This information was collected through interviews with professionals with selected profiles from four countries (Belgium, France, Sweden, Norway). For this, a number of tools were developed; i) map of existing food chain (used as a visual tool for cueing), ii) interview guide document, iii) demographic questionnaire, iv) excel files for collection of data, v) training on performing interviews and qualitative research, and vi) interview try-outs.

The interviews have been performed by three WP1 participants who have received the same training on performing interviews. Twenty interviews have been performed and the information collected by the interviewers was double-checked and validated from the interviewees to ensure credibility. Currently the dataset is being analysed.

WP2: Analysis of food purchase data (M1-M42)

General comment: A number of scientists involved in WP2 have been working full time on the COVID-19 response during spring 2020. Therefore, there has been little progress in some of the tasks (see comments below).

JRP6-WP2-T1: Data availability and barriers (M1-M14)

Completed; see second annual report (2019).

JRP6-WP2-T2: Food purchase data for outbreak investigations (M2-M42)

This aim of this task is to describe the use of consumer purchase data (CPD) as an outbreak investigation methodology. This encompasses the parts described below.

JRP6-WP2-T2-ST1: Identify existing use of CPD for outbreak investigations, including a survey of EU public health institutes, conducted in cooperation with JRP6-WP2-T1.

Task completed; see second annual report (2019).

JRP6-WP2-T2-ST2: Develop Best Practice descriptions for CPD for outbreak investigations.

An international group has been put together and experiences with and ways to promote the use of CPD for outbreak investigations has been collected and discussed. A manuscript describing the results hereof has been submitted (to EuroSurv).

JRP6-WP2-T2-ST3: Describe potential for use as an analytical tool

The part continues particularly as a cooperation between FHI and SSI. Following a series of meetings with Norwegian supermarket chains, a large dataset with purchase data from more than 900,000 households has been obtained. This will form the basis for analyses of simulated outbreaks.

JRP6-WP2-T3: Big data analysis of risk factors for sporadic disease (M1-M42)

This task aims to explore if consumer purchase data can be used for analytical studies beyond the outbreak setting; something which has not previously been done. An electronic web module in which Danish users can be securely invited to sign up and give consent for their purchase data to be used has been built but use has been stalled due to COVID-19.

JRP6-WP2-T3-ST1: Achieve supermarket CPD to study the ways to structure data on foods and run simulation studies for the risk of outbreak/sporadic foodborne infections.

For this, we aim to use the large dataset described above. Work has been initiated but put on hold due to COVID-19.



JRP6-WP2-T3-ST2: Case control study of foods posing a risk for sporadic campylobacter infections.

We will not do a study on sporadic campylobacter infections, but plan to do one on sporadic salmonella infections. However, work has been stalled due to COVID-19 and we are currently not sure if it will be continued this year.

JRP6-WP2-T4: Food distribution data for hospital outbreaks (M1-42)

This task aims to use electronic food purchase data at the institutional level for investigation of nosocomial foodborne outbreaks. A manuscript "Healthcare-associated foodborne outbreaks in high-income countries: a literature review and surveillance study" was submitted to Eurosurveillance in the spring, still awaiting decision. Further, a questionnaire study has been performed within health institutions in Germany and Italy, and a manuscript describing the results is close to submission. Further similar studies are being planned.

JRP6-WP2-T5: Trace back and food risk mapping (M1-42)

This task aims to develop improved tools for food risk mapping and integrate them into the state-of-the-art tracing tool software FoodChain-Lab. In agreement with the work plan, this work has only begun in the second half of 2019.

JRP6-WP2-T5-ST1: Investigate the availability and usefulness of data from WP2, tasks 1-3.

The likelihood model has been implemented and validated on Norwegian outbreak data. Current work focuses on integration with FoodChain-Lab and simulation experiments to explore sensitivity and what if any additional improvements on model structure and/or realistically attainable data quality would be sufficient to make the approach of more practical use.

JRP6-WP2-T5-ST2: Develop the likelihood method so that it handles data on time together with GTIN.

The handover of the likelihood method R script for integration in FoodChain-Lab took place in June 2020, a bit later than planned. The integration of the likelihood method into the analysis of the FoodChain-Lab as a cloud service is the next step. A downgraded implementation of the method is planned by early 2021. We are planning to test the system using outbreak data from other countries (eg Denmark and Norway).

WP3. Syndromic surveillance (M1-M42)

JRP6-WP3-T1: Identify the opportunities for SyS of FBD (M1-M10)

JRP6-WP3-T1-ST1: Food chain mapping (M1-M8)

This task has been completed, see annual report 2018.

JRP6-WP3-T1-ST2: Data source screening: availability, quality and suitability for SyS (M1-M10)

This task has been completed, see annual report 2018.

JRP6-WP3-T2: Univariate syndromic surveillance development for FBD (and AMR) (M11-M30)

This task is completed for two partners (SVA, NHI) and still on-going for Anses. When developing univariate syndromic surveillance systems, several points of discussion about methodologies have been raised, as temporal and spatial scales. The methodological discussion is presented in D.3.4.

This task is completed and a methodological discussion is presented in D.3.4.

JRP6-WP3-T3: Evaluation of multivariate syndromic surveillance for FBD (M11-M42)

We are working on the methods to integrate signals from different univariate syndromic surveillance systems to improve outbreak detection in humans. In Sweden, an explanatory multivariate SyS is in development, in which the value of evidence (Bayesian likelihood ratio) for an outbreak is assessed for each time point (week), by using a Bayesian statistical model. The model can take multiple time series



data (human *Campylobacter* cases, positive *Campylobacter* tests in broilers, and weather data) together and the outcome will be available at national level. The results will be compared with the alerts detected from univariate SyS of humans and chickens, and the system will be further optimized by taking the slaughterbatch size and different abattoirs into account. The Norwegian veterinary and public health institutes are working on adding veterinary data (*Campylobacter* in poultry) and environmental data (rainfall, temperature) to the current syndromic surveillance system for human gastro-intestinal outbreaks. We compare several methods for outbreak prediction. A real-time pilot study was planned for spring/summer 2020 but had to be postponed due to COVID 19 crisis. Since there are no general testing of *Campylobacter* in poultry in Norway during winter, we are not able to do a real time surveillance system during autumn/winter. We are instead using retrospective data from the spring and summer period (and the time period before) for different analysis. We are also making a dashboard which will be used for evaluating the possible usefulness and benefits of having this system running in real-time. The system will be made in such a way that it will be possible to run it in real-time from spring if the results are promising.

In Y3, ANSES completed the univariate analysis of seven animal, food and human databasis in relation with human gastro-enteritis outbreaks. We are working on the development of detection algorithms that can process temporal signals from multiple time series simultaneously. Indeed, multiple univariate analysis considers independence between datasets whereas multivariate analysis takes into account this correlation. It would help acknowledge a statistical link between time series and then improve sensitivity and specificity of detection algorithms.

WP4: Spatial risk mapping (M1-M42)

JRP6-WP4-T1: Identification of spatial relationships and patterns in *Salmonella* prevalence

JRP6-WP4-T1-ST1: Surveillance in high prevalence regions to detect introduction and changes in prevalence (M1-M24)

Task completed. See second annual report 2019.

JRP6-WP4-T1-ST2: Surveillance in low prevalence regions to reduce prevalence (M1-M24)

Task completed. See second annual report 2019. Additional efforts have been done to describe the spatial distribution of *S. Dublin* in Sweden and identify possible risk factors. Results show two areas at high-risk which are more likely to have high numbers of seropositive cattle to *S. Dublin* compared to other regions. These areas are characterized by slightly bigger herd size, high temperature, low precipitation, and fewer contacts with other herds (potential risk factors). It remains unclear which of these factors may be risk factors for spreading *Salmonella Dublin* infections between cattle herds.

JRP6-WP4-T2: Risk of introduction of *Salmonella* in pig farms through animal feed (M1-M42)

The focus of this deliverable was changed due to the decision to ban formaldehyde in the EU. An alternative study has been performed: Use of surveillance AMR data for monitoring emergence of *Salmonella* strains in swine (UCM).

Information about AMR surveillance in *Salmonella* in pigs (2001-2013) have been subjected to several multivariate analytical techniques for exploring their usefulness in the monitoring and detection of emerging antimicrobial resistance (AMR) strains and their spatio-temporal patterns in Spain. Almost 15% (n=170) of the isolates were susceptible to all seven antimicrobials while resistance to other antimicrobials ranged between 0.5 and 51%. In terms of the temporal trend of the prevalence of AMR, several different patterns were identified depending on the specific antimicrobial, including decreasing and changing (decrease followed by increase or viceversa) trends. Hierarchical clustering analysis revealed AMR phenotypes clustered in 6 groups.



JRP6-WP4-T3: Role of the environment in the occurrence and maintenance of Salmonella infection in extensive farming (M1-M42)

A machine learning algorithm (random forest) has been developed to evaluate whether data of salmonella infected farms could be explained by environmental drivers such as temperature, humidity, precipitation or type of soil and vegetation. The model was first successfully validated on surrogate data and subsequently applied to intensive and extensive farming data. No clear link was identified between infected farms and environmental variables. In particular wild boar density was not identified as a driver. In the remaining months, other scenarios will be investigated. In particular, we will apply the same model framework to Salmonella infection in wild boar, to identify potential environmental risk factors.

WP5: Evaluation of surveillance programs & cost efficiency (M1-M36)

JRP6-WP5-T1: Adapt infectious disease models for assessing the effect of surveillance programs in primary animal production on consumer exposure to foodborne pathogens (M1-M42)

An existing transmission model for Salmonella in pigs (part of a previously developed quantitative risk assessment) has been adapted to model different surveillance strategies on the prevalence of infected pigs entering the food chain. The model represents the different production stages, and simulates the impact of surveillance on farm and subsequent control measures on the farms that exceed a threshold number of positive tests. The model has been parameterized for Salmonella Typhimurium in GB. This model has been used to simulate a national control programme in Great Britain, consisting of quarterly sampling of finisher farms using 5 pooled faeces samples, which are tested by PCR. The impact on Salmonella Typhimurium slaughter prevalence in GB and the number of farms that have been required to implement control measures has been modelled for varying the threshold number of positives pooled samples from 1-5.

To estimate the effect of current and alternative sampling schedules for detecting Salmonella infected grand-parent and parent flocks in conventional broiler production, a stochastic dynamic modelling of transmission of Salmonella in parent flocks and combined that with the relation between flock prevalence and test sensitivity for environmental samples in the flock has been developed. The model is parametrized for Danish broiler production. The model has been published in Scientific Report with the title "Using stochastic dynamic modelling to estimate the sensitivity of current and alternative surveillance program of Salmonella in conventional broiler production. This model has been used in the advisory of the Danish broiler industry and food authorities for optimizing the surveillance program in the Danish broiler production.

A manuscript entitled "Modelling spread and surveillance of *Mycobacterium avium* subsp. *paratuberculosis* in the Swedish cattle trade network" was published (<https://doi.org/10.1016/j.prevetmed.2020.105152>). This work defines a method for the modelling of surveillance performance in the primary production using a compartmental infectious disease spread model. This approach leverages the compartment model approach by allowing the model to predict the test status based on test sensitivity and the disease state and storing this information in indicator compartments that hold the test status of each metapopulation over time. A planned delivery of a workshop on this method as part of the 4th International Conference on Animal Health Surveillance, was cancelled, due to the ongoing pandemic.

JRP6-WP5-T2: Assessing the effect of using metagenomics in surveillance of foodborne zoonoses (M1-M36)

Current surveillance of antimicrobial resistance (AMR) in DK is done using minimum inhibitory concentration (MIC) values as an indicator for bacteria isolated from individual animals and isolates of pathogens. To obtain a more robust and representative measure of the occurrence of AMR in animal production, a simulation model for how to utilize metagenomics on samples from very large pools of feces collected at the slaughter line has been developed.



The model is parametrized using existing metagenomics data of AMR in the Danish pig production originating from previous research projects. These data are utilized in a stochastic model, wherein the effect of the number of animals in the pool (including dilution effect), frequency of sampling and sequencing depth in metagenome analysis on i) to detect presence of different resistance genes in the population, ii) time until detection of emerging resistance in the population and iii) power to detect changes in the level of existing resistens in the population. The stochasticity of the model takes into account sampling error arising from randomness at sampling, between animal and between farm variation in presence and abundance of AMR, variation in rate of transmission of emerging AMR and variation in sequencing depth.

JRP6-WP5-T3: Modelling the effect of surveillance programs in the food production on human health (M1-M42)

The work in this task is based on Salmonella in pigs and pork. It is dedicated to risk based sampling and cost-effectiveness of sampling.

The risk based sampling approach for the retail phase focuses on the question how to subdivide sampling capacity the best in order to monitor as much DALYs as possible. The basic idea is that sampling for those pathogen-product combinations is preferred, for which surveillance is cheapest in terms of costs / DALYs monitored. In this study we focus on Salmonella in pig meat in the retail phase. Costs per sample refer to microbiological analysis. The number of samples is set using the criterion of a constant relative uncertainty of estimated pathogen prevalence. The number of DALYS is estimated using an overall pathogen-food animal value, which is subdivided over products using exposure assessment estimates. Risk based sampling methodology was applied to France, United Kingdom, Denmark, Sweden and The Netherlands and this led to two improvements in the methodology, so that the number of food product categories and the number of inhabitants of a country can be taken into account. Ranking of food product groups in terms of costs / DALYs monitored was performed for the respective countries both separately and in combination.

We also explored a method to evaluate the cost-effectiveness of product testing in the pre-retail and retail phase, by food industry and the government, respectively, related to food product withdrawal. The criterion is here costs / DALYs evaded. We focused again on Salmonella in pig meat. Costs refer to microbiological analysis, and the number of samples taken as reported. The effect includes a positive effect on public health, expressed in DALYs, as batches with a positive test will not reach the consumer, or will be heat-treated first. Cost-effectiveness can be compared with a criterion set by WHO. This comparison indicates that the short-term effect of product testing is not cost-effective, the more since it is suspected that the present calculations give too favorable an estimate. On the other hand, uncertainty of the calculations is large. We did not incorporate the long-term effect of product testing on hygiene performance of food industry, which is difficult to quantify.

Finally, we explored a method to evaluate the cost-effectiveness of surveillance in the farm phase, focusing on Salmonella in pigs, and applying it to The Netherlands and Great Britain. The criterion is costs / DALYs evaded. Costs refer to the number of samples taken, and the costs of microbiological analysis per sample. To calculate DALYs evaded, it is assumed that pig meat from Salmonella test-positive farms does not reach the consumer. It is calculated as a decrease in Salmonella prevalence at animal level as a result of surveillance, which is carried through the slaughterhouse- and consumer phase to result in a reduction of DALYs. It is shown that cost-effectivity is independent of the number of farms sampled, and decreases with the number of samples taken per farm. Cost-effectivity is more favorable (lower) for GB than for The Netherlands, 4.78E3 and 8.70E3 euros/DALY, respectively, for comparable scenarios. Both the costs and the DALYs evaded are higher for GB, being a larger country, but DALYs evaded with a higher factor than costs. It must be stressed, however, that the calculations have a high uncertainty. Cost-effectivity of sampling in the farm phase is much more favorable than in the (pre-)retail phase, being about 8,5E3 euro's/DALY vs 6,08E4 or 2,26E5 euro's/DALY (depending on sampling system)(Evers et al., 2020). The value of 8.5E3 euros/DALY is also lower than 4.6E4 euros/DALY, the standard value set by WHO for cost-effectivity of an intervention (WHO Europe, 2014).



Additional data collection and conceptual model improvement will increase reliability of the calculation results.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
06	D-JRP6-2.4	Assessment of the use of the method as an analytical tool, rather than merely hypothesis-generation.	M30		M42		Due to the COVID-19 outbreak and the impact that has had on the possibilities of the SSI to engage in large population studies, this project may need to be redefined. Also, work has been paused throughout the spring of 2020, because the researchers involved have been moved to work on the COVID-19 response exclusively. We would like to propose to move the deliverable and the corresponding milestone (they are part of the same project) to the end of the new project period, i.e. Month 42	4, 7, 9
06	D-JRP6-3.4	Recommendations about the quality standardization of data produced across	M30	2020-06-30			CO	1, 4



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		the food chain for their use in SyS						
06	D-JRP6-5.1	Report comparing performance of surveillance strategies	M30		M42		D-JRP6-5.1 - Report comparing performance of surveillance strategies” and “D-JRP6-5.2 - Recommendations for metrics to evaluate surveillance performance, will be joined into one deliverable. Deadline after extension of the project is M42 (36+6).	1, 5, 7, 9
06	D-JRP6-5.3	An assessment of the public health effects of very different surveillance strategies to detect emerging foodborne infections in a MS or at European level.	M32		M36		Due to lag in recruitment this activity started later than first planned	2, 5, 9
06	D-JRP6-2.6	Case control study of food risk factors for sporadic campylobacter infections.	M34		M42	Yes	Deadline pushed forward as part of extension of the project	
06	D-JRP6-2.10	Description and documentation of software, development of	M34		M42	Yes	Deadline pushed forward as part of extension of the project	



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		tutorials and training material.						
06	D-JRP6-3.5	Contribution of multiple syndromic surveillance components in the FBD surveillance	M36	2020-12-31	M42	Yes	Due to the COVID-19 crisis, NIPH employs two-part time workers for working on NOVA since December 2020.	4
06	D-JRP6-4.6	Assessment of the potential effect of the withdrawal of the use of formaldehyde-based feed treatments done.	M36		M42		Deadline pushed forward as part of extension of the project	
06	D-JRP6-4.9	Potential new environmental surveillance indicators identified.	M36		M42		Deadline pushed forward as part of extension of the project	
06	D-JRP6-5.2	Recommendations for metrics to evaluate surveillance performance	M36		M42		Deadline pushed forward as part of extension of the project	1, 5, 7
06	D-JRP6-5.4	Report assessing the quantitative effect on human health of changing surveillance capacity	M36		M42		Deadline pushed forward as part of extension of the project	5, 6



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		across different sources in an MS						
06	D-JRP6-5.5	A practical risk based sampling approach that combines exposure to zoonoses with disease burden and costs for sampling at a European level.	M30		M40		Deadline pushed forward as part of extension of the project	1, 5, 6

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
06	M-FBZ1-18	Result reporting on hot spot areas for Salmonella transmission between wild boars and low biosecurity systems.	M30	Yes		
06	M-FBZ1.22	Case control study of food risk factors for sporadic salmonella infections.	M30	No	M40	Due to the COVID-19 outbreak and the impact that has had on the possibilities of the SSI to engage in large population studies, this project may need to be redefined. Also, work has been paused throughout the spring of 2020, because the researchers involved have been moved to work on the COVID-19 response exclusively. We would like to propose to move the deliverable and the corresponding milestone (they are part of the same project) to the end of the new project period, i.e. Month 40
06	M-FBZ1.NOV A.23	surveillance layer added to models	M30	Yes		
06	M-FBZ1.NOV A.24	A dynamic modelling layer of surveillance is overlaid the submodels	M30	Yes		
06	M-FBZ1.NOV A.25	initial set of results circulated to project team	M32	Yes	M36	



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
06	M-FBZ1.NOV A.25	initial set of results circulated to project team	M32	Yes	M36	
06	M-FBZ1.NOV A.27	Integration of multiple sources developed and tested	M34	No	M40	
06	M-FBZ1.NOV A.28	Result reporting on risk of Salmonella introduction in pig farms by animal feed and the potential effect of the withdrawal of the use of formaldehyde-based feed treatments	M36	No	M42	Milestone after extension of the project is M42 (36+6).
06	M-FBZ1.NOV A.29	Result reporting on new environmental surveillance indicators.	M36	No	M42	Milestone after extension of the project is M42 (36+6).



4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Identifying emerging trends in antimicrobial resistance using Salmonella surveillance data in poultry in Spain. TBDE, 67(1):250-262. DOI: 10.1111/tbed.13346 https://zenodo.org/record/3660026#.XvyQnGgzZM0	YES		Yes (processing fees: 3750€)
Climatic and topographic tolerance limits of wild boar in Eurasia: Implications for their expansion. Geography, Environment, Sustainability, 13(1):107-114. https://doi.org/10.24057/2071-9388-2019-52 https://zenodo.org/record/4244729#.X6LYN4hKjcc	YES		YES (no processing fee)
Spatial trends in Salmonella infection in pigs in Spain. Frontiers in veterinary science. In Press. (A). ISSN: 2297-1769. DOI: 10.3389/fvets.2020.00345 https://zenodo.org/record/4244797#.X6Li_lhKjcc	YES		YES, processing fee 2048 € (2490 USD)
An outbreak of monophasic Salmonella Typhimurium associated with raw pork sausage and other pork products, Denmark 2018–19 https://zenodo.org/record/4249319#.X6UIWmhKjcc	YES		YES (no processing fee for this publication)
Analysis of consumer food purchase data used for	YES		YES (no processing fee)



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
outbreak investigations, a review https://zenodo.org/record/3747633#.XpCQ_sgZM0			
Non-Typhi, non-Paratyphi Salmonella related hospitalisations in Spain: trends, comorbidities, risk factors for worse prognosis and hospital costs. 10.1007/s10096-018-3433-1 https://zenodo.org/record/4244788#.X6LhGYhKjcc	YES	YES Embargo length = 24 months Manuscript release date = 20 November 2018	
Salmonella Surveillance Systems in Swine and Humans in Spain: A Review. 10.3390/vetsci6010020 https://zenodo.org/record/3635293#.XjlyUGhKhPY	YES		YES (no processing fee)
Using stochastic dynamic modelling to estimate the sensitivity of current and alternative surveillance program of Salmonella in conventional broiler production 10.1038/s41598-020-76514-3 https://zenodo.org/record/4271431#.X65ljTiWxPY	YES		YES, processing fee 1570€
Modelling spread and surveillance of Mycobacterium avium subsp. paratuberculosis in the Swedish cattle trade network https://doi.org/10.1016/j.prevetmed.2020.105152 https://zenodo.org/record/4450338	YES		YES, processing fee 2837€ (3450 USD), excluding taxes



Additional output

Cha, W., Dórea, F., Grøneng, G.M., Rø, G., Hopp, P., Jonsson, M., Dryselius, R. Development of One Health syndromic surveillance for *Campylobacter* in Norway and Sweden. In: Proceedings of the 2nd Annual Scientific Meeting of the One Health European Joint Programme on Foodborne Zoonoses, Antimicrobial Resistance and Emerging Threats; May 27-29, 2020; Online meeting.

Gustafsson, W., Andersson, M.G. Designing multivariate syndromic surveillance for animal diseases in Sweden. In: Proceedings of the 2nd Annual Scientific Meeting of the One Health European Joint Programme on Foodborne Zoonoses, Antimicrobial Resistance and Emerging Threats; May 27-29, 2020; Online meeting.

De la Torre, A; Rodriguez, A; Álvarez, J; Teng, K; Swart, A; Kim H; Beninca, E. Novel approaches for design and evaluation of cost-effective surveillance across the food chain: a story map. ESRI conference. 21-22 october 2020. Madrid, Spain. Online meeting.

Iglesias, M. Martínez, J. Álvarez, K. Teng, A. de la Torre. Spatial distribution and temporal trends for salmonella in pork and humans (2010-2015). I Congreso Virtual de la Sociedad Española de Epidemiología (SEE) y da Associação Portuguesa de Epidemiologia (APE), 21-30 Octubre 2020. Online meeting.

Story Map tool development: This story map highlights main achievements of risk mapping approaches from NOVA project. Available at: <https://arcgis.com/1zHSub>

5. On-going and planned collaborations with national or European projects or networks

Within NOVA, Anses has explored several data sources regarding *Salmonella* detection in farm animals, food and humans in France. We developed a combination of several algorithms and tools for result visualization that will be transmitted to data owners (National Authorities, National Laboratories and private stakeholders) in order to improve their surveillance systems. Specific detection algorithms that can process temporal signals from correlated time series simultaneously are under development. The interest of such algorithms will be discussed with the National Platform for Animal Health Surveillance (ESA) and the Health monitoring platform for the Food Supply Chain (SCA) in France.

Methods developed in NOVA WP3 are also used in a national *Campylobacter* project in Sweden. One of the aims for this project is to assess the temporal correlation between *Campylobacter* surveillance data in broilers and human campylobacteriosis incidence. The work carried out in WP3 on the univariate analysis of broiler data in Sweden and the methodological points discussed (D-JRP6-3.4) will provide a good basis to achieve the aim.

The output from NOVA WP5 (T1) has been used as support for the industry and authorities in revision of the legislation for surveillance of *Salmonella* in the Danish poultry and egg industry. This legislation has to become approved by the EU commission, and thereby the approach is transferred to stakeholders at many different levels. Also in Sweden, output from NOVA has been used to support the Swedish Board of Agriculture and the animal health organisations in their work to adapt surveillance activities and Swedish legislation to the Animal Health Law within the EU. The models have been used to support decisions about the target confidence level of future surveillance and to compare surveillance alternatives (e.g. different sample size, sample type, frequency of testing).

The work conducted in WP5 T2 is a part of the ongoing revision of surveillance of AMR in the Danish animal and food production (DANMAP). In the future, this surveillance will be based on genomics compared to cultivation that is used today, and thereby the approach and opportunities for collection of samples will be changed.



OHEJP MATRIX

A communication for potential collaboration has been initiated between WP1 and the OHEJP Matrix Consortium. Focus is on a questionnaire to ask countries about essential variables in a successful OH surveillance system.

Intermediate results of WP1 (i.e. adapted map of food-chain developed for the purposes of the WP) have also been shared with the Matrix WP2 ("Best-practices and multi-sectorial collaboration").

Expertise from WP4 tasks have allowed WP4 participants to collaborate in salmonella surveillance questionnaires.

OHEJP DISCOVER

This is a project looking at source attribution. The Salmonella modelling carried out in the NOVA project WP5, and in particular the parameter estimation (estimation of prevalence of *S. Typhimurium* at slaughter) will provide useful inputs for the DISCOVER project.

COST ASF-STOP CA15116. Understanding and combating African Swine Fever in Europe.
<https://www.asf-stop.com>

Some of the NOVA WP4 members are participating in this e-cost action. Within this project an assessment of biosecurity measures to prevent the spread of infection diseases has been conducted for intensive, non-commercial and extensive pig farms.

VACDIVA

Outputs from NOVA WP4 related to geographical distribution of animals species evaluated the last two years (2019-2020, pig animals and wild boars) have been shared with the VACDIVA Horizon 2020 European Project to evaluate the vaccination scenarios for ASF.



JRP07-LISTADAPT

1. Summary of the work carried out in year 3

With twenty-one partners, including food, environment, veterinary and public health laboratories, we constructed a dataset of 1575 strains and genomes originating from *Listeria monocytogenes* (Lm) strains collected in 20 European countries. This dataset encompasses a large number of Clonal complexes (CC)s occurring worldwide, covers many diverse habitats and is balanced between ecological compartments and geographic regions. This dataset should contribute to improve our understanding of Lm ecology and should aid in the surveillance of Lm. All the produced genomes in LISTADAPT are now available to the scientific community (umbrella Bioproject submitted to European Nucleotide Archive (ENA)).

We reported for the first time the occurrence of a CC121 strain isolated from a dolphin brain. Genomic comparisons showed that 16 strains isolated from food were the closest to the dolphin strain. Like most of the food strains analysed here, the dolphin strain included genomic features (transposon Tn6188, plasmid pLM6179) both described as being associated with the strain's adaptation to the Food Processing Environment (FPE). It is likely that the infection of a dolphin by Lm may be the result of environmental contamination by anthropogenic activities. The infection of this dolphin by a strain of a clonal complex known to be hypovirulent for human asks the question of the degree of weakness of the animal and its degree of exposure to this clone.

From the 1575 strains, we created a subset of about 200 strains from 34 Clonal Complexes (CC) occurring worldwide. This panel is balanced between three ecological compartments: environment, animal and food. Phenotypic tests were performed to investigate the ability of these strains to survive in the soil. Three groups were identified according to the survival rate (SR): phenotype 1 (SR<2%), phenotype 2 (2%<SR<5%) and phenotype 3 (SR>5%). Genome Wide Association Studies (GWAS) analysis have shown that the ability to survive in the soil was linked to multiple genetic factors. GWAS applied on smaller and more genetically homogeneous subsets (strains from the same CC or from the same origin) successfully identified small effect genomic variations in various transcriptional regulators, stress related genes whose the combination increase significantly the soil survival rate. Moreover, a cluster of 28 prophage-related -genes were associated to soil fitness in the Lineage II. A cluster of 14 phage related genes was associated to poor soil fitness in the CC6 (Lineage I).

We also tested the antimicrobial susceptibility toward 11 antibiotics and 4 biocides. Results revealed that strains isolated from food exhibited overall higher minimal inhibitory concentrations (MIC) for ammonium quaternary compounds (QACs) and peracetic acid (PAC) than strains isolated from animals and natural environments. Conversely, no significant difference was observed among MIC of antibiotics for strains depending on their origins. Interestingly, repeated exposure to QACs recurrently led to a decrease of susceptibility toward ciprofloxacin (CIP), a fluoroquinolone antibiotic, largely used in human and veterinary medicine and considered as a critically important antimicrobial. Moreover, these lower levels of susceptibility to CIP remained stable in most strains even after subcultures without biocide selection pressure suggesting an adaptation involving modifications at the genetic level. PanGWAS analysis have shown e that accessory genomics elements were causally associated with biocide tolerance. These elements were detected across 197 strains. Prophage-related loci and mobile genetic elements (MGEs), including the Tn6188_qacH transposon and the pLMST6_emrC plasmid, were associated with tolerance to benzalkonium-chloride (BC), a QAC widely used in food processing. In addition to BC, the pLMST6_emrC was associated with tolerance to another QAC, the didecyldimethylammonium-chloride, denoting the pleiotropic effect of such marker. Genomics elements encoding for cell-surface proteins were associated with BC or polyhexamethylene-biguanide (PHMB) tolerance, while no associations were detected for chemically reactive biocides (alcohols and chlorines). PHMB tolerance markers were restricted to lineage I strains from animal and the environment. In contrast, the MGEs related to QACs tolerance were widespread across lineage I and II



food strains, emphasizing the high adaptability of such strains, the polygenic nature of tolerance mechanisms and the need to monitor these markers to reduce the risk of tolerant-genotypes in food industrial settings.

2. Work carried out in the JRP, scientific results

WPO: Coordination (M1-M30)

The LISTADAPT leader, Laurent Guillier, left the project during the summer of year 2 (Y2) for another position in ANSES. The project lead has been transferred to Sophie Roussel, the successor of Laurent Guillier. Sophie Roussel is a senior scientist that has maintained close relationships with all the LISTADAPT partners. Indeed, she was a scientist at Anses from 2007 to 2017 working as the head of the molecular typing team of different food-borne bacteria, including *Listeria monocytogenes*. She was closely involved in European projects funded by EFSA and by the European Union (Horizon 2020 PHC7-“COMPARE”). In 2016, with her team, she wrote and set up LISTADAPT. Sophie Roussel worked then during two years (2017-2019) as Research Director at INRAe and was coordinator of the International Centre of Microbial Resources (CIRM) set up by INRAe. In this frame, she was involved in different European programs (H2020 -“CIRCLES” project and H2020 EJP One Health “CARE”).

As LISTADAPT leader, Sophie Roussel coordinated and organised two face-to-face meetings (M25): (i) the first – one day -17th January 2020- meeting ANSES-INRA specifically dedicated to WP3-WP4 (ii) the second - half a day -24th January 2020- with all the partners. Sophie Roussel was invited to present the project (i) during the annual European workshop EURL / NRL *Listeria* in January 2020 (see JRP7-WP5-T4: Dissemination) (ii) during meetings with different scientific partners such as the LIBio, a laboratory of Lorraine University (24th November 2020) and the main professional federations of the French food industry /ADEPALE) (26th November 2020). In addition, Sophie Roussel was invited by the ANSES management team to present the project to the scientific organisation committee of the Anses (26th May 2020 -60 participants). Sophie Roussel is also invited to present the project and all the results during the annual European workshop EURL / NRL *Listeria* in 2021. The seven LISTADAPT NRL partners will attend the meeting. Sophie Roussel will organize a skype closure meeting in May 2021.

WP1: Constitution of a strains collection representative of the different reservoirs of *Listeria monocytogenes* (M1-M12)

JRP7-WP1-T1: Strain collection (M1-M12)

The LISTADAPT strain collection, unique in Europe, is composed of two compartments, one of which (First compartment C1) very original, as it regroups 847 strains isolated in the environment (soil, river, farm environment) along with strains from wild and farm animals (both healthy and animal presenting clinical symptoms)..The second compartment (C2) is composed of 728 strains from five ready-to-eat (RTE) food categories from LISTADAPT partners (Table 1). All the 1575 strains are now centralized, long-term maintained and stored within the ANSES bacterial collection. The future use of some of these strains is protected by MTAs (Material Transfer Agreement). At ANSES, typing data (MLST data and cgMLST data) and associated epidemiological information of all the strains are centralized in a molecular database under the software BioNumerics version 7.6.3. A standardized nomenclature has been established with well-defined categories for all epidata (country, origin, matrix and animal condition) in order to improve the homogeneity of strains IDs.



Table 1: Repartition by compartments and sub-compartments of strains from the whole LISTADAPT collection (1,575)

Animal and environment (C1)				Food products and food production environment (C2)					
Farm animals	Wild animals	Soil and farm environment	Total	Meat	Fish	Dairy	Vegetables	Composite dish	Total
459	194	194	847	246	165	119	95	103	728



JRP7-WP1-T2: Campaigns to collect additional animal and environmental strains (M1-M10)

JRP7-WP1-T2-ST1: External collaborations (M1-M2)

To increase the size and representativeness of the collection, the LISTADAPT consortium performed an extensive review of all recent collections of published and unpublished *Lm* strains and then contacted researchers in charge of these collections. Finally, 14 external partners, food and veterinary laboratories and research institutes, all dealing with *Lm* hazards in Europe, collaborated with the LISTADAPT consortium (Tables 2 and 3). The initial collection included more strains from animals with listeriosis-associated clinical symptoms than without symptoms. In order to reduce the number of strains originating from animals with listeriosis while maintaining maximum diversity of the dataset, we adopted an original method to select the strains based on metadata (e.g., type of sample, geographic location, isolation time, molecular typing data such as PFGE profiles, animal species and geographic sampling location). This method relies on Gower's coefficient (GC), which is a dissimilarity measure: the “distance” between two units is the sum of all the variable-specific distances (associated with metadata categories). Because this collaborative work, we increased the representativeness and the diversity of animal and environmental *Lm* strains (strains from more countries at the European Union (EU) level and more partners at a national level). We constructed a large dataset comprising 344 animal and environmental *Lm* strains from 6 European countries and published collections (Table 2), as well as 373 animal and environmental *Lm* strains from 15 European countries and non-published collections (Table 3).

Table 2: List of 344 animal and environmental *Listeria monocytogenes* strains from published microbial collections.

Partner	Country (country code)	Category	Origin of isolation	No. of strains	Isolation year	References
Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine of Helsinki (n=132)	Finland (FI)	Wild animals	Wild birds, hare, reindeer [NS]	49	1998, 2001	10,14
		Farm animals	Cow, cow milk in bulk tank and pigs [NS]	62	1981–2011	14,43–46
			Cow (aborted fetus) [CS]	4	1984–1987	14,44,45
		Soil and farm environment	Silage ¹ and soil	17	1987–2004	
Faculty of Veterinary Medicine, University of Munich (n=31)	Germany (DE)	Wild animals	Deer and wild boar [NS]	31	2011–2012	6
Norwegian Veterinary Institute (n=29)	Norway (NO)	Wild animals	Slugs	29	2012	11



Department of Applied Microbiology and Human Nutrition ZUT (n=55)	Poland (PL)	Soil and farm environment	Soil from agricultural area	46	2010–2012	26
			Soil from park on city outskirts	9	2015–2016	
Department of Animal Health, NEIKER (n=71)	Spain (ES)	Farm animals	Cow, sheep and poultry feces [NS]	71	2004–2005, 2014–2016	4,47,48
			Farm animals	13	2011–2015	
Veterinary Faculty, University of Ljubljana (n=26)	Slovenia ^a (SI)	Soil and farm environment	Farm environment, water, pond	2	2008, 2014	25
			Wild animals	11	2014	
		Fox brain [NS]	11	2014		

CS, Clinical Symptoms. The reported clinical symptoms included rhombencephalitis, abortion, septicemia and mastitis/subclinical mastitis. The type of clinical samples included cerebellum/brain tissue, aborted fetus, fetal membrane, liver, internal organs, feces and milk. NS, No listeriosis-associated Symptoms¹ Strains isolated from silage were considered as originating from the farm environment since silage mainly includes fermented forage crops collected directly from fields.



Table 3: List of 373 animal and environment *Listeria monocytogenes* strains from non-published collections

Partner	Country	Category	Origin of isolation	No. of strains	Isolation year
Not communicated by the authors (n=96)	Belgium (BE)	Farm animals	Cow [NS]	96	2017–2018
		Farm animals	Cow, pig [NS]	6	2013–2014
Veterinary Research Institute (n=14)	Czech Republic (CZ)	Soil and farm environment	Mud, algae from pond, soil from farm, decaying vegetation	8	2010, 2014
		Wild animals	Gerbil, mouflon [NS]	3	Unknown
State veterinary institute (n=7)	Czech Republic (CZ)	Farm animals	Cow, sheep [NS]	4	Unknown
		Farm animals	Cow, sheep, goat [CS]	24	2014–2018
Veterinary and Food Laboratory (n=25)	Estonia (EE)	Wild animals	Deer [CS]	1	2018
		Farm animals	Cow, pork, goat milk, sheep [NS]	7	1987, 1995, 1998
Faculty of Veterinary Medicine/ Department of Food Hygiene and Environmental Health, Helsinki (n=24)	Finland (FI)	Wild animals	Hare, birds feces [NS]	4	1986, 1987
		Soil and farm environment	Silage ¹ and farm environment	13	2003, 2014–2015
		Farm animals	Cow, poultry [NS], horse [CS]	8	2003, 2014, 2015, 2018
Laboratory for Food Safety ANSES (n=25)	France (FR)	Wild animals	Hare [NS]	3	1986, 1996, 2015
		Soil and farm environment	Manure, soil	14	2004, 2006, 2009, 2012
		Soil and farm environment	Soil, compost, pasture	6	2011, 2012, 2013, 2018
Research Unit OPAALE INRAE (n=6)	France (FR)	Soil and farm environment	Soil, compost, pasture	6	2011, 2012, 2013, 2018
Institute of Food Safety and Food Hygiene, Faculty of Veterinary Medicine, Freie Universität Berlin (n=15)	Germany (DE)	Farm animals	Pig and sheep at slaughterhouse retention area or immediately after slaughter [NS]	15	2009, 2018–2019
Institute of Food Safety, Animal Health and Environment BIOR (n=24)	Latvia (LV)	Farm animals	Cow, goat, sheep, pig [CS]	24	2013–2018
Animal Pathology Laboratory INIAV (n=13)	Portugal (PT)	Farm animals	Cow, goat, sheep, zoo animals [CS]	12	2017–2019
		Soil and farm environment	Corn silage	1	2019
Veterinary Faculty, University of Ljubljana (n=53)	Slovenia (SI)	Farm animals	Cow, sheep, goat [CS]	27	2011–2015, 2018–2019
		Wild animals	Fox [NS]	2	2015
		Soil and farm environment	Cattle farm environment	1	2013
	Croatia (HR)	Farm animals	Cow, sheep, goat [CS]	23	2010, 2016–2017
Department of Biology, Swedish Food Agency (n=16)	Sweden (SE)	Wild animals	Deer, rook, moose, wild boar [NS]	13	Unknown
		Farm animals	Poultry [NS], goat, sheep [CS]	3	Unknown
State Veterinary and Food Institute Dolny Kubin (n=22)	Slovakia (SK)	Farm animals	Sheep, goat [CS]	20	2016–2018
		Soil and farm environment	Feed	2	2015, 2017
Laboratory Feed and Food and Product Safety VWA (n=33)	The Netherlands (NL)	Farm animals	Cow, sheep, goat, poultry [NS]	33	2016–2018

CS, Clinical Symptoms. The reported clinical symptoms included rhombencephalitis, abortion, septicemia and mastitis/subclinical mastitis. The type of clinical samples included cerebellum/brain tissue, aborted fetus, fetal membrane, liver, internal organs, feces and milk ; NS, No listeriosis-associated Symptoms ; ¹ Strains isolated from silage were considered as originating from the farm environment since silage mainly includes fermented forage crops collected directly from fields.



JRP7-WP1-T2-ST2: Sampling campaigns (M1-M10)

Soil, farm, and wild animal samples were collected in eight European countries (Table 4) in 2018 and in 2019. For the collection of soil samples, the LISTADAPT project members raised awareness and organised crowd-sampling campaigns. All the soil samples were collected from agricultural or wild areas according to a common procedure provided to the samplers based on the existing recommendations reported in the literature. The integration of feedback from samplers enabled a continuous improvement of the sampling protocol. The sampling campaigns were conducted in 17 areas in seven EU member states, Norway and Switzerland, namely AT, CH, CZ, FR, IT, NO, SE, SI and SK, resulting in the isolation of 75 *Lm* strains. Out of the 1752 available sampling records, the overall prevalence was 3%. We confirm in the present study the low prevalence of *Lm* in soil reported in the literature (below 1% and up to 6% depending on soil type).

Regarding the subcompartments of farm and wild animal, 55 *Lm* strains were isolated from sampling campaigns. Three campaigns targeting shelled gastropods sampled in IT, SK and CH resulted in the isolation of six strains. Sampling campaigns were also carried out for wild deer and reindeer feces in Southern Norway, and from cattle, roe deer, wild boar, wolf, bear and fox feces in the Abruzzo and Molise regions of Italy. Of the 2577 samples collected from vertebrates during the campaign conducted in IT and NO 40 isolates were detected, with an overall prevalence of 1.5%.



Table 4: List of 130 animal and environment strains from sampling campaigns

Partner	Country (country code)	Category	Origin of isolation	No. of strains	Isolation year
Austrian Agency for Health and Food Safety AGES (n=1)	Austria (AT)	Soil and farm environment	Meadow	1	2018
Veterinary Research Institute (n=21)	Czech Republic (CZ)	Natural and farm environment	Soil, river bank, pond, decaying vegetation, manure	18	2016–2018
	Slovakia (SK)	Natural and farm environment	Soil, river bank	3	2016
Research Unit OPAALE INRAE (n=30)	France (FR)	Soil and farm environment	Soil, compost, pasture	30	2018–2019
Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G.Caporale (n=59)	Italy (IT)	Farm animals	Cow, goat, sheep [CS]	7	2014–2018
		Soil and farm environment	Soil and river water	16	2016–2018
		Wild animals	Fox, wolf, porcupine, badger, bear, snail, crayfish, roe deer, wild boar [NS]	36	2014, 2016–2018
Norwegian Veterinary Institute (n=10)	Norway (NO)	Wild animals	Deer and reindeer [NS]	10	2017–2018
Veterinary Faculty, University of Ljubljana (n=3)	Slovenia (SI)	Soil and farm environment	Water pond and soil	3	2018
State Veterinary and Food Institute Dolny Kubin (n=1)	Slovakia (SK)	Wild animal	Snail	1	2019
Department of Microbiology, National Food Agency (n=1)	Sweden (SE)	Soil and farm environment	Pasture	1	2018
Agroscope (n=4)	Switzerland (CH)	Soil and farm environment	Pasture soil	3	2019
		Wild animal	Snail	1	2019

CS, Clinical Symptoms. The reported clinical symptoms included rhombencephalitis, abortion, septicemia and mastitis/subclinical mastitis. The type of clinical samples included cerebellum/brain tissue, aborted fetus, fetal membrane, liver, internal organs, feces and milk. NS, No listeriosis-associated Symptoms



JRP7-WP1-T3: Strategy for sequencing (M1-M12)

Completed. The last batch of strains to sequence was sent in December 2019.

JRP7-WP2-T1: Purification of Lm DNA from 2000 Lm strains (M2-M14)

JRP7-WP2-T1-ST1: First batch Purification of DNA from Lm strains available (M2-M4)

The first batch of 140 strains has been used to purify DNA at month 10. Four additional batches of strains have been used at months 11-12, for a total number of 546 strains

JRP7-WP2-T1-ST2: Second batch Purification of DNA from additional Lm strains (M13-M14)

The second batch of strains has been used to purify DNA at month 24.

JRP7-WP2-T1-ST3: Purification of DNA from routine surveillance systems at IZSAM, DTU, AGES (M1-M12)

DNA from strains gathered during routine surveillance were also made available by ANSES, IZSAM, DTU and AGES.

JRP7-WP2-T2: Whole Genome Sequencing (WGS) (M3-M14)

JRP7-WP2-T2-ST1: First batch WGS for available Lm strains (M3-M6)

The first batch of 140 strains was sequenced in November 2018.

JRP7-WP2-T2-ST2: Second batch WGS for additional Lm strains (M13-M14)

The second (and last) batch of strains was sequenced in February 2020.

JRP7-WP2-T2-ST3: Ad hoc WGS (M3-M14)

Completed. Four LISTADAPT partners (AGES, IZSAM, ANSES and DTU) mainly performed the sequencing. All the genomes were centralized at ANSES. (See WP2-T3).

JRP7-WP2-T3: Genome Assembling and Annotation (M5-M16)

Task completed in April 2019. The next generation sequencing (NGS) paired-reads (2 × 150 bp) were generated during the project with Illumina platforms. The genomes were all *de novo* assembled and annotated with a harmonized in-house workflow named ARTwork (Assembly of reads and typing workflow) used in the ANSES Laboratory for Food Safety. In addition to *de novo* assembly, the ARTwork pipeline also performs genome annotation using Prokka. This whole genome sequencing (WGS) workflow has been described in detail in previous publications including the integrated bioinformatics tools and their corresponding versions, enabling repeatability and comparability of the results (Table 5). Assembled genome files were made publicly available in FASTA format through Figshare. Different WGS metrics and quality criteria were thus employed in the ARTwork pipeline to ensure high-quality WGS data. Reads with an estimated depth of coverage <30× (as estimated by BBmap⁴⁰) as well as contigs and scaffolds with a length of < 200 bp were excluded (n=22). Draft genomes with a total length outside the range of 2.7–3.3 Mb and with a total number of scaffolds > 200 (n=46) were also excluded. In addition, inter- and intra-species contamination of reads was determined using the recently developed ConFindr software (v0.5.1). Since recently demonstrated, inter-and-intra species contamination of 10 single nucleotide variants (SNVs) assessed by ConFindr in the conserved core genes does not significantly impact cluster analysis. We decided to exclude all genomes presenting SNVs lower than this cut-off (n=12) as well as various read- or assembly-related errors (n=34). The employed WGS metrics and quality criteria of the complete LISTADAPT genome dataset were reported (available at <https://figshare.com/s/6582ab54ce4fabfa1fc8>). After quality control of NGS and WGS data, the final LISTADAPT dataset included 1575 genomes. All metadata and WGS data collected herein were centralized and processed with standardized criteria for common nomenclature and NGS/WGS quality control before sharing between project partners. Reads normalized to 100× coverage, draft



assemblies and annotated genomes were also centralized at the MongoDB database located at ANSES (Maisons-Alfort Laboratory for Food Safety). Raw (non-normalized) reads for all the *Lm* strains sequenced in the LISTADAPT collection (n=1508) were submitted to the NCBI Sequence Read Archive (SRA) for sharing with the LISTADAPT project's partners. Raw (non-normalized) reads for 67 *Lm* food strains obtained from previous publications were submitted to the NCBI Sequence Read Archive (SRA) database and were linked to their existing accession numbers (available at <https://figshare.com/s/6582ab54ce4fabfa1fc8>).

Table 5: Bioinformatics tools implemented in the ARTwork pipeline and their versions

Application	Software	Version
Read mapping	BBMap	38.22-0
Read normalization	BBNorm	38.22-0
Quality assessment of reads	FastQC	0.11.8
Trimming of low-quality reads	Trimmomatic	0.38
<i>De novo</i> assembly	SPAdes	3.13.0
MLST prediction	MLST	2.16.1
Retrieval of the closest reference	Mash	2.0
Reference-based scaffolding	MeDuSa	1.3
Gap closing	GapCloser	2.04
Trimming of contigs <200 bp	Biopython	
Quality assessment of the assembly	QUAST	5.0.2
Genome annotation	Prokka	1.13.3

[WP3 Phenotypic characterisation of *Listeria monocytogenes* strains \(M1-M29\)](#)

JRP7-WP3-T1: Strategy for selection of strains for phenotyping (M1-M12)

A panel of 200 *m* strains among the 1575 collected and sequenced for the project has been selected on the ground of their reservoirs, sub-reservoirs, sampling area and clonal complex (Figure 1). The three LISTADAPT partners (INRA, NVI and ANSES) involved in phenotypic characterization (WP3) received the first set of 100 strains (those isolated from food) in May 2018 then the second (those isolated from environment) in April 2019. The selection was done according to the MLST-CC data, using statistical tests. The most prevalent CCs were selected.

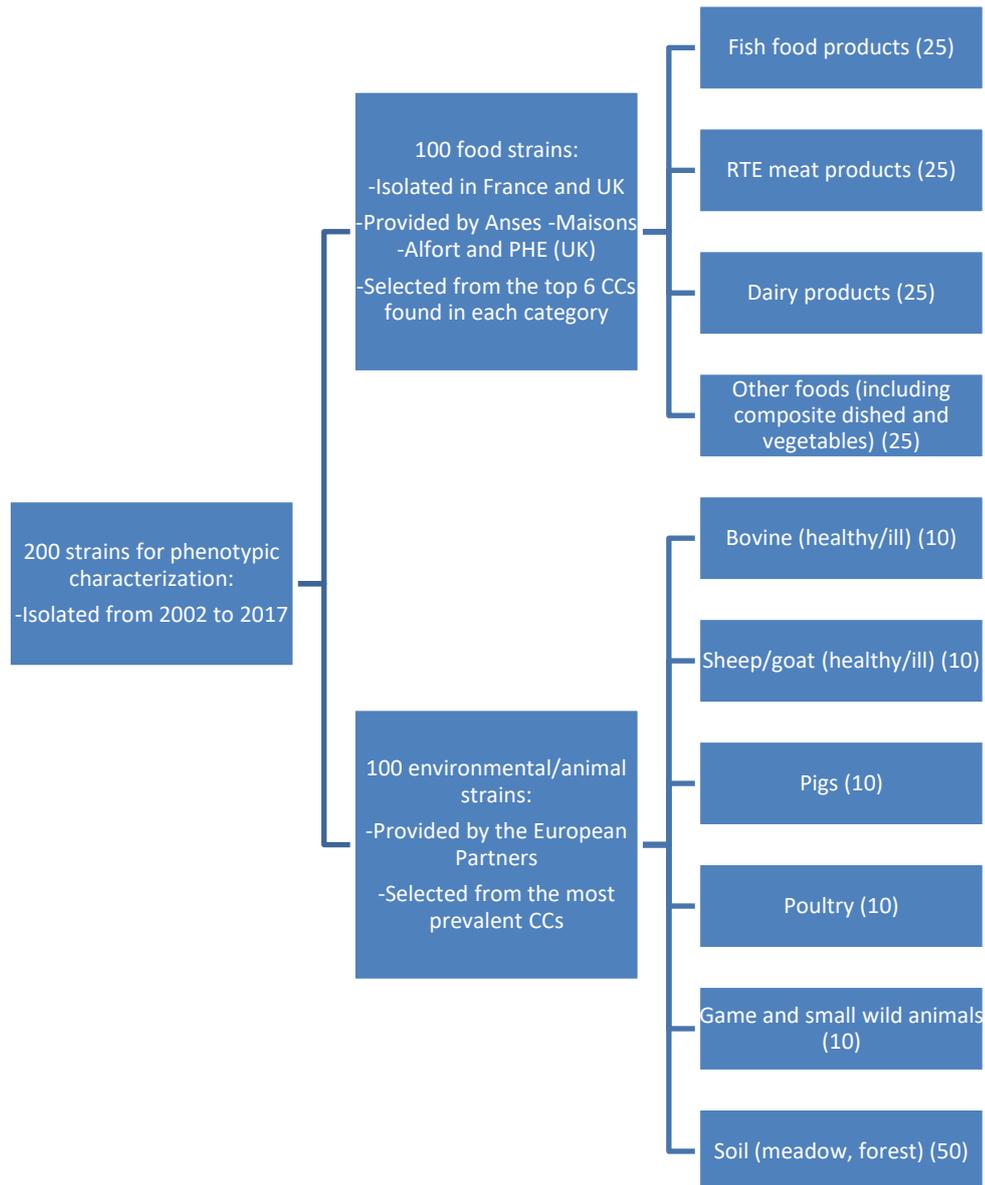


Figure 1: Description of the 200 strains selected for phenotypical tests

JRP7-WP3-T2: The effects of biocides on Lm strains adaptation (M3-M29)

JRP7-WP3-T2-ST1: Antibiotics and biocides resistance profiles of Lm strains (M3-M22)

Completed. The characterization of MICs (minimal inhibitory concentrations) values for 14 antibiotics and 8 biocides was achieved in August 2019. The results were summarized in a paper submitted at the journal Pathogens in December 2020 (cf point 7).

JRP7-WP3-T2-ST2: Adaptation to biocides and cross-resistance development to antibiotics of relevant Lm strains (M12-M22)

Completed. Thirty-four isolates have been tested on three disinfectants (seven different concentrations). The disinfectants used were Didectyl Dimethyl Ammonium Chloride (DDAC), Sodium hypochlorite (HS) and Hydrogen peroxide (Hper).

JRP7-WP3-T2-ST3: The effect of biocides on Lm strains in biofilm (M12-M29)

Completed in May 2020 (M29).



JRP7-WP3-T3: Bacterial adhesion and biofilm formation of Lm strains (M3-M29)

Completed. The ability of strains to adhere and to form biofilm was evaluated by using two complementary approaches: the BioFilm Ring Test™ (BRT) and crystal violet (CV) methods which allow to characterize early and late/mature states of biofilm development, respectively. The BRT device (Chavant et al., J. Microbiol. Meth., 2007) makes it possible to evaluate the capacity of a bacterial strain to adhere to an abiotic support and start forming cellular aggregates. For each strain, the BRT were carried out after 6, 24 and 48 h of culture in BHI medium, in microplate wells incubated at 20°C, with three replicates for each time. The CV method is directly related to the biomass formed by bacterial cells in biofilm. In this case, the biomass of the biofilms formed by the different strains growing in BHI medium at 20°C in microplate wells was evaluated after 24 and 48 h of incubation. Six replicates were carried out for each of the two times.

JRP7-WP3-T4: Survival and persistence of Lm strains in different ecological niches (M3-M29)

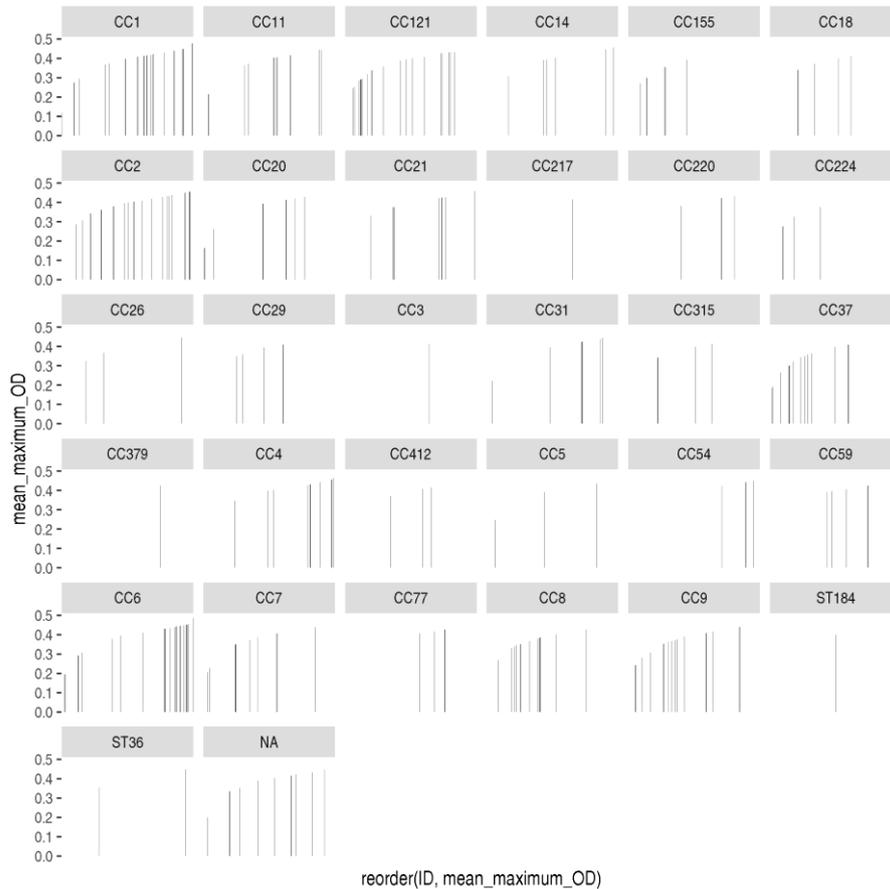
JRP7-WP3-T4-ST1: Survival of Lm in food products and gastro-intestinal environment (M3-M29)

Completed. The primary purpose of the study was to investigate whether the genetic variation had any influence on the responses. The second was to see whether the strains isolated from food responded differently than the strains from nature and animals. An effect was expected as some of the strains from food probably had been isolated from foods with preservatives. All strains grew well in BHIB at 12 °C. The growth was also significant at 4 °C, but much slower, as expected.

At pH 7, no difference was observed between the 200 strains. The additives had no impact on the growth. The latter was reasonable, as the fraction of undissociated acids at this pH was close to zero at neutral pH. At pH 4.5, the strains responded differently, and a clear effect of the additives was observed. Some strains showed a longer lagphase, some a slower increase in optical density, and some did not reach as high maximum optical density. The inhibiting effect of the additives followed this order: high conc of acetate, low cons of acetate, high conc of lactate and finally low conc of lactate. This order was the same as the concentration of undissociated acids in the four cases.

A co-variation between CC groups and the phenotypic response of the isolates was however not seen. The more than 200 isolates studied represented 26 CC groups. Isolates with different sensitivity to 2000 ppm of acetate were present in 16 of the CC groups, some of them often found in food. The reasons for the different responses is therefore connected to other characteristics is the genome, which will be further explored in GWAS analyses. The impact of the results is that challenge studies of foods and development and validation of predictive models should take strain variation into account when targeted to food with low pH.

The impact of CC group, isolation step in the farm-to-fork chain and possible genetic markers for high or low tolerance of preservatives will be systemised and analysed with classical and bioinformatics tools.



The initial results were presented in an oral presentation at the EJP conference in Prague in May 2020 (cf Point 7).

A similar study was carried out to investigate differences in survival rates during in-vitro digestion. Also here, large variations were seen, but the effect of stomach acid pH largely overruled the effect of strain variation.

The deliverable will be uploaded January 2021.

JRP7-WP3-T4-ST2: Survival of *Lm* in soil microcosm (M3-M16)

Completed in November 2019. As shown Figure 1, the ability of *Lm* to survive in soil was strain dependent. Survival ranged from zero to 22% (Figure 2).

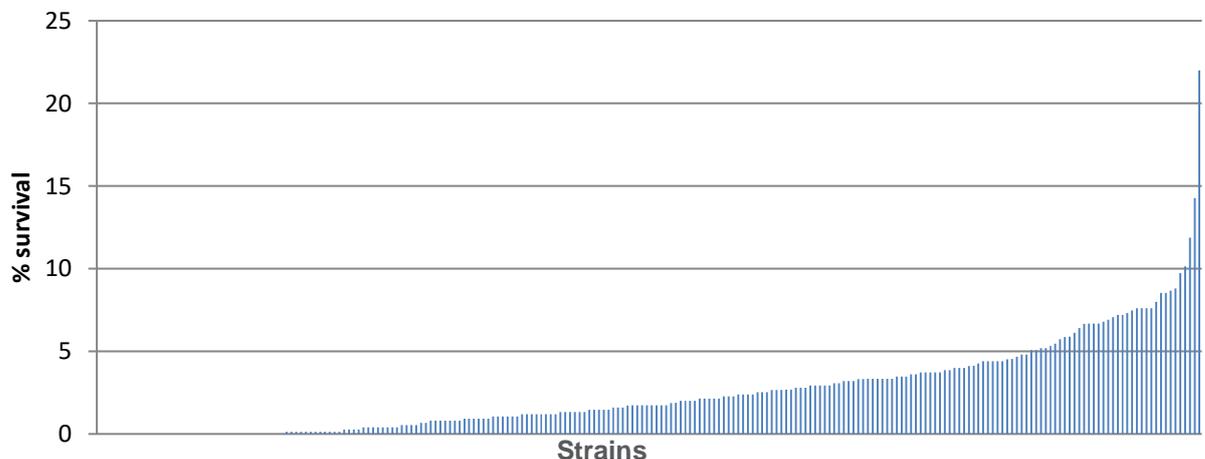


Figure 2. Soil survival phenotype of 230 isolates of *Listeria monocytogenes*.



Ascending Hierarchical Clustering clearly identified 3 groups of phenotypes (Figure 3), possibly indicating that some isolates (Phenotype 3) may be better competitors in complex habitats such as soil.

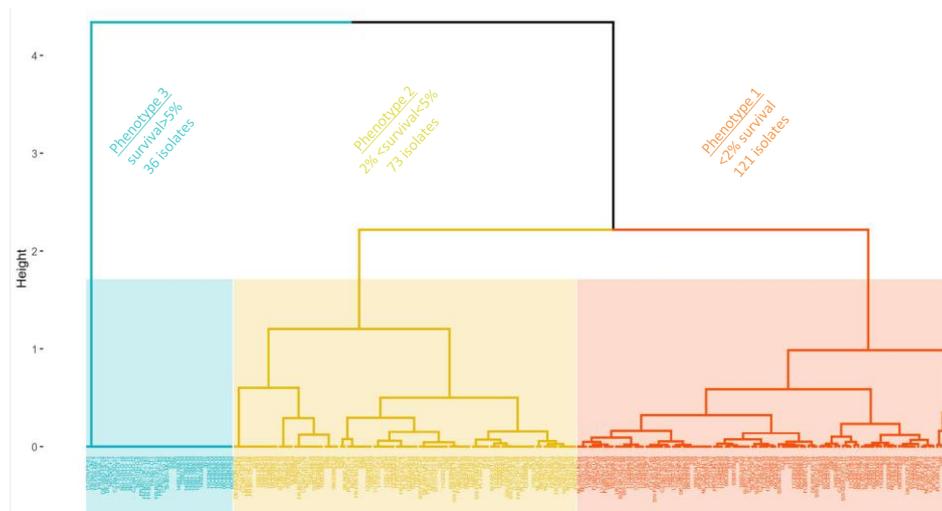


Figure 3. Ascending Hierarchical Clustering of soil survival data.

WP4: Identification of genetic traits in *Listeria monocytogenes* underlying adaptation to the ecological niches (M1-M30)

JRP7-WP4-T1: Analyze the distribution / prevalence of clonal complexes among the reservoirs (M1-M14)

This task has been completed in April 2019. This characterization helped to determine the two sets of strains for phenotypic studies. The 1575 strains clustered into 109 MLST STs, which belonged to 52 CCs and 23 singleton STs. For 38 strains, the allele profile was unknown (a presumable novel ST) or incomplete (When six out of seven MLST alleles were present, a CC was assigned when possible). We analysed in depth the strains genetic diversity between the three compartments and we showed the obtained results during (i) a 2-days-meeting at ANSES in April (2019) including all the partners and some external partners and (ii) the EJP general meeting in Dublin (2019).

JRP7-WP4-T2: Literature search of genes or genetic mechanisms responsible for virulence, adaptation and survival (M9-M12)

Completed. The list of genes involved in adaptation and survival was produced from research data obtained during the H2020 COMPARE project;

JRP7-WP4-T3: Biostatistics analysis of annotated genomes (M6-M29)

JRP7-WP4-T3-ST1: Identification of statistically relevant methods and development of analysis (M6-M16)

Completed. During the kick-off meeting of Y1 (March 2018), a list of relevant tools for identifying markers of adaptation to niches (environment, food industry) was established. The LISTADAPT partners has identified two alternative methods (DBGWAS and machine learning method from DTU) (Jaillard et al., 2018). Within the full lists, at least three methods are tested (Machine learning, GWAS based on presence/absence matrix and TreeWAS for SNP) (Brynildsdud et al., 2016; Collins et al., 2018). For the research of genes identified in JRP7-WP4-T2, the LISTADAPT partners have chosen to use ABRICATE method (<https://github.com/tseemann/abricate>).

- Scoary: <https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-1108-8>
- TreeWAS: <https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005958>
- DBGWAS: <https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1007758>



JRP7-WP4-T3-ST2: Processing of all isolates (M22-M27)

Completed

JRP7-WP4-T4: Comparative analysis of phenotypic data / genotypic data (M24-M29)

The results obtained in tasks WP3-T4-ST1; T3 and T2-ST3 will be compiled with all the genomic data for a mixomics approach. All the data will be centralized in a database.

Antibiotics and biocides resistance profiles of Lm strains

Completed. The results (genomic and phenotypic data) were summarized in a publication submitted at the journal "Food Microbiology" in December 2020 (cf point 7)

Adaptation to biocides and cross-resistance development to antibiotics of relevant Lm strains

Genomic investigations on the strain adapted to biocides revealed important insertions and deletion in the Internalin sequence. Those variations impacted mainly Internalin of unknown function. Several biocide-adapted-strains presented also deletion of transposons or plasmids. An analysis on SNPs revealed that resistance to QACs are likely to be caused by the inactivation of a multidrug resistance efflux pump regulator by the insertion of a stop codon, hence explaining the increased resistance to biocide and to fluoroquinolones. A scientific paper is planned (meeting Anses 18th January 2021)

Survival in soil

Completed. We have investigated the correlation between the phenotypes obtained for survival in soil and the characteristics of the genomes from the 200 isolates. Genome Wide Association Studies (GWAS) analysis did not evidence any link between the origin, the lineage or CC and the fitness in soil. This data suggest that the ability to survive in the soil is linked to multiple small effect genetic factors such as variations of transcriptional regulators, stress resistance proteins and cell wall proteins. However, GWAS applied on smaller and more genetically homogeneous subset (strains from the same CC) successfully identified phage-related- genes associated with soil survival rate. In particular, in the CC6, phenotype 1 was associated with the presence of a lysogenic phage corresponding to LP-030-3 and with variations in the transcriptional regulator BglG. A scientific publication ANSES/INRAE combining the phenotypic and genotypic data is in preparation and will be submitted in February for the review "Frontiers in Microbiology" (cf Point 7)

Bacterial adhesion and biofilm formation of Lm strains

A statistical study of all these results is now undertaken in order to highlight possible correlations between the origin or clonal complex and the ability to rapidly form a biofilm, and/or a significant biofilm. A publication combining the phenotypic and genotypic data is planned (meeting ANSES/INRAE 12th January 2021).

Survival of Lm in food products and gastro-intestinal environment

Genomic analysis are in progress at NVI. A NVI/ANSES meeting was held in March 2020 and another meeting at the end of January 2021. A publication combining the phenotypic and genotypic data is planned.

WP5 : Trainings and dissemination (M1-M30)

JRP7-WP5-T1: Implementation of a workshop (M1-M2)

Statistical and bio-informatic tools useful for the project were discussed during the Kick-off meeting (March 2018).

JRP7-WP5-T2: Trainings (M3-M6)

Completed. A training has been organized by the LISTADAPT coordinator in April 2019, in parallel with the Y2-meeting. It aimed to train 20 participants to R package methods.



JRP7-WP5-T3: Proficiency Testing Trials (M19-M22)

Given the delay in the project, we decided that there will be no Proficiency testing trial WGS as planned. However, as part of the activities carried out by ANSES as EURL / NRL *Listeria*, a PT trial WGS was organized in 2018, then in 2019, during which four LISTADAPT partners (AGES, IZSAM, NVI, ANSES) participated.

JRP7-WP5-T4: Dissemination (M1-M36)

Congresses and Publications –see Point 7

3. Project self-assessment

LISTADAPT has resulted in 1575 new *Lm* genomes available to the scientific community. As planned, LISTADAPT stimulated Reference laboratories to use WGS for the surveillance of *Lm* in their countries, as recommended by EFSA and ECDC. LISTADAPT allows continuing stimulation or implementation of WGS as the method of surveillance of *Lm* in the European countries, as recommended by the EFSA Biohaz panel in 2019 (<https://doi.org/10.2903/j.efsa.2019.5898>). Given the delay in the project, we decided that there will be no proficiency testing trial WGS as planned. However, as part of the activities carried out by ANSES as EURL / NRL *Listeria*, a PT trial WGS was organized in 2018, then in 2019, during which four LISTADAPT partners (AGES, IZSAM, NVI, ANSES) participated. As foreseen, the LISTADAPT's partners now share (i) a well-characterized collection of 1575 strains and genomes representative of the different ecological niches and (ii) a database centralizing genomes, strain metadata and all the phenotypic data.

We had originally planned for WP1 to end in December 2018 (M12). However, it was very challenging to collect *Lm* strains isolated from natural environment. That is why we needed to (i) look for more partners (ii) collaborate with them and (iii) perform additional sampling campaigns. This is the reason why WP1 has been extended until December 2019 (M24). This has led to a substantial delay in the project in particular (i) in obtaining all the genomes assembled and annotated (completed in M28 instead of M16) (ii) in selection of the second batch of strains for WP3 (completed in M16) and (iii) in genomic analysis for WP4. WP4 should be completed in the coming months.

We presented in detail the strain and genome collection in a paper, accepted with minor revision in the review Scientific Data of the Nature journal (Impact Factor: 5,7). This dataset is so important and so original that Sophie Roussel's team will be able to promote its use (i) in several other scientific publications, in collaboration with the LISTADAPT partners (ii) in the OHEJP-CARE project. We have planned two more years to publish all the combined WP3- WP4 results. One of the post-doctoral students that worked on LISTADAPT for two years continues to work on the WP4 (he is funded by One Health EJP CARE until the end of December 2021). In addition, a scientist specialised in genomic analysis has been recently recruited on a permanent scientific position in Sophie's team and is currently working on completing WP4. LISTADAPT allowed us to broaden our network of partners and to discover other scientists. This will allow us to continue to collaborate with them in future research projects on *Listeria monocytogenes*.



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4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories* <i>(1 to 8) (several categories may be applicable)</i>
07	D-JRP7-0.1	Consortium agreement	M1				
07	D-JRP7-0.2	Internal reporting templates	M3	M3		https://zenodo.org/record/3733303#.X_2-W-hKjcc	
07	D-JRP7-0.3	Plan for dissemination and exploitation of results.	M14	M16		The dissemination plan was validated during the face-to-face meeting of April 2019. https://zenodo.org/record/3733315#.X_2-d-hKjcc	
07	D-JRP7-1.1	Description of the panel of strains already sequenced	M1	M1		Sequenced strains were mainly isolated from food industry/ready-to-eat food. These strains were described with metadata https://zenodo.org/record/3733281#.X_2_f-hKjcc	3
07	D-JRP7-1.2	Description of the first panel of strains available to sequence	M3	M3		https://zenodo.org/record/3686994#.X_2_JuhKjcc	3



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories* <i>(1 to 8) (several categories may be applicable)</i>
07	D-JRP7-1.3	Description of the second panel of strains to sequence.	M12	M24		The last panel of strains to sequence was sent in December (11 December 2019) Deliverable uploaded on web site on 18 December 2019. https://zenodo.org/record/3733232#.X_2-tOhKjcc	3
07	D-JRP7-1.4	Report on strain collection and strategy for selection of strains for sequencing.	M14	M14		The strategy was presented during the OHEJP conference in May 2019 (Dublin). Deliverable uploaded on website on 16 December 2019 https://zenodo.org/record/3734093#.X_2-ZuhKjcc	3, 4
07	D-JRP7-2.1	Annotation of <i>Lm</i> genomes already sequenced (genomes available before the start of the project).	M26	M31		Confidential until publication in Scientific data is accepted.	3
07	D-JRP7-2.2	Annotation of the <i>Lm</i> assembled genomes from 1st batch sequencing.	M26	M26		Confidential until publication in Scientific data is accepted.	3
07	D-JRP7-2.3	Annotation of the <i>Lm</i> assembled genomes from 2nd batch sequencing.	M26	M26		Confidential until publication in Scientific data is accepted.	3



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
07	D-JRP7-2.4	Annotation of the <i>Lm</i> assembled genomes from <i>ad hoc</i> WGS	M26	M31		Confidential until publication in Scientific data is accepted.	2, 3
07	D-JRP7-3.1	Resistance profiles to biocide and antibiotics for the 200 <i>Lm</i> strains.	M12	M20		-Deliverable uploaded on web site on 17 December 2019. Poster presented during the OHEJP conference in May 2019 (Dublin) and during the IAFP's European Symposium on Food safety in April 2019 (Nantes). Confidential until publication in Pathogens is accepted https://zenodo.org/record/3686934#.X_2_NehKjcc	3
07	D-JRP7-3.2	Assessment of the ability to adapt to biocides and develop cross-resistance to antibiotics for some illustrative <i>Lm</i> strains.	M25	M26		Confidential until publication in Frontiers in Microbiology is accepted. https://zenodo.org/record/3828737#.X_2-p-hKjcc	9
07	D-JRP7-3.3	Data on the effect of biocides on <i>Lm</i> strains in biofilm.	M29	M26		Confidential until publication in Scientific data is accepted. https://zenodo.org/record/3734064#.X_2-BOhKjcc	9



JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories* <i>(1 to 8) (several categories may be applicable)</i>
07	D-JRP7-3.4	Biofilms phenotypes for the 200 <i>Lm</i> strains.	M33	M37		Task completed in December 2020 (meeting Anses/INRAE 12 th January 2020). Confidential until publication is accepted. Public. Deliverable uploaded on web site on 22 January 2021.	9
07	D-JRP7-3.5	Collection of data on survival of <i>Lm</i> as planktonic cells in various ecological niches.		M27	Deliverable not yet produced. Will be produced at the end of January 2021	Confidential until publication in Scientific data is accepted. Public	9
07	D-JRP7-3.6	Collection of data on survival of <i>Lm</i> in soil microcosms.	M22	M20		-Deliverable uploaded on web site on 17 December 2019. -Poster presented at ISOPOL XX in September 2019 (Toronto). https://zenodo.org/record/3686947#.X_2_TOhKjcc	3
07	D-JRP7-4.1	Bibliographic study on catalogues of genes	M13	M13		https://zenodo.org/record/3733308#.X_2-luhKjcc	3
07	D-JRP7-4.2	Report on prevalence and distribution of clonal complexes	M26	M26		Public https://zenodo.org/record/3734095#.X_2-KOhKjcc	9



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
		among the reservoirs.					
07	D-JRP7-4.3	Software chosen for bioinformatics analysis.	M16	M16		-Validated during the LISTADAPT meeting (April 2019). -Deliverable uploaded on website on 16 December 2019 https://zenodo.org/record/3733311#.X_2_vehKjcc	2
07	D-JRP7-5.1	“LISTADAPT” workshop program.	M2			No workshop was organised. Exchanges and discussions were held during the kick off meeting on methodologies and bioinformatics and statistical tools used in LISTADAPT; https://zenodo.org/record/3733319#.X_2-OOhKjcc	
07	D-JRP7-5.2	Minutes of the training sessions.	M10			There has been no minutes on the training sessions. https://zenodo.org/record/3733322#.X_2-hehKjcc	
07	D-JRP7-5.3	Publications and communications	M36			Public. Cf point 7	8, 1,3,4

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
07	M-JRP7-1	Kick off meeting	M2	Yes		6 th March 2018, located at Anses, Maisons-Alfort, France
07	M-JRP7-2	Selection of the 200 Lm strains based on genomic analyses in WP2.	M3	Yes		100 strains have been selected based on their genomic characteristics and context of isolation. These strains correspond to samples collected along the food production chain. They were sent to WP3 partners in April 2018; For the remaining 100 strains (from environment and from animals), the selection was based on the prevalence of CCs and chosen to best represent the diversity at the European level. The strains were sent to WP3 partners in May 2019.
07	M-JRP7-3	Workshop done	M3	Yes		Discussions on statistical and bioinformatics methods were held during the Kick off meeting.
07	M-JRP7-4	DNA prepared for 1st batch WGS	M4	Yes		The first DNA were prepared in September 2018
07	M-JRP7-5	Strategy for selection of strains for sequencing in place	M5	Yes		An <u>original</u> algorithm was developed for selecting strain based on meta-data describing the context of isolation of the strains



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
07	M-JRP7-6	WGS raw data produced	M6	Yes		The sequencing was reported of many months
07	M-JRP7-7	Face-to-face meeting - 2018	M8	Yes		Kick off meeting / 6 th March 2018, located at Anses, Maisons-Alfort, France
07	M-JRP7-8	First batch Lm genomes assembly completed	M8	Yes		Assembly and annotation with a harmonized in-house workflow named ARTwork. All the genomes are centralized in a database.
07	M-JRP7-9	Identification of Lm strains sequenced to be annotated	M10	Yes		
07	M-JRP7-10	First batch Lm genomes annotation completed	M10	Yes		
07	M-JRP7-11	WGS Training session done	M10	Yes		Done
07	M-JRP7-12	All the strain collected and centralized at ANSES	M12	Yes		<p>All the 1575 strains are centralized, long-term maintained and stored within the ANSES bacterial collection. The future use of some of these strains is protected by MTAs. Typing data and associated epidemiological information of all the strains are centralized in a molecular database under the software BioNumerics.</p> <p>A standardized nomenclature has been established. (country, origin, matrix and animal condition...).</p>



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
07	M-JRP7-13	DNA prepared for 2nd batch WGS	M12	Yes		Done
07	M-JRP7-14	Selection of some representative Lm strains for the study of adaptation to biocides.	M12	Yes		Thirty-four isolates have been tested on three disinfectants (seven different concentrations). The disinfectants used were Didectyl Dimethyl Ammonium Chloride (DDAC), Sodium hypochlorite (HS) and Hydrogen peroxide (Hper).
07	M-JRP7-15	WGS raw data produced.	M25	Yes		Done
07	M-JRP7-16	Second batch Lm genomes assembly completed.	M26	Yes		Assembly and annotation with a harmonized in-house workflow named ARTwork. All the genomes are centralized in a database.
07	M-JRP7-18	Second batch of Lm genomes annotation completed.	M26	Yes		
07	M-JRP7-19	Ad hoc batch of Lm genomes assembly completed	M25	Yes		
07	M-JRP7-20	Bioinformatic analysis done for all the strains	M28	Yes		See Table 5
07	M-JRP7-22	Ad hoc batch of Lm genomes annotation completed	M20	Yes		Annotation with a harmonized in-house workflow named ARTwork



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
07	M-JRP7-23	Face-to face meeting - 2020	M25	Yes		Done in January 2020 (24th). We plan to present all the results during the 2021 European NRL/EURL workshop (involving the Listadapt NRL partners).
07	M-JRP7-24	WGS Proficiency Testing trial (PTtrial) done	M26	No		Given the delay in the project, we decided that there will be no Proficiency testing trial WGS as planned. However, as part of the activities carried out by ANSES as EURL / NRL Listeria, a PT trial WGS was organized in 2018, then in 2019, during which four LISTADAPT NRL partners (AGES, IZSAM, NVI, ANSES) participated.

5. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
First report on the occurrence of <i>Listeria monocytogenes</i> ST121 strain in a dolphin brain. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7601084/ https://zenodo.org/record/4244051#.X6J4fziWxM1	YES		1250.81 EUR



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
A European-wide dataset to uncover adaptive traits of <i>Listeria monocytogenes</i> to diverse ecological niches. In revision for the review Scientific Data (Nature, IF : 5,5) <i>Accepted with minor modifications</i>	YES		1100 EUR
Comparative analysis of genetic determinants encoding cadmium, arsenic and benzalkonium chloride resistance in <i>Listeria monocytogenes</i> of human, food and environmental origin Front. Microbiol., 14 January 2021 https://doi.org/10.3389/fmicb.2020.599882	YES		1000 EU
Palma F, Radomski N, et al. Genomics elements located in the accessory repertoire drive the adaptation to biocides in <i>Listeria monocytogenes</i> strains from different ecological niches. Submitted to the review Food Microbiology in December 2020.	YES		
Guérin A, Bridier A et al. Exposure to quaternary ammonium compounds selects resistance to ciprofloxacin in <i>Listeria monocytogenes</i> . Submitted to the review Pathogens in December 2020	YES		
Sévellec Y, Ascencio Schuttz E, et al. Investigation of genome characteristics underlying fitness of <i>Listeria monocytogenes</i> in soil. In preparation for Frontiers in Microbiology.	YES		
Sévellec Y, Bridier A et al., Assessment of the ability to adapt to biocides and develop cross-resistance to antibiotics for <i>listeria monocytogenes</i> strains In preparation for Frontiers in Microbiology.	YES		



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Torresi M., Orsini M., Acciari V., Centorotola G., Di Lollo V., Di Domenico M., Bianchi D.M., Ziba M.W., Tramuta C., Cammà C., Pomilio F. Genetic characterization of a <i>listeria monocytogenes</i> serotype IVb variant 1 strain isolated from vegetal matrix in Italy. Microbiol Resour Announc. 2020 Aug 13;9(33):e00782-20.DOI: 10.1128/MRA.00782-20	YES		500 EUR



Additional output

Oral communications :

2020

- Skjerdal T, Fagereng T, Osland AM, Lagesen K, Fiskerbeck E, Nesse L, Sévellec Y, Felix B, Roussel S. 2020. Phenotypical responses to stress of in *Listeria monocytogenes* strains of different Clonal Complexes isolated along the nature-to-farm-to-fork chain. 27-29 May. Second annual meeting EJP (virtual meeting)
- Sévellec Y, Ascencio Schuttz E, Félix B, Guillier L, Roussel S, Piveteau P. Comparative Analysis of the genomic diversity of *Listeria monocytogenes* in soil and water and food processing through pan Genome Wide Association Study. 27-29 May. Second annual meeting EJP (virtual meeting)
- Palma F, Guérin A, Radomski N, Bridier A, Sévellec Y, Félix B, Soumet c, Guillier L , Roussel S. Deciphering the Biocide-Resistance of *Listeria monocytogenes* Strains from Europe through Genome-Wide Associations at the pangenomic scale. 27-29 May. Second annual meeting EJP (virtual meeting)
- Guérin A, Palma F Le Grandois P, Bridier A, Soumet C, Sevellec Y, Roussel S. Exposure to quaternary ammonium compounds show resistance to ciprofloxacin for *Listeria monocytogenes* from diverse ecological niches. 27-29 May. Second annual meeting EJP (virtual meeting)
- Sept 2020: Food Micro Next Generation Challenges in Food Microbiology –Athènes. Reported in September 2021/Three abstracts submitted (Y. Sévellec, S. Roussel)
- Sévellec, Y. An insight in *Listeria* genomes. Terramo. annual workshop of the ItNRL Lm. 16th September 2020

2019

- Felix B, Feurer C, Maillet A, Guillier L, Boscher E, Kerouanton A, Denis M and Roussel S (2019). Population genetic structure of *Listeria monocytogenes* strains isolated from the pig and pork production chain in France. 26-27 August 2019, Safepork 2019, Berlin.
- Félix B, Feurer C, Maillet A, Desmots M H, Hickey B, Jankuloski D, Karpíšková R, Skjerdal T, Denis M, Gareis M, Zdovc I, Pietzka A and Guillier L (2019). Typing and persistence of *Listeria monocytogenes* strains in food processing environments, prophages identified as major persistence markers. ISOPOL XX 2019, 24 – 27 September 2019, Toronto.
- Guillier L (2019). Assessment of the Genomic Diversity of a Large Collection of *Listeria monocytogenes* Strains Isolated in EU Natural Environments. OHEJP Annual scientific meeting, Dublin, 22-24 May 2019
- Guillier L (2019). Proposal of an Original Method for Selecting Strains to Include in Source Tracking or Source Attribution Based on their Metadata. OHEJP Annual scientific meeting, Dublin, 22-24 May 2019
- Sévellec, Y. 2019. Listadapt- Study of the Genetic diversity of *Listeria monocytogenes* from environment to human; Terramo. annual workshop of the ItNRL Lm. 4th July 2019

2018

- Felix, B., 2018. Population genetic structure of *Listeria monocytogenes* strains isolated from the pig and pork production chain in France, presented at Food Micro September 2018, Berlin (Germany).
- S. Antoci, V. A. Acciari, V. Di Marzio, I. Del Matto, G. Centorotola, M. Torresi, C. Marfoggia, G. Iannitto, A. Ruolo, G. A. Santarelli, G. Migliorati, F. Pomilio. Preliminary results on



prevalence and persistence of *Listeria monocytogenes* in different dairy and meat processing plants in Central Italy, presented at “International meeting on emerging diseases and surveillance” November 2018, Vienna (Austria).

Posters:

2020

- Toresi M, Rinaldi A, Marzio D et al. Genomic features of two main Clonal Complex groups of *Listeria monocytogenes* strains isolated from European wild animal. 6th World One Health Congress; 30 October - 3 November 2020.

2019

- Torresi M., Rinaldi A., Cammà C., Di Pasquale A., Skjerdal T., Lagesen K., Felix B., Sevellec Y., Guillier L., Leroux A., Ricao M., Boysen M., Lindström M., Castro H., Korkeala H., Gareis M., Frank E., Bulawova H., Brychta M., Amar C., Grant K., Pate M., Zdovc I., Pomilio F (2019). Diversity of *Listeria monocytogenes* associated with wild animals: focusing on CCs with a wide capacity of adaptation. International Symposium on Problems of Listeria and Listeriosis ISOPOL XX. 24-27 September 2019 Toronto
- Oevermann A, Hurtado A, Papić B, Karpíšková R, Piveteau P, Wullings B, Bulawova H, Castro H, Lindström M, Korkeala H, Šteingolde Ž, Bērziņš A, Avsejenko J, Kramarenko T, Cabanova L, Szymczak B, Torresi M, Leroux A, Sevellec Y, Guillier L and Félix B (2019). European-wide study reveals high prevalence of hypervirulent *Listeria monocytogenes* clones in farmed ruminants and their environment. ISOPOL XX -24 – 27 September 2019 Toronto
- Skjerdal T, Sevellec Y, Guillier L, , Zdovc I, Pate M, Torresi M, Ricao M, Boysen M, Lindstrøm M, Castro H, Gareis M, Bulawova H, Amar C, Grant K, Leroux A, Pomilio F, Camma C, Di Pasquale A, Lagesen K, Osland Mohr A, Rinaldi A, Karpiskova R, Pietzka A, Ruppitsch W, Szymczak B, Ascencio-Schultz E, Piveteau P and Felix B (2019). Occurrence and diversity of *Listeria monocytogenes* strains in environment and wild life in Europe. ISOPOL XX -24 – 27 September 2019 Toronto
- Ascencio-Schultz E, Gal L, Garmyn D, Szymczak B, Karpiskova R, Pietzka A, Ruppitsch W, Boysen M, Pomilio F, Torresi M, Camma C, Di Pasquale A, Pate M, Skjerdal T, Sevellec, Y., Felix B, Guillier L and Piveteau P (2019). Investigation of genome characteristics underlying fitness of *Listeria monocytogenes* in soil. ISOPOL XX -24 – 27 September 2019 Toronto
- Sévellec Y, Torresi M, Orsini M, Centorotola G, Bilei S, Senese M, Terracciano G, Felix B, Guillier L and Pomilio F (2019). Investigation of a dolphin infection by *Listeria monocytogenes* CC121. ISOPOL XX -24 – 27 September 2019 Toronto
- Vranckx K, Sevellec Y, Deneweth J and Felix B. Phages in Listeria: Who are they, what do they do? ISOPOL XX -24 – 27 September 2019 Toronto
- Guérin A, Le Grandois P, Bridier A, Soumet C (2019). Evolution of antibiotics and biocides resistance of *Listeria monocytogenes* from diverse ecological niches following in vitro exposure to biocides disinfectants. ISOPOL XX -24 – 27 September 2019 Toronto
- Guérin A, Le Grandois P, Bridier A, Soumet C (2019). Evolution of antibiotics and biocides resistance of *Listeria monocytogenes* from diverse ecological niches following in vitro exposure to biocides disinfectants. IAFFP’s European Symposium on Food Safety, Nantes, 24-26 April 2019
- Guérin A, Le Grandois P, Bridier A, Soumet C (2019). Evolution of antibiotics and biocides resistance of *Listeria monocytogenes* from diverse ecological niches following in vitro exposure to biocides disinfectants. OHEJP Annual Scientific meeting, Dublin, 22-24 May 2019

2018

- Felix B, Feurer C, Maillet A, Guillier L, Boscher E, Kerouanton A, Denis M, Roussel S. 2018. Population genetic structure of *Listeria monocytogenes* strains isolated from the pig and pork production chain in France. IAFFP EU Stockholm 25-27 April 2018



6. One Health Impact

The LISTADAPT project makes it possible bridging the gap between “Med” and “Vet”: this is the first time a project has focused on such a large and diverse collection of *Lm* strains isolated from farming/wild animals and farming/wild environment in different European countries. Most of these strains and genomes will be useful for the One Health EJP Project “CARE” (2020-2023) (Cross-sectoral framework for quality Assurance Resources for countries in the European Union), in close relation with the French National and European Reference Laboratory (NRL and EU-RL). The results obtained in WP3-T4-ST1 will be valuable for EURL activities such as selection of strains for challenge testing of low pH foods, and for risk assessments of foods with preservatives. Further, these results can be used to assess the validity of predictive models that have been developed with few strains for strains with other genetic characteristics.

With LISTADAPT project, we are looking for molecular markers of interest such as for instance mobile genetic elements harbouring antimicrobial resistance factors as well as provide insight into the population structure and evolutionary history of *Lm* for epidemiologic investigation. This information could be used for the development of **new diagnostic tests** to screen food, processing environment and animal reservoirs for contamination by *Lm* strains. These new tests represent **key tools** to improve the *Lm* surveillance system and to assist the food industry decision-making around food processing for improving food safety. For instance, a professional federation of the French food industry is interested in results obtained in W3/WP4. Following the presentation of the project in November 2020, they asked Sophie to give them a presentation in next May on the results obtained in WP4. They want to understand the mechanisms of adaptation of the strains in the plant chain from the soil to the finished plant products and wish to set up a research collaborative project with Sophie’s team on this subject.

The detailed characterization of these strains at phenotypic and genotypic level will help to assess the true importance of these strains as sources of foodborne infections for public health. Mechanisms for the survival and adaptation of *Lm* (i) in food processing environment (ii) in wild and farming animals (iii) in natural and farming environments will be identified. The genes identified will be used as targets for developing rapid monitoring tests. With a view to controlling risks in agricultural and agri-food systems, this project will make it possible to assess the relevance of monitoring plans, for instance in agricultural soils.

JRP08-METASTAVA

1. Summary of the work carried out

OHEJP-JRP8-METASTAVA investigated how to bring diagnostic use of metagenomic high throughput sequencing methods (mNGS) for unbiased pathogen detection closer to public vet/med/food labs. Work focused on providing guidelines for informed mNGS experimental design, standardised methods for generating and analysing data (WP1); providing quality control metrics, tools and interlaboratory assessment (WP2); and assessing the analytical properties of mNGS for pathogen/sample matrix combinations relevant to the OHEJP (WP3). In addition, cross-cutting work packages ensured integration with other ongoing research efforts and dissemination (WP4) as well as project management (WP5).

WP1 standardised the methods for mNGS data generation (D-JRP8-1.1) and data analysis (D-JRP8-1.2) in Metastava. An investigation of publicly available NGS raw data indicated that mNGS public datasets on pathogen/sample matrix combinations relevant to Metastava lacked sufficient sample and methodological metadata (D-JRP8-1.3), necessitating a focus on the datasets produced within the project. A general guidance document for informed implementation of mNGS methodologies in a diagnostic context was published (D-JRP8-1.6), pointing scientists to the wealth of published information and past and ongoing research efforts on mNGS, as well as documenting possible validation and quality monitoring measures for metagenomics methods.



WP2 thoroughly evaluated a commercial standardised spike-in external control for mNGS of viruses (D-JRP8-2.2), offering QC metrics during the workflow (on extracted RNA) and during data analysis (using normalised control read numbers). In addition, the detection of RNA viruses in technical replicates of swine faeces was shown to be reproducible and insensitive to the effect of different reagent batches (D-JRP8-2.3). A Metastava proficiency test (D-JRP8-2.5) evaluated the interlaboratory reproducibility and tested the robustness of the methods for data generation and analysis selected in WP1. Aliquots of swine faecal samples naturally infected with several porcine astrovirus species were dispatched to all participants, which handled the data generation using Metastava methodological modules described in D-JRP8-1.1, while a standardised data analysis methodology was used by all (D-JRP8-3.2). The results indicated excellent reproducibility in terms of astrovirus species detection, while considerable variation was detected in the reported normalised read counts, due to varying methods for data generation. Metastava protocols, expertise and resources were used in parallel research and diagnostic efforts by the different partners (T2.4). This research included the characterisation of the virome in farms with consistent neonatal piglet diarrhea, the characterisation of Sars-CoV-2 during the ongoing pandemic; the validation of Metastava protocols for the characterisation of animal coronaviruses, and the investigation of animal samples giving false positive results in Ag LFD (antigen detection lateral flow devices) quick tests for SARS-CoV-2.

WP3 investigated the analytical properties of mNGS for model pathogens based on suitable sample collections (D-JRP8-3.1) and using a standardised procedure for data analysis (D-JRP8-3.2). Based upon the characteristics of the different model pathogens, the required taxonomic resolution, and the complexity of the relevant sample matrices investigated, differences were found for the detection using completely random and enrichment free mNGS workflows for hepatitis E virus (in human and animal samples & food, D-JRP8-3.3), Norovirus (in human samples and food, D-JRP8-3.4), animal zoonotic pox viruses (in animal samples, D-JRP8-3.5), Shiga toxin expressing E. coli (food samples D-JRP8-3.6), and antibiotic resistance genes (in animal samples D-JRP8-3.7). This suggests that the most important power of random mNGS (its access to nucleic acids from all pathogens in a sample) is also its Achilles' heel, indicating that for some diagnostic applications enrichment methods or targeted sequencing approaches are needed.

2. Work carried out in the JRP, scientific results

WP: Collect reference data from other metagenomic projects, select the metagenomic methods to be used for the project, and provide guidance data for informed metagenomic workflow design (M1-M30)

JRP8-WP1-T1: broad survey to collect information about sample selection and data generation methods for metagenomics (M1-M12)

We successfully organized several meetings about data generation methods, including one live meeting (during the project kickoff meeting) and three teleconferences. A detailed survey was finalized in m6 and listed all methodologies used by the partners (integration ensured by partners also participating in COMPARE and EFFORT, as well as the integration activities with these projects undertaken in WP4). A conclusion report listed the methods that would be used as standardized methods for the Metastava project and was discussed in a follow-up teleconference. A modular approach was selected, offering alternative technical solutions for sample disruption or homogenization, nucleic acid extraction, cDNA synthesis and double stranding, library preparation, and sequencing. Partner FLI generated extensive comparative validation data for the cDNA synthesis modules (IonS5 NGS sequencing of 15 samples with both alternative modules). The objective is to maximize the use of the selected methodology modules. Where their use is not possible, parallel validation of in-house methods to the selected modules should be provided.

D-JRP8-1.1 includes a report on the questionnaire + a conclusion with selected methods for generating short read metagenomic NGS data from clinical methods.



JRP8-WP1-T2: broad survey to collect information about data analysis methods for metagenomics (M1-M12)

Data analysis methodology was initially discussed in a session during the kickoff meeting. A detailed questionnaire (finalized 15.06.2018) collected information about IT capacity, tools, and databases used by the partners and was discussed in a follow-up teleconference. It was decided to select one fast mapping approach (Kraken) and one more inclusive iterative assembly/mapping approach (Riems) as standardized tools. Due to time constraints and availability problems for all partners, commercial solutions were not evaluated (CLC genomics was the most prevalent commercial solution).

D-JRP8-1.2 includes a report on the questionnaire + a conclusion with selected methods for the metagenomic analysis of short read NGS data.

JRP8-WP1-T3: identifying available sequence datasets (M1-M36)

An initial survey of publicly available short read NGS data (NCBI short read archive; SRA) indicated that although raw randomly generated data for relevant animal and human sample types is publicly available they lacked sufficient sample and methodological metadata to reconstruct the exact biological context of the sample and the way the data was generated. Moreover, the combination of model pathogens/sample matrices studied in Metastava was rare (cf. D-JRP8-1.3). as a result, we focused on listing shared datasets that were generated by Metastava, and for which we have high quality metadata. At the date of this report, these include the data related to D-JRP8-2.2 (publicly available in the Sequence Read Archive (SRA) under BioProject accession number PRJNA615303). Reproducibility data from the Metastava proficiency test will be included with a manuscript that is currently being written, as will selected datasets from WP3 and WP2. Currently, this already includes all raw datasets related to task 2.2 regarding the use of endogenous internal process controls (NCBI SRA BioProject accession number PRJNA615303).

JRP8-WP1-T4: Propose a standardised framework for the description of the application scope and analytical properties of a metagenomics assay (M18-M36)

Scheduled for the last 6 months of the project (prolongation), and is heavily linked to task 2.1. Metastava jointly decided to deliver a guidance document (D-JRP8-1.6) for labs considering diagnostic use of metagenomic workflows (joining the original intention of deliverables 1.4, 1.5, and 2.1) referring to the richness of recently published studies, norms, and guidelines since the start of the project. The resulting opinion document by Metastava avoids being “yet another diagnostic metagenomics review” and guides readers interested in diagnostic implementation of metagenomic NGS methods to:

- High quality review papers on the topic
- Relevant norms and validation guidelines for metagenomic NGS, as well as for targeted diagnostic use of NGS methods
- Past and ongoing collaborative research projects on diagnostic metagenomics
- Suggestions on quality control measures and metrics to include in mNGS workflows.

The goal of this guidance document, entitled “Key considerations for the implementation of high throughput sequencing based metagenomics (mNGS) in diagnostic clinical, food and veterinary labs : a no-nonsense pointer” (D-JRP8-1.6) is to promote informed decisions on metagenomic workflow choices, as well as careful interpretation and follow up research on metagenomic results. The document will not be published as a classical review article, but rather as an interactive document with clickable links to the information sources. It is Intended to draw diagnostic scientists into critical reflections on how they should set up and interpret mNGS methods. It will be published on the OHEJP website and further dissemination will be investigated with the OHEJP communications team.



WP2: Quality assurance tools for the validation and interpretation of metagenomics (M1-M30)

JRP8-WP2-T1: The development of quality metrics to evaluate the significance of the outcome of a metagenomics experiment (M10-M36)

As stated above, and in the context of increasing availability of high quality research and publications on the topic, Metastava decided to join the objective of this tasks with heavily linked task 1.4. An extensive overview of quality metrics to monitor mNGS workflows and to ensure proper interpretation of mNGS results is included in our guidance document entitled “*Key considerations for the implementation of high throughput sequencing based metagenomics (mNGS) in diagnostic clinical, food and veterinary labs : a no-nonsense pointer*” (D-JRP8-1.6).

JRP8-WP2-T2: development and evaluation of external controls for metagenomics (M1-M18)

Deliverable report D-JRP8-2.2 uploaded on 15.06.2020 and publication accepted in J.Vir.Meth. PMID: 32574649 DOI: 10.1016/j.jviromet.2020.113916.

Metagenomic next generation sequencing (mNGS) is increasingly recognized as an important complementary tool to targeted human and animal infectious disease diagnostics. It is, however, sensitive to biases and errors that are currently not systematically evaluated by the implementation of quality controls (QC) for the diagnostic use of mNGS. We evaluated a commercial reagent (Mengovirus extraction control kit, CeraamTools, bioMérieux) as an exogenous internal control for mNGS (after reviewing the literature on available exogenous controls for viral metagenomics). It validates the integrity of reagents and workflow, the efficient isolation of viral nucleic acids and the absence of inhibitors in individual samples (verified using a specific qRT-PCR). Moreover, it validates the efficient generation of viral sequence data in individual samples (verified by normalized mengoviral read counts in the metagenomic analysis). We show that when using a completely random metagenomics workflow: (1) Mengovirus RNA can be reproducibly detected in different animal sample types (swine feces and sera, wild bird cloacal swabs), except for tissue samples (swine lung); (2) the Mengovirus control kit does not contain other contaminating viruses that may affect metagenomic experiments (using a cutoff of minimum 1 Kraken classified read per million (RPM)); (3) the addition of 2.17×10^6 Mengovirus copies/ml of sample does not affect the virome composition of pig fecal samples or wild bird cloacal swab samples; (4) Mengovirus Cq values (using as cutoff the upper limit of the 99% confidence interval of Cq values for a given sample matrix) allow the identification of samples with poor viral RNA extraction or high inhibitor load; (5) Mengovirus normalized read counts (cutoff RPM>1) allow the identification of samples where the viral sequences are outcompeted by host or bacterial target sequences in the random metagenomic workflow. The implementation of two QC testing points, a first one after RNA extraction (Mengoviral qRT-PCR) and a second one after metagenomic data analysis provide valuable information for the validation of individual samples and results. Their implementation in addition to external controls validating runs or experiments should be carefully considered for a given sample type and workflow.

JRP8-WP2-T3: reproducibility and batch effect evaluation (M1-M12)

The repeatability between technical replicates (including the effect of different production batches of critical reagents) was investigated for the metagenomic detection of RNA viruses in swine fecal material (D-JRP8-2.3). Reproducibility needs to be considered when performing metagenomic diagnostics and research studies. Indeed batch effects may mask true biological differences between groups, e.g., healthy vs diseased individuals, due to variations in reagents/kits lot, personnel, or laboratory procedures. Of particular importance for metagenomics with its wide scope, kit contamination may result in false discovery of causative agents. Two technical replicates were mNGS-sequenced from 12 swine fecal samples, showing reproducible detection (but variation in terms of the normalised number of reads reported) of RNA viruses. A follow up investigation looked at technical replicates of porcine astrovirus infected swine feces serially diluted in fecal suspension collected from a specific pathogen free (SPF) pig. This demonstrated reproducible detection, although the limit of detection was as expected higher than for astrovirus specific realtime RT-PCR. Near the mNGS detection limit, repeatability issues were evident between technical replicates, showing the value of



including either technical or biological replicates in mNGS studies where low viral titres are expected in the tested samples. A final investigation looked at technical replicates of swine fecal samples using different library prep and sequencing kit reagents showed very reproducible virus detection for astroviral species. At the virus detection level, only questionable read numbers (1-4) reads for a species were not reproduced in the technical replicate using the other sequencing reagents batches. As our guidelines (D-JRP8-1.6 and specific cutoff of RPM>1 in D.JRP8-2.2) indicate, such low read numbers should always be critically investigated, certainly in a diagnostic context.

JRP8-WP2-T4: evaluation of QC metrics on additional parallel datasets (M13-M24)

The methodologies and expertise developed in Metastava was used by several partners in parallel diagnostic and research efforts. The methodologies and expertise has been proven particularly relevant in response to the ongoing COVID-19 pandemic.

- Exogenous IC (cf. D-JRP8-2.2) and Metastava sequencing and analysis methods (D-JRP8-1.1 and D-JRP8-1.2) were used for the study of the virome in swine farms with persisting neonatal piglet diarrhea (Van Borm et al. published in Virus Genes).
- In response to the COVID-19 pandemic, partner Sciensano set out to validate the Metastava metagenomic protocols on clinical samples and isolates of animal coronaviruses (avian infectious bronchitis virus, genus Gammacoronavirus), where we proved the modules for RNA virome analysis set out in D-JRP8-1.1 (Trizol/Rneasy – SSIV/NEBNext – NexteraXT – Kraken) allowed the determination of complete Gammacoronavirus (Infectious Bronchitis virus) genomes from chicken clinical samples (tissue pools and swabs), while at the same time allowing robust characterisation of co-infecting chicken Astrovirus, Sicinivirus, and avian leucosis virus (Alpharetrovirus).
- Metastava methodologies and expertise was helpful for the characterisation of swine astroviruses from swine fecal samples (partner WUR).
- Similar coronavirus protocol validation was done on porcine epidemic diarrhea virus (PEDV, Alphacoronavirus) and avian coronaviruses from Guineafowl (by partner Anses).
- Metastava expertise was used in response to the COVID-19 pandemic for sequencing of human (partner ErasmusMC) and animal (partner WUR) SARS-CoV2
- More indirectly, several Metastava experts were recruited into crisis response projects including the development and use of targeted molecular diagnostic assays, as well as targeted whole genome sequencing methodologies. This highlights the relevance of the developed expertise for emerging threats, but resulted in operational delays for the research tasks planned in Metastava.

JRP8-WP2-T5: Metagenomic proficiency test (M13-36)

Within METASTAVA, modules suitable to build standardized metagenomics next-generation sequencing (mNGS) workflows were selected and tested. These modules represent the main steps of the mNGS workflow, namely sample disintegration, RNA extraction, double strand cDNA synthesis and library preparation. For each of the modules, two methods were chosen as outlined previously in report D-JRP8-1.1. Likewise, common procedures for analysis of sequencing data were chosen (D-JRP8-1.2 and D-JRP8-3.1 v1.3). To finally evaluate the selected modules in terms of (i) suitability to detect viruses in native samples and (ii) simultaneously assess their reproducibility, a proficiency test (PT) was carried out as part of METASTAVA. For this PT, a pre-analysed swine faeces sample containing a porcine astrovirus and RNA extracted from that sample were distributed among the participants. Most importantly, using the abovementioned methods comprised in the METASTAVA modular mNGS workflow, all participants identified the same viral species belonging to the family *Astroviridae* in both the clinical sample and in the distributed RNA. In detail, all participants classified the majority of viral reads to two species of porcine astroviruses (PAstV2 and PAstV4), regardless of the used analysis method (Kraken and Bowtie2). Moreover, participants performed well in terms of repeatability when



the faecal sample was tested in duplicate, resulting in a low coefficient of variation. Therefore, this PT showed that the methods selected as METASTAVA mNGS modules are (i) well suited for virus identification even from challenging matrices like faeces and (ii) the selected methods can reproducibly be applied.

Detailed results of this proficiency test can be consulted in deliverable report D-JRP8-2.5, while a manuscript is currently being written to publish the results.

WP3: evaluation of the analytical properties of metagenomics workflows (M1-M30)

An inventory of relevant samples available in sample repositories for the different model pathogens was made with the involvement of all participants in the task teams. The emphasis was put on naturally infected samples with different pathogen loads. Where this was not available, samples were spike with a model pathogen. The inventory of samples to be sequenced for each task was finalised during the first year of the project (D-JRP8-3.1).

JRP8-WP3-T1: analytical sensitivity, HEV (M1-M36)

Here the potential of the METASTAVA workflow to detect HEV in samples from various origins was investigated and positively demonstrated for some matrices (serum, cell culture and meat pork) but not for highly complex matrices like pig feces. We were able to detect HEV in samples until about 100,000 IU/ml (Cq of 26 with our protocol). Bioinformatics analyses showed that Bowtie2 is more sensitive than Kraken for analysis. Nevertheless, a proper reference genome is essential to detect the target otherwise false negatives may arise. For instance, this can be seen in the Kraken analysis when the RefSeq sequence used in the Kraken database poorly represents HEV diversity, resulting in a poor sensitivity. Another issue are low complexity regions, which induced false positive detections of HEV. This lack of specificity was partially improved by masking poly-A/T/N tails longer than 15 nucleotides in the reference genomes and by trimming reads with Prinseq. Despite these adaptations, false positive reads were still detected in the negative control samples and it is essential to tackle these in the future. In particular, more detailed experiments, requiring more starting material, are essential to delineate a reliable cut-off for the presence or the absence of HEV in a sample.

For the very complex matrices that are pig feces, the metagenomics approaches in this study has failed to identify the HEV for real life samples corresponding to loads of 10E6 genome per ml. The reduction of the sample complexity by molecular technics, for example DNase SISPA, as demonstrated by WBVR, can be an alternative procedure but the successful detection of HEV in human positive serum suggests blood samples are probably a best alternative for HEV detection in swine if a viremia is induced. However, Metastava protocols have shown reproducible detection of astroviruses (D.JRP8-2.5: metastava proficiency test) as well as diverse other swine intestinal viruses (Van Borm et al 2020b) from swine feces, and Norovirus (D-JRP8-3.2) in human feces. Assay sensitivity should always be verified for a specific model pathogen/sample matrix combination and that the specific conditions of each disease should be considered (e.g. asymptomatic carrier such as HEV in swine vs. symptomatic animal such as diarrhetic pigs with astrovirus infection).

Importantly, high amounts of HPgV (human pegivirus) reads were detected in a HEV-negative patient serum sample, showing that our approach also allows for the identification of pathogens we did not hypothesize for.

D-JRP8-3.3 reports the detailed methodologies and results of the HEV investigations in Metastava

JRP8-WP3-T2: analytical sensitivity, norovirus (M1-M36)

This study has shown the potential of metagenomics to detect norovirus in fecal suspensions and in food matrices such as strawberries, but also raised some issues needing further improvements.

The direct RNA extraction (NucliSens® easyMAG™ platform, Biomérieux) for virus extraction from human stool suspensions, and the ISO 15216 method for virus extraction from food are the most suitable for viral metagenomics sequencing purposes.



The limit of detection obtained by metagenomics approach was estimated in this study for fecal suspensions to $1.4E+05$ - $1.8E+05$ cg of NoV/g of stool. This concentration is rather low compared with median viral loads detected in the stools of NoV-infected patients ($8.4E+05$ and $3.0E+08$ for NoV GI and NoV GII respectively) (2).

On the other hand, the limit of detection obtained by metagenomics approach was estimated in this study for food (NoV-spiked strawberries) samples to $5.6E+03$ - $7.3E+03$ cg of NoV/g of food sample, which is a high contamination level compared to naturally contaminated food samples. Our laboratory established that 71% of NoV-positive food samples suspected in foodborne outbreaks had NoV genomic levels $\leq 1E+03$ cg/g of sample (3).

The Metastava project assessed generic metagenomics workflow modules for viral and bacterial nucleic acids preparation and sequencing, harmonized for all sample types. Although this assessment deviated from the Metastava selected nucleic acid extraction methods due to laboratory organizational restrictions, it has shown that direct nucleic acid extraction allows metagenomic detection of NoV in patient samples with a relevant analytical sensitivity to contamination levels seen in the diagnostic lab. However, these direct nucleic acid extraction methods do not seem the most suitable for the detection of RNA viruses in food samples. Even using the ISO 15216 nucleic acid extraction method for food samples, the analytical sensitivity of metagenomics methods was poor in relation to diagnostically (qRT-PCR) observed contamination levels. These findings stress the importance of a careful workflow design and validation fit-for-purpose, i.e. fit for a combination of pathogen and sample matrix in a diagnostic context.

D-JRP8-3.4 documents the detailed methodologies and results of the Norovirus investigations in Metastava.

JRP8-WP3-T3: analytical sensitivity, large DNA viruses (M1-M36)

Several DNA viruses of the family *Poxviridae* pose a zoonotic risk. These include members of the genus *Parapoxvirus* that can result in skin lesions (Orf or ecthyma contagiosum) in humans handling the small ruminant animal reservoir (goat and sheep). Cow pox (genus *Orthopoxvirus*) represent another zoonotic occupational risk. This task investigated the diagnostic value of metagenomic methods for the detection and characterisation of Orf virus in small ruminant clinical samples (work at partner Sciensano) as well as other pox viruses in naturally infected samples (work at partner FLI).

This report presents the data on *Parapoxvirus* and needs to be updated as additional data need to be added.

Although randomly generated metagenomic datasets generated from *Parapoxvirus* skin lesion samples are dominated by host (small ruminant) DNA sequences, this study suggests sufficient *Parapoxvirus* specific sequence information is generated both quantitatively and qualitatively. High normalized *Parapoxvirus* read numbers do not only allow for confident identification, but yield high genome coverage both in clinical samples and in clinical samples diluted in negative tissue homogenate (until 1/100). Complete open reading frame coverage for most commonly used *Parapoxvirus* phylogenetic markers (B2L, E3L, I3L, F1L, A4L) directly from clinical samples. Where a loglinear relationship was observed between *Parapoxvirus* realtime PCR Cp values and normalized readcounts in samples diluted in negative tissue homogenate, this was not the case for the panel of clinical samples tested (most likely due to the diverse preservation and sampling history with samples from 2003, 2011, and 2018). Altogether these data suggest that randomly generated mNGS data from poxviral skin lesion samples (1) allows a confident identification of poxviruses with an analytical sensitivity covering the observed range of virus concentrations in tissue samples and (2) yields high resolution genetic information including near-complete genome sequences and good coverage of all frequently used phylogenetic markers.

D-JRP8-3.5 documents the detailed methodologies and results of the *Poxviridae* investigations in Metastava.



JRP8-WP3-T4: analytical sensitivity, STEC (M1-M24)

During foodborne outbreak investigations, the required response time can be long due to the time needed for isolation/cultivation of the responsible pathogen(s). Moreover, often the isolation of these pathogens from food is not possible. Metagenomics could significantly reduce the detection time. The goal of this task is to evaluate the generic harmonized METASTAVA workflow to generate and analyse metagenomics data for the foodborne bacterial pathogen Shiga toxin-producing *Escherichia coli* (VTEC/STEC), and to compare the analytical sensitivity and specificity with standard diagnostic methods (i.e. qPCR).

Minced beef meat purchased at a local store and free of prior STEC contamination was artificially spiked at different concentrations (10 colony forming units (CFU) (representative of a naturally occurring contamination load), 10^8 CFU and 10^{10} CFU) of a culture of STEC in 25g of matrix previously stomached with 225ml buffered peptone water. mNGS data (on average $2.6 \cdot 10^6$ reads per sample) was generated and analysed using Metastava protocols (WP1). *E. coli* reads were identified in all samples, even in the blank meat that contains only the DNA extracted from the matrix. However, only in the sample spiked with 10^{10} CFU could reads be assigned to a lower taxonomic level *E. coli* O157:H7. Finally VirulenceFinder and SerotypeFinder could only identify NGS reads mapping to virulence genes in the sample with the highest spiking level.

Although completely random mNGS on complex food matrices generates data allowing species-level detection of *E. coli*, the required taxonomical resolution in this particular diagnostic context necessitates higher sensitivity. Indeed, natural STEC contamination levels in food samples are in the range of 10 CFU, while only extremely high contamination levels allowed the needed taxonomic resolution and detection of virulence genes using the completely random metastava protocols on complex food matrices. As a consequence, targeted approaches (or extremely high sequencing effort per sample) are needed to allow the necessary strain typing (culture enrichment or targeted NGS approaches). These targeted approaches are outside of the scope of Metastava, which critically investigated the potential diagnostic value of truly random mNGS approaches.

D-JRP8-3.6 documents the detailed methodologies and results of the STEC investigations in Metastava.

JRP8-WP3-T5: analytical sensitivity, detection of ABR genes (M1-M36)

Detection of antimicrobial –resistant bacteria from animals can be performed by culture. Then the resistant bacteria can be studied by phenotypic or genomic methods. However, the diversity of the bacterial species and resistance genes possibly present in a sample makes such an approach a challenge. For this reason, global approaches, such as metagenomics, seem a relevant option. The aim of this task of the METASTAVA project was to evaluate the robustness and sensitivity of the metagenomics for antimicrobial resistance and to compare the analytical sensitivity and specificity with bacteriological methods including isolation on supplemented media. For this purpose, fecal samples containing bacteria with resistances of public health importance were used.

In a first step, the workflows for generating mNGS data and bioinformatic steps to identify ABR genes were validated on isolates of ABR bacteria, resulting in the confirmation of all phenotypically determined ABR markers. Secondly, spiking of pig fecal samples with *E. coli*, *Campylobacter coli* and *Staphylococcus aureus* strains with characterized ABR spectra at concentrations ranging from 10^2 to 10^7 colony-forming units per 1g of feces indicated the presence of a diverse spectrum of ABR genes that were not specific to the spike strains, while only at the highest concentration (10^7 colony-forming units per 1g of feces) spike strain specific ABR genes were detected. Of note, high sequencing efforts were done per sample (>130 million reads per sample). Lastly, mNGS on fecal samples from swine in vivo inoculated with ABR *E. coli* strains yielded similar results with the documentation of a huge diversity of circulating ABR genes, but poor sensitivity for the detection of ABR genes specific to the inoculum strain.

The highly diverse microbial community in swine fecal samples, with a diversity of circulating AMR genes in the swine population, represent a difficult “needle in the haystack” situation for direct detection of AMR genes using random mNGS sequencing from fecal samples, where the sensitivity



seems to be limited to 10^7 colony-forming units per 1g of feces (spiking experiment) and where inoculum specific ABR markers are not detected in experimentally infected pigs. As a result, studies envisaging specifically the detection of ABR genes (unlike Metastava that studies hypothesis free detection of all pathogens in a sample) may require specific sample enrichment, pretreatment, or sequencing approaches.

D-JRP8-3.7 documents the detailed methodology and results of the ABR gene detection investigations in Metastava.

JRP8-WP3-T6: bioinformatics and statistical analysis of analytical performance experiments (M1-M24)

After concertation between the different partners involved in WP3, a standardised workflow for the data analysis was decided (M-FBZ2.metastava.4) and an SOP was written (current version D-JRP8-3.2 v1.3 after consecutive minor updates). In addition, a standardised database for metagenomic classification was build and shared between partners to avoid bias introduced by different databases used.

Overview of the analytical procedure

The Next-Generation Sequencing (NGS) data of a sample generated by means of the Illumina platform is analysed by following the sequential steps listed below, with the tools used in parentheses:

- Raw reads quality check (FastQC)
- Reads poly A/T tail trimming (Prinseq)
- Reads quality trimming (Trimmomatic)
- Trimmed reads quality check (FastQC)
- Reads taxonomy profiling (Kraken)
- Taxonomy profile report generation (Kraken)
- Optional: Taxonomy profile visualization (Krona)
- Read mapping against target species (Bowtie2)
- Target species reads counting (Samtools)

The detailed information about each step is described in deliverable report/SOP (D-JRP8-3.2)

WP4: Concertation with ongoing efforts and dissemination (M1-M36)

JRP8-WP4-T1: concertation with ongoing initiatives (M1-M24)

The concertation with other initiatives investigating the use of NGS for the detection or characterization of microorganisms (viruses and bacteria) was ensured in two ways. First, several Metastava partners were actively involved in the research consortia COMPARE, EFFORT against AMR, Global microbial identifier, relevant ISO workgroups etc. (details listed in D-JRP8-4.1). The continuation of the developed expertise was ensured by participation of Metastava partners in OHEJP second call projects including OHEJP-JRP16-TeleVIR and OHEJP-JRP12-Farmed. Secondly, public deliverables of other initiatives were taken into account and Metastava participated in interactive workshops with other initiatives. . Steven Van Borm participated in three cogwheel workshops organized by the OHEJP, one with COMPARE (12.04.2018), and one with EFFORT (26.10.2018), and one with IRIDA and INNUENDO. Participation in a cogwheel workshop with VEO (versatile Emerging Infectious Disease Observatory) is planned (25.20.2021). Closest fit was observed with COMPARE. However, the exchange possibilities (e.g. protocols) were limited by reporting restrictions of COMPARE (e.g. COMPARE deliverables had to be validated by EC before becoming public). Public deliverables of COMPARE were taken into account when published (<https://www.compare-europe.eu/library>) and where still possible integrated in the Metastava conclusions. However, given the short time frame available to Metastava,



we could not reconsider e.g. the decisions we made regarding methodologies (WP1). It should be stressed that several Metastava partners also participate in COMPARE, resulting in an indirect integration of e.g. methodologies and participation of several Metastava partners in the COMPARE proficiency tests. Of particular interest was the participation of Steven Van Borm in the Second conference on NGS for adventitious agent detection in biologics for human and animal use (IABS – International Association of Biological Standardisation), generating new insights from the pharmaceutical and quality assurance of biological products fields and giving access to the latest insights of IABS' Advanced Virus Detection Technologies Interest Group (AVDTIG). An integral report of the later meeting, including a summary of our presentation on Metastava was published (<https://pubmed.ncbi.nlm.nih.gov/32660862/>).

JRP8-WP4-T2: formal dissemination (M1-M36)

Metastava produced both procedures, SOP's, and guidelines, as well as formal scientific publications. The later are listed in the dedicated section of the present report. The SOP's and guidelines are listed in D-JRP8-4.2, and include methods for the generation of metagenomic data, for their analysis, for the use of external controls, and guidelines for informed design and interpretation of metagenomic experiments and suggested metrics for their quality assurance.

JRP8-WP4-T3: dissemination of recommendations to stakeholders (M1-M24)

Metastava output was disseminated to the following stakeholders at scientific meetings:

- Presence and presentations at the OHEJP kickoff meeting (30-31/01/2018), annual scientific meetings (2019, 2020), and programme owners committee meeting (19/06/2019) and programme management committee (9/05/2019).
- Presentation at Workshop: ESCV Next Generations Sequencing in Clinical Virology (European Society for Clinical Virology). 20-21 nov 2018
- Presentation at the International Association for Biological Standardization(IABS)) conference on next generation sequencing for adventitious virus detection in biologics for humans and animals, Ghent, 13-14 Nov. 2019
- Presentation at the Belgian branch of the World Veterinary Poultry Association (WVPA). Ghent, 27/02/2020.
- Presentation at the SVA science day (12 november 2019);
- 2x (2019, 2020) OHEJP@Sciensano day with invited Belgian stakeholders (food safety agency, ministry of health, etc...)
- Presentation at Joint 11th International Congress for Veterinary Virology and 12th Annual meeting EPIZONE. (27-30 aug 2019)

JRP8-WP4-T4: Organization of a scientific meeting (M20-M24)

We focused on maximum participation in the OHEJP Annual Scientific meetings to take advantage of a wider forum. D-JRP8-4.3 reviews the scientific discussion during our annual scientific meetings and the presentations at the OHEJP ASM's. A separate scientific dissemination meeting was originally planned for the last 6 months of the project. However, the ongoing COVID-19 meeting made the organisation of a physical meeting impossible, while several key scientists were recruited into crisis-response teams. As a result, Metastava could not organise a scientific dissemination meeting.

WP5: Project management (M1-M36)

JRP8-WP5-T1: Consortium agreement (M1-M6)

The grant agreement of the entire Onehealth EJP covers all necessary agreements between partners and includes the work plan of our project as it was submitted. There is no need for a joint research project – level consortium agreement.



JRP8-WP5-T2: Internal communication (M1-M24)

Pre-kickoff meeting phone calls with work package leaders. Kickoff meeting (21.02.2018, Brussels). WP1 phone calls (coordinator- WPL) about standardization. Internal WP1 questionnaires on data generation and data analysis + follow up teleconferences. Mailings to all collaborators or partner contacts about general EJP-OH information. Teleconference on WP1 standardization (end of M6). Internal WP1, WP2 (19/07/2018) and WP3 (5/10/2018) teleconferences. Internal WP3 questionnaire to document the sample collections. WPL/general assembly progress teleconference (11/12/2018). Yearly annual meetings + periodic WP specific teleconferences (summarized in D-JRP8-5.2)

The Metastava general assembly managed severe shifts in responsibilities (changes in work package leadership and deputy workpackage leadership).

Moreover, the integration of a Third partner (Erasmus Medical Center) via WUR was formalized, while two no-cost extension requests were submitted and obtained to manage project-related delays (initial 6 month extension) as well as serious COVID-19 crisis related delays (extension until December 2020).

JRP8-WP5-T3: reporting and liaising with the EU (M20-M24)

Completed, see first, second, and third intermediate reports (2018 and 2019 and 2020) and the present final report.

3. Project self-assessment

In our opinion, all scientific objectives of the Metastava project were met as documented in the present final report and more detailed in the corresponding deliverable reports. These objectives include building a small One Health community of labs investigating diagnostic use of metagenomics, standardisation of methodologies, validation of analytical aspects of metagenomics, and assessment of control strategies and formulation of guidelines for informed implementation and interpretation of metagenomics results. Importantly, the generated results have indicated a huge potential of mNGS methods but have also highlighted the importance of a critical investigation and informed decisions in the implementation of NGS based assays.

However, the long-lasting impact of the current COVID-19 pandemic seriously hit all partners. This included lock-downs and reduced access to laboratories, as well as the direct involvement of Metastava staff in the management of varying aspects of the pandemic, including the implementation of molecular diagnostic SARS-CoV2 assays, contribution to diagnostic capacities, sequencing of SARS-CoV2 genomes, etc. Insights, methods and expertise developed in Metastava were useful in these crisis response efforts. For some partners this resulted in a high investment in pandemic management activities for the most of 2020, without an immediate view on improvement during the first half of 2021.

As a result of this unprecedented situation, Metastava struggled to formally disseminate its fulfilled deliverables in the form of scientific publications (several are seriously delayed) as well as its dissemination to the wider scientific and stakeholder community. Importantly, we did not manage to organise the planned scientific dissemination meetings due to practical constraints as well as unavailability of key personnel as a consequence of the pandemic. This limited the dissemination activities of the project to what was already achieved in 2018 and 2019. Realising this weakness, Metastava published an easily accessible document (D-JRP8-1.6) regrouping guidelines for the diagnostic implementation and validation and the correct interpretation of metagenomic methods aimed at (1) scientists envisaging mNGS diagnostic applications and (2) stakeholders envisaging to estimate the potential diagnostic value and complexity of implementing mNGS for diagnostic purposes.



4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories* (1 to 8) <i>(several categories may be applicable)</i>
08	D-JRP8-1.1	Dataset: reference data metagenomics data generation	12	2/10/2018		OHEJP: available Zenodo: https://zenodo.org/record/4486754#.YBgYmuhKjcc https://zenodo.org/record/4486763#.YBgY9OhKjcc	
08	D-JRP8-1.2	Dataset: reference data metagenomics analysis	12	28/06/2018		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486767#.YBgZO-hKjcc https://zenodo.org/record/4486769#.YBgZguhKjcc	



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08	D-JRP8-1.3	List of sequence datasets	27	16/12/2020		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486773#.YBgZ5uhKjcc	3
08	D-JRP8-1.4	SOP: guidelines for the description of scope and analytical properties of a metagenomic method in a diagnostic context	30	36 as D-JRP8-1.6		D1.4, D1.5, D2.1 will be joined in a single guidance document for labs envisaging mNGS as a diagnostic method = D-JRP-1.6	2
08	D-JRP8-1.5	Review paper	30	36 as D-JRP8-1.6		D1.4, D1.5, D2.1 will be joined in a single guidance document for labs envisaging mNGS as a diagnostic method	2
08	D-JRP8-1.6	Review document with guidelines on diagnostic applications of metagenomics: Metastava guidelines	New D	36 as D-JRP8-1.6		Public OHEJP: available Zenodo: https://zenodo.org/record/4486779#.YBgapOhKjcc	2



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
		for informed mNGS implementation					
08	D-JRP8-2.1	SOP: use of quality metrics for metagenomics dataset evaluation	30	36 as D-JRP8-1.6		D1.4, D1.5, D2.1 will be joined in a single guidance document for labs envisaging mNGS as a diagnostic method	2
08	D-JRP8-2.2	Report and guidelines for the use of exogenous process controls in metagenomics	26	30		Public OHEJP: available Zenodo: https://zenodo.org/record/4486781#.YBga5OhKjcc	2&3
08	D-JRP8-2.3	Report on batch and contamination effects in metagenomics	12	16		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486807#.YBghSuhKhMO	
08	D-JRP8-D-2.5	Report on proficiency test	M24	34		Confidential OHEJP: available Zenodo:	



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						https://zenodo.org/record/4486809#.YBgdjOhKjcc	
08	D-JRP8-3.1	Spiked sample panels ready for analysis	6	m12	12	Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486821#.YBgd6-hKjcc https://zenodo.org/record/4486823#.YBgeJ-hKjcc	
08	D-JRP8-3.2	Procedure for analyzing analytical sensitivity and robustness datasets	12	17		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486827#.YBgecOhKjcc	
08	D-JRP8-3.3	Report: analytical sensitivity and robustness , hepE	30	04.01.2021		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486829#.YBgeqehK	2 & 3



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						jcc	
08	D-JRP8-3.4	Report: analytical sensitivity and robustness , NoV	30	04.01.2021		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486834#.YBge5ehKjcc	2&3
08	D-JRP8-3.5	Report: analytical sensitivity and robustness , pox	30	06.01.2021	34	Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486840#.YBgfMuhKjcc	2&3
08	D-JRP8-3.6	Report: analytical sensitivity and robustness , STEC	30	30		Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486844#.YBgfa-hKjcc	2



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
08	D-JRP8-3.7	Report: analytical sensitivity and robustness , ABR genes detection	30	06.01.2021	35	Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486850#.YBgfvehKjcc	2&3
08	D-JRP8-4.1	Report of meeting with ongoing initiatives to assure input in WP1: Metastava integration with ongoing research efforts in diagnostic metagenomics and diagnostic NGS use	7	9.05.2018 Cogwheel workshop report		Confidential Cogwheel workshop report =EJP deliverable 4.3. https://zenodo.org/record/4486854#.YBggB-hKjcc	
08	D-JRP8-4.2	SOP's, guidelines, scientific papers, presentations	30	17/12/2020	36	Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486860#.YBggTOhKjcc	2



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
08	D-JRP8-4.3	Minutes of scientific meeting	30	Not realised		Organisation impossible: COVID19	10
08	D-JRP8-5.1	Consortium agreement	6			Not needed: OHEJP level	8
08	D-JRP8-5.2	Progress and final meeting minutes	30	36		We focused on producing a complete list of all metastava meetings throughout the project instead of providing detailed minutes of individual meetings (which are documents for internal use in the consortium) Confidential OHEJP: available Zenodo: https://zenodo.org/record/4486864#.YBggjOhKjcc	8
08	D-JRP8--5.3	Half term report	M13	13		Not delivered on group website: report to PMC	8
08	D-JRP8-5.4	Final report	30	36		Not delivered on group website: report to PMC	8



* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
08	M-JRP8-M1	Concertation meeting with ongoing initiatives	6	Yes		Cogwheel workshop with COMPARE and EFFORT. Additional contacts are ongoing.
08	M-JRP8-M2	Public and own dataset identified	30	Yes	36	see D-JRP8-1.3
08	M-JRP8-M3	Proficiency test panel ready for shipping	20	Yes		
08	M-JRP8-M4	Procedure for analysing analytical sensitivity and robustness datasets agreed	12	Yes 17		
08	M-JRP8-M5	Progress meeting	12	Yes 13		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
08	M-FBZ2.metastava.6	Scientific meeting	M30	No		COVID-19 impact
08	M-JRP8-M7	Scientific meeting	30	Yes		3x Metastava annual meeting + 2x participation OHEJP ASM
08	M-JRP8-M8	Dissemination to various stakeholders including joint communication with ongoing initiatives	30	Yes		Various presentations to national and international stakeholder's meetings
08	M-JRP8-M9	Final meeting	30	Yes		online meeting on 30/11/2020

5. *Publications and patents*

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Evaluation of a commercial exogenous internal process control for diagnostic RNA virus metagenomics from different animal clinical samples https://doi.org/10.1016/j.jviromet.2020.113916	YES		GOLD



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
COVID-19 in health-care workers in three hospitals in the south of the Netherlands: a cross-sectional study https://doi.org/10.1016/S1473-3099(20)30527-2	YES		GOLD
Monitoring SARS-CoV-2 circulation and diversity through community wastewater sequencing https://doi.org/10.1101/2020.09.21.20198838	YES		GOLD
Increased viral read counts and metagenomic full genome characterization of porcine astrovirus 4 and Posavirus 1 in sows in a swine farm with unexplained neonatal piglet diarrhea. 10.1007/s11262-020-01791-z https://zenodo.org/record/4244782#.X6LfzYhKicc	YES	GREEN, 12 months	
Detection of Norovirus Variant GII.4 Hong Kong in Asia and Europe, 2017-2019. doi: 10.3201/eid2701.203351.	YES		GOLD
Rapid SARS-CoV-2 whole-genome sequencing and analysis for informed public health decision-making in the Netherlands. doi: 10.1038/s41591-020-0997-y	YES		GOLD
In preparation: "A proficiency test of metagenomics as a diagnostic tool for the detection of RNA viruses in swine fecal material". Lihong Liu, Mikhayil Hakhverdyan, Kevin Vanneste, Pierrick Lucas, Yannick Blanchard, Bas B. Oude	YES		



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Munnink, Sander van Boheemen, Claudia Wylezich, Dirk Hoepfer, Alex Bossers, Marcel Hulst, Steven Van Borm			
In Preparation: Metagenomic sequencing of avian coronavirus positive clinical chicken samples: complete genome characterization of circulating infectious bronchitis vaccine strains and detection of co-infecting poultry viruses including avian leucosis virus, chicken astrovirus, sicinivirus, avibirnavirus and chicken calicivirus. Steven Van Borm, Mieke Steensels, Elisabeth Mathijs, Frank Vandenbussche, Thierry van den Berg, Bénédicte Lambrecht	YES		
In preparation: Metagenomic characterization of Orf virus circulating in small ruminants, Belgium, 2003-2018. Steven Van Borm, Andy Haegemans, Kevin Vanneste, Raf Winand, Kris De Clercq, Frank Vandenbussche.	YES		



Additional output

- Liu L, Hakhverdyan M, Leijon M. The influence of sample preparations on high-throughput sequencing detection of viruses in clinical samples. The 11th International Congress for Veterinary Virology. Vienna, Austria, 27-30 August 2018.
- Sander van Boheemen. Sample Pretreatment: Challenges in Virology. Workshop: ESCV Next Generations Sequencing in Clinical Virology. 20-21 November, 2018
- Van Borm S. OHEJP-METASTAVA: Joint efforts in standardization and analytical validation of diagnostic metagenomics approaches in public (animal) health laboratories. 2nd IABS (International Association for Biological Standardisation) conference on next generation sequencing for adventitious virus detection in biologics for humans and animals, Ghent, 13-14 Nov. 2019
- Van Borm S. Next Generation Sequencing from sample to result : how does it work, what is the current status, and what is the added diagnostic value? World Veterinary Poultry Association, Belgian Branch. Study day 27/02/2020.

6. One Health Impact

Metastava has provided important guidelines, standardised protocols and results indicating the potential diagnostic added value of metagenomic NGS based workflows, as well as current hurdles and the importance of properly informed selection of workflows and careful interpretation of results. These results and guidelines provide important information for stakeholders considering the potential inclusion of such methodologies as complementary wide-scope orientation diagnostics to supplement highly sensitive and specific targeted diagnostic assays.

The developed methodologies and insights in principle bridge the med/vet/food boundaries as they can be applied to any pathogen and sample type. However, careful consideration is needed to assess the suitability of the approach for a particular research or diagnostic question. Metastava's results not only echo the huge potential of metagenomic methods but also highlight where current hurdles still prevent their widespread use as catch-all diagnostic methods. The output of the project contributes to clarifying for which particular diagnostic questions metagenomics provide a huge added benefit (e.g. detection of novel emerging viruses) and for which diagnostic questions metagenomics' current analytical properties do not provide added benefit (e.g. detailed typing of bacterial strains in a complex food sample matrix with low pathogen loads). Especially our "Metastava guidelines for informed mNGS implementation" urges diagnosticians, researchers, policy makers, and stakeholders alike to critically consider the potential benefits and remaining hurdles when considering metagenomics as a diagnostic tool and to select methods that suit the diagnostic question.

JRP09-AIRSAMPLE

4. Summary of the work carried out

During the first year, we harmonized the methodologies within the consortium and made a repository list of Campylobacter strains and technologies to detect rapidly Campylobacter in bio secured chicken houses. We made a preliminary trial in each country using the existing air sampling protocols and equipment from Sartorius GmbH, which made reagents and equipment available to the consortium. During the second year, we modified the protocols and harmonized the actual flock sampling, pre-analytical sample treatment and methods for analyses across the member institutions in order to enable data comparison. In addition, the second year included development of implementation of



metagenomics diagnostic using filter samples. Substantial work was devoted to DNA bioinformatics that used different software at different partner labs. Finally, we conducted a multi-centre evaluation of our harmonized protocols using samples taken in the five participating countries. The third year has focussed on producing publications, SOPs and guidelines. An online video clip was prepared for the education of broiler industry (<https://www.youtube.com/watch?v=S9mapXSM8tw&t=95s>). Substantial work has been put into writing the second publication that has been published in AEM. The final air sampling protocol has been converted into a user guideline for wider communication to the public and stakeholders (<https://youtu.be/LMD03JAAPUw>). The final guideline has been also disseminated to all relevant authorities in the EU, because the results showed that air sampling together with real-time PCR improved detection. The project has conducted field-testing in five European countries in Northern, Southern, Central and Western Europe with different prevalence of Campylobacter in their broiler production; from a low prevalence (Norway) to countries with higher prevalence. In Norway (low-prevalence country), the air sampling together with real-time PCR gave the same results as the cultivation method indicating that it is also suitable in countries with low prevalence of Campylobacter. The results also show that the likelihood of detecting Campylobacter in a chicken flock has quadrupled with the new method (air sampling together with real-time PCR). That is, up to four times more chicken flocks show signs of Campylobacter being present when the new method is used compared to sock samples. The method is especially useful to farmers to confirm the cleanliness of their house BEFORE inserting new chick.

5. Work carried out in the JRP, scientific results

WP1: Method Development (M1-M13)

JRP9-WP1-T1: Sampling activities and creation of a sample bank (air and boot-swab samples) from different regions (M1-M6)

For the creation of a sample bank of air samples, the method of culturing, size of gelatine filters, temperature, time, volume of samples and samples preparation were optimised. The work has been done by inoculating the gelatine filters with different levels of Campylobacter jejuni.

The same results were obtained for the incubation of ½ filters in Bolton Broth for 44±4 h and the incubation of boot socks ISO 10272-1. It were no false results from gelatin filters. In addition, the PCR results from the filters corresponded to the results obtained with the culture-based method.

Optimisation of protocols for boot swab samples were provided by NVI. The protocols were optimised with regard to ISO 10272-1 for the detection of Campylobacter in the boot socks of own choice. Both protocols were shared among the partners. The protocols include the sampling step, cultural method, optimised PCR step and the storage (Figure1).

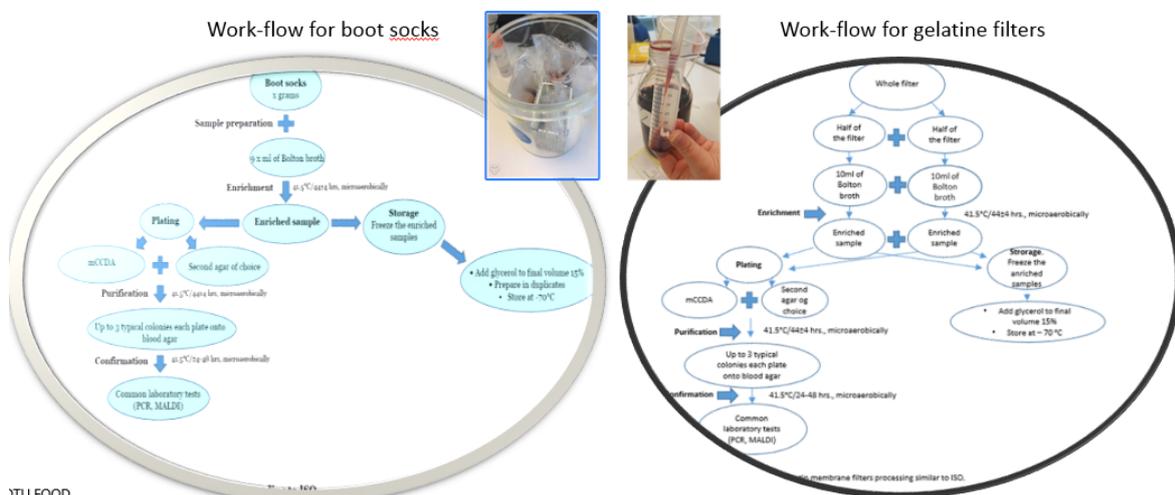




Figure 1. Protocols for culture-based detection of Campylobacter from chicken farms.

For the sample bank, a “Farm questionnaire” was designed. The metadata associated with sample collection includes:

- Type of farm with biosecurity measures: organic or conventional farm (open housing farms must be excluded)
- Size of the farm (number of houses)
- Size of the house (square meters; or length x breadth)
- Size of flock (number of chicken per house)
- Type of bedding: Shavings, sawdust, straw, paper, peat, etc.
- Age of chicken coming in and out the house
- Feed composition or feed compound type
- Additional equipment (floor heating, fly net, etc.
- Type of chicken breed
- Chicken supplier
- Number of breeding cycles per house and year
- Type of disinfection
- Biosecurity measures (rodent and insect control, dead-bird disposal, water sanitation, litter removal, visitor’s control, owner’s pets on the farm)
- Health care anamnestic data (use of antibiotics; first week mortality)
- Date
- Age of animals (days)
- Outdoor temperature
- Indoor temperature
- Farm region (not exact address, because identity of farms participating in the sampling plans are not to be revealed.)

The data was stored on the drives of the participating institutions. All stored samples were linked to the farm’s questionnaire lists. The 1mLx2 of each positive enriched samples and the extracted DNA were stored at minimum -70°C in the facilities of each partner. The data was shared among the partners. The data (results) are associated with the analyses of electronic journals/ reports stored in the institutional servers of the participating countries. The raw data was stored in Mx3000P and Mx3005P QPCR Systems instruments, Microsoft Excel files, Microsoft Word in the institutional servers of the project participants.

In the first sampling round, the samples (boot socks and air samples) were all analysed by cultivation. The use of real-time PCR was voluntary, and not all partners carried out PCR. It was also variation between the partners that used real-time PCR on which steps PCR was used.

Sampling activities for creation Sample bank.

The DTU sampled at three farms with five different houses. One house was found to be positive for both boot socks and air filter. Overall, it was found that the results from the incubation of ½ filters in Bolton broth for 44±4 h are the same as the results of the incubation of boot socks (ISO), with no false results from gelatin filters. In addition, the PCR results from the filters corresponded to the results obtained with the culture based method.



In June 2018, the NVI sampled eight flocks from seven farms by collecting one pair of sock samples and one air filter sample from each flock. In September, NVI also sampled two flocks from one farm. Here, one pair of sock samples and two air filter samples were collected from each flock. NVI found three flocks positive for boot socks, while all the air filters were negative.

The VRI sampled ten different houses from four farms. From each house, two pair of socks and two air filters were sampled. According to the cultivation method, all the flocks were negative. Nevertheless, the molecular methods retrieved two positive samples out of the ten samples. Gelatine filters do not seem to be able to keep campylobacters alive, and a 15-minute air sampling does not seem sufficient. The cultivation methods do not seem sensitive enough, whereas the PCR method (direct DNA extraction) is more effective in some filter samples.

The NVRI sampled from two farms with three houses at eight different time points. The first two samplings showed positive PCR results from filters, but they could confirm the results through strain isolation only from socks for sampling no. 2, and only after 24h incubation in Bolton broth. For the remaining samplings, two out of six were positive in culturing from socks but only in one of the air filters *C. jejuni* was isolated. Finally, three filters showed positive PCR results. In conclusion, for the socks samples, plating out after incubation in Bolton broth for 24h, seems better, perhaps due to a high level of background microflora present in direct plating. Air filter-culture based detection gave one positive result, while in real-time PCR, the air filters resulted in three positive results. In one case (sampling no. 3), for the positive samples, 100% correlation was observed (the same results for socks, filter culturing and real-time PCR). For the negative samples, four times (sampling numbers 4, 5, 7 and 8) resulted in 100% correlation for different type of samples. Three samplings gave divergent results for various kind of samples.

The IZSAM sampled in ten different houses from two farms. From each house, two boot socks pairs and two air filters were sampled. The culturing was performed using direct plating and enrichment using the Bolton broth. The DNA was extracted directly from air filters and boot socks. For the first five houses the culturing from boot socks revealed one positive sample using the direct plating while all the enriched samples were negative, and the air filters were all negative. The other five houses were positive to the direct plating and negative to the enrichment method, while one air filter was positive to the enrichment. Direct plating for boot socks was shown to be the best option while enrichment with Bolton Broth was not the appropriate method for the isolation of *Campylobacter* from boot socks.

Summary results of field studies shown in Table 1 below:



Country	No of samples (n)	Number of positive samples							
		Boot socks				Air filters			
		cultivation methods		real-time PCR		cultivation methods		real-time PCR	
		direct plating	enrichment	Direct	enrichment	direct plating	enrichment	direct	enrichment
Italy	10	6	0	7	5	0	1	8	5
Czech R.	10	-	0	-	0	-	0	2	0
Norway	10	-	3	-	-	-	-	-	-
Poland	8	-	3	-	-	-	1	3	-
Denmark	6	-	1	-	-	-	1	1	-

JRP9-WP1-T2: Development of a protocol for non-complex DNA extraction for diagnostic qPCR and metagenomics analysis from gelatine-filter samples (M3-M13)

Based on the intensive in-house studies at several participating labs, the consensus protocol enclosed was agreed. It consists of two steps: Pre-treatment of gelatine filters taken by AirPort8 device, and DNA extraction using QIAgen DNeasy Blood&Tissue Kit, according to manufacturer’s instruction with several modifications. The main step in the filter preparation is the addition of the Protex protease to completely dissolving the gelatine. The modifications that we found to be important to performance of the commercially available DNA extraction kit were: 1) Addition of RNase (due to our intended later use of the DNA product for downstream metagenomics analysis); 2) The replacement of kit elution buffer with TE-EDTA buffer; 3) The addition of AL buffer. A metagenomics pilot project was performed that included a standardized MOCK community. Based on the pilot project sequencing technology, sequencing depth and bioinformatics analysis for a larger metagenomics project were agreed upon, including samples from all partners. The samples from all partners were collected and sequenced. The bioinformatics analysis and interpretation of data including writing manuscripts has been carried out by NVI (Norway). Two metagenomics manuscripts are in preparation, one for the pilot study and one for a large metagenomics study. The pilot study was performed by NVI and a preliminary title for this manuscript is “[Detection of *Campylobacter* in air samples from poultry houses using shot-gun metagenomics – a pilot study](#)” by the following authors; Thomas H.A. Haverkamp, Bjørn Spilsgberg, Gro Johanessen, Mona Torp, Camilla Sekse to be submitted to BMC Microbiome (Gold Open Access). The second manuscript will involve all partners.

WP2: Validation and Standardization (M13-M30)

JRP9-WP2-T1: Validation of air sampling and DNA extraction methods.

Three harmonized protocols were prepared for 1) field air sampling, 2) DNA extraction, 3) PCR testing. The protocols were evaluated on field air samples from chicken farms around Europe collected during the summer 2019. A sample bank was established for later use for shot-gun metagenomics. The protocols were planned to make the basis for draft standards to be submitted to CEN and EFSA for consideration as part of the continued standardization work for *Campylobacter* detection in chicken farms. This part of the work was planned for Spring 2020. A second manuscript was planned based on the outcome of the summer sampling in 2019 and was published autumn 2020 (see 7. Publications).



JRP9-WP2-T2: Statistical analysis, Standardization and dissemination.

The work in 2020 departed from the achievements of the first annual period and took place over the second annual period and extended in 2020. Validation is important to regulatory approvals before the methods can be used. Hence, the air sampling protocol from WP1 was evaluated. Following statistical data analysis of the results obtained, the best performing protocol was drafted as a Standard Operating Procedure (SOP). This was planned to be demonstrated among the EJP partner laboratories involved in testing of *Campylobacter*, but due to the Covid-19 pandemic and cancellation of the physical meeting in Prague, the physical demonstration was cancelled. Following agreement by the OHEJP coordinators, this has been done as an online video demonstration (<https://youtu.be/LMD03UAAPUw>). The project and SOP were presented in Sept 2020 by Gro S. Johannessen at the virtual EU Reference Lab meetings for *Campylobacter*. A manuscript describing the final results has been published in AEM: Hoorfar J, Koláčková I, Johannessen GS, Garofolo G, Marotta F, Wiczorek K, Osek J, Torp M, Spilsberg B, Sekse C, Thornval NR, Karpíšková R. [Foodborne *Campylobacter*: A multi-center proposal for a fast screening tool in biosecured chicken flocks.](#) Appl Environ Microbiol. 2020 Aug 7:AEM.01051-20. doi: 10.1128/AEM.01051-20.

Online video animation material for the education of broiler industry: <https://www.youtube.com/watch?v=S9mapXSM8tw&t=95s>

Possible dissemination through a hands-on, wet-lab workshop, in the case the pandemic is eased.

3. Project self-assessment

The project has followed more or less the initial plan, naturally by some modifications. The outcome has been more profound than envisaged, since the new protocol can detect up to four times more colonized flocks than the stool sampling. It was initially planned to submit a draft standard to CEN/ISO, however, due to the Covid-19 pandemic situation no meeting was held in 2020. For this reason, our effort mostly focussed on publications and virtual demonstration through YouTube, which is completely open to the public. In addition, a targeted effort was done with the professional help by the OHEJP coordinators to communicate the results to EU regulators, EFSA and ECDC. This deviated slightly from the original plan that involved more interaction with the standardization organizations. The project and results have been presented at the annual workshop for EURL *Campylobacter* with positive response.

An interesting observation from the first round of sampling was the different interpretations of the initial dilution steps of boot swabs among the partners, although a standardized method (ISO 10272-1) was used. This was discussed in the first paper (Johannessen et al, 2020) and has been brought to the attention of both EURL *Campylobacter* and ISO TC 34/SC 9. A short-coming with this project is that the detection of *Salmonella*, which is important in broiler production, is not included. It is believed that this sampling technique and the samples can be expanded to include more pathogen targets thus being a simple tool for sampling of several relevant organisms. This could be followed up by further studies if funding opportunities arise.



4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number <i>(Original number, if different from the actual one)</i>	Deliverable name <i>(Original name, if different from the actual one)</i>	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments <i>(Please mention: Zenodo reference and other comments)</i>	Proposed categories* <i>(1 to 8) (several categories may be applicable)</i>
23	D-JRP9-1.1.	Prototype laboratory method to detect and enumerate <i>Campylobacter</i> in air samples.	M9	M8		Public https://zenodo.org/record/3676374#.Xk-zfqaWwj9	2
23	D-JRP9-1.2.	Prototype metagenomics method for characterization of <i>Campylobacter</i> in air samples.	M12	M12		Public https://zenodo.org/record/3754239#.YBQ1vOhKhM0	2
23	D-JRP9-2.1.	Online video demonstration of air sampling for farmers.	M24	M18		YouTube demonstration video https://youtu.be/S9mapXSM8tw	1
23	D-JRP9-2.2.	Standard Operating Procedure (SOP) for air sampling.	M24	M24		Public https://zenodo.org/record/3676376#.Xk-ztaaWwj9	2
23	D-JRP9-2.3	Publication in AEM.	M30	M30		Confidential; to be made public after the end of the embargo (Green Access with 6 months embargo) TD	8
23	D-JRP9-2.4	Hands-on, wet-lab workshop for relevant EJP	M29			This deliverable was cancelled – the workshop in Prague was not held because of Covid-19.	5



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
		partners.				As alternative solution, a video demonstration was prepared: https://youtu.be/LMD03UAAPUw	

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
03	M-JRP9-1	Sample bank is established	M9	Yes		A decentralized sample bank at partner organizations involved.
03	M-JRP9-2	Sample preparation method is selected	M9	Yes		QiaAMP Tissue&Blood kit
03	M-JRP9-3	Local field studies completed.	M18	Yes		All partners have conducted local field sampling in chicken farms, as described the project plan. (Initially M15, adapted in AWP-Y2)
03	M-JRP9-4	Statistical analysis completed.	M29	Yes		Delivered as part of the manuscript submitted.



5. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Prevalence and antimicrobial resistance of Campylobacter isolated from carcasses of chickens slaughtered in Poland – a retrospective study 10.1016/j.foodcont.2020.107159 https://zenodo.org/record/3676306	Yes	No	Yes (3 060 €)
Campylobacter in chicken – critical parameters for international, multicentre evaluation of air sampling and detection methods https://doi.org/10.1016/j.fm.2020.103455 https://zenodo.org/record/3663545#.XkPEvGhKiUk	Yes	Yes – 6 months.	No
A multi-center proposal for a fast screening tool in biosecured chicken flocks for the foodborne pathogen Campylobacter 10.1128/AEM.01051-20 https://zenodo.org/record/4244138#.X6KEbDiWxM1	Yes	Yes – 6 months	No
MLST-based genetic relatedness of Campylobacter jejuni isolated from chickens and humans in Poland 10.1371/journal.pone.0226238 https://zenodo.org/record/3628110#.X6FsFjiWxPY	Yes	No	Yes (PloS One)



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Thornval, N., Hoorfar, J. (2021). Progress in detection of <i>Campylobacter</i> in the food production chain. <i>Current Opinions in Food Science</i> (In Press).	Yes	Yes – 6 months	No
T.H.A. Haverkamp, B. Spilsberg, G. Johannessen, M. Torp, C. Sekse. (2021). Detection of <i>Campylobacter</i> in air samples from poultry houses using shot-gun metagenomics – a pilot study. (In prep)			To be submitted to BMC Microbiome
All partners (2021). Large metagenomics study (In prep). Contact person: Gro S. Johannessen			
Marotta, F., Janowicz, A., Di Marcantonio, L., Ercole, C., Di Donato, G., Garofolo, G., & Di Giannatale, E. (2020). Molecular Characterization and Antimicrobial Susceptibility of <i>C. jejuni</i> Isolates from Italian Wild Bird Populations. <i>Pathogens</i> , 9(4), 304.	Yes	No	Yes



Additional output:

Dissemination to EFSA and EC (enclosed).

6. One Health Impact

For the first time, the EU has published a guideline for the maximum number of *Campylobacter* in chicken meat (1000 CFU/g). This is a good start. However, controlling the pathogen at the farm level is hampered by the lack of a low-cost and harmonized protocol that can be handled by farmers themselves. The case is that farmers are reluctant or not willing to let outsiders in to sample for *Campylobacter*, or any other pathogen. This is due to the risk of flock contamination by avian flu, coronaviruses, or other externally introduced pathogen risks. The protocol we have presented here enables the use of a smooth and hand-held device, similar to a vacuum cleaner that can be easily handled by farmers. The filter can be taken out and shipped by ordinary mail to any service lab that does qPCR. The advantage of such protocol is that it can gradually be expanded to include an increasing number of pathogen targets, depending on the need of chicken farmers. Furthermore, the sample pre-analytical DNA/RNA extraction method can be more or less used for other pathogens. Thus, the protocol presented may be a game-changer in rapid diagnostics.

What is missing in our approach is the inclusion of detection of *Salmonella*, which is highly important in chicken production. We acknowledge this shortcoming but this was not the main aim of this small project. It is thus recommended to follow up on the inclusion of *Salmonella* (and other pathogens) as a possible future effort.

The project results and SOP have been presented at the EURL *Campylobacter* workshop in 2020 and was well received. The project partners have been in contact with national authorities and stakeholders to inform about the project and also the results to make them aware of this new approach for *Campylobacter* testing.

JRP10-MOMIR-PPC

1. Summary of the work carried out in year 3

The **MoMIR-PPC project** aims to develop new approaches to predict, identify and prevent the appearance of animal and human super-shedders based on immune response and gut microbiota composition. In order to achieve this aim the project will focus on four objectives.

1. Defining **predictive markers** that will signal the risk of both animals and *Salmonella* isolates becoming a super-shedder of *Salmonella*.
2. Immune and microbiota **biomarkers** to detect super and low-shedders.
3. **Preventive measures and /or control measures** of this zoonotic problem by the characterisation of prebiotics, probiotics and nutraceutical products.
4. Development of **mathematical models to provide new risk management tools**. These tools will lead to creation of a **pool of biosecurity measures** at the farm levels, each with a **cost effectiveness** consideration.

To date the project team has undertaken *in vivo Salmonella* infection studies in both chickens and pigs. Serological analysis of these studies has now been completed, with immunological and microbiome analyses currently ongoing. Numerous experiments in field conditions as well as experiments with humans have been delayed, particularly because of the COVID-19 crisis. Nevertheless, to date (January 2021), these studies have enabled the consortia to identify predictive biomarkers based on gut



microbiota composition in the chicken. Some of the predictive immune biomarkers remain to be confirmed. Joint articles have been accepted or are in preparation on this topic. Other biomarkers, which can indicate the super- or the low-shedder phenotypes have been identified both in pigs and in chickens. The first results showed that the number of circulating blood immune cells could not be a predictive marker for the appearance of the low and super-shedder phenotypes as well as the non-specific antibody levels. Experiments have suggested that the low and super-shedder phenotypes could not be explained by a modification of the virulence levels of *Salmonella in vivo*. Recruitment of participants in the human aspect of the project is also now ongoing, with analysis being carried out on a rolling basis.

Numerous putative probiotic strains have been isolated and characterised by the Partners. Four (2 chicken and 2 porcine) have been sent to Bulgaria for evaluation in chickens and pigs. Unfortunately the experiments were delayed. However, the experiment with the 4 groups of day-old chicks that received the test probiotics and prebiotic are complete and the pig studies are nearing completion. Once the bacteriology results have been analysed a sub-sample of the 3600 faecal/gut samples will be subjected to 16S metagenomic studies at the UoS to determine how the interventions and *Salmonella* status influence the gut microbiome. Chicken studies undertaken (INRAE) to determine the influence of commensal bacteria on *Salmonella* shedding status and immune response have also been completed.

The comparison of the gut microbiota compositions and the immune parameters of the probiotic-inoculated and control groups revealed, in experimental inoculations, an overall impact of the inoculated strains. A mix of four commensal bacteria can, in part, protect chickens from *Salmonella* colonization. Similarly, chickens fed with fermented defatted 'alperujo' were in part protected against *Salmonella* colonization when they were infected at 7 or 21 days of age. At this moment the metagenomics data are under statistical analysis.

A first version of a generic mathematical model of the dynamic interplay between the gut microbiota, the pathogen and the host's immune response at the within and between-host scale has been developed. These models are based on the data obtained from the pig and chicken studies. New experiments will also enable us to validate (and possibly refine) a mathematical model describing indirect transmission by testing its predictions in an experimental setting. Finally, a draft inventory of relevant intervention measures against *Salmonella* in laying hens has been developed within the framework of a HACCP analysis, and the cost effectiveness (utility) of intervention strategies using probiotics has been calculated.

The project extension has been accepted by the OHEJP Project Management Team and the Scientific Steering Board. The Partner 23 (University of Surrey) will host the final project meeting.

2. Wok carried out in the JRP, scientific results

WPO: Management (M1-M30)

JRP10-WP0-T1: Draft and agree Consortium Agreement (M1-M6)

Completed.

JRP10-WP0-T2: Produce project-planning, control documentation and Data Management Plan (M1-M12)

Completed. See second annual report, 2019.

JRP10-WP0-T3: Control and manage activity progresses, the timely delivery of project tasks and outputs (M1-M36)

This task is ongoing. Two exceptional extensions of the project of 12 months and then of 6 months at no extra expense has been obtained by the EJP board. The new extension was motivated by the delay in several experiments due to the Covid19 crisis:



Concerning the data obtained in human: Until now, no scientific results are available. Sample collection is complete. Laboratory analyses of the collected stool samples are ongoing. Metagenome sequencing and analysis of the results should be finished in the beginning of 2021.

JRP10-WP0-T4: Control and manage the project closure and outputs (ends: M30)

A final meeting has been planned in Surrey, the date and the condition of this meeting will depend of the evolution of the Covid19 crisis.

WP1. Risk prediction for Super-shedder animals and human asymptomatic carriers through the use of gut microbiota and immune status analyses (M1-M12)

JRP10-WP1-T1: Predictive immunological markers associated to the high and low-shedders in chickens and pigs (M1-M12)

Partner Anses has performed a trial as already described in a previous report. Immune response has been analysed by **ANSES** and **INRAE**. One predictive biomarkers and several biomarkers able to characterize the low- and super-shedder phenotypes have been identified. Similarly, as indicated in the previous report, **Partner ISS** and **Partner IZLER** have performed experiments in pigs. In these studies blood and fecal sampling were performed over a period of weeks/days. For the **ISS** study, tissue samples were also taken at *post-mortem* examination. Blood samples were analysed by **ANSES** and **ISS** to determine if immunological markers could be linked to the *Salmonella* shedding status of pigs. NFS and ELISA analysis, performed by **ANSES** showed that some blood cells or cytokines are over or under produced within a few days after infection (Day 1, 2 or 3) and that the production is significantly different between high and low shedders. However, these differences are not prolonged over time; they disappear as one moves away from the infection. The **ISS** analysis is ongoing and will be reported in the coming months.

In order to shed some light on the differences in the immune response of low and super-shedders, **INRAE** experimentally infected four groups of chickens with *S. Enteritidis*. Two groups (Group-1, 3) were inoculated at one days of age with a mix of four commensal bacteria described in previous work; Group-2 and 4 were inoculated at one days of age with buffer solution. At 7 days of age Group-1 and 2 were infected with *S. Enteritidis*. Hierarchical clustering performed on the level of *Salmonella* in faecal and caecal samples identified super and low-shedder phenotypes. Kinetic of the number of blood immune cells was determined by Flow cytometry before and after infection.

The results showed that the number of blood immune cells circulating in the blood could not be a predictive marker for the appearance of the low and super-shedder phenotypes. Moreover, the analysis has shown no differences, after infection, when low and super-shedders are compared with the control group. In the same way, the immuno-histological work performed by **Partner VISAVET-UCM** on caecal samples did not show any major histopathological changes between low and super-shedders. However, the monocytes/macrophages and the heterophils numbers were significantly increased at 7 dpi in low-shedders and super-shedders, respectively, when animals were inoculated with the probiotic flora. These results suggested that inoculation of the 4 commensal bacteria has modified the number of immune cells circulating in the blood during *Salmonella* infection and thus has most likely modified the immune response. To analyse the immune response before and after infection, RNA from blood samples and from internal organs were taken super and low-shedders have been extracted and analysed with the Biomark. An article is in preparation.

JRP10-WP1-T2: Predictive microbiota markers associated to the high and low-shedders in chickens and pigs (M1-M12)

The previous works performed by **INRAE** and **VRI** demonstrated the role of gut microbiota in the susceptibility to *S. Enteritidis* infection and in the appearance of the low and super-shedder phenotypes. We especially demonstrated that (1) axenic and antibiotic-treated chicks are more prone to become super-shedders; (2) super or low-shedder phenotypes can be acquired through microbiota



transfer; (3) specific gut microbiota taxonomic features determine whether the chicks develop a low- and super-shedder phenotype after *Salmonella* infection in isolator. This study demonstrates the key role plays by gut microbiota composition in the heterogeneity of infection. An article describing this work has been accepted for publication in Microbial Biotechnol. These results have been presented at the OHEJP-ASM (virtual) conference (2020).

For pigs, **ANSES** has performed a trial as described in previous report. All the faecal samples recovered before and after pig infection have been sequenced by the **UoS**. 16S data from the gut microbiota composition have been analysed by **UoS** and by **INRAE**. The results have indicated that numerous taxa are specific to the low and super-shedder phenotypes. A joint article is in preparation. Similarly, as indicated in the previous report, **ISS** and **IZLER** have performed experiments in pigs and sent the samples to **UOS** for metagenomic analysis. The data have been analysed in conjunction with metadata related to the animal's overall health and shedding status, to test hypotheses regarding the association of the gut microbiome with *Salmonella* shedding status. It should be noted that both the studies were carried out using pigs with different genetic backgrounds and *Salmonella* strains, therefore an opportunity to test the association between the microbiome and *Salmonella* shedding in two different scenarios.

Figure 1 illustrates the bioinformatic pipeline used to analyse the gut microbiota composition undertaken at the **UoS**. An overview analysis, conducted at **UoS** and **INRAE** with the **ANSES** experimental data highlighted a difference in microbial diversity (measured as the distribution of operational taxonomic units (OTUs) in different parts of the intestinal tract) Fig.2. Moreover, statistically significant differences in the OTUs detected in the microbiome of animals classified as high, intermediate and low *Salmonella* shedders are detected Fig. 3. These significant differences are observed using small- and large-size of OTUs at several timepoint post infection, between Low and high-shedders Fig. 3. The results of this analysis have been reported in a poster presented at the OHEJP-ASM (virtual) conference (2020) and the abstract is available in the conference proceedings. Further analysis is ongoing.

A Co-inertia analysis between the immune genes dCt and microbiota features abundances, conducted at INRAE showed a very good correlation (0.98 and 0.68, respectively) at Day 1 post infection.

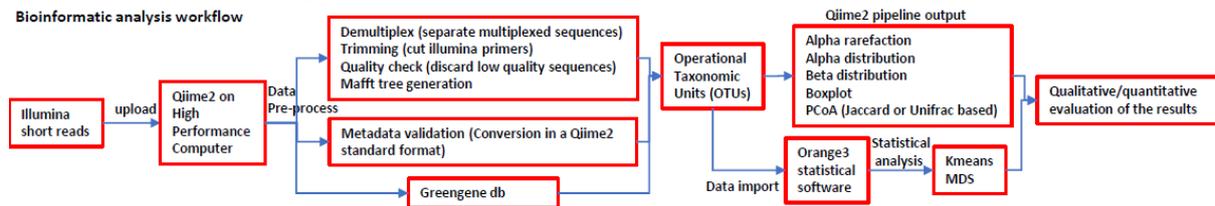
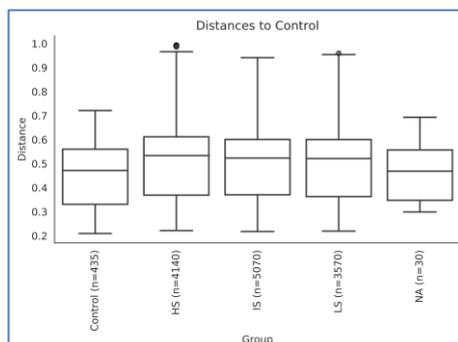


Fig. 1: Bioinformatic pipeline used at the UoS for the metagenomic analysis of pig gut microbiota.



Pairwise permanova results

Group 1		Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
Control	HS		100	999	6.391340	0.001	0.003333
	IS		100	999	7.542294	0.001	0.003333
	LS		140	999	5.018066	0.001	0.003333

Fig.2: β -distribution of samples according to *Salmonella* shedding class. Small, but statistically significant differences (highlighted in red in the table) can be noted.



	Small OTUs			Large OTUs		
	LS vs SS	LS vs Int	SS vs Int	LS vs SS	LS vs Int	SS vs Int
D0	0	0	3	0	0	0
D1	0	1	0	0	0	0
D2	45	13	26	10	1	2
D3	18	0	0	25	6	0
D7	6	1	4	0	0	0
D14	0	4	13	0	0	0
D17	1	0	3	0	0	1
D21	0	0	2	0	0	0
D22	1	0	3	0	1	1

Numbers in red include OTUs assigned to *Salmonella*

Fig.3: Number of differentially abundant OTUs, at each timepoint, between Low and high-shedders.

JRP10-WP1-T3: Risk factors associated with prolonged convalescent *Salmonella* shedding in humans (M1-M12)

In order to comply with research ethics in medical research and implementation of GDPR in 2018, we first needed all relevant permissions for collection and processing of patients' data (obtained in the last quarter of 2018). . Sample collection started on the 1st of January 2019. The recruitment of study participants continued until the 31st of December 2019, to complete a full year of sampling. Currently, stool samples and questionnaires from 323 study participants in total have been collected. Direct culture of all the stool samples has been performed. In addition, all isolates received at the NIPH have been sequenced. Data analysis and metagenome sequencing will be carried out throughout the first half of 2021. Publication of research results is foreseen in the second quarter of 2021.

JRP10-WP1-T4: Virulence of *Salmonella* strains originated from high and low-shedders (M7-M12)

In vitro virulence of *S. Typhimurium* and *S. Enteritidis* collected from super-shedders and low-shedders pigs and chickens have been evaluated by INRAE in pig and chicken epithelial and macrophage cell lines. Cell entry and cell response after infection of *S. Typhimurium* and *S. Enteritidis* collected from super-shedders and low-shedders pigs and chickens, respectively, was evaluated. *Salmonella* strains from two super-shedders and two low-shedders animals were used in each experiment in comparison with the strain which was inoculated. *Salmonella* adhesion, invasion and intracellular multiplication to an intestinal pig epithelial cell line (IPEC-1) and a pig macrophage cell line (3D4) were measured as well as with a chicken epithelial cell line (LMH). No differences were observed between *Salmonella* strains for any of the parameters evaluated (see example figure 4).

In addition, the gene expression of pro-inflammatory and anti-inflammatory genes was evaluated. No differences were observed in the gene expression of CXCL8, IL8 or TGFβ for any of the conditions evaluated (see example figure 5).

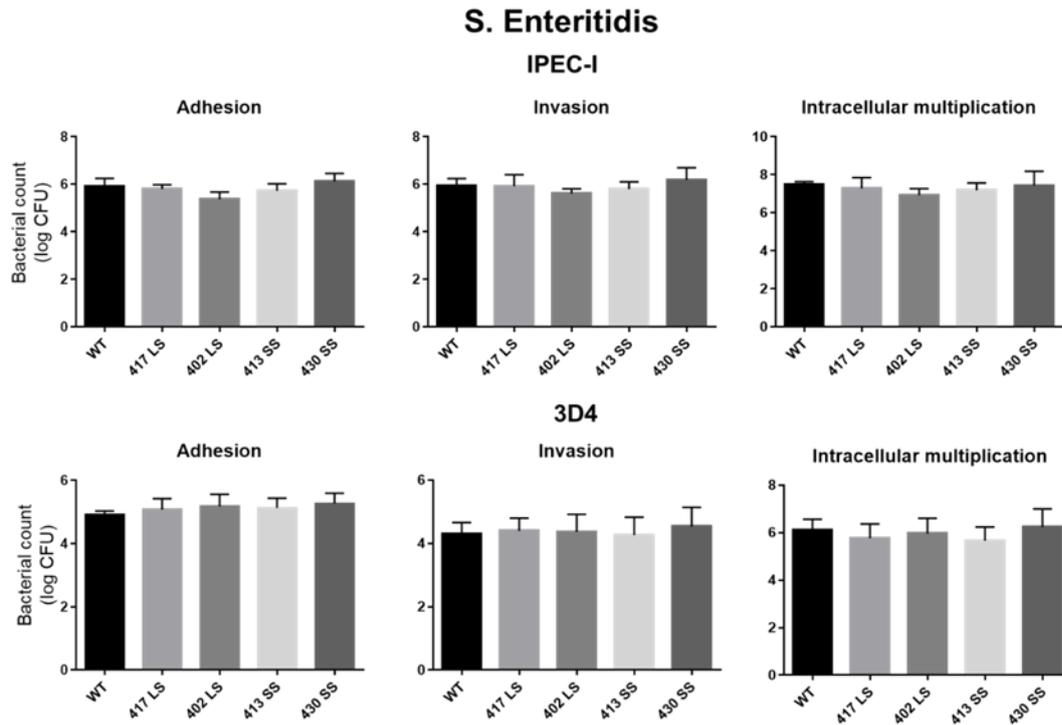


Figure 4: *S. Enteritidis* strains from super-shedder and low-shedder origin do not present any difference in terms of their adhesion, invasion and intracellular multiplication in a pig intestinal cell line (IPEC-I) or a macrophage cell line (3D4). SS represents super-shedder; LS represents low-shedder; WT represents the original *Salmonella* strain inoculated into the animals.

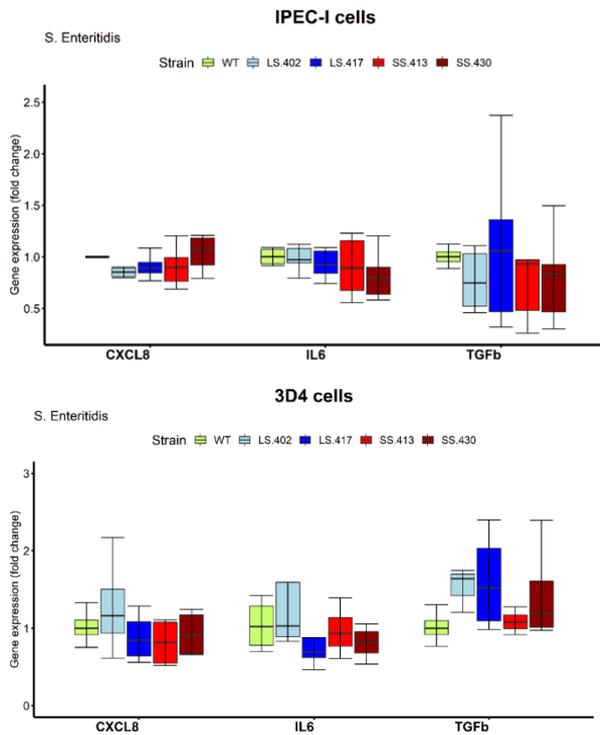




Figure 5. Real-time RT-qPCR analysis of IPEC-I and 3D4 cells infected with *S. Enteritidis* strains recovered from super-shedder and low-shedder origin. SS represents super-shedder; LS represents low-shedder; WT represents the original *Salmonella* strain inoculated into the animals. Gene expression levels are shown relative to the WT group.

In conclusion, our data do not show any alteration in the *in vitro* virulence of *Salmonella* strains from super-shedder and low-shedder origin. These results strongly suggested that the super-shedder and low-shedder phenotypes are not related to a modification of the virulence level of the bacterial strains.

WP2. Prevention of the appearance of Super-shedder animals and asymptomatic carriage in humans and animals by modifying feed and/or microbiota (M1-M12)

JRP10-WP2-T1: Use of probiotics in chicken and pig (M7-M12)

VRI continued in the systematic culture of chicken gut anaerobes and laboratory collection, which currently consists of more than 450 isolates with known genomic sequences (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP101913&o=acc_s%3Aa). These strains are gradually used for oral inoculation of chicks on the day of hatching followed by verification of their presence 7 days later. The repeated observation and conclusion is that in this type of experimental design, bacterial species expressing outer membrane, i.e. Bacteroidetes, Proteobacteria and Veillonellaceae, can efficiently and persistently colonise chicken caecum. Rather unexpectedly, they never succeeded with the colonisation of newly hatched chicks with Gram positive bacteria from phylum Firmicutes and families Lachnospiraceae, Ruminococcaceae or Lactobacillaceae, despite the fact that these species are common microbiota members. Recently they have changed the protocol for culture of gut anaerobes and this seems to provide novel opportunities for culture of yet unculturable species. This modification consisted of culture under microaerophilic conditions and using Karmali agar. Several experiments have been performed to understand 1- why the inoculation of Lachnospiraceae, Ruminococcaceae or Lactobacillaceae which are otherwise common in gut microbiota on chickens 1 to 2 weeks old, were unable to colonise chicks after oral inoculation and 2- the origin of these bacteria in the caecum of chickens.

Other experiments were performed to determine whether the colonisation of chicken intestine with some strains modified on the long term the faecal and/or caecal microbiota composition. For this purpose defined mixtures consisting of bacterial species which can colonise chicken caecum after a single dose on day of hatch were gradually tested in real commercial farms. Altogether, over 130 000 chickens were treated with different mixtures consisting mainly of different Bacteroides, Prevotella and Megamonas species. Due to the financial scopes, these experiments were co-funded also by other projects of VRI. Central issue when upscaling from laboratory to field level was the administration of chicken gut anaerobes to flocks consisting of more than 10, 000 chickens. They tested oral administration via drinking water, resuspension of liquid cultures to pre-starter feed, jellyfying liquid probiotic cultures before their spread over the pre-starter feed or even feed fermentation by the probiotic strains. Since they collected chickens for control of their colonisation, they know that tested probiotic strains persist in the caeca of treated broilers till day 35 of life. Moreover, since some of the flocks were formed by reproductive birds, they monitored these flocks till week 20 of their life and also at this time point that could detect bacterial species from tested probiotic mixture in their caeca. So the persistence of selected and tested species seem to be quite long if not permanent.

UoS isolated a large panel of potential probiotic candidates from pig faeces. Following identification by 16s and basic *in vitro* characterisation a subpanel was selected for further study. 20 porcine probiotic *Lactobacillus* strains were further characterised both genotypically and phenotypically. These studies included growth curves, survival in acid/bile, whole genome sequencing and tissue culture studies (pig cell line) to determine safety and inhibitory ability of whole cells and CFS against *Salmonella* adhesion/invasion (ongoing). To date the studies have indicated that a number of strains are suitable probiotics that meet the EFSA requirements. Four probiotic strains (2 chicken) and (2 pig)



were sent to Bulgaria for efficacy evaluation in chickens and pigs, respectively.. If required, strain will also be shared with the AVANT H2020 project."

NDRVMI has analysed the effect in field conditions of several probiotics obtained from the **UoS**. they used 4 groups of day-old chicks o (18,500 in each group). In groups with probiotics 1 and 2, the birds were raised without antibiotics, as well as any other side effects. Growth rates and food consumption were also excellent and mortality was lower than in the control group (where antibiotics were still used). In the third experimental group, where probiotics 1 and 2 were used and a prebiotic was added to stimulate probiotic microorganisms, there were some issues in the middle of the experiment with a sharp increase in mortality observed, this was due to the presence of a pathogenic strain of *E. coli* and for 5 days they were treated with an antibiotic after which the mixture of probiotic 1 and 2 and prebiotic were delivered until the time of slaughter. This group was not a linear hybrid Ross like the other groups, but a linear hybrid Gobb, which is much more susceptible to disease. The experiment is being repeated only with this compromised third group and the control, as the linear hybrid is Ross.

The experiments with pigs started on September 3 2020, when 3 experimental groups of freshly weaned pigs were formed, 25 in a group and transferred to individual boxes without a connection between boxes. Other pigs of the same age, reared in the usual way on the farm, are used as controls. The pigs in all four experimental groups received the same feed, with the only difference being the respective probiotic or a mixture of the two probiotics tested with the addition of prebiotic fed through the drinking water. Particular attention was paid to the safety of supplying a sufficient amount of water containing at least one billion living cells in a milliliter of broth culture dissolved in drinking water. In practice, each pig receives 3 millimeters of broth culture daily with the appropriate probiotic or a mixture of probiotics and prebiotics. However, it seems that the pigs did not like the taste of water containing broth culture and took time to drink. One after drinking this water containing probiotic bacteria, they are given another drinking water.

It should be noted that in 4 experimental groups the presence of *Salmonella* Typhimurium monophasic, as well as *Salmonella* GIVE and Infantis was detected. At the same time, in the past 3 months there were no sick pigs from the three experimental groups treated with probiotics, and the other parameters are to be determined (growth, efficiency in food absorption, nutritional ratio, histological findings in the internal organs). At the end of December or the beginning of January 2021, the slaughter of fattened pigs will take place (105-110 kg body mass), where samples will be taken from the caecum of the slaughtered animals and samples for histology from the internal organs.

After completion of the experiment with the broilers and the pigs, all samples will be sent to the **UOS** for 16s metagenomic analysis. This is likely to happen in January 2021.

Previous works performed by **INRAE** demonstrates the role of gut microbiota in the susceptibility to *S. Enteritidis* infection and in the appearance of the low and super-shedder phenotypes. During the last period we showed that partial protection can be conferred by inoculation of four commensal bacteria prior to *Salmonella* infection (figure 6). This study paved the way for developing a protective mix of probiotics. An article describing this work has been accepted for publication in Microbial Biotechnol. The impact of the four commensal bacteria on the gut microbiota composition and the immune response is under investigation by a 16S metabarcoding approach in collaboration with **VRI** and by the Biomark approach.

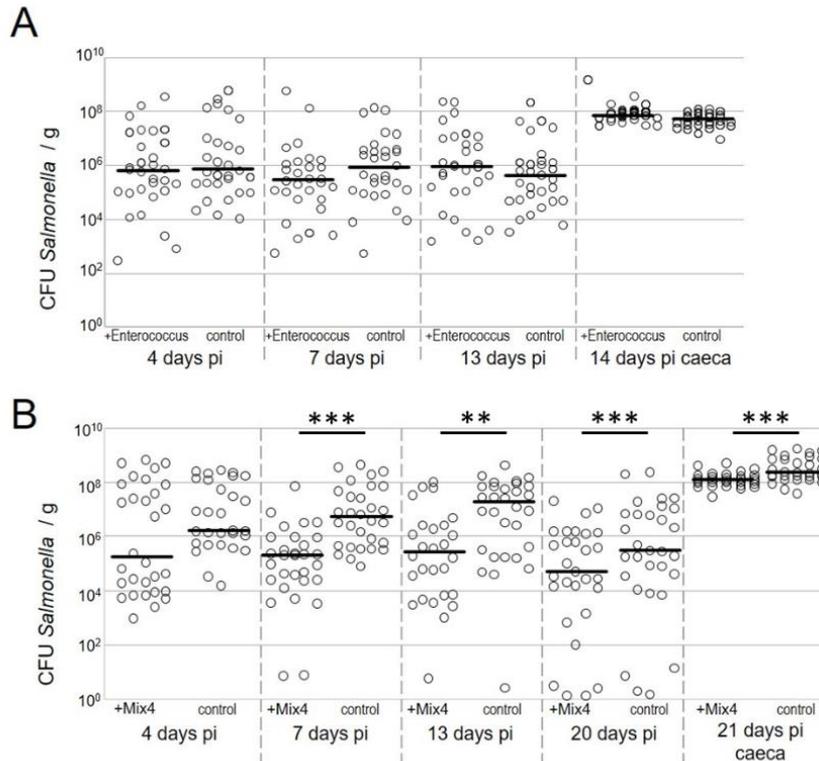


Figure 6: Level of *S. Enteritidis* after inoculation at one days of age of *Enterococcus faecium* (A) and the mix of four commensal bacteria (B).

JRP10-WP2-T2: Use of pre-biotics and nutraceutical already defined by the consortium partners in chicken and pig (M1-M12)

VISAVET-UCM has completed data analysis detailed in the last report, with both traditional culture and qPCR approaches.

Salmonella Typhimurium Colonisation in the Cecum: In 7-day-old challenged chickens, at 7 dpi (14 days old), the load of *Salmonella* spp. in the cecum was significantly lower than controls by culture ($p = 0.008$), and almost by qPCR ($p = 0.056$). At 14 dpi (21 days old), there were significant differences in the *Salmonella* spp. cecal load between the control and treated group by qPCR ($p = 0.032$) but not by culture ($p > 0.05$). At 21, 28, and 35 dpi (28, 35, and 42 days old, respectively) there were no significant differences in the cecal *Salmonella* spp. load among groups by culture ($p > 0.05$) or by qPCR ($p > 0.05$) (Fig. 7).

In 21-day-old challenged broilers, all cecal content challenged was negative for *Salmonella* spp. either by culture or qPCR. At 7 dpi (28 days old), the *Salmonella* spp. load in the cecum was significantly reduced in the treated group by qPCR ($p = 0.016$), and by culture, which was not significant ($p = 0.075$). At 14 dpi (35 days old), there were no significant differences in the cecal *Salmonella* spp. load among groups by culture ($p > 0.05$), or by qPCR ($p > 0.05$). Finally, at 21 dpi (42 days old) there was a significant reduction in treated broilers by qPCR ($p = 0.016$), not by culture ($p = 0.076$) (Fig. 7).

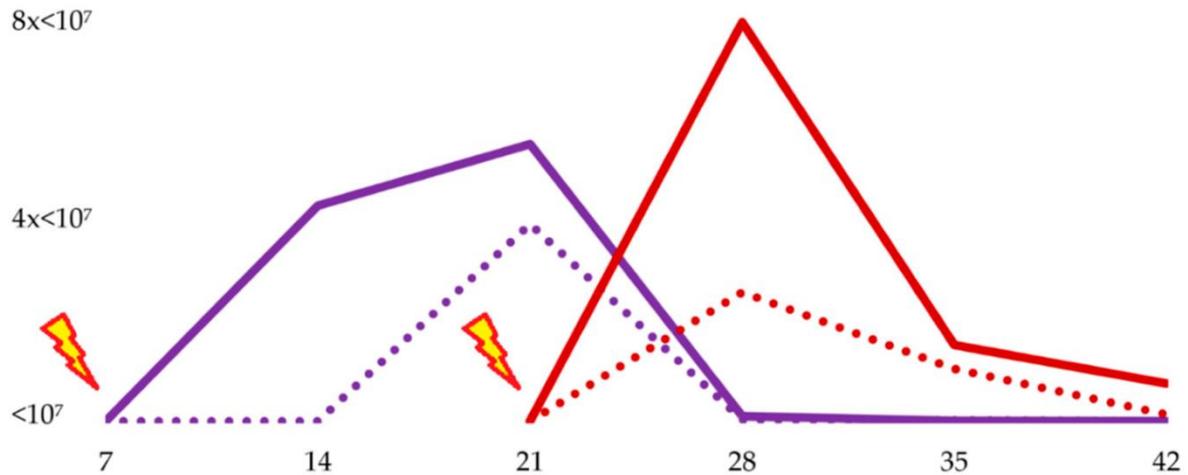


Fig. 7. Results of the *Salmonella* spp. count in selective agar in broilers challenged with *Salmonella* Typhimurium at day 7 (purple lines) and day 21 (red lines) of life. Continuous lines represent control groups, whereas discontinuous lines represent treated groups, fed with fermented defatted 'alperujo'. The *Salmonella* spp. count by culture (CFU/g) is shown on the vertical axis, and samplings (7, 14, 21, 35, or 42) on the horizontal axis. Figure retrieved from <https://doi.org/10.3390/ani10101931>.

Intestinal histopathology: In both control groups, in 7- or 21-day-old challenge, the duodenum at 7 dpi (14 or 28 days of life) displayed a moderate atrophy and stunting of the villi with mild epithelial desquamation and a slight increase in intraepithelial lymphocytes. The lamina propria was moderately to severely expanded by an inflammatory infiltrate composed of lymphocytes, heterophils, and macrophages that partially distorted the crypt structure. At 14 dpi (21 or 35 days of life), in the control group the lesions were similar, with additional mild crypt distortion and mild gut-associated lymphoid tissue (GALT) hyperplasia. In the treated group, there was a reduction in the severity of villous stunting and lymphocytic infiltrate. In the cecum, at 7 dpi (14 or 28 days of life), control groups presented a mild epithelial desquamation and the lamina propria was slightly expanded by an infiltrate composed of lymphocytes and plasma cells that moderately to severely distorted the crypt structure. GALT hyperplasia was moderate to severe. By 14 dpi (21 or 35 days of life), all those changes were maintained in both control groups with an additional increase in intraepithelial lymphocytes. In the treated group, there was a reduction in the intensity of lamina propria lymphocytic infiltration and GALT hyperplasia compared to the control group.

Intestinal morphology: In 7-day-old challenged chickens, duodenum villi height was significantly improved in treated chickens on days 7, 14, 28, 35, and 42 ($p < 0.05$). Similarly, the crypts in the duodenum were deeper in all treated samplings ($p < 0.05$). Regarding ceca morphology, crypts were seen to be deeper in 7-, 21- and 42-day-old treated chickens ($p < 0.05$). At 28 days of life, controls displayed a higher value for crypt depth ($p < 0.05$). Broilers in the treated group challenged at 21 days of age showed a significant improvement in the duodenum villi height at 28, 35, and 42 days of life ($p < 0.05$). The depth of the crypts in the duodenum was significantly improved by the treatment on days 28 and 42 ($p < 0.05$). The cecum crypt was deeper in treated chickens on days 35 and 42 of life ($p < 0.05$).

Cecal microbiota: In chickens challenged at 7 days of age, there were no statistically-significant differences among the groups established. The most abundant bacterial family at days 7, 14, and 21 of life was Enterobacteriaceae in both groups. At day 28, Enterobacteriaceae drastically decreased and was replaced by Lachnospiraceae and Ruminococcaceae in similar abundance, and these were also the most prevalent families at 35 days of life, with a higher abundance of Lachnospiraceae. Finally, at day 42 of life, Bacteroidaceae, Ruminococcaceae, and Lachnospiraceae were the most prevalent families in both groups. In chickens challenged at 21 days of age, there were no statistically-significant differences among the groups established. The most abundant bacterial family at day 7 was Enterobacteriaceae in the control group and Lactobacillaceae in the treated group. At day 14 of life,



Enterobacteriaceae was still prevalent in the control group, whereas in the treated group Enterobacteriaceae and Ruminococcaceae were more abundant, being substituted by Lachnospiraceae as the second-most-abundant family in both groups at 21 days of life. In 28-day-old broilers, Lactobacillaceae and Ruminococcaceae were the most abundant families in the control group, whereas Lachnospiraceae and Ruminococcaceae were predominant in treated chickens. On days 35 and 42 of life, Ruminococcaceae and Lachnospiraceae were the most abundant families, with slightly higher values of Ruminococcaceae over Lachnospiraceae in the control groups.

NDRVMI finished the experiment with the 4 groups of day-old chicks that received the tested probiotics and prebiotic. After the slaughter, materials and samples were taken and analysed by **NDRVMI** and **UoS**. During the experiment, the condition of the birds were taken into account, and photos were taken during sampling.

The experiment with pigs has been performed. Analysis of data is ongoing.

[WP3. Modelling the transmission of zoonotic agents to improve intervention strategies on livestock farms \(M1-M12\)](#)

JRP10-WP3-T1: Transmission modelling at within-host and between-host scales (M1-M12)

JRP10-WP3-T1-ST1: Within-host scale: modelling individual responses and shedding (M1-M12)

Completed, see second annual report 2019.

JRP10-WP3-T1-ST2: Between-host scale: modelling transmission, linked to within-host results (M1-M12)

The PhD student of **CVI/DLO** has carried out modelling analyses of the outcomes of an experiment studying the indirect transmission of *Campylobacter* between broilers, and of other relevant historical and new data. In previous research consisting of a combination of experiments and mathematical modelling, a mathematical model of indirect transmission of bacteria between broilers was developed. This model assumes that bacteria are transferred from inoculated animals (source animals) to spatially separated susceptible animals (recipient animals) through random displacement of infectious material in the environment in combination with a loss of viability of the bacteria in time. Technically this model uses diffusion equations to describe the random displacement of material in the environment between the source and recipient animals. The experiment served to validate and refine the existing model, and to do so consisted of three different spatial setups that were each studied in two repeat animal rooms. The results showed no transmission at longer distances (above 130 cm), which is consistent with the existence of a threshold distance. A new experiment has been carried out, studying the indirect transmission of *Campylobacter* and *Salmonella* between broilers. Whereas the previous experiment used three different spatial setups, this experiment used one spatial setup but in this setup simultaneously studies the transmission from source animals to both animals in direct contact as well as to spatially separated animals. This enables us further validate (and possibly adjust) the mathematical model(s) describing indirect transmission by testing predictions in an experimental setting. The model can be used for designing and quantitatively assessing candidate bio-security based intervention strategies against indirect transmission of *Campylobacter* and *Salmonella*. The results of the experiment are currently being analyzed using the model(s) that it serves to validate. In this analysis, that has been delayed by COVID-19, also simplified models have been developed to aid in the interpretation of the more detailed model(s). The model validation is being extended by applying the model to not only the latest experiment but also to all relevant historic experiments together. This modelling subtask is planned to run until the end of the project (M42).

JRP10-WP3-T2: Interventions strategies: Identification and evaluation tools (M1-M42)

JRP10-WP3-T2-ST1: Systematic inventory of relevant intervention measures (M7-M42)

A draft inventory of relevant intervention measures against *Salmonella* in laying hens has been developed within the framework of a HACCP analysis and involved both literature study and elicitation



of expert opinion. This draft still needs to be worked out to a systematic inventory. This was planned in M25-M30, but due to illness it is postponed to 2021, M35-M42.

JRP10-WP3-T2-ST2: Inclusion of potential interventions into the modelling (M35-M42)

At within-host scale, **INRAE-Jouy** completed the realization of a C++ software and the corresponding Matlab and R plugins allowing to analyse time series of microbial concentrations. We have encountered problems for frequency time series analyses (see second annual report 2019) that are currently being fixed. Based on these tools, we have recently started analysing microbiota time series from WP1 (ANSES pig experiment), in collaboration with UoS and INRAE-Tours. Our next step will be to include measures on the inflammation and immune response. Our objective is to contribute to the data analyses by detecting species or features associated to each phenotype. The resulting knowledge, as well as knowledge from probiotic strategies in WP2 will be used to include probiotic based strategies in the model developed in WP3-T1-ST1. This work is planned to last until M42.

JRP10-WP3-T2-ST3: Development of economic analysis tools (M1-M12)

Completed, see second annual report 2019. An article has been published on this part.

WP4: Communication and Dissemination for Impact (M1-M12)

JRP10-WP4-T1: Dissemination of data within the project and management of data (M1-M12)

Completed, see second annual report 2019.

Participation to the OHEJP-ASM (virtual) conference (2020) with several posters and conferences.

As described in the report, Partners have exchanged for analysis numerous samples and thus the corresponding data from animal experiments. **UoS** and **VRI** have sequenced the samples from pigs and chickens, respectively. **UoS**, **INRAE-Tours** and **VRI** Have analysed the data and thus the gut microbiota composition of the different samples. **VISAVET-UCM** has performed histological analyses, **INRAE-Tours** has performed virulence assays with strains recovered by other Partners. **INRAE-Tours** and **ISS** have performed immune responses analyses. UoS, IZLER and ISS have sequenced and analysed the data and thus the gut microbiota composition of the different samples. **IZLER** and **NDRVMI** have performed field experiments with strains of **UoS**. Data obtained by other Partners during experimental infections have been sent to INRAE-Jouy and to **U. Wageningen**. Consequently, an impressive collaboration has made it possible to analyse many parameters of the *in vivo* experiments performed with pigs and chickens.

JRP10-WP4-T2: Dissemination of data outside the project and management of data (M9-M12)

Participation to several congresses. Due to the Covid19 crisis the high strategic meeting has been cancelled and the budget devoted to this activity has been reimburse to EJP (**INRAE**) and (**NCOH**)).

Several joint articles have been accepted. Others s are in preparation.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
10	D-JRP10-1.04	In vitro virulence levels of different Salmonella strains recovered from high and low-shedders in animals and humans (first round)	M30		M36		this deliverable has been merged with D-JRP10-1.05 Confidential	8 report
10	D-JRP10-1.05	In vitro virulence levels of different Salmonella strains recovered from high and low-shedders in animals and humans (second round)	M30		M36		CO: until the publication. For strain of human origin, no available results yet. The microbiome analyses of the human stool samples are planned to start after the completion of the laboratory analyses of the stool samples, by June 2020.	8 report
	D-JRP10-1.06	Definition of predictive immunological markers associated to the high and low shedders in chickens and in pigs.	M34		M36		this deliverable has been merged with D-JRP10-1.07	



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
	D-JRP10-1.07	Definition of immunological markers associated to the high and low shedders in chickens and in pigs.	M34		M36		CO: until the publication.	
10	D-JRP10-1.09	Definition of predictive microbiota markers associated to the high and low-shedders in chickens and in pigs.	M32		M36		CO One publication accepted, others are in preparation	1 ; 7 ; 8 report
10	D-JRP10-1.10	Definition of microbiota markers associated to the high and low-shedders in chickens and in pigs.	M32		M36		One publication accepted, others are in preparation	1 ; 7 ; 8 report
10	D-JRP10-1.11	Recovery of all human samples	M32		M42	Yes	The sampling has been achieved as well as the sequencing of fecal samples. Data are being analyzed	10
10	D-JRP10-1.14	Identification, from in vitro studies, of immune parameters related to high and low-shedders	M30		M36		CO No differences have been detected in chicks. One joint publication is in preparation	8 report
10	D-JRP10-2.01	In vitro effect of already characterized probiotics on Salmonella growth and cell	M30		M40	Yes	In vitro growth inhibition studies are complete, but tissue culture studies have been delayed by COVID-19.	8 report



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		invasion						
	D-JRP10-2.02	Description of the microbiome and resistome in farms	M36		M42	Yes	The experiments have been completed. Data analysis is ongoing	
	D-JRP10-2.03	Characterization of protective commensal bacteria able to inhibit Salmonella colonization (second rounds)	M34		M40		One article accepted for chicken, One joint publication on pigs is in preparation This deliverable has been merged with D-JRP10-2.04	
10	D-JRP10-2.04	Characterization of protective commensal bacteria able to inhibit Salmonella colonization (two rounds)	M30		M40		CO An in vivo test performed in chicks demonstrated the protective activity of a mix of 4 commensal bacteria. The article has been uploaded	7, 8 report
10	D-JRP10-2.05	Determine the influence of defined and undefined probiotics on the microbiome signature, the immune response, gut physiology and welfare of pig and/ or chicken	M30		M42		This deliverable has been merged with D-JRP10-2.06 The experiments have been completed. Data analysis is ongoing	7



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
10	D-JRP10-2.06	Impact of defined and undefined probiotics on Salmonella colonization in pig and chicken	M26		M42	Yes	Done for chickens. One article accepted for publication. On farm studies with pigs and chickens are done. Gut microbiota analyses are ongoing, but delayed by COVID-19.	7

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
10	M-JRP10-10	In vitro infection of cell lines and organoids with the <i>Salmonella</i> strains recovered from high and low-shedders in animals	30	yes	36	



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
		<i>and humans (from the first experiments)</i>				
10	M-JRP10-12	Comparison of immune response of high and low-shedders in chickens and pigs	26	yes	36	
10	M-JRP10-21	First version of economic analysis tools completed	24	yes	33	One publication accepted
10	M-JRP10-22	Organization of consortium meetings (intermediate and closure)	24	No	40	The intermediate meeting has been done. The final meeting is planned.
10	M-JRP10-28	In vitro infection of several cell lines and organoids with the different Salmonella strains recovered from high and low-shedders in animals and humans (second round)	30	yes	36	
10	M-JRP10-32	Final inventory of intervention measures completed	30	No	39	In progress



4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
A Multi-Scale Epidemic Model of Salmonella infection with Heterogeneous Shedding. A first draft was accessible on HAL repository. https://doi.org/10.1051/proc/202067015 https://zenodo.org/record/4244169#.X6KF4TiWxM0	YES	GREEN 0 MONTHS	
Reduction of Salmonella Typhimurium Cecal Colonisation and Improvement of Intestinal Health in Broilers Supplemented with Fermented Defatted 'Alperujo', an Olive Oil By-Product. 10.3390/ani10101931 https://zenodo.org/record/4114070	YES		GOLD – 1,334.20 €
Cost-effectiveness analysis of using probiotics to control Campylobacter in broilers (submitted). https://doi.org/10.1016/j.psj.2020.05.003 https://zenodo.org/record/4244748#.X6LaalhKjcc https://doi.org/10.1016/j.psj.2020.07.015	YES		GOLD – 2000 €



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
<p>Dietary Supplementation with Fermented Defatted 'Alperujo' Induces Modifications of the Intestinal Mucosa and Cecal Microbiota of Broiler Chickens.</p> <p>https://doi.org/10.1016/j.psj.2020.07.015</p> <p>https://zenodo.org/record/4017817#.X6LRjYhKjcc</p>	YES		GOLD – 1 720 €
<p>Effects on Intestinal Mucosal Morphology, Productive Parameters and Microbiota Composition after Supplementation with Fermented Defatted Alperujo (FDA) in Laying Hens.</p> <p>https://doi.org/10.3390/antibiotics8040215</p> <p>https://zenodo.org/record/3648214#.XvyTJygZM0</p>	No		GOLD – 435.89 €
<p>Gut microbiota composition before infection determines the <i>Salmonella</i> super- and low-shedder phenotypes in chicken.</p> <p>https://doi.org/10.1111/1751-7915.13621</p> <p>https://zenodo.org/record/4005830#.X0kCUMqzbcc</p>	YES		GOLD – 2 100 €



Additional outputs

Participation to the ASM virtual EJP OneHealth meeting (UoS, INRAE)

Posters/

Metagenomic Analysis of The Pig Gut Microbiota and association with Salmonella status. Poster.

https://www.researchgate.net/publication/342123925_Metagenomic_Analysis_of_The_Pig_Gut_Microbiota_and_association_with_Salmonella_status/stats

Metagenomic Analysis of The Pig Gut Microbiota and Association With *Salmonella* Status. Presentation. RCE 2020 event at UoS.

https://www.researchgate.net/publication/346943667_Metagenomic_Analysis_of_The_Pig_Gut_Microbiota_and_Association_With_Salmonella_Status

5. On-going and planned collaborations with national or European projects or networks

Probiotic strains will be shared with the AVANT H2020 project.

Member of ECDC and AFSA will be invited at the final meeting. We will contact members as soon as a date for the final meeting is decided.

JRP11-MEDVETKLEBS

1. Summary of the work carried out

Klebsiella pneumoniae (Kp) is a bacterial pathogen of increasing public health concern given the emergence of highly multidrug resistant strains. Kp is also a model of ubiquitous bacteria that should be analysed in a One Health perspective, given its broad ecological distribution in animals (including humans) and the environment, and its capacity to contaminate food. The MedVetKlebs project aimed at enhancing Kp research and surveillance by developing and harmonizing study methods and by investigating its ecology and transmission across sources.

Protocols were developed for *Klebsiella* isolation from various sources (food, water, animal and human faecal material) and for molecular detection through a highly sensitive qPCR method, the ZKIR assay, which detects Kp and its closely related species from soil, food, faeces and other complex matrices. Novel taxonomic classifications (four novel *Klebsiella* species and subspecies) and novel biomarkers and laboratory identification tools (MALDI-TOF mass spectrometry) were also defined. Our protocols were disseminated through publications and the open platform protocols.io. Further methodological developments will be finalized in the short term, including a combined qPCR method for detecting and differentiating at the same time the medically most problematic *Klebsiella* species, *K. pneumoniae sensu stricto*, from related Kp species. An additional short-term goal stemming from MedVetKlebs is the setting-up a publicly accessible MALDI-TOF *Klebsiella* identification web site.

Using our harmonized protocols, broad sampling of varied sources was accomplished in order to investigate the presence of Kp. In total, nearly 4000 samples from different sources (food, environment, healthy animals and humans) were analyzed. Among the food sources tested, chicken meat and vegetables (mainly ready-to-eat salads and onions) presented the highest Kp recovery rate (35% and 20%, respectively). In the environment, all sources tested showed a high prevalence of Kp. Based on this broad sampling campaign, we have sampled more deeply some specific sources of potential relevance for One Health transmission and of scientific interest: chicken meat, ready-to-eat salads, onions, seawater and soil, as well as human carriage.



Genomic sequencing of Kp isolates from the chicken/salads and soil has been performed. A very high strain-level genetic diversity was found but importantly, with low prevalence of antimicrobial resistance and virulence genes. We have also started to analyse by genomic sequencing, strains from other sources. In addition, we are still exploring Kp prevalence and diversity in human gut colonization using a targeted metagenomics approach.

Finally, we have explored a mathematical modelling approach to simulate the diversification of a bacterial lineage that contaminates food, as a function of time and mutation rate of the bacteria. This approach will be used to define clusters of related Kp isolates that result from single contamination and transmission chains. The model can be generalized to all foodborne or environmental bacterial species, and will help defining short-term transmission of pathogens to animals and humans.

So far, 10 manuscripts and one preprint related to MedVetKlebs were published, and 10 more are being prepared. Although COVID-19 restrictions have hampered physical meetings and some experimental work, the MedVetKlebs project was highly successful in delivering novel analytical methods and scientific knowledge on Kp ecology and transmission. By achieving its scientific tasks and dissemination activities, fostering novel collaborations among partner institutions, and integrating and harmonizing *Klebsiella* study strategies among partners and beyond, the MedVetKlebs project has contributed significantly to enhance our collective research and surveillance capacity on this important pathogen.

2. Work carried out in the JRP. scientific results

Klebsiella: taxonomy and definitions

Bacteria of the genus *Klebsiella* are human and animal pathogens with wide ecological distribution. The genus *Klebsiella*, a member of the family *Enterobacteriaceae*, encompasses a huge diversity in terms of phylogenetic lineages, genomic content, pathogenic properties, and ecological distribution. Two major subdivisions of *Klebsiella* are the *K. pneumoniae* species complex (KpSC) and the *K. oxytoca* species complex (KoSC). The KpSC currently includes five taxonomic species, two of which comprise two subspecies. These taxa were initially defined as seven distinct phylogroups, Kp1 to Kp7.

K. pneumoniae (phylogroup Kp1) is one of the most problematic pathogens associated with antibiotic resistance worldwide. This species is phylogenetically closely related to *K. quasipneumoniae* [subsp. *quasipneumoniae* (Kp2) and subsp. *similipneumoniae* (Kp4)], *K. variicola* [subsp. *variicola* (Kp3) and subsp. *tropica* (Kp5)], '*K. quasivariicola*' (Kp6) and *K. africana* (Kp7).

In turn, the KoSC comprises 6 species and 7 phylogroups (4): *K. michiganensis* (Ko1), *K. oxytoca* (phylogroup Ko2), *K. spallanzanii* (Ko3), *K. pasteurii* (Ko4), *K. grimontii* (Ko6) and *K. huaxiensis* (Ko8). *K. oxytoca* is well known as a cause of opportunistic human infections and post-antibiotic hemorrhagic diarrhea.

WP1: Methods for Kp detection and isolation

JRP11-WP1-T1: Evaluation and optimization of culture-based approaches (M1-M12)

Productivity and specificity tests – two partners were involved in this task (IP, IZSAM). The tests were performed in accordance with the ISO 11133:2014 and using a collection of 57 reference panel of strains from our IP internal collection, that included members from the Kp complex and also other related species (*K. oxytoca*, *Raoultella* spp., *K. aerogenes*). Three different media - SCAI, *Klebsiella* Selective ChromoSelect Agar Base from Sigma and other not yet commercialized from Liofilchem - were compared with a non-selective agar media. The results showed similar recovery rates (productivity) for the three different media tested, but higher specificity for SCAI compared to the other selective and differential media tested. In addition, SCAI appeared easy to prepare and to identify *Klebsiella* colonies from. Furthermore, productivity tests with SCAI at two different incubation temperatures (37°C and 44°C) were also performed. This was done because at 44°C, we detect less



interferences of other species besides *Klebsiella* spp., and we wanted to be sure that all Kp complex members are able to growth at 44°C. Our results revealed similar productivity rates in SCAI at both temperatures. All this work will be disseminated via a publication in preparation.

Klebsiella isolation tests from different sources - We designed protocols for the detection and isolation of *Klebsiella* strains using SCAI medium from different sources (human and animal fecal carriage; food; water; soil). Seven partners were involved in this task (AGES, ANSES, IP, IZSAM, INRA, SSI, NUIG). In the case of human and animal fecal carriage, the protocol established includes an enrichment step in lysogeny broth (LB) plus ampicillin (or amoxicillin) (10 µg/mL) at 37°C/18-24h and then streak to isolate single colonies using a 10 µl loop in SCAI plates (37°C/48h) (<https://www.protocols.io/view/isolation-of-klebsiella-strains-from-human-or-anim-662hhge>). The protocol for soil samples, is the same described for human and animal fecal carriage with the only difference being the incubation temperature in the enrichment step (28-30°C instead of 37°C). In the case of food samples, the protocol was optimized using chicken meat (legs and chest with and without skin from free-range and not free-range chickens). The best strategy achieved includes an enrichment in buffer peptone water (BPW) at 37°C/18-24h and then plating 10 µL of the enrichment in SCAI (44°C/48h) (<https://www.protocols.io/view/isolation-of-klebsiella-strains-from-food-samples-baxtifnn>). For water samples the strategy is similar to the one for food samples, with differences in the incubation temperatures (enrichment in BPW at 42°C/18-24h, and SCAI incubation at 37°C/48h) (<https://www.protocols.io/view/isolation-of-klebsiella-strains-from-water-samples-baxuifnw>). Following the four protocols mentioned above, all suspected colonies typical of *Klebsiella* spp. (large, yellow, moist colonies) are then identified using MALDI-TOF mass spectrometry.

IN SUM, we validated the SCAI medium and defined source-adapted strategies to isolate *Klebsiella* from complex sources. This work represents a foundation for the harmonization of *Klebsiella* isolation and detection for surveillance and research. It represented a milestone for subsequent tasks of the project dedicated at looking for *Klebsiella* in a variety of sources.

JRP11-WP1-T2: Detection and quantification (M1-M12)

Although the culture strategies outlined above represent important advances for *Klebsiella* ecology and One Health transmission studies, molecular detection methods have higher throughput. We have therefore also developed a real-time PCR (qPCR) assay for the direct detection of Kp in complex samples. Two partners were involved in the methodological development component (IP, INRA). The targets for the qPCR were chosen based on a *K. pneumoniae* pan-genome analysis. Candidate genes exclusive for, and conserved within, the KpSC were searched, leading to define six optimal target genes (target for KpSC; and targets for phylogroups Kp1, Kp2, Kp3+5, Kp4 and Kp6). A sequence between *zur* (zinc uptake regulation protein) and *khe* (annotated as coding for a haemolysin) intergenic region (ZKIR) was used with a SYBR green strategy. The ZKIR qPCR for detection of KpSC members in different matrices was published in March 2020 (Barbier *et al.* Applied Env Microbiol). Besides, we had disseminated the protocol ahead of publication (<https://www.protocols.io/view/detection-of-klebsiella-pneumoniae-and-closely-rel-7n6hmhe>).

The protocol metrics show nearly 1000 views and 100 downloads. The Norwegian consortium Nor-Kleb-Net (<http://www.nor-kleb.net/>) has adopted the ZKIR method for quantification and detection of Kp in gut samples of a large cohort (project Kleb-GAP), testifying on the rapid adoption of our methods by the international community.

Additionally, we sought to combine the detection of the entire KpSC (using the ZKIR target) with a specific detection of phylogroup Kp1, its most abundant subgroup. The methodological component was optimized by our INRA (Dijon) partner, and tested at SSI (finished) and NUIG (in progress). This qPCR assay has already been tested on a panel of environmental, food and sewage samples and worked very well. One initial issue was the non-detection of a minor phylogroup, Kp6, due to a specific Kp6 DNA polymorphism where the probe hybridizes. To address this lack of detection, INRA developed a second probe (ZKIR P2), specific to Kp6, and the problem was solved. The protocol has already been distributed to all the consortium members and as soon as all tests are completed it will be posted on



the protocols.io platform. This novel method will be useful for studies aiming at differentiating Kp1, the clinical most relevant KpSC member, from other phylogroups which are rare and less well understood ecologically and epidemiologically.

Finally, we have tried to implement a second duplex qPCR using the previous defined targets in different combinations (Kp3+5 and Kp2; Kp3+5 and Kp4) but we were not successful. In fact, the specific probes for each of the species of the complex work in simplex and were validated in a panel of reference strains at INRA, but the combination in a single multiplex does not give good results.

Due to its complexity, the task of quantification of Kp in samples was not addressed during the project. However, the developed qPCR will allow this to be done in future dedicated studies.

IN SUM, we have developed two novel molecular detection methods useful for One Health studies of *Klebsiella* ecology and epidemiology.

JRP11-WP1-additional tasks: taxonomic updates and MALDI-TOF MS

To achieve precise detection and identification of *Klebsiella* members, it is important to define the target species precisely. Although not initially planned, taxonomic updates of the genus *Klebsiella* were therefore performed. First, two novel taxa of the KpSC were described: *K. africana* and *K. variicola* subsp. *tropica* (<https://doi.org/10.1016/j.resmic.2019.02.003> and <https://www.sciencedirect.com/science/article/pii/S0923250819300956>). Second, in collaboration with the JPIAMR-funded SpARK project (<https://www.jpiaamr.eu/supportedprojects/third-joint-callresult/>), two novel species previously misidentified as *K. oxytoca* were described: *Klebsiella pasteurii* and *Klebsiella spallanzanii* (<https://doi.org/10.3389/fmicb.2019.02360>).

To facilitate and improve laboratory identification of *K. pneumoniae*, we evaluated and validated the potential of MALDI-TOF mass spectrometry (MS), a fast and cost-effective technique that is well established in clinical microbiology laboratories for microbial identification (<https://www.ncbi.nlm.nih.gov/pubmed/30581423> and both taxonomic publications above). As reference spectra of novel *Klebsiella* taxa are not readily incorporated into the reference MALDI-TOF MS databases used in microbiology laboratories, we have developed a publicly-accessible web-based tool, *Klebsiella* MALDI TypeR (<https://maldityper.pasteur.fr>), a user-friendly application that enables users to upload their MALDI-TOF data and identify *Klebsiella* isolates using our reference taxonomic database. Our database also includes new *Klebsiella* biomarkers. The *Klebsiella* MALDI TypeR tool will improve identification of *Klebsiella*.

IN SUM, although these tasks were not initially planned as deliverables of our project (because the novel species were not discovered yet, and because the successful application of MALDI-TOF MS was not anticipated), they represent important advances for the refinement of *Klebsiella* definition and identification in One Health studies. For example, the SpARK consortium (<https://gtr.ukri.org/projects?ref=MR%2FR00241X%2F1>) has adopted our novel nomenclature and MALDI work to characterize *Klebsiella* isolated from a massive (> 6000 samples from diverse environmental, animal and human sources) sampling effort, and a high-profile bioinformatics tool that aims at harmonizing the study of *Klebsiella* genomes has incorporated our nomenclature outputs (<https://www.biorxiv.org/content/10.1101/2020.12.14.422303v1>).

JRP11-WP1-T3: Harmonization and alignment (M1-M24)

Protocols for Kp isolation by culture from various food sources were disseminated/made publicly available through the protocols.io platform in a project called '*Klebsiella* Research and Surveillance' (<https://www.protocols.io/workspaces/klebsiella-research-and-surveillance/publications>).

We have pursued our additional objective of setting-up a MALDI-TOF MS based identification web site for *Klebsiella*. A pilot web site is functional (<https://maldityper.pasteur.fr>), and the work was submitted for publication and is available at BioRxiv platform (<https://doi.org/10.1101/2020.10.13.337162>).



IN SUM, our novel culture, molecular and MALDI-TOF methods for *Klebsiella* detection and identification have been implemented within the consortium partner Institutions and beyond.

WP2: Sampling

JRP11-WP2-T1: Broad sampling of potential reservoirs and sources of Kp (M1-M12)

To define the ecological distribution and reservoirs of Kp, broad sampling from varied sources was performed. In total, 3781 samples from food, environment, healthy animals and humans were analyzed by the MedVetKlebs partners. Among animals, swine and rabbits showed the highest prevalence (to be confirmed on more samples in the future), whereas in poultry and bovine samples the prevalence was around 20%. Among the food sources tested, chicken meat and vegetables (mainly ready-to-eat salads and onions) presented the highest recovery rate of Kp (35% and 20%, respectively), whereas in the environment all sources tested (seawater, fresh water, wastewater and soil) showed a high prevalence of Kp (>40%). Carriage among healthy humans was between 15-20%, similar to rates reported from other projects (SpARK and Nor-Kleb-Net projects, pers. comm.). Sampling results will be disseminated via several publications in preparation (soil prevalence was published in part in the ZKIR qPCR [Barbier et al.] publication).

IN SUM, our project demonstrated the ubiquity of Kp in the environment, food and mammalian animals including humans, and demonstrated the higher prevalence of phylogroup Kp1.

JRP11-WP2-T2: Deep sampling of selected sources (M13-M24)

To study in more depth *Klebsiella* from particularly Kp-rich sources, we have further focused on six sources of potential One Health relevance and of scientific interest: chicken meat and ready-to-eat salads (multicentric study), soil, veal calves, seawater and onions. Sampling of onions and seawater has been slowed by COVID-19, but is re-starting currently. Sampling of veal calves turned out to be mostly negative and this sub-study was therefore abandoned (veal calves results will be disseminated within our future broad sampling reports). One partner (IP) has also been looking at Kp carriage and transmission in patients from a rehabilitation hospital.

In the multicentric study (five sampling centers involved), a total of 305 samples were analyzed, with 160 from chicken meat and 145 from ready-to-eat salads. Chicken meat showed a prevalence of Kp twice as high as salads (58% versus 30%, accordingly to ZKIR qPCR results). A total of 131 Kp isolates were recovered. The antibiotic susceptibility tests revealed that the majority (82%) of the isolates presented a wild-type phenotype, being susceptible to all the antibiotics tested (except ampicillin, for which all Kp are resistant).

To analyze the transmission and ecology of Kp in a local “One Health” context, we have focused on a restricted geographic setting, Burgundy in France. We first collected 664 environmental samples in a single French administrative locality (Department of Côte d’Or, Burgundy, France) from July 2018 to July 2019. Most samples were collected monthly in a market gardener farm (n = 329) and in an organic cattle farming (n = 304) and consisted in soil/mud (n = 219), roots (n = 189), leaves (n = 106), water (n = 34), cow bedding and faeces (n = 50) and fertilizer/compost (n = 35). In addition, water and sludge were sampled in 31 wastewater treatment plants (WWTP) in the same department (n = 31). For comparison purposes, 47 clinical isolates collected in 2018 and 2019 by the Department of Bacteriology from the University Hospital of Dijon (the capital city of Côte d’Or), France, were included. Environmental samples screening with ZKIR qPCR assay followed by culture on SCAI media allowed the isolation of Kp strains in 24.7 % (164/664) of the environmental samples. Phylogenetic and genomic analysis are ongoing to evaluate the diversity of Kp in these environmental niches across one year, and to compare them with sewage and clinical isolates in terms of strain subtype, virulence factors and antimicrobial genes.

In order to understand better the pattern of transmission of ESBL *K. pneumoniae* in health care settings, we used data from i-Bird (Individual-Based Investigation of Resistance Dissemination, PI



Didier Guillemot, IP; Collaboration with Dr Lulla Opatowski), a 4-month study that took place in 2009 in a rehabilitation hospital and followed up more than 600 patients and health care workers. Weekly rectal swabs and human-human proximities recorded from wireless captors were recorded. In total, 604 ESBL-*Enterobacteriaceae* were isolated from 84 patients. Within the project, we have sequenced 61 *K. pneumoniae* and the data is being processed.

Additional work: *Klebsiella* sampling and characterization work: Some MedVetKlebs partners have engaged in additional *Klebsiella* sampling and characterization work, which was not initially planned but was nevertheless closely related to the project by its goals, and personnel supported by MedVetKlebs contributed to these studies. We describe these completed studies below:

Work by Huynh et al.: prevalence of Kp in gut carriage. Although most Kp infections are caused by endogenous intestinal carriage, little is known about the prevalence and microbiological characteristics of Kp in asymptomatic human carriage, and attached risk factors including environmental sources exposure. In this work, 911 pregnant women from communities in Madagascar, Cambodia, and Senegal were screened for gut colonization by Kp. Characteristics of Kp strains (antimicrobial susceptibility, genomic diversity, virulence, and resistance genes) were defined, and associated risk factors were investigated. Kp carriage rate was 55.9%, and Kp populations were highly heterogeneous. One third of Kp isolates had acquired antimicrobial resistance genes. MDR-Kp (11.7% to 39.7%) and extended spectrum beta-lactamase (ESBL)-producing Kp (0.7% to 14.7%) varied among countries. Isolates with virulence genes were detected (14.5%). Environmental exposure factors including food, animal contacts, or hospitalization of household members were associated with carriage of Kp, antimicrobial resistance and hypervirulence. However, risk factors were country-specific and Kp subpopulation-specific. This work was published: <https://doi.org/10.1080/19490976.2020.1748257>

Work by Loncaric et al.: Analysis of resistant *Klebsiella* in horses. The aim of this study was to investigate the diversity of broad-spectrum cephalosporin-resistant clinical *Klebsiella* isolated from horses in Austria. A total of seven non-repetitive cefotaxime-resistant *Klebsiella* isolates were obtained during diagnostic activities from autumn 2012 to October 2019. Antimicrobial susceptibility testing was performed. The isolates were genotyped by whole-genome sequencing (WGS). Four out of seven *Klebsiella* isolates were identified as *K. pneumoniae*, two as *K. michiganensis* and one as *K. oxytoca*. All isolates displayed a multi-drug resistant phenotype. The detection of resistance genes reflected well the phenotypic resistance profiles of the respective isolates. Besides resistance genes, a variety of virulence genes, including genes coding for yersiniabactin were detected. Considering the high proximity between horses and humans, these results undoubtedly identified a public health issue. This work was published: <https://pubmed.ncbi.nlm.nih.gov/32093201/>

Work by Lepuschitz et al.: Analysis of resistant *Klebsiella* in rivers. This study reports on the characterization of carbapenem resistant and ESBL-producing *K. pneumoniae* isolates from Austrian river water samples, compared to 95 clinical isolates recently obtained in Austrian hospitals. Ten water samples were taken from four main rivers, collected upstream and downstream of major cities in 2016. The presence of AMR genes, virulence genes and plasmids was extracted from whole genome sequence (WGS) data. In total three ESBL-producing strains and two carbapenem resistant strains were isolated. WGS based comparison of these five waters isolates to 95 clinical isolates identified three clusters. Cluster 1 (ST11) and cluster 2 (ST985) consisted of doublets of carbapenem resistant strains (one water and one clinical isolate each). Cluster 3 (ST405) consisted of three ESBL-producing strains isolated from one water sample and two clinical specimens. The cities, in which patient isolates of cluster 2 and 3 were collected, were in concordance with the water sampling locations downstream from these cities. The genetic concordance between isolates from river water samples and patient isolates raises concerns regarding the release of wastewater treatment plant effluents into surface water. From a public health perspective these findings demand attention and strategies are required to minimize the spread of multiresistant strains to the environment. This work was published: <https://pubmed.ncbi.nlm.nih.gov/30690357/>



Work by Wisgrill et al.: analysis of an outbreak and clinical management of infections because of a highly virulent yersiniabactin-producing, non-multiresistant *K. pneumoniae* strain in a neonatal intensive care unit. An outbreak investigation and effectiveness assessment of multidisciplinary infection control measurements to prevent patient-to-patient transmission of highly pathogenic *K. pneumoniae* were undertaken. Outbreak cases were identified by isolation of *K. pneumoniae* from blood or stool of infants. Clinical data were abstracted from medical charts. *K. pneumoniae* isolates were genotyped using whole genome sequencing, and yersiniabactin production was evaluated by luciferase assay. Fourteen cases were confirmed with 8 symptomatic and 6 colonized patients. Symptomatic patients were infants of extremely low gestational and chronologic age with fulminant clinical courses including necrotizing enterocolitis and sepsis. Whole genome sequencing for bacterial isolates confirmed the presence of an outbreak. All outbreak isolates produced yersiniabactin. Yersiniabactin-producing *K. pneumoniae* can display a high pathogenicity in extremely premature infants with low chronologic age. This outbreak also underlines the considerable potential of today's infection control systems for recognizing and controlling nosocomial infections in highly vulnerable populations. This work was published: <https://doi.org/10.1097/INF.0000000000002258>

Work by Chiarelli et al.: Most clinical Kp isolates produce capsule as a major virulence factor. Here, we explored the genetic causes of *in vitro* switching from capsulated, mucoid to non-mucoid, non-capsulated phenotype in eight clinical carbapenem-resistant Kp isolates. We compared capsulated, mucoid colony variants with one of their non-capsulated, non-mucoid isogenic variant. The two colony variants were distinguished by their appearance on solid medium. Whole genome comparison was used to infer mutations causing phenotypic differences. The frequency of phenotypic switch was strain-dependent and increased along with colony development on plate. We observed, for 72 non-capsulated variants that the loss of the mucoid phenotype correlates with capsule deficiency and diverse genetic events, including transposition of insertion sequences or point mutations, affecting genes belonging to the capsule operon. Reduced or loss of capsular production was associated with various *in vitro* phenotypic changes, affecting susceptibility to carbapenem but not to colistin, *in vitro* biofilm formation and autoaggregation. These results suggested heterogeneous selective advantage for capsular loss according to the strain and the mutation. Based on our results, we believe that attention should be paid in the phenotypic characterization of Kp clinical isolates, particularly of traits related to virulence and carbapenem resistance. This work was published: <https://pubmed.ncbi.nlm.nih.gov/33109078/>

IN SUM, we have launched and largely completed, several large studies on the ecology and transmission of Kp in specific settings. These works provide original data on the ecological diversity and transmission of Kp across sources.

WP3: Genomics and Modelling

JRP11-WP3-T1: Analyses of genomic sequences (M13-M24)

Genomic analysis of Kp isolated from our above studies will enable defining medically and ecologically relevant features such as strain subtypes, virulence genes and resistance genes, and their distribution across sources.

We have completed the genomic analyzes of the 131 Kp strains from the chicken-salad multicentric study, of 338 Kp isolates from the Burgundy project and of 61 Kp from the ESBL study. Genomic sequencing of Kp from other sources including onion and seawater is in progress. Overall, a high genetic diversity was found among these sources.

Additional task: Analyses of metagenomic sequences. We are also exploring Kp prevalence and diversity in human gut colonization using an innovative 'targeted metagenomics' approach. In short, in parallel to single colonies, the bacterial mass growing on the SCAI medium plated from fecal material were harvested, DNA extracted, and Illumina sequenced *en-masse*. Sequence data analysis algorithms developed in the SpARK project (Ed Feil, PI) will be applied to the generated 'targeted metagenomics'



data to quantify reads from *K. pneumoniae* or other *Klebsiella* species, and hopefully to define the diversity of clones within the samples. This project involves the coordinator center (IP) and UMCU partner (Rob Willems) and is performed in collaboration with SpARK partners.

Additional task: Analyses of an ST66 isolate. *Klebsiella pneumoniae* (Kp) reference strain Kp52.145 is widely used in experimental *Klebsiella* pathophysiology. Since 1935, only one other strain of the same sublineage (sequence type ST66, capsular serotype K2) was isolated (AJ210, Australia). In the course of the MedVetKlebs project, we have described a community-acquired invasive infection caused by a ST66-K2 Kp strain (SB5881) in France. The ST66-K2 strain presented a full antimicrobial susceptibility profile, including to ampicillin, and was more closely related to strain AJ210 (135 SNPs) than to reference strain Kp52.145 (388 SNPs). Colibactin and yersiniabactin gene clusters were present on the integrative and conjugative element ICEKp10 in the chromosome. The two plasmids from Kp52.145 were detected in SB5881. In addition to carrying genes for virulence factors RmpA, aerobactin and salmochelin, plasmid II has acquired in SB5881, the conjugation machinery gene cluster from plasmid I. We thus reported the first case of community-acquired infection caused by a hypervirulent ST66-K2 Kp strain in Europe and demonstrated the long-term persistence of the high-virulence and laboratory model ST66-K2 sublineage. The combination of a conjugative apparatus and major virulence genes on a single plasmid may contribute to the co-occurrence of hypervirulence and multidrug resistance in single Kp strains. This work was published: <https://pubmed.ncbi.nlm.nih.gov/32749955/>.

IN SUM, genomic sequencing was slowed down by the COVID-19 crisis but a majority of the data was obtained. A prominent result is that different from the clinical setting, a low number of antimicrobial resistance and virulence genes are found in environmental or food isolates. This is a very significant result in the perspective of Kp antimicrobial resistance ecology and transmission. Analysis and publication of the results are still in progress.

JRP11-WP3-T2: Modelling and source attribution (M1-M24)

Based on our genomic sequence data, a huge diversity of Kp subtypes (strains) was present in all sources where we have isolated Kp strains. As a consequence, the initially planned source attribution approach, which relies on the differential distribution of subtypes across sources, was deemed impossible to follow. While we are still exploring what best could be done in this direction (partners IP and SSI), we have decided to adapt our initial objectives and address a related, less risky and high impact objective: we are exploring models to simulate the evolutionary diversification of a bacterial lineage that contaminates food, as a function of time and mutation rate of the bacteria. This approach will be used to define in an innovative and evolutionary biology-informed way, isolates that belong to a cluster (for example an outbreak of infections, or a set of cross-contaminated food items). The high potential impact of this approach lies in the generalization to the modelling work to all foodborne or environmental bacterial species, and in the fact that it addresses a long-standing but still unresolved question in molecular epidemiology: 'what is a strain?'

This task started in December 2019 with the recruitment of Audrey Duval, a post-doctoral fellow in mathematical modelling. We propose a forward model of bacterial evolution to simulate mutation within a population maintaining its size in a given source during a certain time. An algorithm is being implemented to return the expected distribution of SNP (or cgMLST) distances given the specificities of a given contamination event. In addition, we will provide a web site for users to define the expected genetic distance threshold. This forward model will help defining short-term transmission of pathogens from food to animals or humans. We are currently preparing the corresponding publication.

IN SUM, classical approaches for modeling transmission and source attribution in a One Health context are difficult to apply given the huge diversity of Kp subtypes disclosed in the MedVetKlebs project. We have therefore shifted our initial objectives into providing a novel tool to define short-term transmission of Kp or any other bacterial pathogen that contaminates food or a given environmental source.



WP4: Management, dissemination, exploitation

JRP11-WP4-T1: Implementation of the project management structure (M1-M24)

Nothing specific to report here.

JRP11-WP4-T2: Administrative, legal, financial and ethical support to the consortium (M1-M24)

Nothing specific to report here.

JRP11-WP4-T3: Exploitation of results and Intellectual Property rights management (M1-M24)

Publications citing support from the MedVetKlebs project and One Health EJP funding:

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3. Barbier E, Rodrigues C, Depret G, et al. The ZKIR Assay, a Real-Time PCR Method for the Detection of *Klebsiella pneumoniae* and Closely Related Species in Environmental Samples. *Appl Environ Microbiol.* 2020;86(7):e02711-19. doi:10.1128/AEM.02711-19.
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10. Rodrigues C, Passet V, Rakotondrasoa A, Brisse S. Identification of *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, *Klebsiella variicola* and Related Phylogroups by MALDI-TOF Mass Spectrometry. *Front Microbiol.* 2018 Dec 7;9:3000. doi: 10.3389/fmicb.2018.03000.

Preprint:



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3. Project self-assessment

The initial proposed MedVetKlebs aims were to:

- develop and harmonize methods of detection, isolation and quantification of Kp.
- define the major environmental niches that act as reservoirs of Kp, and more specifically its high-risk subtypes and associated antimicrobial resistance and virulence genes and plasmids;
- define the main vehicles of contamination, quantify the role of different foods and feeds in exposure of humans and animals to Kp; and trace the transmission of Kp subtypes, resistance genes and plasmids;
- inform the scientific community and decision makers in order to optimize current practices.

As testified in this report, we consider that most initial objectives were achieved. Regarding ongoing work, MedVetKlebs partners have agreed to continue to meet regularly after the project end date, and to finalize ongoing collaborations (and possibly define novel ones). Therefore, the ongoing tasks will be completed in the near future.

Some tasks of the project were not achieved: quantification of Kp in sources, and modelling of Kp transmission across sources. The first one was deemed too complex and not feasible within the project. However, we have developed tools that will facilitate this task in future work; and in fact, quantification of Kp in some sources is already ongoing in at least one other consortium (KlebGAP) using our ZKIR qPCR. The second unachieved task turned out to be scientifically impossible based on findings from the initial tasks, as the huge diversity of Kp subtypes means statistical power in tracing sources of subtypes will be very low. An alternative related task was therefore addressed.

Of note, in addition to initially planned tasks, the MedVetKlebs consortium has achieved supplementary tasks: MALDI-TOF identification of *Klebsiella*, description of four novel *Klebsiella* species or subspecies, additional sampling and characterization studies, a targeted metagenomics study, and the novel modeling approach to define transmission.

The significant scientific output of MedVetKlebs is testified by 10 publications, one preprint and 10 additional publications in preparation. MedVetKlebs experimental procedures were posted on open web sites, have high consultation metrics and have been adopted by ongoing consortia and One Health *Klebsiella* research projects.

As a consortium, we therefore consider that the MedVetKlebs project was highly successful in achieving its scientific tasks, fostering novel collaborations among partner institutions, providing novel analytical methods and understanding of Kp diversity, ecology and epidemiology, and integrating and harmonizing *Klebsiella* study methods among partners and beyond.



4. Progress of the research project: milestones and deliverables

Deliverables

JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
11	D-JRP11-1.1	Optimized culture protocols	M12	M10		PUBLIC. Productivity and specificity of SCAI medium tested. Best enrichment steps and incubation temperatures defined according to the different sources. Protocols disseminated/made publicly available through the protocols.io platform in a project called ' Klebsiella Research and Surveillance ' (https://www.protocols.io/workspaces/klebsiella-research-and-surveillance/publications).	1, 2
11	D-JRP11-1.2	qPCR protocols	M12	M11		PUBLIC. qPCR for the detection and identification of Kp complex members designed and validated. Article published and protocol made publicly available through the protocols.io platform in a project called ' Klebsiella Research and Surveillance ' (https://www.protocols.io/workspac	1, 2



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						es/klebsiella-research-and-surveillance/publications).	
11	D-JRP11-additional-1	MALDI-TOF MS	M12	M12 M36		<p>PUBLIC. Demonstration and validation of the potential of MALDI-TOF MS to correctly identify <i>Klebsiella</i> strains at the phylogroup level. Articles published. (http://doi.org/10.5281/zenodo.3678153)</p> <p>PUBLIC. Additionally, we have set-up a MALDI-TOF MS based identification web site for <i>Klebsiella</i>. A pilot web site is functional (https://maldityper.pasteur.fr), and the publication has been submitted and it's available at BioRxiv platform (https://doi.org/10.1101/2020.10.13.337162).</p>	1, 2, 3
11	D-JRP11-2.1	List of high-Kp occurrence sources	M12	M24		<p>PUBLIC. Multiple (3781 in total) samples were screened by the consortium. This task gained impetus due to the availability of the qPCR detection method. Some sources where Kp is prevalent were already identified including wastewater, seawater, soil, chicken meat,</p>	1, 2, 5



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						vegetables and ready-to-eat food (http://doi.org/10.5281/zenodo.4499266)	
11	D-JRP11-2.2	Prevalence in selected sources	M24	M24		PUBLIC. Multicentric study – chicken meat and ready-to-eat salads. Manuscript in preparation. (http://doi.org/10.5281/zenodo.4499275)	1, 5
11	D-JRP11-2.3	Prevalence in selected sources	M30	M37		PUBLIC. Soil project. Includes 664 environmental samples recovered in a single French administrative locality (Department of Côte d’Or, Burgundy, France) from July 2018 to July 2019. In addition, water and sludge were sampled in 31 wastewater treatment plants (WWTP) in the same department (n = 31) (http://doi.org/10.5281/zenodo.4499292).	1, 5
11	D-JRP11-2.4	Prevalence in selected sources	M30		M39	The sampling of onions and seawater has been impacted by the COVID-19 crisis, and is re-starting currently.	1, 5
11	D-JRP11-2.5	Genome sequence data	M28		During 2021	PUBLIC. Genome sequence data are available for internal analyses so far (see deliverable;	3



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						http://doi.org/10.5281/zenodo.4499296); a few were published in the Barbier et al paper and put on public repositories (ENA, BioProject PRJEB34643). Sequences will be released once the corresponding publications are accepted.	
11	D-JRP11-2.6	Quantification of Kp in selected sources	M30			Due to its complexity, the task of quantification of Kp in samples was not addressed during the project. However, the developed qPCR will allow this to be done in future dedicated studies.	
11	D-JRP11-3.1	Source distribution of clonal groups, plasmids and genes	M30		During 2021	PUBLIC. Although some data are available already (see deliverable, http://doi.org/10.5281/zenodo.4499308), more data is still being analysed and will be added to each publication.	4
11	D-JRP11-3.2	Source attribution models by microbial subtyping and comparative exposure assessment	M30		M40	PUBLIC. This objective will be rediscussed among involved partners given (i) the huge diversity found in our sampling surveys (see deliverable 3.1) and (ii) the presence of <i>K. pneumoniae</i> in all sources	4



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						where we looked (deliverable 2.1, January 2020). Therefore, we will provide an alternative deliverable, before the end of the project. It will consist of a document (D-JRP11-3.2alt) containing a description of scenario for source attribution approaches as a function of pathogen ecological distribution and genotypic diversity.	
11	D-JRP11-3.3	Computer program for dynamic models simulation	M30		During 2021	PUBLIC. Objectives corresponding to deliverables 3.3., 3.4 and 3.5 were abandoned given (i) the huge diversity found in our sampling surveys (see deliverable 3.1) and (ii) the presence of <i>K. pneumoniae</i> in all sources where we looked (deliverable 2.1, January 2020). Instead, we are proposing a novel deliverable , which will consist of a computer programme and attached publication, useful to model genomic diversification within food as a function of time and mutation rate (D-JRP11-3.345alt).	7



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						https://zenodo.org/record/4576821#.YD-o0GhKhM0	
11	D-JRP11-3.4	Estimates of the attribution proportion for different food, animal and environmental sources for the countries included.	M30		During 2021	<p>PUBLIC. Objectives corresponding to deliverables 3.3., 3.4 and 3.5 were abandoned given (i) the huge diversity found in our sampling surveys (see deliverable 3.1) and (ii) the presence of <i>K. pneumoniae</i> in all sources where 11we looked (deliverable 2.1, January 2020).</p> <p>Instead, we are proposing a novel deliverable, which will consist of a computer programme and attached publication, useful to model genomic diversification within food as a function of time and mutation rate (D-JRP11-3.345alt).</p> <p>https://zenodo.org/record/4576821#.YD-o0GhKhM0</p>	7
11	D-JRP11-3.5	Estimates of the relative contribution of different transmission routes for exposure to Kp in the different	M30		During 2021	<p>PUBLIC. Objectives corresponding to deliverables 3.3., 3.4 and 3.5 were abandoned given (i) the huge diversity found in our sampling surveys (see deliverable 3.1) and (ii)</p>	7



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
		countries included.				the presence of <i>K. pneumoniae</i> in all sources where we looked (deliverable 2.1, January 2020). Instead, we are proposing a novel deliverable , which will consist of a computer programme and attached publication, useful to model genomic diversification within food as a function of time and mutation rate (D-JRP11-3.345alt). https://zenodo.org/record/4576821#.YD-o0GhKhM0	
11	D-JRP11-4.1	Project Periodic Reports	9, 13, 21, 25, 30	9, 13, 21, 25, 30		PUBLIC	8
11	D-JRP11-4.2	Consortium meetings – Review of work done/progress made and definition of priorities for next period	1,12,24	1,12,35		PUBLIC (http://doi.org/10.5281/zenodo.4499317) Regarding consortium meetings, in the impossibility of a live meeting planned for April 2020 due to COVID-19 restrictions, we have organized a one-day video meeting (17/11/2020) where all partners presented the advances in the project in this last year. A final after-project follow-up	8



JRP/JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Comments (Please mention: Zenodo reference and other comments)	Proposed categories* (1 to 8) (several categories may be applicable)
						meeting is prevue on April-May 2021.	
11	D-JRP11-4.3	Communication strategy plan	M12	M24		PUBLIC. A first version has been produced, validated by all partners and sent to the One Health EJP responsible on Nov 20 th , 2019. http://doi.org/10.5281/zenodo.4451400	8
11	D-JRP11-4.4	Plan for the dissemination and exploitation of results (data management plan)	M18	M16 (1st version DMP) M24 (2nd version DMP) M37 (DMP final version)		PUBLIC. A second version has been produced, validated by all partners and sent to the One Health EJP responsible on Nov 20 th , 2019. The final version of the DMP has been sent to One Health EJP coordinators. http://doi.org/10.5281/zenodo.4451409	8
11	D-JRP11-4.5	Final Report	M36	M37		PUBLIC	8

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities); 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
11	M-JRP11-1	SCAI medium culture protocol validated on sites	M3	Yes		Best enrichment steps and incubation temperatures defined according to the different sources. Protocols made publicly available through the protocols.io platform in a project called ' Klebsiella Research and Surveillance ' (https://www.protocols.io/workspaces/klebsiella-research-and-surveillance/publications).
11	M-JRP11-2	Preparation of draft of the strategic communication plan	M10	Yes		A first version has been produced, validated by all partners and sent to the One Health EJP responsible.
11	M-JRP11-3	Project reporting template	M10	Yes		All periodic and final reports have been delivered to One Health EJP coordinators.
11	M-JRP11-4	Standard culture methods defined	M12	Yes		Productivity and specificity of SCAI medium tested. Best enrichment steps and incubation temperatures defined according to the different sources. Protocols made publicly available through the protocols.io platform in a project called ' Klebsiella Research and Surveillance ' (https://www.protocols.io/workspaces/klebsiella-research-and-surveillance/publications).
11	M-JRP11-5	qPCR protocol available for detection, isolation and quantification	M12	Yes (partially)		qPCR for the detection and identification of Kp complex members designed and validated. Article published and protocol made publicly available



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
						through the protocols.io platform in a project called ' Klebsiella Research and Surveillance ' (https://www.protocols.io/workspaces/klebsiella-research-and-surveillance/publications). Due to its complexity, the task of quantification of Kp in samples was not addressed during the project. However, the developed qPCR will allow this to be done in future dedicated studies.
11	Additional	MALDI-TOF MS	M12	Yes		Demonstration and validation of the potential of MALDI-TOF MS to correctly identify strains of the Klebsiella at the phylogroup level. Articles published. Additionally, we have set-up a MALDI-TOF MS based identification web site for Klebsiella. A pilot web site is functional (https://maldityper.pasteur.fr), and the publication has been submitted and it's available at BioRxiv platform (https://doi.org/10.1101/2020.10.13.337162).
11	M-JRP11-6	Broad survey of Kp in multiple sources complete	M24	Yes		Multiple (3781 in total) samples were screened by the consortium.
11	M-JRP11-7	Development of model frameworks for dynamic modelling and source attribution	M29	No		Please see comment for Deliverable 3.2 to 3.5
11	M-JRP11-8	Initial prevalence, quantification and genomic data for model refining	M28	Yes		



JRP/JIP Code	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
11	M-JRP11-9	1st batch of clonal groups, plasmids and genes defined, for refinement of models	M24	Yes		
11	M-JRP11-10	Compilation and integration of the data produced in WP1 and WP2 to be used in the dynamic and source attribution models	M31	No		Please see deliverables 2.4, 3.1 and 2.1
11	M-JRP11-11	Identification of a list of scenarios for control measures to be assessed through model simulations	M30	No		Please see comment for Deliverables 3.2 to 3.5
11	M-JRP11-12	Consolidated prevalence, quantification and genomic data for modeling of Kp transmission	M28	No		Deliverables 2.4, 3.1 and 2.1
11	M-JRP11-13	Application of dynamic and source attribution models to data collected from different countries	M30	No		Please see comment for Deliverables 3.2 to 3.5
11	M-JRP11-14	Reporting of transmission and source attribution estimates	M30	No		Please see comment for Deliverables 3.2 to 3.5



5. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Community-acquired infection caused by the uncommon hypervirulent <i>Klebsiella pneumoniae</i> ST66-K2 lineage. https://doi.org/10.1099/mgen.0.000419 https://zenodo.org/record/4436269#.X_8DxS_5Q6U	YES		GOLD 1795€
Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant <i>Klebsiella pneumoniae</i> isolates from Austrian rivers and clinical isolates from hospitals 10.1016/j.scitotenv.2019.01.179 https://zenodo.org/record/4249232#.X6UhA2hKjcc	YES		GOLD 2765€
Diversity of mucoid to non-mucoid switch among carbapenemase-producing <i>Klebsiella pneumoniae</i> https://doi.org/10.1186/s12866-020-02007-y https://zenodo.org/record/4456731#.YBtD7C_5TjA	YES		GOLD 1870€
Broad-spectrum cephalosporin-resistant <i>Klebsiella</i> spp. isolated from diseased horses in Austria https://doi.org/10.3390/ani10020332 https://zenodo.org/record/3929408#.Xv8m_vgzZM0	YES		GOLD 1482€
Description of <i>Klebsiella africanensis</i> sp. nov. <i>Klebsiella variicola</i> subsp. <i>tropicalensis</i> subsp. nov. and <i>Klebsiella variicola</i> subsp. <i>variicola</i> subsp. nov. https://doi.org/10.1016/j.resmic.2019.02.003 https://zenodo.org/record/3660879#.XkEmO2hKiUk	YES	GREEN 12M	
Description of <i>Klebsiella spallanzanii</i> sp. nov. and of <i>Klebsiella pasteurii</i> sp	YES		GOLD 2399€



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
https://doi.org/10.3389/fmicb.2019.02360 https://zenodo.org/record/3660875#.XkEICGhKiUk			
Identification of Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola and Related Phylogroups by MALDI-TOF Mass Spectrometry. 10.3389/fmicb.2018.03000 https://zenodo.org/record/3660887#.XkEm5mhKiUk	YES		GOLD 2399€
Klebsiella pneumoniae carriage in low-income countries: antimicrobial resistance, genomic diversity and risk factors https://doi.org/10.1080/19490976.2020.1748257 https://zenodo.org/record/3929396#.Xv8lrSgzZM0	YES		GOLD 2399€
Outbreak of Yersiniabactin-producing Klebsiella pneumoniae in a Neonatal Intensive Care Unit 10.1097/INF.0000000000002258 https://zenodo.org/record/3661013#.XkFq-OSWwj9	YES	GREEN 12M-	
The ZKIR Assay, a novel Real-Time PCR Method for the Detection of Klebsiella pneumonia and Closely Related Species in Environmental Samples 10.1128/AEM.02711-19 https://zenodo.org/record/3730608#.Xn3Kk4hKi70	YES		GOLD 2033€



Additional output

One Health EJP ASM (2019, 2020) and IMMEM-XII:

1. Carla Rodrigues, Sylvain Brisse on the behalf of MedVetKlebs consortium. The MedVetKlebs project: *Klebsiella pneumoniae* from Ecology to Source Attribution and Transmission Control (poster).
2. Carla Rodrigues, Virginie Passet, Andrianiaina Rakotondraso, Sylvain Brisse. Suitability of MALDI-TOF Mass Spectrometry to Discriminate Species within the *Klebsiella pneumoniae* Complex (poster).
3. Carla Rodrigues, Marisa Haenni, Maxime Bour, Cécile Ponsin, Jean-Louis Pinsard, Virginie Passet, Jean-Yves Madec, Sylvain Brisse. Genomic Diversity, Antimicrobial Resistance and Virulence of *Klebsiella pneumoniae* from Healthy Food-producing Animals and Horses (oral).
4. Elodie Barbier, Carla Rodrigues, Géraldine Depret, Virginie Passet, Laurent Gal, Pascal Piveteau and Sylvain Brisse. Design, Development and Validation of a Real-Time PCR Assay for Detection of *Klebsiella pneumoniae* Complex in Environmental Matrixes (poster).
5. Elodie Barbier, Juan Sebastian Lopez-Fernandez, Carla Rodrigues, Virginie Passet, Laurent Gal, Sylvain Brisse, Pascal Piveteau. Development of Phylogroup-Specific Taqman Real-Time Assays for Identification of Members of *Klebsiella pneumoniae* Complex (poster).
6. Małgorzata Ligowska-Marzeta, Katrine Grimstrup Joensen, Carla Rodrigues, Sylvain Brisse and Eva Møller Nielsen. Broad Sampling for Presence of *Klebsiella pneumoniae* in Different Sources from Denmark (poster).
7. Violeta Di Marzio, Gabriella Centorotola, Cristina Marfoggia, Alessandra Cornacchia, Maria Antonietta Saletti, Carla Rodrigues, Sylvain Brisse, Francesco Pomilio. A Comparative Study of Productivity, Selectivity and Specificity of Three Selective Culture Media for *Klebsiella* spp. Detection (poster).
8. Carla Rodrigues, Marisa Haenni, Maxime Bour, Cécile Ponsin, Jean-Louis Pinsard, Virginie Passet, Jean-Yves Madec, Sylvain Brisse. Genomic Diversity, Antimicrobial Resistance and Virulence of *Klebsiella pneumoniae* from Animal Carriage: a Comprehensive Analysis in an One Health Perspective (oral).
9. Alessandra Cornacchia, Maria Antonietta Saletti, Violeta Di Marzio, Aurora Ciarrocchi, Gabriella Centorotola, Cristina Marfoggia, Carla Rodrigues, Sylvain Brisse, Francesco Pomilio. Detection of *Klebsiella* spp. in chicken meat: methods performance study (poster).
10. Violeta Di Marzio, Gabriella Centorotola, Aurora Ciarrocchi, Cornacchia Alessandra, Cristina Marfoggia, Maria Antonietta Saletti, Carla Rodrigues, Sylvain Brisse, Francesco Pomilio. Evaluation of antimicrobial resistance of *Klebsiella pneumoniae* strains in foods (poster).

6. One Health Impact

A significant impact of the MedVetKlebs project results is anticipated in several directions.

First, our methodological developments and validations (culture and isolation methods, molecular detection, MALDI-TOF identification, taxonomic descriptions) will improve laboratory work, and thereby impact positively laboratory capacity in detection, identification, surveillance, epidemiology and ecology of *Klebsiella*. Our novel methods and tools (e.g., *Klebsiella* MALDI-TypeR) have already been adopted by research consortia on *Klebsiella* and may be adopted by microbiology laboratories, including reference laboratories, in their workflows.



Second, the data on the prevalence and resistance/virulence of *Klebsiella* in varied sources, generated by the MedVetKlebs project, will contribute to better assess the risk associated with potential sources or reservoirs of *Klebsiella*.

Third, our modelling approach may provide a very useful tool to define outbreaks of Kp and other food-borne or environmental pathogens.

Finally, the genomic data generated in this study, integrated into the *Klebsiella* genomic library and nomenclature server BIGSdb-Pasteur, will contribute to define *Klebsiella* subtypes and track their spread more efficiently.

Examples of interactions with relevant stakeholders are described below:

- **ECDC and EFSA** we contacted along with multiple international scientists by the MedVetKlebs coordinator in the frame of the organisation of the KlebNET project meeting (April 2020, which was cancelled due to COVID19). We have reinitiated contacts with ECDC and have agreed on collaborating on genomic nomenclature of strains for pan-European tracking purposes.
- **The international network Kleb-NET** – H2020 EU JPIAMR 7th call on surveillance - <https://research.pasteur.fr/en/project/kleynet-a-one-health-network-bridging-science-and-surveillance-on-antimicrobial-resistant-klebsiella/> (PI: Sylvain Brisse) was successfully built upon initial networking activities within the MedVetKlebs project.
- **SpARK** – JPIAMR 3rd Joint Call on Transmission Dynamics - <https://www.jpiamr.eu/wp-content/uploads/2016/11/SpARK.pdf> (PI: Edward Feil): This project includes S Brisse as partner, and benefitted from our MedVetKlebs validated SCAI culture method – more than 6000 samples from soil, animals, humans and food were searched for Kp presence by culture and metagenomics methods.
- **KLEB-GAP** - Bergen Research Foundation grants - <https://www.vetinst.no/en/research-and-innovation/ongoing-research-projects/kleb-gap-klebsiella-pneumoniae-a-key-driver-in-the-global-spread-of-antimicrobial-resistance-and-a-target-for-new-approaches-in-diagnostics-surveillance-and-alternative-therapeutics> (PI: Arnfinn Sundsfjord and Co-PI Iren Høyland Løhr). S Brisse is a partner in this project, and provided expertise, culture and qPCR protocols developed in the MedVetKlebs project, ahead of public dissemination.
- **NOR-KLEB-NET** – funded by the Norwegian Research Council - <http://www.nor-kleb.net>. Sylvain Brisse is a partner in this project, and provided expertise, culture and qPCR protocols developed in the MedVetKlebs project, ahead of public dissemination. The Kp sampling performed through this project has benefited from MedVetKlebs outputs.

JRP12-FARMED

1. Summary of the work carried out in year 3

FARMED aims to assess the feasibility of long-read sequencing to rapidly characterise the metagenome and resistome of samples, on-site, in order to provide investigators with the correct information to apply the most appropriate control measures. A survey of the consortium identified the different methods and experiences of institutes with both short- and long-read sequencing technologies, and also highlighted the different matrices from a variety of human, animal, and environmental sources examined, DNA extraction methods, and bioinformatics tools used to detect bacterial species and/or AMR.

The first task of WP1 was to identify the most common simple and complex sample matrices typically analysed by the consortium, which were water and faeces, respectively. The consortium decided on the sample matrices, which were relevant for each institute and included simple (water and saliva) and



complex (human/animal faeces, feed additives, and boot swabs). We agreed that a defined microbial community (DMC) would be used to inoculate a simple (wastewater) or complex (animal faeces) matrix to test the effectiveness of the DNA extraction methods, used by each institute, to perform long-read sequencing.

In May 2020, FARMED were able to progress WP1 and avoid further delay, following the COVID-19 related lockdowns. BfR and Sciensano (WP1 leaders), prepared a detailed plan for the defined microbial community (DMC) which will be used to assess the feasibility of long-read metagenome sequencing. The consortium agreed on six bacterial species (harbouring AMR), the concentration of inoculated bacteria and the sample matrices (pond-water or animal faeces from BfR collection) to be used, which were preserved in nucleic acid preservation solution. Two bacterial isolates (DTU/Sciensano) were supplied to BfR for inclusion in this sample set. These inoculated matrices were sent to each institute in July 2020. Generation of DMC data is nearing completion.

To enable comparison of the DNA extraction methods only, WP2 decided on the initial bioinformatics workflow to be used for the DMC analysis in WP1. The WP2 institutes held a phone meeting to discuss and decided on the bioinformatics analysis methods, which were to be used by participants in WP1 to analyse the DMC samples. To ensure the bioinformatics methods performed at each institute were the same, a published dataset was included. The bioinformatics analysis of the results from the DNA extraction methods used by each institute are underway after some technical delays, which are now planned for January 2021. Our first face-to-face online meeting postponed to October 2020, discussed the experiences of institutes for long-read sequencing as well as comparison of the requirements of on-site DNA extraction methods (WP3).

As laboratory work was being initiated in spring, the COVID-19 pandemic was starting across Europe, which resulted in lockdowns in many countries and reprioritisation of work at the various institute laboratories. The majority of the FARMED project is based on wet laboratory work; therefore, the lockdowns or limited laboratories access has severely affected progress, losing approximately 3-4 months. Many institutes are not yet back to pre-COVID-19 activities and are involved in COVID-19 testing, which has further impacted progress.

2. Work carried out in the JRP, scientific results

WP1: Assess feasibility of Long-read metagenome sequencing on exemplar matrices. Investigate the use of Hi-C metagenomics (M25-M44)

JRP12-WP1-T1 - Assess feasibility /perform long-read metagenomics MinION from ‘defined’ microbial community (M25-M40)

To assess the approach of long-read metagenome sequencing, the consortium planned and discussed in the teleconferences from the 09/01/2020 and 20/05/2020, establishing a defined microbial community (DMC) consisting of six different microbial isolates, with different AMR profiles, in two different sample matrices. BfR conducted a literature search as well as screened and analysed isolates from the BfR (9) strain collection to find feasible isolates for composing the defined microbial community (DMC). A DMC of four Gram-negative isolates and two Gram-positive isolates with known complete genome sequences and different AMR profiles was designed and approved by the consortium, which are summarised in the table 1.

Table 1 – The concentration of bacteria used to spike complex and simple matrices.

Bacteria	Control	Fixed Concentration DMC (CFU/ml)	Mixed Concentration DMC (CFU/ml)
<i>Acinetobacter</i>	0	10 ⁵	10 ³



<i>baumannii</i>			
<i>Escherichia coli</i>	0	10 ⁵	10 ³
<i>Salmonella enterica</i>	0	10 ⁵	10 ⁵
<i>Staphylococcus epidermidis</i>	0	10 ⁵	10 ⁵
<i>Vibrio cholerae</i>	0	10 ⁵	10 ⁷
<i>Bacillus subtilis</i>	0	10 ⁵	10 ⁷

Two different sample matrices, water buffalo faeces (complex matrix: WP1-T3) and pond water (simple matrix: WP1-T2) were chosen as they were the most common sample matrices used by the consortium. The samples were screened for *Salmonella*, and ESBL and/ or Carbapenemase producing Enterobacteriaceae as well as isolates harbouring the *mcr-1* to *mcr-9* genes, and found to be free and suitable for the DMC analysis.

The *S. epidermidis* was obtained from DTU (12) in June and the *B. subtilis* from SCIENSANO (4) in July. All 6 isolates have known complete genome sequences as well as different AMR profiles. Furthermore, all 6 isolates were characterised with respect to their growth in liquid LB medium for consequent calibration of the final concentrations of each bacterial species in the defined mock community. These six isolates were assembled into two differently composed communities, either with a fixed concentration of 10⁵ CFU/ml or with a range from 10³ to 10⁷ CFU/ml. These communities were spiked into either a simple or a complex matrix, according to project partner preference. In order to preserve the integrity of the DNA present within the defined mock community and the matrices themselves, two different nucleic acid preservation solutions were tested for their efficacy and deactivation properties. To that extent, both a commercial and a self-made solution were trialled by storing the defined mock community in them and consequently extracting DNA and comparing the resulting DNA fragment lengths. Consequently, the commercial nucleic acid preservation solution (DNA/RNA Shield, Zymo Research) was selected and used to store the spiked mock community and their matrices. The DNA extraction protocol at the BfR was tested separately on the defined mock community and on the complex matrix to ensure a sufficient DNA output was extracted for sequencing procedures. These spiked and un-spiked matrices samples were sent as duplicates to project partners in July 2020 ('simple' matrix: BfR, Sciensano and UCM, 'complex' matrix: BfR, SSI, DTU, APHA, Sciensano, WBVR, and IZSAM).

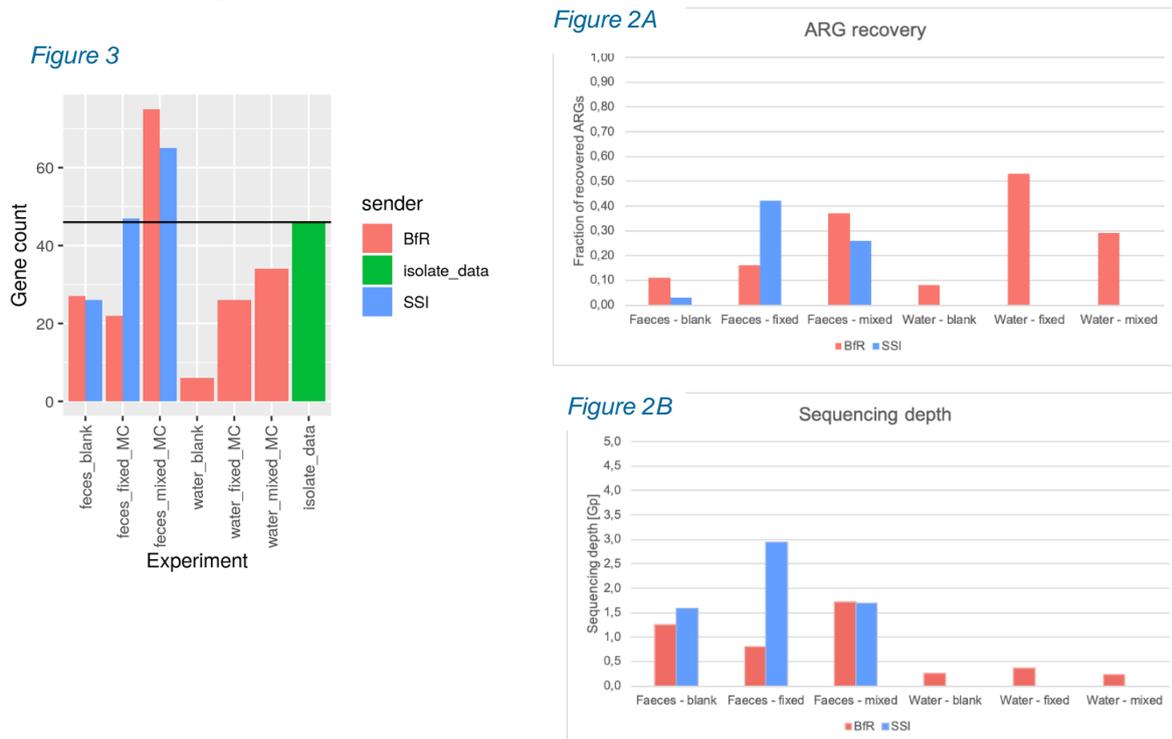
Each partner used the DNA extraction protocols (mainly commercially available kits) already used/present in their laboratories, which include bead beating, enzymatic lysis or a mixture of both to extract DNA. The concentration of DNA extracted by the various methods ranged from 1 to 60 ng/ μ l of DNA, with satisfactory 260/280 ratio (1.6-1.8). The range of DNA fragment sizes (3-50kb) also varied between the methods. The project partners used the same long-read sequencing kits (ONT Ligation sequencing kit (SQK-LSK109) and native barcoding Expansion) in order to obtain comparable sequencing results between the consortium. DNA was sequenced with Illumina short-read (these varied between the consortium) and ONT-MinION (Flow Cell R9) long-read sequencing technologies, aiming to achieve 5Gbp per sample.

During the face-to-face meeting on 29th October 2020, it was decided to collate and share the technical and quality control details from the differing DNA extraction protocols, library preparation, and sequencing amongst partners of the work package. Furthermore, it was agreed to complete sequencing by Illumina short-read and ONT long-read MinION technologies by mid-December. Currently, bioinformatic analyses regarding species and AMR genes are ongoing at individual project partners and it has been agreed that for the comparison of results, raw sequencing data will be shared and collated by mid-December, unfortunately due to technical and purchasing delays this was not completed by all partners. Individual partners will share their raw (MinION and Illumina) sequences as well as their KMA analysis, so that the various DNA extraction methods can be compared, this analysis will be performed by DTU.



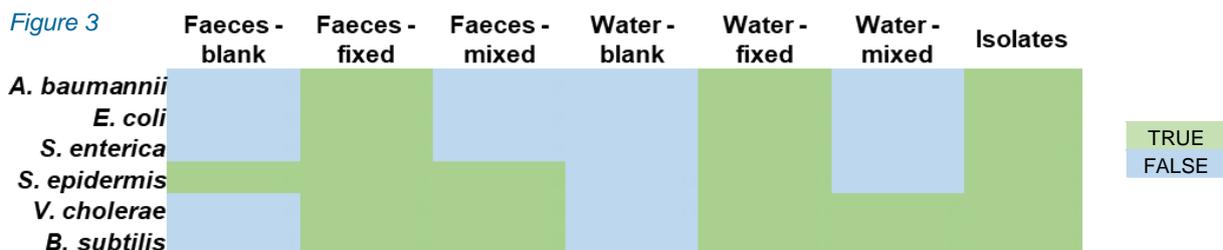
Preliminary comparisons of the long-read sequencing results between project partners (BfR and SSI) were presented to the consortium on 29th October 2020. These results were obtained from the KMA (k-mer alignment) bioinformatics pipeline by DTU (WP2.1), which will be utilised for the planned comparison between all project partners. These exemplary analyses included a comparison of how many of the antibiotic resistance genes (ARGs) that were present in the defined mock community were identified through sequencing.

Figure 1 - ARG count post MinION sequencing (incl. duplicates and taking variants into account) between BfR and SSI, Figure 2 - Comparison of ARG recovery (ignoring duplicates and comparing ARG names, not variants) (2A) and sequencing depth (2B) between BfR and SSI



Initial analysis on the BfR sequencing dataset to assess whether the presence of the six species of the DMC could be identified within the two matrices (figure 3). These preliminary long-read sequencing results and comparisons indicate that long-read sequencing and subsequent analyses were capable of identifying some, but not all, of the DMC bacterial species and their ARGs that were spiked into the two matrices. Bioinformatics analyses could correctly predict the presence of all 6 bacterial species in the faecal and water sample with the DMC at a fixed concentration but not when at a mixed concentration, particularly *E. coli* and *A. baumannii* at 10^3 or *S. enterica* at 10^5 , in either matrix.

Figure 3 - Prediction of DMC species' absence or presence within the two different matrices (TRUE = presence, FALSE = absence) as found at BfR.

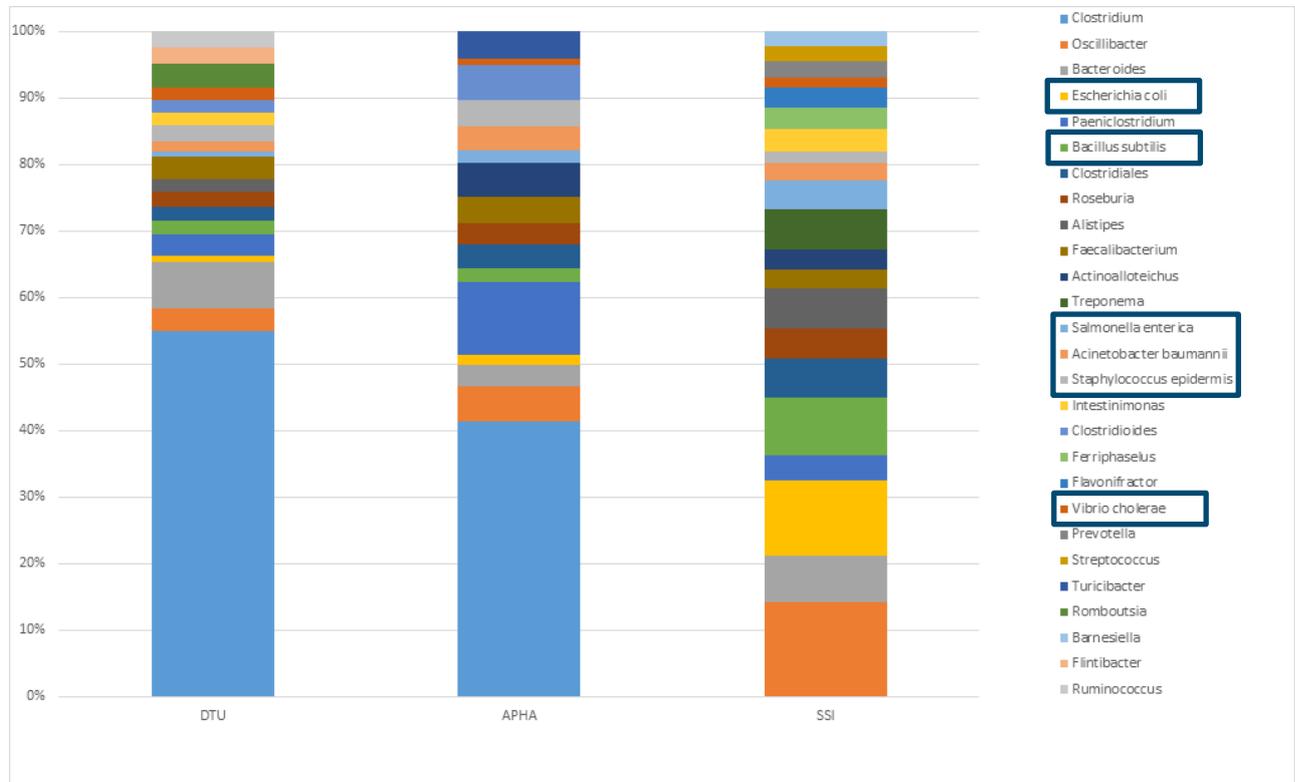


Preliminary comparison (figure 4) of the fixed DMC samples (faeces) from DTU, APHA and SSI, shows the variation of the microbiome between the different methods employed. The spiked-in bacteria (table 1) were identified in the long read sequences of three communities but at lower levels compared



to the other bacteria. Analysis of the complete DMC dataset will be undertaken using tools in WP2, to assess the different extraction methods.

Figure 4 - Profile of top 20 most abundant bacterial genera in fixed DMC samples from 3 institutes, spiked-in bacteria have also been included (percentage abundance).



In addition to MinION, this task was investigating use of PacBio (Pacific Biosciences, Sequel II) long read sequencing technology, which was available at BfR. Using the same ‘complex’ DMC spiked samples, BfR have completed the PacBio sequencing and are currently analysing these results for further comparison with the other two sequencing (Illumina and MinION) technologies.

Due to COVID impact there have been delays at most institutes, although both short- and long-read sequencing data is currently being generated and analysed. In January 2021, a thorough evaluation of the data from all project partners, inter-partner comparisons will be completed by DTU in order to assess the success of long-read metagenome sequencing of this defined microbial community. The detailed analyses should reveal whether long-read sequencing and subsequent bioinformatics can successfully identify the spiked-in species from the mock community including their concentrations and identify the AMR genes present within the defined mock community. Moreover, based on these results, an optimal DNA extraction protocol will be defined for the work packages WP1-T2 and WP1-T3.

JRP12-WP1-T2 - Assess feasibility /perform long-read metagenomics MinION from ‘simple’ sample matrices (M27-M42)

This task is delayed. During first face-to-face meeting, the consortium discussed the analysis of ‘simple’ sample matrices, i.e. water and saliva. However, this cannot progress until finalisation of the DMC samples (task WP1.1), to determine which DNA extraction and bioinformatics analysis methods should be explored further, with institutes starting to analyse their own ‘real’ simple samples.



JRP12-WP1-T3 - Assess feasibility/perform long-read metagenomics MinION from 'complex' sample matrices (M29-M44)

This task is delayed. During the first face-to-face meeting, the consortium discussed the analysis of 'complex' sample matrices, i.e. human/animal faeces, feed additives, and boot swabs. As with WP1.2, this cannot progress until finalisation of the DMC sample analysis (task WP1.1), then institutes can begin to analyse their own 'real' complex samples. Sciensano started with the analysis of the complex feed additive matrix using shotgun metagenomics. Feed additives (e.g. vitamins) are often produced through the use of genetically modified microorganisms (GMMs) to replace chemical synthesis methods, as this is more practical and requires fewer resources. The presence of a genetically modified microorganism (GMM) or its DNA, often harbouring antimicrobial resistance (AMR) genes, in microbial fermentation products is prohibited by European regulations. GMMs are currently screened for through qPCR assays targeting AMR genes and vectors, and then confirmed by targeting known specific GM constructs/events. However, when the GMM was not previously characterized and an isolate cannot be obtained, its presence cannot be proven. A metagenomics approach is then the only way to demonstrate the presence of a GMM in a microbial fermentation product, with characterization based on detection of AMR genes and vectors, species and unnatural associations in the GMM genome. We based the evaluation of the feasibility of this new approach on the investigation of a previously analysed sample containing a GMM *Bacillus subtilis* overproducing vitamin B2 (riboflavin), isolated and fully characterized at that time (RASFF 2014.1249). The method was then applied to a sample positive for some qPCR markers but for which no isolate could be obtained. The short and long reads sequencing technologies were compared for their performances, including the newly released Flongle, as a smaller and cost-effective alternative. The analysis is currently ongoing.

JRP12-WP1-T4 - Perform Hi-C metagenomics (M33-M42)

BfR started with initial development of a Hi-C analysis workflow (comparison of different Hi-C analysis workflows) and performed initial experimenting with the Hi-C wet lab protocol. BfR have prepared Hi-C libraries from two poultry faecal samples, from the EFFORT project and a 'defined' microbial community, containing four bacterial species (with complete genome sequences), consisting of *Salmonella enterica*, *E. coli*, *A. baumannii* and *V. cholerae* and harbouring different AMR plasmids. The DMC samples from WP1-T1.1 will also be characterised using Hi-C sequencing and compared to the short-read and long read sequencing data.

WP2: Bioinformatics tools to analyse the sequencing data and defining the characteristics within the sample (M28-M45)

Supplementary – to enable sharing and storage of sequencing data between the consortium, which could remain private before being published, APHA and SCIENSANO researched the available options, as this facility was not provided by OHEJP. We anticipated that for each task comparison we would generate up to 120GB of MinION data and 192GB of Illumina data between the consortium. Three options were considered for sharing and storage of data (Google Cloud, Google Drive and ownCloud), each with advantages and disadvantages, the consortium decided to use ownCloud (<https://farmedejp12.owncloud.online/>), which was already utilised by the Full-Force EJP consortium. Data from all DMC analysis (WP1.1), as well as data generated from simple (WP1.2) and complex (WP1.3) sample matrices would be shared between the consortium, unless confidential data was included that could lead to privacy concerns.

JRP12-WP2-T1 - Development/adaptation of a pipeline that can predict species within sample/matrix (M28-M45)

The KMA (k-mer alignment) pipeline is implemented and used for bacterial community assignments from long-read as well as short-read sequences, and will be considered the standard workflow for FARMED data analysis. It is tested for Oxford Nanopore sequences. DTU hosted a training session to communicate KMA usage amongst all FARMED partners. During this session, the different institutes



were also introduced to the KMA protocols to assign both long and short reads to bacterial taxa. This is particularly useful for more standardized comparisons between both technologies across different institutes. All partners mapped DMC sequencing outputs (task WP1.1) using KMA and all the aligned reads were assigned to bacterial taxa using the same parameters. To ensure consistency of results, DTU will perform KMA analysis using all the raw sequencing data from the consortium. The entire DMC dataset, which includes the raw sequencing data files as well as the KMA output files, will be compiled for evaluations and comparison, analysis to be conducted by DTU in January 2021. This analysis will evaluate the feasibility of long-read metagenome sequencing to identify pathogens.

JRP12-WP2-T2 - Development/adaptation of a Resfinder-'like' pipeline for identification of AMR for long-read sequences (M30-M51)

ResFinder is compatible with long reads for AMR assignments after KMA mapping. KMA and ResFinder are also compatible with short-read sequences, which facilitates a more consistent AMR comparison of the same samples using both technologies: short- and long-read. All the involved partners are now familiar with ResFinder and KMA pipeline for long read analyses, and DMC sequences (task WP1.1) were fed into KMA and ResFinder for bacterial composition and AMR profiling using the same parameters. To ensure consistency of results, DTU will perform KMA analysis using all the raw sequencing data from the consortium. The entire DMC dataset, which includes the raw sequencing data files as well as the KMA output files will be compiled for evaluations and comparison, analysis to be conducted by DTU in January 2021. At this stage, the primary output to compare between partners is bacterial composition and AMR profiling. In future analysis, the consortium will consider the capability of finding links between bacteria and AMR genes, however this may be hard to accomplish using current sample reads, unless reads are assembled to identify co-linkage of AMR, plasmids and bacteria.

WP3: Implementation of on-site protocols for long-read metagenomic DNA sequencing (M25-M54)

JRP12WP3-T1 - Literature search & Harmonisation of on-site DNA isolation (M25-M29)

WBVR (31) and APHA (21) designed and shared a questionnaire within the consortium to survey the DNA extraction, DNA short- (isolates and metagenomes) and long-read sequencing methods, as well as which matrices/samples were used by each institute. The bioinformatics analysis methods were also surveyed. All institutes contributed to this methods survey and this was collated by WBVR. This information along with literature searches on long read metagenomics and metagenomics analysis from animal faecal samples and food products will be undertaken. Based on these findings, any additional methods that are suitable for on-site DNA extraction will be tested and compared to the methods tested in WP1 and WP3.

JRP12-WP3-T2 - Investigate the use of Voltrax (M30-M41)

This task is not yet initiated.

JRP12-WP3-T3 - Assess DNA isolation methods for suitability, test on matrices (faeces, blood, dust, (environmental), milk) (M33-M54)

This task is not yet initiated.

JRP12-WP3-T4 - Test protocols on-site (M37-M54)

This task is not yet initiated.

WP4: Project management, coordination, and training workshop (M25-M54)

JRP12-WP4-T1 - Annual physical project meetings (M30-M54)

The first physical day meeting was arranged for 19th March but had to be postponed due to the start of COVID-19 and travel restrictions across Europe. Online meetings were held in May 2020 where initial discussions and plans were decided for the first FARMED task (WP1.1). The consortium had provisionally planned to meet in October 2020; however, travel bans/restrictions were still in place due to the COVID-19 pandemic. Finally the face-to-face daylong meeting was held online on the 29th of October, via go-to-meeting (hosted by SCIENSANO), see screenshot below. The consortium discussed in detail the activities carried out for the WP1-T1, WP2 for the develop of bioinformatics tools that could be used for long read sequencing, and APHA and SSI gave an overview on existing methods and current challenges regarding DNA extraction from faecal samples for the WP3 –Onsite DNA extraction/sequencing. Giusy Sannino (MicroGEM) Paul Butler (FORENTEQ LIMITED) presented a portable automated extraction system PDQex, showing its capability to extract high quality DNA from various sample matrices, which is applicable to the FARMED project; participants were enthusiastic about its potential.

Figure 5 - The FARMED consortium during October meeting.



JRP12-WP4-T2 - Teleconferences will be organised every 3 months, between partners (M25-M54)

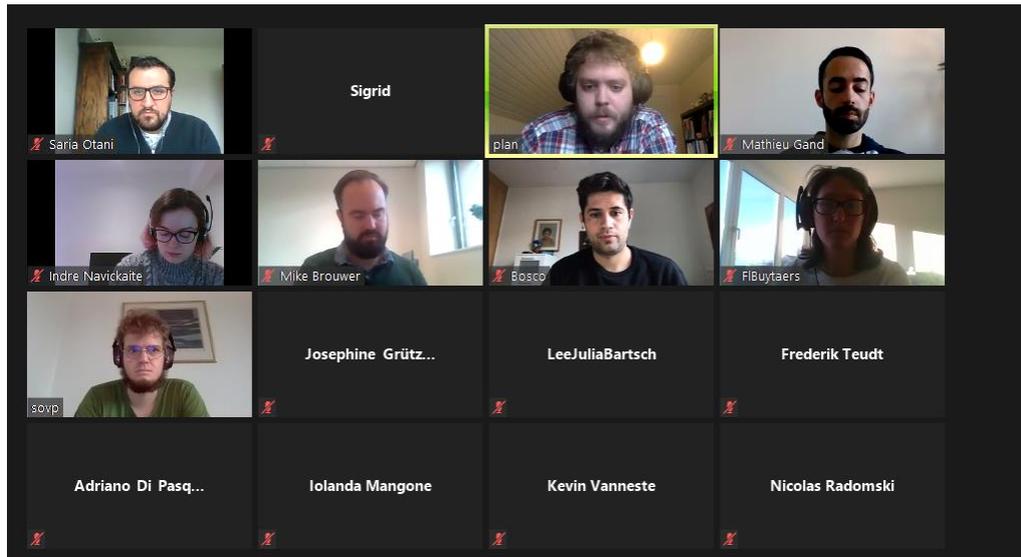
The consortium gathered on the phone on 9th January for a kick off meeting. Due to the COVID-19 situation, and not adding unnecessary stress on already closed labs, we had the next teleconference on 20th May when laboratories had a better idea of post COVID-19 activities. The consortium agreed a plan to start lab work and report findings in October 2020. There were sub-group teleconferences on separate topics/WPs (e.g. WP2 on 23/06/2020).

JRP12-WP4-T3 - Training/dissemination of developed protocols

DTU (Philip T. L. C. Clausen) held an online hands-on training session (6th November) on the use of the KMA aligner to analyse the data generated from the DMC samples (task WP1.1). The session aimed to provide experience in analysing Nanopore and Illumina generated data using the KMA pipeline as well as go over installation requirements/steps, parameter set-up and troubleshooting. Following the training session, all collaborators will share their KMA analysis results (.mapstat and .res files) by uploading them onto the BfR cloud server.



Figure 6 - The FARMED consortium during KMA workshop in November 2020.





3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
12	D-JRP12-WP3.1	Review on current scientific literature and overview of commercially available methods for on-site DNA isolation.	M30		M36	Yes	Delay due to national lockdowns and reprioritisation/redeployment of staff to essential/statutory work, including involvement with COVID-19 testing.	8 (Summary of available methods, not publication)

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
12	M-JRP12-01	Review on current scientific literature and overview of commercially available methods for on-site DNA isolation presented to FARMED consortium.	M29	No	M36	Delay due to national lockdowns and reprioritisation/redeployment of staff to essential/statutory work, including involvement with COVID-19 testing.

4. Publications and patents

Additional output

Poster of project “FARMED: Fast Antimicrobial Resistance and Mobile-Element Detection using metagenomics for animal and human on-site tests” presented at the One Health EJP Meeting, held in virtually in Prague 27-29th May.



5. On-going and planned collaborations with national or European projects or networks

Several members of the FARMED consortium are involved in other OH-EJP projects. In particular the Full-Force EJP is relevant as the project is utilising long-read sequencing to characterise mobile genetic elements which are associated with driving AMR in commensal and pathogenic Enterobacteriaceae. From their experience, the FARMED project chose to create an ownCloud.online data repository to share the large sequencing data files (<https://farmedejp12.owncloud.online/>). Now we have some experience and long-read sequence data, direct communication and discussion between the project will likely drive and improve outputs of our respective projects.

Oral presentation of project “FARMED: Fast Antimicrobial Resistance and Mobile-Element Detection using metagenomics for animal and human on-site tests” by Manal AbuOun at the Cogwheel workshop with OHEJP and JPIAMR on 28th April (held online). As a result of attending this meeting we identified the K-Star project from jpiamr coordinated by Derek MacFadden, as they use very similar approaches to the analysis of sequencing data. We will contact this group once we have started developing the FARMED analysis workflows.

IZSAM and DTU colleagues participate in EURL activities, both of which relate to whole genome sequencing to detect and characterise pathogens, and have a growing interest in metagenomics.

JRP13-WORLDCOM

1. Summary of the work carried out in year 3

The OneHealth WorldCOM project aims to develop on-site diagnostic tools, linked with mobile referencing technology, for detection of AMR zoonotic pathogens (*Escherichia coli*, *Salmonella*, *Campylobacter*) in agricultural and environmental settings. The impact for OHEJP and stakeholders will be rapid on-site detection of AMR pathogens in animal and human populations. Technologies under development will facilitate investigation of potential emerging resistances at earlier stages than currently feasible. Data generated will be used to develop machine-learning algorithms to predict AMR in various environments.

Recruitment of staff has been completed by most partners, and considerable progress made on a number of tasks. All *bla* antimicrobial resistance genes present in *Salmonella*, *E.coli*, *Klebsiella* and *Acinetobacter* genomes were extracted from the NCBI Pathogens database (<https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/>). All types and subtypes of Extended Spectrum β -Lactamases (ESBLs) and plasmid-mediated colistin resistance genes have been analysed for frequency among reported and extracted sequences (UoS). High frequency resistance gene subtypes have been highlighted for further sequence analysis to illustrate geographic distribution and geography-specific single nucleotide polymorphisms (SNPs). The prevalence of ESBL subtypes in bacterial pathogens and a sequence database of the selected alleles have been published in Zenodo (DOI: 10.5281/zenodo.4019839).

Targeted and whole-genome sequencing of AMR strains has been undertaken. This includes: *de novo* sequencing and genome assembly of AMR *E.coli* strains; 88 *Campylobacter* spp. strains have been genome sequenced, genomes assembled, and MLST types assigned (UT); aminoglycoside and beta-lactam resistant Enterobacteria from companion animals, leading to identification of new epidemic plasmids that will be studied within the Consortium in different countries; 50 plazomicin-resistant Enterobacteria isolated from a single location from animals, sewage, wastewater, humans and food (UCM); *E.coli* strains resistant to 3rd and 4th generation cephalosporins isolated from swine and alpaca hosts; a subset of 30 strains, characterised with respect to phylogeny, antimicrobial-resistance



patterns and plasmid profile and whole-genome-sequenced (FLI); *E. coli* CTX-M environmental samples (NUIG); and *E. coli* isolates resistant to 3rd and 4th-generation cephalosporins and/or carbapenemes and/or colistin from human and animal origin (INSA).

LAMP assay development has been substantially progressed including the development and validation of a duplex CTX-M-1/15 LEC-LAMP assay for the differential detection of *E. coli* CTX-M-1 and CTX-M-15 isolates (NUIG). The duplex assay has been transferred to another WorldCOM partner (FLI) for further testing and validation using veterinary samples. LAMP assays targeted at plasmid-mediated colistin resistance (*mcr-1* gene), KPC-mediated carbapenem resistance, oxacillin-hydrolyzing β -lactamases (OXA-48 gene) and the most frequent alleles of the OXA-48-like variants, have also been developed and validated (UoS), demonstrating detection of AMR target genes within 3-5 minutes. Good progress has also been made on the development and evaluation of rapid DNA extraction protocols for use in the field. This includes the detection of AMR genes from water samples, demonstrating the detection of the *mcr-1* target gene with a detection limit of 10² cfu/mL as confirmed by culture. LAMP assays targeting AMR genes coupled with optimised sample-preparation methods will be crucial for on-site detection of AMR in different settings.

2. Work carried out in the JRP, scientific results

WP1: Generation of up to date sequence information for selected pathogens and antimicrobial resistance genes (M25-M36)

Task 1 (WP1-T1): Analysis of publically available sequences for antimicrobial resistance genes associated with *Salmonella*, *Campylobacter* and *E. coli* (M25-M28)

At the UoS, all *bla* antimicrobial resistance genes present in *Salmonella*, *E. coli*, *Klebsiella* and *Acinetobacter* genomes have been extracted from NCBI Pathogens database (<https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/>). For the initial phase of this work package, we have focused on ESBL-related AMR genes. As these genes are absent from *Campylobacter*, we have not included this bacterium in these analyses, and have instead, used the important pathogens *Klebsiella* and *Acinetobacter*. Later work will include antibiotic resistances relevant to *Campylobacter*. All types and subtypes of Extended Spectrum β -Lactamases (ESBLs) and plasmid-mediated colistin resistance genes have been analysed for frequency among reported and extracted sequences. High frequency resistance gene subtypes have been highlighted for further sequence analysis to illustrate geographic distribution and geographic-specific single nucleotide polymorphisms (SNPs). The Prevalence of ESBL subtypes in bacterial pathogens and a sequence database of the selected alleles have been published in Zenodo (DOI: 10.5281/zenodo.4019839). This task has been completed.

Task 2 (WP1-T2): Targeted and whole genome de novo sequencing of phenotypically characterised isolates from various settings (M25-M42)

In the UK, no whole genome sequencing has been performed due to the COVID-19 lock down and it is currently postponed. However, the task is still ongoing.

In Estonia 120 *E. coli* strains have been *de novo* sequenced and genomes assembled. The bioinformatic analysis of these genomes, i.e. annotation and prediction of the genes, is ongoing. In addition, 88 *Campylobacter* spp. strains have been *de novo* sequenced, genomes assembled and MLST types assigned. A manuscript is planned for submission in Jan-Feb 2021 based on preliminary analysis of these *Campylobacter* sp. strains.

At UCM, plazomizin-resistant isolates are being analysed. Interestingly, studying plazomizin resistance, an emerging resistant gene has been identified, *npmA*. This gene is present in a novel Transposon within a new Integrative Conjugative Element (ICE). Further, emergence of plazomizin resistance in pig-farms is being identified in enterobacteria highly resistant to aminoglycosides and beta-lactams



that have been sequenced using Illumina and Nanopore sequencing, leading to the identification of new epidemic plasmids that will be studied within the Consortium in different countries. Further, a new IncR epidemic plasmid has been identified in a horse *Enterobacter hormaechei* ST171 -clone. This is an epidemic clone, responsible for several epidemics in pediatric Hospitals in the USA. Tracking of this plasmid-world-in the Consortium is envisaged.

At FLI, *E. coli* strains resistant to 3rd and 4th generation cephalosporins were isolated from swine and alpaca hosts. A subset of 30 strains was selected, characterized with respect to phylogeny, antimicrobial resistance pattern and plasmid profile and is currently being whole-genome-sequenced (Illumina) and analysed.

NUI Galway provided genomic nucleotide sequence information from locally isolated *E. coli* CTX-M environmental samples to a WorldCOM AMR gene sequence database for use in assay development. This nucleotide sequence information included 2 x CTX-M-1, 1 x CTX-M-9, 4 x CTX-M-4, 24 x CTX-M-15, 5 x CTX-M-27 and 1 x CTX-M-65 *E. coli* isolates.

At INSA, *E. coli* isolates resistant to 3rd and 4th generation cephalosporins and/or resistant to carbapenemes and/or resistant to colistin from human and animal origin were sequenced using a MiSeq Illumina platform. Bioinformatic analysis was performed using command line pipelines and the respective results are being shared with the other partners.

Task 3 (WP1-T3): Development of machine learning algorithms for the prediction of anti-microbial resistance. (University of Surrey, VISAVET Health Surveillance Centre UCM) (M37-M42)

The start date for this task has been partially delayed as Dr Gardner only recently joined the UoS School of Veterinary Medicine. At UCM using the inhouse tool ARUflow together with targeted search in world-wide sequencing projects, we have identified and epidemic in a Ducth Hospital of a new emerging plazomicin-resistant bacterium.

WP2: Assay Development (M36-M45)

Task 1 (WP2-T1): Development and performance evaluation of singleplex isothermal amplification assays for selected AMR gene targets (M31-M39)

NUI Galway completed the design of singleplex isothermal nucleic acid amplification assays for the detection of the selected AMR associated CTX-M-1 and CTX-M-15 gene targets. This initial assay design was achieved using existing genomic sequence data of *E.coli* isolates from environmental samples collected and processed by NUI Galway. These *E. coli* isolates contained CTX-M Group 1 variants, type 1 and 15. Sequence alignment analysis was used to identify single nucleotide polymorphism (SNP) differences between the type 1 and 15 variants. These SNP differences were subsequently used to develop loop-primer endonuclease cleavage loop-mediated isothermal amplification (LEC-LAMP) assays to detect and differentiate the type 1 and 15 variants. Standard LAMP assays were also designed to detect both CTX-M Group 1 variants. Evaluation of these assays successfully identified an optimal CTX-M-1 LEC-LAMP assay that detects CTX-M-1 targets while differentiating CTX-M-15 targets. The oligonucleotide probe used in the CTX-M-1 LEC-LAMP was modified to contain a different fluorophore label to enable detection of the CTX-M-15 target, while differentiating CTX-M-1. For development of an internal amplification (IAC) control assay, a synthetic DNA template and corresponding LEC-LAMP assay was developed. All three singleplex LEC-LAMP assays for the detection of CTX-M-1, CTX-M-15 and the IAC template were then optimised and evaluated. The analytical sensitivity of each assay demonstrated low limits-of-detection with 10 genome copies detected in under 30 min. Each assay demonstrated 100% analytical specificity with 10 *E. coli* CTX-M-1 isolates and 25 *E. coli* CTX-M-15 isolates correctly identified by each corresponding LEC-LAMP assay. These assays would subsequently be used in WP2 Task 3 to develop multiplex LEC-LAMP assays.

At the UoS, *mcr*, *bla*_{OXA}, *bla*_{CTX-M}, *bla*_{NDM} and *bla*_{KPC} gene sequences have been extracted from genomes listed in the NCBI Pathogens database to represent all gene alleles. This showed the conserved regions



across the different alleles of each *mcr*, *bla_{KPC}* and *bla_{OXA-48}* subtypes and the homology across the selected subtypes. The extracted sequences showed a species-specific trend across *mcr* gene subtypes with *mcr-1* being the most predominant in *E. coli*, *mcr-8* in *Klebsiella* and *mcr-9* in *Salmonella*. MSA of *bla_{KPC}* genes showed 99% homology among *bla_{KPC-2}* gene sequence alleles, while MSA of all *bla_{KPC}* genes and corresponding alleles showed 93% conserved homology. Thus, conserved regions were used to design LAMP primer sets for *mcr-1*, *mcr-8*, *mcr-9*, all *bla_{KPC}*, *bla_{OXA-48}* and some of the *bla_{OXA-48}*-like genes (OXA-48, OXA-232, OXA-181 and OXA-54 encoding genes) using both LAMP Designer 1.15 and Primer Explorer V5.

After optimisation, the KPC LAMP assay was able to detect all KPC positive strains including two *K. pneumoniae* clinical strains, *E. coli* and *E. cloacae* within 4 min, while all control strains, including a diverse range of Gram-negative and -positive bacteria, remained negative. The KPC LAMP assay demonstrated good sensitivity and a detection limit of approximately 10 pg DNA (successfully amplified within 9 mins). The *mcr-1* LAMP assay was also able to detect all *mcr-1* positive isolates including *E. coli* and *S. Typhimurium* within 4 min with good specificity with regards to all control strains. The *mcr-1* assay demonstrated a detection limit of approximately 1 pg DNA, which was successfully amplified at 11 min. The OXA-48 LAMP assay was tested against a wide range of *bla_{OXA-48}* and *bla_{OXA-48}*-like *K. pneumoniae* and *E. coli* isolates and successfully detected the target gene within 3-5 min. The test panel included some of the *bla_{OXA-48}* alleles, including OXA-181, OXA-232 and OXA-244 and they were successfully detected. The panel also included OXA-48 negative controls of *K. pneumoniae* and *E. coli* isolates, and *A. baumannii* OXA-23, OXA-25 and *P. aeruginosa* OXA-50. All negative controls were negative except for OXA-25 and OXA-50 isolates, which were amplified at a very low rate, suggesting the amplification was not specific. Further optimisation is ongoing to improve the assay specificity, assess the detection limit and design specific LAMP assays for *bla_{OXA-23}*, *bla_{OXA-25}* and *bla_{OXA-50}* if possible.

The task is still ongoing.

Task 2 (WP2-T2) : Evaluation and selection of sample preparation methods for use with laboratory and on-site tests (M31-M39)

NUI Galway has developed and evaluated a modified Chelex® 100 heat lysis extraction method for rapid on-site DNA extraction from bovine and porcine faecal samples. Optimisation and evaluation of this extraction method was carried out using *E. coli* CTX-M-1 and *E. coli* CTX-M-15 isolates spiked into bovine and porcine faecal samples. This extraction protocol can be carried out using portable equipment in approximately 15-20 min and the current resulting LEC-LAMP limit-of-detection is approximately 50 CFU/uL for *E. coli* CTX-M-1 and *E. coli* CTX-M-15 spiked into bovine or porcine faecal samples. Further evaluation of the modified Chelex® 100 heat lysis extraction method will be carried out using alpaca and porcine faecal samples previously confirmed to be positive for *E. coli* CTX-M-1 and *E. coli* CTX-M-15, supplied by FLI.

UoS has initiated work on the development and evaluation of water sample preparation methods for the detection of targeted AMR LAMP assays. Initial LAMP testing was performed using *mcr-1* LAMP and spiking of an *mcr-1* positive *E. coli* strain in tap water for validation of the sample preparation methods. After optimisations, a water sample preparation method has been finalised. The method involves centrifugation of the water samples to precipitate the bacterial content followed by resuspension in extraction buffer and heat shock treatment for bacterial lysis. The method is advantageous in being rapid, only taking 30 min. Spiked water samples were successfully amplified within 30 min of sample preparation and the *mcr-1* LAMP assay successfully detected up to 10² cfu/mL (as confirmed by culture) at 11 min of amplification. Further validation is ongoing to confirm detection limit and assess the sample preparation method using different water sources and AMR targets. The task is still ongoing.



Task 3 (WP2-T3) : Development and performance evaluation of multiplex assays for pathogens and resistance genes (M36-M45)

At NUIG the singleplex CTX-M-1, CTX-M-15 and IAC LEC-LAMP assays were combined to develop various duplex LEC-LAMP assays. The IAC LEC-LAMP assay was separately combined with the CTX-M-1 and CTX-M-15 LEC-LAMP assays, creating internally controlled two-target assays. No cross reactivity was observed between the IAC LEC-LAMP assay and the CTX-M-1 or CTX-M-15 LEC-LAMP assays, and no significant differences observed in limit-of-detection or time-to-positivity values between the singleplex and duplex assays. Combination of the CTX-M-1 and CTX-M-15 LEC-LAMP assays to develop a duplex CTX-M-1/15 LEC-LAMP assay required minor primer re-design. The duplex CTX-M-1/15 LEC-LAMP assay demonstrated similar limit-of-detection and time-to-positivity values to the corresponding singleplex assays, and also successful differentiation between all 10 *E. coli* CTX-M-1 isolates and all 25 *E. coli* CTX-M-15 isolates. Minor IAC LEC-LAMP probe redesign involving alteration of the fluorophore labelling is required for incorporation of the IAC LEC-LAMP assay with the duplex CTX-M-1/15 LEC-LAMP, to develop a triplex internally controlled CTX-M-1/15 LEC-LAMP assay. Future work will involve evaluation of this multiplex LEC-LAMP assay on-site.

WP5: Project Management (M25-M54)

Task 1 (WP5-T1) - Project Management (M25-M54)

A WorldCOM consortium KOM was held on January 26th and 27th 2020 in Brussels, at which WP and Task priorities were agreed. Subsequent WorldCOM consortium meetings have taken place via Zoom meetings held monthly from March 2020 (March 24th; April 28th; May 20th; June 2nd; October 2nd). The consortium had planned a further consortium meeting to review progress and for future project planning in Prague, during the OHEJP ASM in May. However, the planned meeting was replaced with a Zoom meeting on June 2nd.

A WorldCOM DMP based on the Horizon 2020 FAIR DMP was developed according to guidance provided by the OHEJP WP4 team in their D4.7 Guidelines for Data Management Plan implementation. The DMP was submitted (deliverable D-JPR15-AMR2.1-WP5.2) and is available on the DMP group of the OHEJP website. WorldCOM DMP leader Liam Burke attended online training on August 5th 2020 for the new OHEJP online data management platform CDP. A data management guide and DMP Excel template for WorldCOM has been prepared for task leaders to help them comply with the DMP. Liam Burke will update the CDP application with details of WorldCOM data throughout the project, with information provided to him by task leaders on their datasets using the Excel template. The OHEJP WP4 team reviewed and approved WorldCOM's online DMP in December 2020.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
13	D-JPR13-AMR2.1-WP1.1	Generation of a sequence database comprising publically available and newly generated sequences for E. coli, Salmonella and Campylobacter spp. and resistance genes CTX-M-15, NDM-5, KPC-2, OXA-48 and MCR-1.	M30 – June 30 th 2020		M33 – 30th September 2020		Zenodo link: https://doi.org/10.5281/zenodo.4019840 . Confidential on relevant WorldCOM OHEJP website section, and already shared with all consortium members during development.	3
13	D-JPR15-AMR2.1-WP1.2	Novel machine learning algorithms for the prediction and detection of AMR from genomic sequences.	M 36 – December 2020		M42 – June 2021	Delayed recruitment & and laboratory work	Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept	4



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
							confidential, in line with the OHEJP guidelines.	
13	D-JPR15-AMR2.1-WP5.1	Kick-off meeting organised	M26 – February 2020	M25 – January 27 th 2020	N/A	N/A	Confidential	Other – Project Management
13	D-JPR15-AMR2.1-WP5.2	Project Specific Data Management Plan prepared	M30 – June 30 th 2020	M33 – September 9 th 2020	N/A	No – due to CMP platform delay	Confidential on relevant WorldCOM OHEJP website section, and already shared with all consortium members during development.	3
13	D-JPR15-AMR2.1-WP5.3	Project review meeting organised	M33	M33 – September 9 th 2020	September 2020		Virtual Meeting held due to COVID-19 restrictions	Other – Project Management
13	D-JPR15-AMR2.1-WP5.4	Technical and financial reports prepared for submission	M 36 – December 2020		M 38	No	Confidential to WorldCOM and OHEJP	Other – Project Management



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
13	M-JPR13-AMR2.1-01	Generation of sequence information for pathogens and resistance genes of interest.	M30 – June 30th 2020	No	M42 – June 2021	Delayed due to COVID-19 and country-wide lockdowns.
13	M-JPR15-AMR2.1-02	Transfer of relevant sequence information for development and training of novel machine learning algorithms.	M36 – December 2020	No	M42 – June 2021	Delayed due to COVID-19 and late recruitment
13	M-JPR15-AMR2.1-03	Singleplex assays for the detection of pathogens including <i>Salmonella</i> , <i>Campylobacter</i> and <i>E. coli</i> and CTX-M-15, KPC-2, NDM-5, OXA-48 and MCR-1 genes	M36	No	M39 – March 2021	Singleplex assays for CTX-M-1/15, KPC, NDM-5, OXA-48, OXA-48-like and MCR-1/8/9 genes representing major AMR determinants. Delayed due to COVID-19 and country-wide lockdowns.
13	M-JPR15-AMR2.1-04	Sample preparation methods evaluated	M36	No	M39 – March 2021	Delayed due to COVID-19 and country-wide lock-downs.



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
13	M-JPR15-AMR2.1-05	Organisation of kick-off meeting	M26 February 2020	M25 – January 27 th 2020	N/A	N/A
13	M-JPR15-AMR2.1-06	Preparation of technical and financial reports for submission	M36	No	M38 – February 2021	

4. Publications and patents

No publications yet.



5. *On-going and planned collaborations with national or European projects or networks*

NUI Galway participates in the MedVetKlebs OHEJP project, and is a member of the Med-Vet-Net association. Prof Morris is part of a JPIAMR network aimed at identifying robust, measurable surveillance indicators and methodologies for the monitoring of antimicrobial resistance (AMR) in the environment.

FLI participates in the JPIAMR consortia HECTOR and OASIS. HECTOR aims to identify determinants of host restriction of *Escherichia coli* strains and their potential association with AMR transmission and prevalence. OASIS aims to develop an AMR surveillance strategy, based on the Lot Quality Assurance Sampling approach, in a One Health context, and applicable in high-, middle-, and low-income countries.

UCM participates in the AVANT EU project, in ARDIG and FARMED, both from OH EJP, as well as in the MSCA CARTNET. Currently, UCM is preparing a Project for the JPI-AMR call on One health interventions (13th call). Input from these projects will be relevant for WORLDCOM, and active participation between OHEJP projects will be encouraged for the benefit of OHEJP. Further, several TCs were organized between Dr. Burke and Prof. Gonzalez-Zorn to organize a visit to Madrid for training and organizing common research projects. The application for Short Term Missions in OH EJP has been approved, and Dr. Burke will visit the lab at UCM in Summer 2021. We hope that the epidemic situation will allow for this visit.

UT participates in the FED-AMR from OH EJP and Intereg Baltic Sea Region seed money project VETMED (Veterinary medication and its impact on antimicrobial resistance in the environment).

INSA also participates in other OHEJP projects, such as FED-AMR and FULL-FORCE.

We are fully convinced that at the end of the project the tool obtained will be very useful, so it will be presented to EFSA, ECDC, and EURL AMR.

JRP14-FULLFORCE

1. *Summary of the work carried out in year 3*

The goal of the Full Force project is to supply 17 EU partners with a technological toolbox and hands-on training in Single-Molecule Real-Time (SMRT) sequencing, and to apply this knowledge on six study cases and applications in metagenomics and AMR transmission models. Using this state-of-the-art technology, public health and veterinary labs will have the capacity to perform full-length sequencing, and gain detailed insight in mobile genetic elements (MGEs) which carry antimicrobial resistance and virulence genes within and across species.

Unfortunately, this project set-up has been hit hard by the Covid-19 pandemic. The basis of this project was supposed to be an on-site, three day workshop on SMRT sequencing, held at the State Serum Institute (SSI, DK), followed by a proficiency test to analyse each partner's capacity to perform SMRT sequencing. Due to restrictions imposed by all EU governments, we had to **postpone and reoriented this workshop to an online course held from September 7-8**. Moreover, all research activities were suspended for more than two months during lockdown, and many consortium members were reoriented towards Covid-19 surveillance. Likewise, the physical kick-off meeting, planned during ECCMID 2020 in Paris, was cancelled and replaced by a meeting in Brussels on October 8. It goes without saying that all this is causing significant delays in deliverables and milestones, as elaborated more in detail in the sections below. However, we are still confident that all goals of the Full Force project are still within reach.



- Applying intensive workload during the SMRT course, we still hope that our consortium partners with limited SMRT skills will be able to reach a sufficient technical level in plasmid sequencing. To maximize the output from this project, SSI scientists involved in WP1 are developing an easy-to-use software package (tentatively named Full Force Plasmid Assembler – FuFoPA), which will automatically perform hybrid assemblies through the build-in UniCycler program from a combination of short and long sequence reads.
- A proficiency test, to assess each institute’s capacity for SMRT sequencing, has been organised by SSI, using reference material from BfR. Samples are sent out in November 2020, and results are expected by the end of February 2021.
- Although WP2 (five cases studies implementing long-read sequencing on existing datasets) has suffered some delays, substantial progress has been made. The focus will be hypothesis generation based on short-read sequence data for the five defined case studies (T2.1-2.5). Short-read Illumina data will allow comparison of both AMR profile and total plasmid content, as well as relatedness of isolates . Based on these results a subset of isolates will be chosen for MinION runs.
- WP5 has not been impacted by the pandemic, as no lab work is required. The design of a transmission spread model of pAMR in the simulation framework SimInf has been initiated by collaboration between consortium partners.



2. Work carried out in the JRP, scientific results

WPO: Project Management (M25-M54)

This overarching WP ensures proper coordination at both the overall project and the individual WPs, as well as timely reporting of results and budgets according to the formal EU requirements.

JRP14-WP0-T1: Meetings and telcalls (M25-M54)

The two major consortium meetings, planned during ECCMID (Paris) and the SMRT sequencing workshop (SSI), were **postponed** due to Covid-19 measures. These were replaced by one-to-one teleconferences held by the PI with each individual partners, and by specific calls organized by task and WP leaders. An online workshop on SMRT sequencing (See WP1) was held is planned on September 7-8, and the online kick-off/progress meeting was organized on October 8 in Brussels.



The meeting outline was as follows, giving an overview of all ongoing work packages:

A full meeting report was published online at [10.5281/zenodo.4275887](https://zenodo.org/record/4275887)

JRP14-WP0-T2: Reporting (M25-M54)

Five deliverables from WPO and WP1 were uploaded to Zenodo. The nine month report was submitted in due time.

JRP14-WP0-T3: Central data repository (M25-M36)

FULL_FORCE will use a centralized data repository to upload sequence- and metadata which are generated during WP1 and WP2. Originally, we planned to use the AMR data hub of the European COMPARE Consortium and the European Nucleotide Archive (ENA). However, we were not successful in reaching an agreement with ENA, who is still negotiating single-subcontractor model for hubs created during COMPARE. Therefore, we have decided to use a commercial cloud tool (OwnCloud) for temporal data storage during Full Force. The platform was presented during the online workshop with a virtual tour of Owncloud, and an overview of the various groups and possibilities. This presentation was recorded, and shared online at [10.5281/zenodo.4277545](https://zenodo.org/record/4277545).



Full Force meeting – October 8th

- 10h State of the Full Force & Some admin issues (Pieter-Jan)
- 10h15 Long-read implementation (Henrik)
 - KEY DISCUSSION POINTS • wet lab protocol • FFPA installation • Proficiency test set-up • Workshop Part 2
- 11h Case Studies (Muna) and *in vitro* plasmid typing (Benoit)
 - KEY DISCUSSION POINTS • task hypothesis / strain selections • planning *in vitro* studies
- 13h30 Culture-independent approaches (Saria)
 - KEY DISCUSSION POINT • Validation - planning
- 13h50 AMR Transmission models (Stefan)
 - KEY DISCUSSION POINT • How to integrate with the rest of Full Force?
- 14h15 Short-term Planning and Closure (Pieter-Jan)



Interestingly, other EJP projects (e.g., ADONIS, FARMED) were inspired by Full Force, and chose to use the same platform for data sharing.

JRP14-WP0-T4: Data management plan(M25-M54)

In September 2020, a new Data Management Platform based on the CDP software was embraced by OHEJP. Full Force's PI followed the training coordinated by the EJP WP4 responsible, Géraldine Boseret. In September 2020, a complete DMP of Full Force was created and uploaded to <https://apps.lisam.com/app/#Apps/CDP>. This plan will be updated annually. As important part of the DMP, a framework agreement on Material Transfer was drafted, circulated and signed among all 18 participating institutions. This agreement covers all transfer of data and strains during Full Force.

Strain ID	Category	Type of data	Species	Matrix	Purpose	Custom	Dissemination	Sustainability
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1
[JRP14-WP0-T4]	Sequence data	Raw and processed data	Strep. pneumoniae	Genome	Genetic epidemiology	Genetic epidemiology	The results of this work... 2023-12-31	2 1

WP1: SMRT implementation (M25-M36)

In the pre-pandemic planning of Full Force, we scheduled three-day workshop on practical implementation of long-read sequencing in Copenhagen. The main goals was to get less advanced users of SMRT sequencing up to speed, and to use the technological know-how in WP2-4. However, as explained below, we were forced to postpone this workshop and suffer from delays in deliverables and milestones.

JRP14-WP1-T1: Methodology for MGE sequencing (M25-M27)

In two rounds of teleconferences (January and March 2020) led by RIVM, task participants shared experiences in SMRT sequencing. In short, SSI results are between N50 of 15-20k, RIVM results N50 of 35k. Regarding DNA extraction methodologies, there was a choice between faster (semi-)automated protocols using magnetic beads (as used by Sciensano, APHA and SSI), and more elaborate protocols based on DNA precipitation as used by RIVM. As N50 of the RIVM protocol is clearly higher, it was decided to go for the longer procedure to produce highest-quality data. Both protocols can be compared during the proficiency testing of Task 1.3.

A final consensus protocol for SMRT sequencing was elaborated by RIVM, based on the rapid library generation protocol from Nanopore. It is published under embargo at 10.5281/zenodo.4277521 and contains the following parts:

1. DNA isolation from strains cultured in liquid medium, using DNA/RNA shield and QuickExtract Bacterial DNA Extraction Solution
2. A classical DNA precipitation step, using 3M sodium acetate pH 5.2 for ethanol precipitation
3. SMRT run set-up using the Rapid Barcoding Sequencing system, using the manufacturers' recommendations.

This protocol (JRP-WP1.D3) was shared among all participating institutes alongside the instructions of the proficiency test (Task 1.2)

JRP14-WP1-T2: SMRT sequencing workshop (M28-M30)

The basis of this project was supposed to be an on-site, three day workshop on SMRT sequencing, held at the Statens Serum Institut (SSI, DK) in Q2 of 2020, followed by a proficiency test to analyse each partner's capacity to perform SMRT sequencing. Due to restrictions imposed by all EU governments, we had to postpone and reorient this workshop to an online course to be held on September 7-9, 2020.



A	B	str
Post-sequencing reporting	strain1	
Institution: <i>Please insert your institution here!!</i>		
DNA purification method (RIVM/other)	RIVM	RIV
Flow cell total output		
Total raw output from entire flow cell in gigabasepair?		15.2
Number of barcodes/samples in flow cell?		5
raw ONT output before filtering (NanoPlot on sequencing_summary.txt)		
Guppy version used for basecalling?		v4.0.14
basecalling configuration? (fast, high-accuracy, high-accuracy methylation-aware)		hac_m
number of reads?		200.000
number of bases?		3.000.000.000
median fragment length?		6.200
median quality?		10.1
read length N50?		15.000
longest read?		150.000
ONT after filtering to q≥8 (NanoPlot on filtered barcodeXX.fastq.gz)		
tools used for filtering? (program, version, used options)		NanoFilt
number of reads?		160.000
number of bases?		270.000.000
median fragment length?		6.200
median quality?		10.1
read length N50?		15.000
longest read?		150.000
Short read technology used? (Illumina/Ion Torrent)		
		Illumina
raw short reads before filtering		
number of reads?		1.500.000
number of bases?		225.000.000
short reads after filtering		
tools used for filtering? (program, version, used options)		rimmomatic v3.6
adaptor removal? (yes/no)		yes
minimum quality for filtering?		q20 end-trim
minimum length for filtering?		140
number of reads?		800.000
number of bases?		120.000.000
hybrid assembly		
tools used for assembly? (program, version, used options)		FFPA_v1.py
number of contigs? (circular)		4
number of contigs? (linear >10 kb)		1
number of contigs? (linear <10 kb)		3
total length of contigs? (sum of contig lengths)		5.3
identified ESBL/pAmpC gene		CTX-M-15
identified replicon of plasmid ESBL/pAmpC gene		incI1
exact length of contig carrying ESBL/pAmpC gene		112.456
name, which you choose to give the contig carrying ESBL/pAmpC gene to submit for OwnC		p1_SSI_RIVM_R
ONT coverage calculation		
Short reads coverage calculation		50.943.396
Did you hand-polish the plasmid sequence?		42.452.830
Did you run other polishing tools on the plasmid sequence?		yes
If yes, which polishing tools and how?		no
		n/a

Figure. Some post-sequencing QC parameters, which will be assessed during the EQA.

WP2: Genome studies (M25-M54)

In WP2, the acquired SMRT toolbox will be applied in the (re-)sequencing of AMR strains from various research and surveillance projects in WP2 including EU projects such as EFFORT, COMPARE, ENGAGE and ARDIG, as well as national and EU surveillance activities for which short read sequences are available. Given the postponed SMRT workshop, focus in WP2 in the first months of Full Force was lead on **hypothesis generation based on short-read sequence data** for the five defined studies cases (T2.1-2.5). Short-read Illumina data will allow comparison of total plasmid content and phylogenetic relatedness of isolates and based on those results choose a subset of isolates for MinION runs.

JRP14-WP2-T1: MGE evolution in longitudinal sample sets (ARDIG, ABRES) (M25-M54)

During a teleconference coordinated by Muna Anjum (APHA, UK), task participants decided to focus on plasmid evolution in longitudinal datasets from livestock and human samples. It was agreed to focus on plasmid evolution within the IncI1 plasmids encoding bla_{CTX-M-15}, with main research question being:

- What is the European diversity in the complete sequences of these plasmids?
- Can specific factors be recognized in the most successful plasmids?
- How have these plasmids evolved over the past decade?

All partners will examine short read sequence data from their databases to enable selection of isolates harbouring IncI1 plasmids encoding bla_{CTX-M-15} for long-read sequencing. Based on the available data, it will be decided if isolates before 2010, other ESBLs and non-ESBLs will be included in the final dataset.



JRP14-WP2-T2: MGE evolution in cross-sectional data sets (EFFORT, ENGAGE & National Surveillance) (M28-M54)

Jens-Andre Hammerl (Bfr, GER) coordinates the group studying cross-sectional datasets. In a series of teleconferences, it was decided to focus on IncK plasmids (with/without CMY-2). The main issue was the preclassification as IncB/O/K/Z-positive by PlasmidFinder, while this task would focus only on IncK plasmids. Therefore, more discussion is needed on the identification of reliable detection markers for IncK classification, like RNAI or phylogeny.

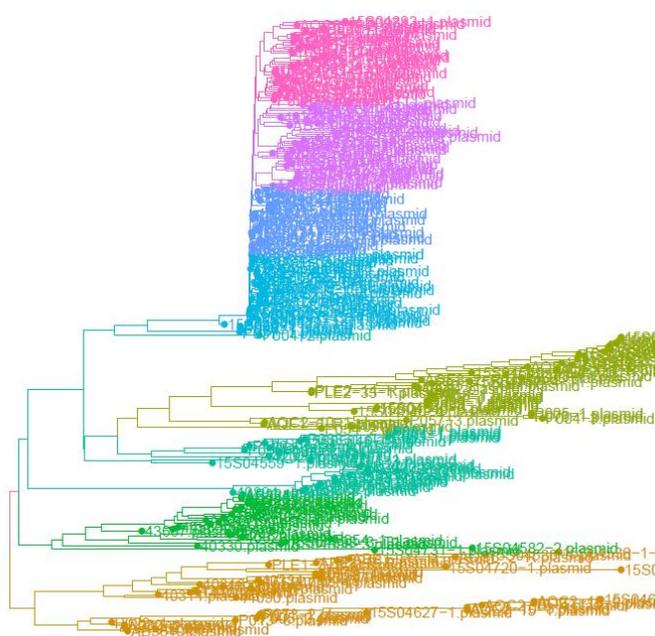
JRP14-WP2-T3: Klebsiella pneumoniae: the canary in the coalmine (M28-M54)

Alma Brolund (PHAS) organised teleconferences with task participants, in which it was decided to focus on *K. pneumoniae* isolates with reduced susceptibility to carbapemems. Participants from Norway, Denmark, Sweden, The Netherlands and Portugal agreed that isolates with proposed high variation in genetic context were seen as most interesting to study. A separate work group will be initiated where *Klebsiella* isolates from the animal (and environmental?) sector can be further discussed. A first analysis of diversity/overlap between these is performed by RIVM. The task leader sent around a metadata sheet serving as basis for isolate inclusion.

JRP14-WP2-T3: ESBL-producing Enterobacteriaceae in horses – A separated epidemiology of plasmids? (M28-M54)

SVA (Stefan Borjesson) coordinated a teleconference with task participants, in which it was decided to focus on *E. coli* isolates from horses encoding bla_{CTX-M-1} and bla_{SHV-12} genes. All short-read sequencing has been performed, and data has been shared on OwnCloud.

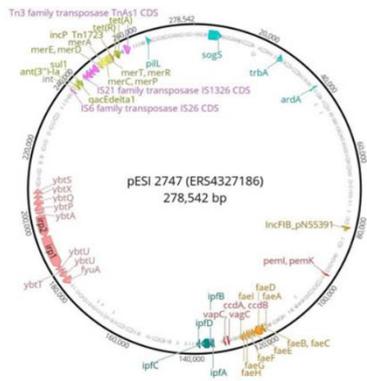
A first rough analysis has been performed by Aldert Zomer (Utrecht University), which will serve to select representative isolates of each cluster for long read sequencing (see figure on the left).



It is also worth noting that task leader (Stefan Borjesson) has been replaced by Joost Hendrickx (RIVM, NL) due to changes in job positions.

JRP14-WP2-T5: Salmonella Infantis and S. Kentucky across reservoirs: role of MGEs (M28-M54)

ISS (Laura Villa) coordinated a teleconference among task participants, in which it was decided to focus on the pESI virulence plasmid of *Salmonella infantis* from animal and human origin. All task participants completed a metadata sheet, and samples were selected for short-read sequencing which should be completed by M36. It was decided that each partner selects 20-30 *S. Infantis* isolates from their collection, with maximal diversity in selection (year/source), and focusing on the pESI markers: SMX-TET-SUL resistance (NAL/CIP), and/or the presence of IncFIB(pN55391), tet(A), sul1 and dfrA14.



JRP14-WP2-T6 :Evaluation of publicly available and in-house tools for MGE typing (M30-M54)

This task has not yet been initiated at the time of writing.



WP3: Culture-independent typing and metagenomics (M30-M54)

The development of culture-independent methods to detect, quantify and identify bacterial plasmids carrying antimicrobial resistance genes is greatly encouraged for future surveillance efforts. This WP will focus on (i) enhanced mining of existing metagenomics data, including those from the EFFORT and COMPARE projects, and correlate this to AMR-gene abundance, and (ii) development of diagnostic tools for direct identification of MGE/plasmid identifications from various sample types.

JRP14-WP3-T1: MGE Analyses in metagenomics datasets (M30-M54)

In the first annual year, it was planned to develop, evaluate and make available a database of MGEs detection from single isolates and metagenomics datasets. In research performed by Markus H K Johansson (DTU, DK), this databases was established and consists of ~4450 MGE sequences that originate from ~1050 different species. They contain several types of mobile elements:

- Insertion sequences (ISs) are among the smallest types of iMGEs. They are often composed of a transposase gene flanked by two inverted repeats (IRs). They are notable for their ability to modulate gene expression and promote mobility by forming composite transposons (ComTns), translocatable units (TUs) and in the case of elements from the IS26 family pseudo-composite transposons (PCTs).
- Unit transposons (Tns) are generally flanked by IRs and carry a transposase gene. They usually carry a resolvase gene, accessory genes and/or additional iMGEs. Miniature Inverted Repeats (MITEs) are non-autonomous ISs or Tns that have undergone deletions in their core genes but have retained the IR and can form ComTn-like structures.
- Integrative Conjugative Elements (ICEs), Cis-Mobilizable Elements (CIMEs) and Integrative Mobilizable Elements (IMEs) are larger iMGEs capable of conjugation. They can either conjugate independently or be co-mobilized by conjugation of other elements. These elements carry many accessory genes and other MGEs.

In the following years, this database will be updated and implemented in the evaluation of bioinformatics pipelines for quantification of MGE in metagenomics datasets, and in determining the presence and abundance of MGEs in public and de novo generated metagenomics datasets.

JRP14-WP3-T2: Culture-independent methods for plasmid identification (M30-M54)

Genomic Epidemiology, DTU, DK (Saria Otani *et. al.*) has developed a plasmidome-DNA extraction protocol from complex biomes (*e.g.*, sewage and faeces). The protocol allows plasmid DNA isolations, degrades linear gDNA and enriches circular elements in metagenomics samples. In short, plasmid DNA isolation was performed on individual sewage pellets (420 mg) using Plasmid Purification Mini Kit (Qiagen, Cat No./ID: 12123) following the manufacturer's instruction with the following modifications: protein precipitation with P3 buffer mixture was incubated on ice for 20 minutes, elution buffer QF and EB buffer were preheated at 65°C prior applications, and the DNA pellet washing step was done using ice-cold 70% ethanol after isopropanol precipitation. LyseBlue dye for cell lysis indication was added, and all buffer volumes were adjusted to sewage pellet weight. The plasmid DNA pellet was dissolved in 25 µl EB buffer for 1 hour at room temperature. Linear chromosomal DNA was reduced by Plasmid-Safe ATP-Dependent DNase (Epicentre, USA) treatment for 24 hours at 37°C. The DNase was inactivated at 70°C for 30 minutes. Circular DNA was enriched using phi29 DNA polymerase (New England Biolabs, USA) following the manufacturer's instructions, similar to as previously described. This can be combined with an assembly workflow, utilizing the long-read length of Oxford Nanopore sequencer. The pipeline was already tested at DTU using sewage samples from 22 countries (5 continents) as part of DTU global sewage surveillance project. 105 Gpb Oxford Nanopore data were obtained and 159.322 circular contigs were assembled. Data annotation is in progress to further validate the pipeline.



WP4: Functional characterization of AMR mobile genetic elements (MGE)-carrying AMR genes and bacterial host associations (M25-M54)

The overall goals of this WP are to (i) gain knowledge on molecular mechanisms of spread and persistence of main MGEs carrying critically/highly important antimicrobial resistances, (ii) identify key molecular interactions between AMR-MGEs and bacterial host important for dissemination and maintenance.

JRP14-WP4-T1: Selection of MGEs and host strains for detailed characterization (M25-M42)

Given the delay caused by postponing both the kickoff meeting as well as the workshop on SMRT sequencing, it was decided that the selection of MGEs will be performed within tasks 2.1-2.5. Therefore, a current focus lies on pESI of *S. Infantis*, pKpQIL of KPC-producing *Klebsiella pneumoniae*, IncX3-SHV and IncHI1-CTX-M plasmids from horses, Inc1-AmpC/ESBL plasmids of *E. coli*. To allow smooth exchange of reference strains and/or donor-acceptor strains for conjugation experiments, a Material Transfer Agreement has been approved by all WP4 partners. A meeting on available reference strains and protocol is planned in early 2021. Drafting a common conjugation protocol is in progress that will be available on January 2021 for discussion/improvement and then to be shared among involved partners.

JRP14-WP4-T2: Functional characterization of MGEs (M31-M54)

This task has not yet been initiated at the time of writing.

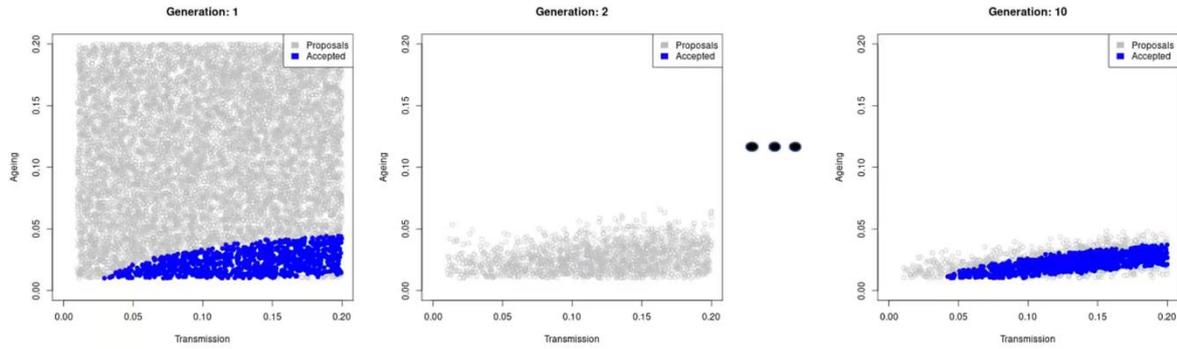
WP5: Modelling (M25-M54)

The objectives of WP5 are to address: i) gaps in quantitative knowledge on the spread of pAMR which will be essential to direct future focused research, ii) insight in the uncertainty around the effect of measures reducing pAMR prevalence in the food production chains, and iii) identification of key elements in the production chains to mitigate the risk of human exposure.

JRP14-WP5-T1: Model design for AMR transmission (M25-M42)

The design of a transmission spread model of pAMR in the simulation framework SimInf has been initiated. SVA (Stefan Widgren) has coordinated two teleconferences with task participants. The first teleconference was a startup meeting and the second teleconference was a meeting to discuss horizontal vs. vertical AMR transmission.

A necessary but challenging step in stochastic modelling is to determine parameters such that the model generates data that are consistent with observations. Parameterization is preferably conducted within a Bayesian framework and in WP5 we are focusing on using Approximate Bayesian computation (ABC), a recent computational approach for simulation-based inference. In the first annual year, development is underway to add ABC functionality to the open-source SimInf modelling R package (<https://github.com/stewid/SimInf>). The figure below illustrates using ABC in SimInf to fit parameters from data of infected chicken broilers published in Dame-Korevar et al. (2017), and a model with susceptible (S) and infected (I) chicken, showing (for example) that the ageing parameter has a tighter



posterior distribution compared to the transmission parameter. Work is ongoing to identify and include other sources of broiler data for parameterisation of more complex models.

JRP14-WP5-T2: Exposure assessment of horizontal and vertical transmitted AMR (M25-M54)

This task has not yet been initiated at the time of writing.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
14	D-JRP14-WP0.D1	Start-up meeting report	M27	M34		YES	Confidential due to research updates; 10.5281/zenodo.4275887	8
14	D-JRP14-WP0.D2	Financial and activity report Y3	M36	M36			The annual 12M will be published on time.	
14	D-JRP14-WP0.D3	Recorded webinar tutorial ENA AMR data hub	M27	M33		YES	Public; 10.5281/zenodo.4277545	3
14	D-JRP14-WP1.D1	Teleconference to assess required protocols and infrastructure	M25	M26			Public; 10.5281/zenodo.3733393	8
14	D-JRP14-WP1.D2	Teleconference to discuss proposed protocols and infrastructure (follow-up)	M26	M26			Public; 10.5281/zenodo.3759335	8
14	D-JRP14-WP1.D3	Completion of final protocol for SMRT sequencing	M27	M34		YES	CONFIDENTIAL UNTIL PUBLICATION; 10.5281/zenodo.4277521	2
14	D-JRP14-WP1.D4	Invitation to workshop delivered to all participating institutions	M28	M27			Public; 10.5281/zenodo.3693741	8



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
14	D-JRP14-WP1.D5	Completion of workshop organization plan including selection of course material	M29	M35		YES	CONFIDENTIAL UNTIL PUBLICATION; 10.5281/zenodo.4290698	8
14	D-JRP14-WP1.D6	Selection of proficiency test data	M32	M35		YES	CONFIDENTIAL UNTIL PUBLICATION; 10.5281/zenodo.4290707	8
14	D-JRP14-WP1.D7	Delivery of analysis results of proficiency test by partners to SSI	M33		M39	YES	CONFIDENTIAL UNTIL PUBLICATION; Proficiency test will be organised as follow-up of the postponed online course. Therefore, this delivery dates shifts backwards.	8
14	D-JRP14-WP1.D8	Completion of final report of proficiency tests	M36		M46	YES	Proficiency test is ongoing, but suffered from some delays due to COVID-19	
14	D-JRP14-WP2.D1	Submission of sequence- and metadata of longitudinal samples at ENA hub	M33		M38		CONFIDENTIAL UNTIL PUBLICATION; Given the lab closures in M28-30, we expect all short-read sequencing now to be done by M38.	3
14	D-JRP14-WP2.D2	Submission of sequence- and metadata of cross-sectional samples at ENA hub	M33		M38	YES	CONFIDENTIAL UNTIL PUBLICATION; Given the lab closures in M28-30, we expect all short-read sequencing now to be done by M38.	3
14	D-JRP14-WP2.D3	Submission of sequence- and metadata of K. pneumoniae samples at ENA hub	M36		M38	YES	CONFIDENTIAL UNTIL PUBLICATION; Given the lab closures in M28-30, we expect all short-read sequencing now to be done by M38.	3



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
14	D-JRP14-WP2.D4	Submission of sequence- and metadata of <i>S. enterica</i> samples at ENA hub	M36		M38	YES	CONFIDENTIAL UNTIL PUBLICATION; Given the lab closures in M28-30, we expect all short-read sequencing now to be done by M38.	3
14	D-JRP14-WP2.D5	Submission of sequence- and metadata of horse-related samples at ENA hub	M36		M38	YES	CONFIDENTIAL UNTIL PUBLICATION; Given the lab closures in M28-30, we expect all short-read sequencing now to be done by M38.	3
14	D-JRP14-WP2.D6	List of relevant publicly available and in-house developed tools.	M36		M42		Taskgroup will assembly first time at the beginning of 2021	2
14	JRP14-WP3.D1	Database construction tailored at MGEs	M36	M36			Public; 10.5281/zenodo.4305711	3
14	D-JRP14-WP3.D2	Protocol for plasmid DNA extraction from environmental samples	M34		M42	YES	Delay caused by lab closure, due to COVID-19	2
14	D-JRP14-WP4.D1	First collection of type-materials (MGEs and strains) to be shared between involved partners	M36		M42		Taskgroup will assembly first time at the beginning of 2021	3
14	D-JRP14-W5.D1	Source code of the implementation of a SimInf model designed for pAMR transmission.	M34	M36			Public; 10.5281/zenodo.4305750	1

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance



data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
14	M-JRP14-M1	Creation of specific data hubs in ENA AMR hub	M27	Yes		Password-protected data hubs for each individual task were created at OwnCloud (not ENA)
14	M-JRP14-M2	A teleconference or physical meeting on horizontal vs. vertical AMR transmission	M27	Yes		
14	M-JRP14-M3	Selection of MGEs and host strains to be studied T4.2	M28	Yes		
14	M-JRP14-M4	A teleconference or physical meeting on input/output relationship between SimInf and sQMRA	M29	Yes		
14	M-JRP14-M5	Publication of first version of data management plan	M30	Yes		
14	M-JRP14-M6	3-days workshop on SMRT sequencing event	M30	Yes		
14	M-JRP14-M7	Shipment of proficiency test data and strains	M32	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
	M-JRP14-M8	Sharing of protocols, recipient- and host-strains, molecular tools	M33	No	M42	Due to delays in WP2
14	M-JRP14-M9	An implementation of a SimInf model designed for pAMR transmission to be studied in T5.1	M33	Yes		
14	M-JRP14-M10	Final selection of longitudinal samples for SMRT sequencing	M34	No	M39	Will be based on phylogenetic analyses of short-read data, foreseen for M39
14	M-JRP14-M11	Final selection of cross-sectional samples for SMRT sequencing	M34	No	M39	Will be based on phylogenetic analyses of short-read data, foreseen for M39
14	M-JRP14-M12	Final selection of <i>K. pneumoniae</i> samples for SMRT sequencing	M34	No	M39	Will be based on phylogenetic analyses of short-read data, foreseen for M39
14	M-JRP14-M13	Final selection of <i>S. enterica</i> samples for SMRT sequencing	M34	No	M39	Will be based on phylogenetic analyses of short-read data, foreseen for M39
14	M-JRP14-M14	Final selection of horse-related samples for SMRT sequencing	M34	No	M39	Will be based on phylogenetic analyses of short-read data, foreseen for M39
14	M-JRP14-M15	Analysis of proficiency test data by all partners	M35	No	M38	New deadline for proficiency test
14	M-JRP14-M16	Individual reports of proficiency test sent to partners	M36	No	M40	Update reporting date



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
14	M-JRP14-M17	Successful adaptation of plasmid purification protocol to field samples	M36	No	M42	Delay due to lab closure (COVID-19)

4. Publications and patents

No publications & additional output thus far.



5. *On-going and planned collaborations with national or European projects or networks*

The SOLIDNESS network (JPIAMR, 2019-2020) which grouped experts in sequencing, plasmid biology and bioinformatics, and aims to streamline procedures for MGE sequencing. We build on their expertise to organise the PT.

- Cross-sectional and longitudinal bacterial samples of ENGAGE, EFFORT (Horizon 2020, 2013-2018) and ARDIG (OHEJP, JRP2, 2018-2020) projects will be selected for long-read sequencing during WP2.
- The KENTUCKY PhD project (OHEJP, 2020-2022) will use fully sequenced *S. Kentucky* strains (T2.4) to focus on the cell biology behind MGE transfer.
- Potential collaborations with ECDC and EFSA might be envisioned, for sustainable implementation of long-read sequencing technology in surveillance of AMR in Europe.

JRP15-FEDAMR

1. *Summary of the work carried out in year 3*

In the FED-AMR project, extracellular DNA (exDNA) is presented as an important environmental reservoir for antimicrobial resistance genes (ARGs). Furthermore, bacterial transformation contributes to the horizontal gene transfer (HGT) of antimicrobial resistance genes (ARGs). However, empirical data on the impact of bacterial transformation in the environment are lacking.

Since the beginning of the project on 1st January 2020, and to fulfil its aims, we are conducting a longitudinal study over a one-year crop-growing period by monitoring and comparing 11 different matrices (“Compartments”) from agricultural research areas (or alternatively, from production units) located in four European regions. Indeed, the FED-AMR project aims are 1) to analyse microbial biodiversity and ARGs along food/feed chain, 2) to evaluate the relevance of free exDNA in the HGT of ARGs over ecosystem boundaries 3) to identify points for intervention to reduce the spread of antimicrobial resistance (AMR) via exDNA 4) to compare geographical differences and trends in AMR and antimicrobials in the natural environment 5) to put a focus on multidrug and emerging resistances.

Overall, we have been able to coordinate the sampling (WP1) and generate all sampling protocols (WP2) for the 11 compartments, the general culture protocol (WP2) and the *C. difficile* culture protocol (WP3), as well as the three protocols for analysis of antibiotics, elements and herbicides in environmental samples (WP4). Also, we finalized the description of the catchment areas and collectors within the four European regions (WP2), the guidelines for sample distribution, transportation and conservation (WP2), the sampling timeline for each collector (WP2) and the unified sample references (WP2). In addition, protocols including extracellular DNA extraction, Whole Genome Sequencing (WGS) of bacterial strains and shotgun metagenomics are finished as well (WP2). The latter will produce results that will be used to get quantitative data instead of qPCR.

All FED-AMR partners have been affected by COVID-19 as many have been directly involved in the work and laboratory activities, which have been shut down to handle only essential work. Additionally, in many cases, sample collection, laboratory work and analysis of the samples were delayed, as well as the recruitment of post-doctoral fellows, PhD students or technicians. However, very recently, 10-FLI and 13-SSI have recruited a PhD student each (Ines Dost and Semeh Bejaoui, respectively), 25-NUIG and 36-INSA a MSc student each (Charitini Nikolaidou and Rita Castro, respectively). Likewise, a PhD student (Krõõt Arbo) and two technicians (Jelena Kiprovskaia and Viia Kõiv) have been recruited by 14-



UTARTU. Two Postdocs (Marwa Hassan and Brian Gardner, respectively) are already well integrated in the FED-AMR project at 23-UoS.

As described in WP1 and WP2 (see corresponding WPs below), the huge amount of experimental and sampling protocols needed exceeded the expectations. However, for these and all WPs, we are committed to compensate the delay in milestones and deliverables. Nevertheless, all partners hope that the period of the project can be extended after June 2022, as this will help in weighting decisions, in more detailed analysis of results and in a greater dissemination, so that the project can have an improved impact on decision makers.

2. Work carried out in the JRP, scientific results

All WPs have started, but with delays in several tasks, due to the staff shift to prioritized COVID tasks. The recruitment of the initially planned research staff has not happened until recently. The budget adjustments are an ongoing task due to necessary improvements in the analytic field. This is an ongoing process to be finalised after the twelve month report (to be expected in early 2021, Y4) in accordance with all partners and the SSB. Additional information on the progress can be found below for each task and subtask in the different WPs.

WP1: Project Management and Communication (M25-M54)

AGES is responsible for the project management and for all communication within FED-AMR (WP1). The leader of the WP1 is now Werner Ruppitsch and his deputy leader is Adriana Cabal Rosel.

JRP15-WP1-T1: Scientific Management (M25-M54)

Manuela Caniça (36-INSA) is now the responsible person for the scientific management of the project, acting as leader of task 1 within this WP (**WP1-T1**). She is supported by her deputy leader Adriana Cabal Rosel (2-AGES). This task is **ongoing** and it will comprise the whole project duration (M25 to M54). Up to now, this task together with task **WP1-T2** has involved the creation of a Scientific Supervisory Board (SSB) composed by experts within the consortium and the nomination of the local administrative representatives. The outcome of both tasks can be seen as part of the deliverable D-JRP15-FED-AMR - WP1.1

JRP15-WP1-T1.1: Coordination of sampling, laboratory experiments and building a database (M25-M33)

Manuela Caniça (36-INSA) is now the responsible person for this **ongoing** sub-task, and her sub task deputy leader Adriana Cabal Rosel (2-AGES) supports her. Initially, this subtask was planned to start in M25 and to finish in M28. The task started on the expected month (M25), but it ended in M33. This was partially due to SARS-CoV-2 crisis that prevented many partners to hire staff, work in the laboratory or coordinating sampling campaigns.

The deliverable associated to this sub-task (now named as D-JRP15-FED-AMR-WP1.2) was delayed as well because it is strongly associated with WP2 and its corresponding deliverable (D-JRP15-FED-AMR-WP2.1). Both deliverables were finished by M33.

Contributing partners collaborated to generate new guidelines and harmonized protocols for sampling and experimental analysis. The high amount of newly designed protocols exceeded the initial expectations and contributed to this delay, even when using some of the available protocols from the EFFORT and COMPARE projects and from reference institutions (e.g. DTU in Denmark). Protocols included those related with sampling in the 11 compartments, those for the molecular techniques or bacterial culture, among others.

In this sub-task (WP1-T1.1), the leader and the deputy leader, coordinated the sampling and the experimental protocols. Within the sampling, project partners were asked about the type of samples they could collect. As stated in the project proposal, 11 different compartments were selected for each



of four European Regions and other countries were also associated to complement sampling. However, not all partners could collect samples from each of the compartments, as planned in the proposal. All the experimental protocols are now finished. For additional information, see WP2.

JRP15-WP1-T1.2: Webinar forum and Skype meetings for instant scientific interactions (M30-M50)

Task WP1-T1.2 is **ongoing** and four webinars were already held. The first webinar on the topic “Environmental reservoirs of antimicrobial resistance genes” took part of the deliverable related to this task (D-JRP15-FED-AMR-WP1.4), which took place in M31. Professor Elisabeth Wellington was proposed as the first speaker. The second webinar was celebrated in M33 and it collected information about the first metagenomics and gene enrichment tests carried out with samples from the FED-AMR project. For the third webinar, which also took place in M33, the task leader (Mónica Oleastro, 36-INSA) proposed Markus Wögerbauer as speaker with the topic “Extracellular DNA in natural environments: a neglected source for antibiotic resistance?” and the fourth presenter Professor David Weissbrodt shared his experience in M36 on the topic “Biotechnology and Safety: Tracking and Analyzing Free-floating Extracellular DNA across Urban Waterways”. All presentations were recorded with permission of speakers and participants. The recordings were made available to the consortium and are confidential, as they are only meant for the consortium members that could not take part or would like to revisit the webinar. As a general rule, the dissemination outside of the FED-AMR project is not permitted.

Regarding the online meetings, the vast majority of project partners have attended through a different online platform other than Skype at the beginning of each month, since the kick off meeting. Minutes of the meetings were registered and shared with the project partners for approval. A definitive version incorporating all suggestions received was distributed and published in the AGES site (<https://fed-amr.ages.at>). In addition, weekly calls were arranged between the deputy leader of WP1 and the leader of WP1-T1.1 to discuss the evolution of the different WPs and tasks.

JRP15-WP1-T1.3: Project Meetings (M25-M52)

The Kick off meeting took place in Vienna at the end of the M25. The next one is planned in Lisbon in M41. This task is **ongoing**.

JRP15-WP1-T2: Administrative Management (M25-M54)

The administrative management (AM) is supported by the infrastructure of the AGES Academy and the secretariat of the AGES knowledge transfer department. The coordination of joint activities in the frame of the FED-AMR project is being coordinated by AGES. Additionally, each partner had appointed an Administrative Representative who is and will be in direct contact with the AGES AM whenever necessary.

The AM is also responsible for the internal communication and a proactive time and risk management of the project. An important part of the administrative management is the coordination of the project and the implementation of sound project management practices, such as an accessible communication structure and facilitating internal communication, assistance of the partners with different administrative tasks and overall assistance of the project and the WP leads. Furthermore, considerable effort was put in the assistance with budgetary issues for the entire project and specific partners, which was conducted in close cooperation with the project lead, the scientific manager and the partners in question.

A risk management on a daily basis is also taken care of by the administrative team, in coordination with the leading staff. An overarching risk management strategy for the project is being put in place by the AM and the Scientific Manager (SM), in consultation with the Scientific Supervisory Board (SSB) to ensure that adverse situations are properly handled along the course of the project, which will be highlighted in the Data Management Plan. This task is **ongoing**.



JRP15-WP1-T3: Data and Protocol Management (M25-M52)

The Data and Protocol Management Plan was delayed due to the new platform made accessible by OHEJP WP4 and therefore, its deliverable was sent to OHEJP by M34. The DMP leader attended online training on August 5th 2020 for the new OHEJP data management platform CDP, provided by the OHEJP WP4 team. The CDP application was adapted and updated with details of FED-AMR data throughout the project, with information provided to the leader and deputy leader by task leaders on their datasets. The first DMP was generated and it will be regularly updated till the end of the project with the data obtained. This task is **ongoing**.

WP2: Field experiments: Determination of the naturally occurring ARG background load and microbial biodiversity in the tested environmental compartments (M25-M50)

WP2 takes place over the first, second and third year of the project (Y3, Y4 and Y5). The end of this WP has been postponed to M50. Thus, tasks WP2-T1 and WP2-T2 took place in the first year (Y3). Tasks WP2-T3 and WP2-T4 take place over the first and second year of the project (Y3 and Y4). Tasks WP2-T5 and WP2-T6 take place over the second year (Y4). Task WP2-T7 was delayed up to the third year, so now is planned to take place over the three years of the project (Y3, Y4 and Y5).

In this WP the overall prevalence, quantity and movement of AMR via free exDNA will be monitored along different compartments of the food/feed chain within the HOAL catchment: “human/animal gut -> manure -> soil -> crop -> drainage -> surface water -> groundwater -> human/animal”. All matrices (pig faeces, manure, agricultural soil, crop plants, drainage, surface and ground-water) will be analysed for the presence of clinically relevant ARGs encoded on free exDNA taking into special account antimicrobial treatments of the pig herds. Cultivable bacteria from soil and gut will be characterized with standard microbiological methods. The results will be compared with data obtained from similar testing locations and environmental compartments from different regions. The establishment of the bacterial biodiversity in the tested compartments will be carried out, as well as the identification of the most prevalent naturally transformable species in agricultural soils and the monitoring of their fate in different environmental compartments.

JRP15-WP2-T1: Assemble list of sampling compartments and points. Determination of test areas representative for the European regions (North, West, East, South) (M25-M30)

The consortium members contributed to compile the final list of sampling compartments and points, having been carried out according to the collectors from East (Czech Republic, Poland), West (Austria, Ireland and Great Britain), North (Estonia and Norway), and South (Portugal). The final list of compartments according to partners was achieved and is already available to the participants via the AGES site (<https://fed-amr.ages.at>), as well as the sample timeline by collectors, the sample distribution, transport and conservation of sampling by compartment. A unique identifier by compartment and time point was given to all samples planned to be collected in the frame of the FED-AMR project, ensuring a correct traceability of all samples from their collection until their processing and analysis at the laboratory. The description of the HOALs and main catchment areas within FED-AMR was elaborated. Participants of WP2 (2-AGES, 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI, 36-INSA) provided input and advice according to their expertise and involvement in the sampling. The sampling list provided in this task supports harmonization of testing procedures and enhances comparability of the results obtained from those regions of Europe. The leader (36-INSA, Manuela Canica) and deputy leader (2-AGES, Adriana Cabal) of this task provided preparatory and final work and the remaining participants took part, namely during the teleconferences made monthly by the project leader, with all members of the consortium. The end month was delayed to 30. This task is **finished**.



JRP15-WP2-T2: Establish common protocol for sampling and data analyses to facilitate comparability of the results between European test areas (North, West, East, South) and local sampling locations (M25-M33)

These common protocols support harmonization of testing procedures and enhance comparability of the results obtained from different regions of Europe. The task was finished later than planned (M33) due to the numerous compartments and procedures involved. 2-AGES, 7-SZU, 14-UT, 23-UoS, 25-NUIG, 33-NVI and 36-INSA were the partner institutes that participated more actively in the execution of this task. An example of cooperation of non-WP2 partners in this task exists for one WP5 Postdoc, who was engaged in helping revising WP2 protocols in which 23-UoS was involved (e.g. pig feces, manure and culture, and in susceptibility testing protocol).

First protocols for sample collection were made available by the leader (36-INSA) and the deputy leader (2-AGES). Therefore, all compartments have already their respective protocol(s) of sampling, which were uploaded and regularly updated on AGES FED-AMR internal website (<https://fed-amr.ages.at>), as well as in the OHEJP website, available for all FED-AMR partners at both. Culture and antimicrobial susceptibility testing protocols provided a harmonizing framework in the microbiology laboratory, for bacterial isolation and identification, as well as for antibiotic resistance determination in the strains obtained from the different collected samples, such as faeces (from pigs, wild animals and farmers), manure, soil, water, crops and feed. This procedure is applied to samples that may harbour bacterial strains of human, veterinary, zoonotic or environmental origin and aims at identifying six bacterial species of clinical relevance for humans (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp, *Staphylococcus aureus* methicillin resistant, and *Enterococcus faecium* and *E. faecalis* vancomycin resistant). The protocol for DNA extractions (eDNA and total DNA) was finished for samples of all compartments (pig and wild animals feces, and feces from farmers, manure, soil, crops, river water, groundwater, wastewater and feed). The protocols for molecular and genomic analysis were finished (see task WP2-T3, WP2-T3.2 and WP2-T4). All these protocols were part of the deliverable D-JRP15-FED-AMR-WP2.1. By now, the FED-AMR consortium has decided after voting to dispense with qPCR; the main reason is that in order to carry out the detection of resistance genes in extracellular DNA, the gene enrichment technique can target more than 8,000 ARGs per sample while a qPCR supports only few AMR markers. In addition, all the institutes are dedicating much time of their work in COVID diagnosis and therefore this new strategy will now provide both the consortium and the project not only with more data but also with faster results, which is actually quite relevant due to the time constraints we are facing (due to COVID). Moreover, the planned budget for 16S metagenomics and target enrichment was restricted, not covering the total amount of samples collected by the partners. Furthermore, the original project proposal included only 4 countries as collectors of samples, now there are additional 4 countries that also provide samples from individual compartments, as considered an asset. Therefore, the new expected budget for the analysis of these samples with 16S/AMR target enrichment has been adjusted. For additional information, see JRP15-WP2-T3 and WP2-T4.

This task was **finished** in M33.

JRP15-WP2-T3: Assess microbial and ARG diversity with NGS in the selected test environments (metagenomics). Compare microbial and ARG diversity between ecosystems and over ecosystem boundaries. Characterization of cultivable environmental bacteria on complete nutrient and minimal media (M27-M48)

Due to the situation caused by the COVID pandemic and the tight budget available for genomic analysis, the FED-AMR consortium decided to introduce changes with respect to this task, as also indicated in task JRP15-WP2-T2. First, it was agreed by all WP2 participants to modify the aims of the culture protocol to be able to identify in all collected samples six bacterial species of clinical relevance for humans (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp, *Staphylococcus aureus* methicillin resistant, *Enterococcus faecium* and *E. faecalis* vancomycin resistant), instead of focusing on all



cultivable environmental bacteria. This unanimous decision was taken to favour the identification of human, animal and environmental bacteria that may be resistant to one or more critically important antimicrobials, and that can have equal or similar genetic traits than human pathogenic bacteria. Antimicrobial resistance is now being evaluated through diverse antimicrobial susceptibility test depending on the availability of each test in the participant countries and includes determination of the minimum inhibitory concentration (MICs) by broth microdilution, E-test and/or disc diffusion. Secondly, microbial diversity was agreed to be evaluated and compared by detecting the entire 16S region (V1-V9) through 16S metagenomics, which is more sensitive than 16S amplicon sequencing. Third, we agreed on the evaluation and comparison of ARGs using a novel methodology based on gene capture probes (see task WP2-T2 and subtask WP2-T3.2). This novel methodology allows us to dispense with both shotgun metagenomics (WP2-T3.1 task) and qPCR (WP2-T4). As main advantage, target enrichment will analyze the presence or absence of several thousands ARGs, in contrast to qPCR, which only detect few of them. Moreover, we will avoid performance bias, since all samples will be analysed by the same persons and the same devices. In addition, quantitative data will be inferred from the number or reads that cover each detected ARG. Hence, we will invest tangibly more of the currently unspent budget in this novel methodology and therefore we decided to divert additional funds to target enrichment and 16S metagenomics. This task is **ongoing**.

JRP15-WP2-T3.2: Gene enrichment with gene capture probes (M31-M42)

The start of this sub-task was delayed to M31. As explained before, this subtask was recently re-evaluated regarding the *pros* and *cons* of achieving a better detection of ARGs in samples with complex bacterial communities and of different exDNA sources (e.g. soil), which needed to be well defined. To do so, we performed a pilot study involving four Austrian wastewater samples, which were processed between AGES and an external company and that helped on the above-mentioned decision-making. Briefly, the methodology included exDNA and total DNA detection through conventional shotgun metagenomics and the novel target enrichment assay in the collected samples. Results showed a better performance of the latter in regards of ARG detection. The outcome of this pilot study was shared with the FED-AMR consortium, which voted in favour of this methodology as a replacement of qPCR and conventional shotgun metagenomics. The first batch of DNAs from 2-AGES was analysed through target enrichment and 16S metagenomics in the current month (M36) and the remaining countries will send their DNA samples to the external company in Austria in year 4, as AGES did. This task is **ongoing**.

JRP15-WP2-T4: Quantify clinically relevant ARGs in the tested compartments (qPCR; qPCR arrays) (M34-M42)

As explained above, the detection of ARG through qPCRs was eliminated from the project. The Scientific Supervisory Board (SSB) contributed to this decision-making process. However, the quantification will be performed, as it will be inferred from the number or reads that cover each detected ARG.

JRP15-WP2-T7: Isolate and assess quantity, diversity and stability of free extracellular ARG encoding DNA in the tested environments. Sequence comparisons (M34-M50)

Following the available DNA extraction protocol, WP2 participants have extracted the exDNA and total DNA from all samples immediately after their collection. Since the sampling campaign finishes in Spring 2021, DNA extraction will be finished by April next year. As stated before, the target enrichment and 16S metagenomics assays are starting in M36 for the first batch of DNA samples.

E. coli, *K. pneumoniae*, *Salmonella spp*, *S. aureus* methicillin resistant, *E. faecium* and *E. faecalis* vancomycin resistant strains have been retrieved from the collected samples. Antimicrobial susceptibility testing and Whole Genome sequencing is being performed in some institutes in a selection of strains, where the characterization of the ARGs harboured by those strains is also being investigated.



The start and end of this task was delayed to M34 (year 3) and M50 (year 5), respectively.

WP3: Elucidating the role of *Clostridium difficile* as an ARG transfer platform over ecosystems boundaries and its linkage between human and non-human (zoonotic) reservoirs (M25-M50)

There is increasing evidence that *C. difficile* may have a foodborne or zoonotic aetiology, challenging the One Health paradigm. *C. difficile* has also been suggested as a reservoir/receptor of resistance genes that might be transferred to other species in the host gut as well as in the environment. WP3 aims therefore to investigate the epidemiology of zoonotic *C. difficile*, the genetic overlap between human and non-human *C. difficile* lineages and the role of *C. difficile* as an ARG transfer platform over ecosystems boundaries.

JRP15-WP3-T1: Epidemiological survey of zoonotic ribotypes across participant countries. (M25-M40)

In task **JRP15 -WP3-T1** the task leader (Mónica Oleastro, 36-INSA), her deputy leader (Søren Persson, 13-SSI) and other WP3 participants aimed at investigating the epidemiology of zoonotic *C. difficile* through the identification zoonotic types of *C. difficile* across the WP3 countries by generating an epidemiological survey of zoonotic ribotypes. The task started in M25 at the kick-off meeting and finished in M36 and comprised a collection of genomic data (WGS reads) and associated metadata by the consortium partners on potential zoonotic types of toxigenic *C. difficile* isolates from various sources (human, animal and environment). Metadata included demographic and epidemiological data, as well as strain type, namely ribotype, toxin profile and AMR profile, when available. Discrimination between zoonotic and non-zoonotic types was based on *C. difficile* ribotypes and other genetic markers described in the literature.

Each participant partner started a sampling campaign in order to enrich the collection of *C. difficile* isolates available from different sources, more specifically from diverse animal and environmental sources. Until now, *C. difficile* isolates have been obtained from reptiles, lamas, poultry carcass, pets, pigs, food and manure. Due to COVID pandemic, the sampling will be extended until April 2021.

Prior to the selection of zoonotic types among the human isolates, an exhaustive search was made in the peer-reviewed literature.

Based on this inventory, each partner has selected the human isolates from zoonotic types, from the existing collections, isolated between 2016-2020. The overall set of *C. difficile* isolates from different sources was used for the construction of the database. The first version of the database was uploaded in the project site. This database will be updated when necessary.

A harmonized protocol for *C. difficile* isolation and characterization was developed by WP3 partners.

This task is **finished**.

JRP15-WP3-T2: WGS and AMR characterization of human and non-human *C. difficile* isolates (M34-M46)

At the moment, all the new *C. difficile* strains collected by WP3 participants are being tested to identify their AMR profiles and whole genome sequenced to identify ST and resistance genes. In addition, ribotyping is also being conducted. This task is **ongoing**.

JRP15-WP3-T3: *C. difficile* / AMR dissemination between the human, animal and the environment: pig farm as a proof of concept (M34-M50)

This task started on M34 although it was planned to start on M37. Two sampling campaigns have been undertaken, one in the summer and the other in autumn, in the HOALs from Austria and Portugal. For *C. difficile* study, samples were taken from pig barn, pig manure, farmers, wastewater treatment plant, groundwater and superficial water according to the sampling scheme from WP2.3. The partners that



could not perform *C. difficile* isolation sent their samples to other partners. Several *C. difficile* strains have already been isolated and currently under study. This task is **ongoing**.

WP4: Determination of the selection pressures in the tested compartments of human, animal and environmental ecosystems (M25-M50)

Due to the COVID-19 crisis, the start of tasks WP4-T2 to WP4-T7 was delayed. The main reason was the impossibility of shipping samples to the laboratory performing the analysis (34-PIWET), due to national COVID-19 restrictions. Meanwhile, 191 samples from HOAL Austria were shipped on June, September 2020 and October 2020 to 34-PIWET (antibiotics), UBA Vienna (herbicides) and 23-UoS (elements). All other samples were shipped from other HOALs between September and October 2020. Analyses of the first set of collected samples of soil (WP4-T5 and WP4-T6), manure (WP4-T3 and WP4-T6) and water (WP4-T2, WP4-T6, WP4-T7) has been finished. Results related to the analysis of antimicrobials, elements and herbicides are available for a total of 108 samples.

JRP15-WP4-T1: Selection of essential antimicrobials to be quantified in the tested compartments (published antibiotic consumption data, farmers' questionnaire, personal experience, expert interviews (veterinarians) (M25-M30)

This task is **finished**. Task WP4-T1, was planned for M25 to M26. The task was delayed, but it has been finished in M30 and the corresponding deliverable (D-JRP15-FED-AMR-WP4.1) was uploaded into the members area of the OHEJP website; this deliverable contains three protocols as annexes on the quantification of antibiotics, elements and herbicides in the different compartments.

Corresponding to the ARGs [*tet(M)*, *tet(W)*, *tet(Z)*, *sul1*, *sul2*, *sul3*, *erm*-like genes, PMQR-encoding genes] to be investigated in the environment (faeces, manure, agricultural soil, drainage, surface and ground-water; see WP2), the four antimicrobial classes to be tested in these compartments were selected: tetracyclines, macrolides, sulphonamides, trimethoprim and fluoroquinolones (task JRP15-WP4-T1). From these antibiotic groups the most important (according to published antibiotic consumption data and EFSA report on antibiotic residues in live animals and food) were included in the analytical method by liquid chromatography-tandem mass spectrometry (LC/MSMS) performed by 34-PIWET (WP4-T2 to WP4-T5).

Herbicides were chosen in the same Task, among those that are often used in agriculture, such as glufosinate and glyphosate, as well as its degradation product aminomethylphosphonic acid (AMPA), 2,4-Dichlorophenoxyacetic acid (2,4-D). Quantification of these substances (Task WP4-T6) will be performed by an AGES associated sister company (UBA Vienna).

Heavy metals and trace elements were also already chosen among those that are triggering co-selection and that have been used in co-selection studies: Cd, Cr, Cu, Ni, Hg, Co, Pb, Zn. The samples will be analysed by inductively coupled plasma mass spectrometer (ICP/MS) carried out by 23-UoS (Task WP4-T7).

JRP15-WP4-T2: Quantification of five antimicrobial classes (tetracyclines, macrolides, sulphonamides, fluoroquinolones and diaminopyrimidines) in aqueous matrices (water) (M31-M50)

Samples from some HOALs were taken and collected: 5 water samples from different compartments including wastewater (inlet and outlet), river water, ground water and drainage water.

Due to the COVID-19 crisis, the start of tasks WP4-T2 to WP4-T7 has been delayed. The main reason was the impossibility of sending samples to the laboratory performing the analysis, due to national COVID-19 restrictions. Meanwhile, 10 samples from HOAL Austria were shipped on 22th June and 1st September 2020. Next 5 water samples (HOAL Austria) were shipped on October 2020. The LC-MS/MS analyses of first set of collected samples (10 samples) were conducted before the end of September 2020. Moreover, the range of substances analysed in the LC-MS/MS method was extended by 2



analytes: azithromycin (macrolides) and trimethoprim (*diaminopyrimidines*). Obtained results were presented in monthly TC (2020.12.01). The analysis of second set of collected water samples will be conducted before the end of December 2020.

JRP15-WP4-T3: Quantification of five antimicrobial classes (tetracyclines, macrolides, sulphonamides, fluoroquinolones and diaminopyrimidines) in manure (M31-M50)

The expected starting date for this task was M27. The start date for analyses is now M31 (see task WP4-T2). However, samples from some HOALs were already collected. 4 samples from HOAL Austria were shipped on June and September 2020.

The LC-MS/MS analyses of first set of collected samples (4 manure) the analyses were carried out by October 2020. In this case, also the range of substances analysed in the LC-MS/MS method was extended by 2 analytes: azithromycin (macrolides) and trimethoprim (*diaminopyrimidines*). Obtained results were presented in monthly TC (December/2020). Next 2 manure samples were shipped on October 2020. The analyses of second set of samples are planned to be completed by the end of December 2020.

JRP15-WP4-T4: Quantification of five antimicrobial classes (tetracyclines, macrolides, sulphonamides, fluoroquinolones and diaminopyrimidines) in faeces (M35-M50)

The expected starting date for this task was M30. The new starting date is delayed to M35. The range of substances analysed in the LC-MS/MS method was extended by 2 analytes: azithromycin (macrolides) and trimethoprim (*diaminopyrimidines*).

JRP15-WP4-T5: Quantification of five antimicrobial classes (tetracyclines, macrolides, sulphonamides, fluoroquinolones and diaminopyrimidines) in soil (M31-M50)

The expected starting date for this task was M27. The start date for analyses was delayed to M31 (see task WP4-T2). Samples (soil with crop, soil before/after harvest, forest soil, meadow soil) from some HOALs have been already collected. 28 soil samples from HOAL Austria were shipped on June and September 2020. The LC-MS/MS analyses of first set of collected samples (28 samples) were conducted before the end of September 2020. In this case, also the range of substances analysed in the LC-MS/MS method was extended by 2 analytes: azithromycin (macrolides) and trimethoprim (*diaminopyrimidines*). Obtained results also, were presented in monthly TC (2020.12.01). The second set of soil samples (8 samples) were shipped on October 2020. The analyses of this samples will be completed by the end of December 2020.

JRP15-WP4-T6: Quantification of herbicides in agricultural soil (M31-M50)

The expected starting date for this task was M27. The start date for analyses is delayed to M31 (see task WP4-T2).

56 samples (36 soil, 5 manure, 15 water) from HOAL Austria were shipped on June, September 2020 and October 2020. The range of substances analysed in the LC-MS/MS method was extended by 7 analytes: bentazon, metolachlor and its degradation products metolachlor ESA and metolachlor OA, metazachlor and its degradation products metazachlor ESA and metazachlor OA. All 56 samples were analysed: in 8 of 36 soil samples, 4 of 5 manure samples and 14 of 15 water samples herbicides were detected (mainly AMPA and glyphosate).

Other HOALs are shipping samples since September 2020.

JRP15-WP4-T7: Measurement of the concentration of trace elements in environmental samples gathered across participants countries (M31-M50)

Since the start of the project, the work has been done in order to design a sampling strategy suitable for the analysis of trace elements in variety of samples. These include soils, crops and animal feed,



water, manure and sewage sludge. The researchers have worked very closely with the leaders of WP2 in order to harmonize the sampling strategy and make sure that the sampling procedures and processing were compatible with all types of analyses across the consortium. The team has also completed the protocol for sample preparation for solid and liquid samples, as well as the procedures for instrumental analysis and the sourcing of the certified reference material for validation. Arrival of the first set of samples to the ICP-MS Facility at Surrey was planned for March 2020, however this was postponed due to the lock-down and restrictions to research activities at the University of Surrey, starting on March 2020. The labs reopened gradually since June, and after assessing the risk posed by COVID-19 epidemics, the 1st set of samples is expected to be completed by the end of September 2020.

Samples from HOALs have been already taken, namely at the Austrian HOAL, where 78 samples (36 soil, 5 manure, 15 water, 3 feed, 19 crop) were collected and shipped on June, September 2020 and October 2020; 10 water samples were analysed for the elements Cd, Cr, Cu, Ni, Hg, Co, Pb, Zn by ICP/MS.

The expected starting date for this task was M27. The start date for analyses was delayed to M31.

WP5: Identification of environmental conditions modulating transformation frequencies in soil microcosms and an in vitro porcine gut model (poGutMo) (laboratory studies) (M32-M54)

The start of this work package has been delayed for two reasons. First, it took longer than anticipated for 23-UoS to recruit the PDRA to work on this work package. Second, the laboratories at 23-UoS have been closed due to national COVID-19 restrictions since March. Marwa Hassan was recruited successfully to the project at the end of April 2020. In the second week of June, the first members of staff have begun a phased return to the laboratories. M. Hassan was able to access the laboratories early in July. From mid-June, she has focussed on preparing the protocols and ordering the consumables to start work on WP5 in M32. This represents a seven-month delay to the anticipated start of WP5. As such, WP5-T1 will now be extended into year 4, as will WP5-T1-ST3 and WP5-T1-ST4. We anticipate getting the project back on track by M45.

Acinetobacter was proposed as a model organism for transformation experiments; however, our preliminary experiments proved the inability of this pathogen to grow anaerobically. After consultation with the team and the FEM-AMR consortium, it was agreed that the transformation experiments will be performed using *E. coli* as a model organism in the anaerobic gut model. Currently, the selection of strains to use for the transformation and conjugation experiments has been finalized. *E. coli* J53 (a derivative of K-12) and 912 (isolated from pigs) will be kindly provided by AGES and Ana Herrero-Fresno (University of Copenhagen), respectively, to be used for the transformation experiments. For the conjugation experiments, *C. difficile* strains 630 and CD37 will be kindly provided by Prof. Peter Mullany, University College London, in the near future. We also finalised our risk assessments including risk assessments for handling genetically modified organisms and are currently finalising the protocols for WP5, including procedures for analysis of trace elements and heavy metals in samples from the pig gut model. We also prepared a rifampicin mutant of *E. coli* J53 (*E. coli* J53-Rif^{r1}) to be used as a donor DNA in the gut model transformation experiments. We are currently performing natural transformation experiments using *E. coli* J53 as a recipient strain and DNA amplicon of part of the *rpoB* gene from *E. coli* J53-Rif^{r1} for comparison with the *in vitro* gut model and demonstration of successful transformation using recipient strain and donor DNA.

JRP15-WP5-T1: Establish baseline levels of HGT in the model organism (*E. coli*) arising from transformation in the poGutMo (M32-M47).

Preliminary experiments proved the inability of *Acinetobacter* to grow anaerobically; thus, *E. coli* was chosen as a model organism for the transformation experiments in the anaerobic gut model.

Start date delayed to M32. New end month: 47.



JRP15-WP5-T1-ST1: Ability of *E. coli* and Clostridial strains to acquire AMR to serve as a donor DNA (M36-M38).

Start date delayed to M36. New end month: 38

E. coli J53 has been obtained from AGES and is used as a reference strain in transformation experiments both *in vitro* and in the gut fermentation model where sodium azide is used as the first selection marker. Using the spontaneous mutant generation method, *E. coli* J53 was used to generate rifampicin resistant mutants so rifampicin can be used as a second selection marker. Six rifampicin resistant mutants were generated and named *E. coli* J53-Rif¹⁻⁶ and a growth curve experiment was performed for both rifampicin resistant strains and the parental J53 strain to confirm the growth rate of the mutant strains and ensures the lack of any intrinsic fitness burden associated with the mutations. All rifampicin mutant strains grew successfully with minimum or no effect on the growth rate. *E. coli* J53-Rif¹ was chosen, cultured and DNA extracted to be used as a donor DNA. PCR primers were designed targeting part of the RNA polymerase β subunit (*rpoB*) gene (2250 bp) including all RNA polymerase β subunit clusters, where mutation frequently occur. Targeted *rpoB* sequence of both *E. coli* J53 and *E. coli* J53-Rif¹ were successfully amplified, purified and quantified.

Preliminary natural transformation experiments were performed using *E. coli* J53 strain as the recipient strain (rifampicin sensitive) and the *rpoB* DNA amplicon (0.2-0.5 μ g) from *E. coli* J53-Rif¹ as the donor DNA (rifampicin resistant). Our preliminary results showed the successful recovery of *E. coli* J53 that is both sodium azide and rifampicin resistant with controls showing colonies only on sodium azide/MacConkey agar plates, which confirms the suitability of the strain to be used for transformation experiments in the gut model. This completes the first part of this sub-task.

C. difficile strains are still to be obtained in January 2021. This delay has been caused by the sending lab currently undergoing refurbishment. As the chosen strains have previously been used for conjugation we do not anticipate any further problems in completing this sub-task and the Clostridial strains should be suitable to be used in the gut fermentation model.

New end month: 38. This task is **ongoing**.

JRP15-WP5-T1-ST2: Determine the optimal growth parameters for cultivating *E. coli* strains within the gut model (M38-M43).

We are currently working on setting up the *in vitro* gut model to include 6 fermentation vessels (usually uses 2), which will allow for more experimental conditions to be tested. The task has started and is still on-going.

Start date delayed to M38. New end month: 43 (Year 4).

JRP15-WP5-T1-ST3: Rates of transformation calculated by taking samples from the gut model and plating on TSC agar plates supplemented with the appropriate antibiotics (M44).

Start date delayed to M44. New end month: 44 (Year 4).

JRP15-WP5-T1-ST4: DNA transfer rates via bacterial conjugation will be calculated using the endpoint method (M43-M47).

Start date delayed to M43. New end month: 47 (Year 4).

WP6: Probabilistic and mechanistic models of the links between antimicrobial usage in animals, AMR in the environment, and the risks for public health (M32-M54)

The start of this work package has been delayed as it took longer than anticipated for 23-UoS to recruit the PDRA to work on this work package. A Postdoc (Brian Gardner) was recruited successfully to the project at the beginning of May. Thus, the project and milestones were delayed by two months.



JRP15-WP6-T1 Build a probabilistic mathematical model of the emergence of AMR in target bacteria and the relative contribution of transformation and conjugation to ARG acquisition (M32-M54).

Gardner has started to define the protocol for a systematic search of the literature on environment and AMR. The output of the search will also provide the data to be used as input for the machine learning approach. An initial preliminary search returned about 3000 papers, the protocol is essentially finalised pending further comments from other members of the team. Davide Messina from UOS has agreed to help to co-lead the paper with Gardner. In few weeks we will start to screen title and abstract and we hope in the next month or so to allocate papers for the review. As Barnaghi has recently resigned from the University, Mirek Bober has agreed to help with WP6-T1, mentoring Brian Gardner and advising the group.

JRP15-WP6-T1-ST1 Data Integration, Annotation and Association Analysis (M32-M37)

Start date delayed to M32.

Due to certain challenges in obtaining the data required for setting up a machine learning model (in part related to COVID-19 restrictions), the workflow of WP6.1 was since changed to initially carry out a systematic review of the literature regarding environmental factors of AMR prevalence. The idea is that the data extracted as part of this systematic review will be used to inform the design of a future machine learning model. This revised deliverable provides the protocol for such a systematic review.

Specifically, a mini-scoping review was conducted to establish the novelty of this topic. Feedback gained from collaborators involved with related FED-AMR projects, as well as from UoS and PHE.

In-line with the goal of this WP6.1, a key outcome is to identify the relative importance of HGT mechanisms associated with the spread of antibiotic resistance, i.e. transformation vs. conjugation. The searched databases, specific search strategy, plans for data management and categories of extracted data types are specified in the linked protocol. This deliverable provides this protocol documentation on the UoS GitLab website at the following link: <https://gitlab.eps.surrey.ac.uk/bg0013/systematic-review-protocol-amr>. This is a private repository, and will remain confidential until publication of the systematic review or registration of the protocol. The protocol can be shared with all members of the FED-AMR or other One-Health EJP members and they can access to the GIT repository if requested.

JRP15-WP6-T2: Develop mechanistic models to address key questions regarding the spatio-temporal changes observed in microbiological communities (M32-M54)

Since May, Gardner has been fully engaged in reviewing the relevant literature. He has been in contact with the team at UoS (Chambers, La Ragione, Horton and other members of their group) to explore different sources of data that can be used as input/validation for the modelling approach and to refine the research questions. A model has been formulated and Gardner is currently developing computational approaches (e.g. Ridge regression, Bayesian approaches) to infer relevant parameters of the model.

JRP15-WP6-T2.1 - Modelling microbial communities I (M34-M41)

Start date was delayed to M34.

In a preliminary analysis performed on the dynamics of microbial community, we showed that these can critically switch from one state to another depending on how antibiotics are administered. Further steps are the application of the model using our novel data (from UoS or from other FED-AMR partners) rather than the one in the literature



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
15	D-JRP15-FED-AMR - WP1.1	Scientific Supervisory Board (SSB) installed. Local administrative representatives nominated (T1, T2)	M25	M25			Confidential (contains e-mail addresses of the members of the consortium) OHEJP: available Zenodo: to be uploaded once public	10
15	D-JRP15-FED-AMR - WP1.2	Unified sampling and experimental protocols (T1.1.)	M27	M33			Public OHEJP : available Zenodo: to upload	2
15	D-JRP15-FED-AMR - WP1.3	Data and protocol management plan (T3)	M27	M34			Public OHEJP : available Zenodo: to be uploaded	8
15	D-JRP15-FED-AMR - WP1.4	Webinars (T1.2.)	M30	M31			Public OHEJP: available Zenodo: uploaded	5
15	D-JRP15-FED-AMR - WP1.5	Annual project report	M36		M38		Public	8



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
15	D-JRP15-FED-AMR-WP2.1	List of sampling compartments, points and European test areas and harmonized protocols in alignment with EFFORT project protocols available in data repository (T2.1, T2.2)	M26	M33			Public OHEJP: available Zenodo: to be uploaded	2
15	D-JRP15-FED-AMR-WP2.2	Preliminary data collection on ARG prevalence and ARG background load in the compartments analysed so far (T2.4)	M36	M37			Public	10
15	D-JRP15-FED-AMR-WP3.1	Database of zoonotic Clostridium difficile isolates across participant countries (task 3.1)	M36	M36			Public OHEJP: available Zenodo: to be uploaded	3
15	D-JRP15-FED-AMR-WP4.1	Standardize protocols for sampling and testing of environmental samples	M26	M30			Public OHEJP: available Zenodo: to be uploaded	2
15	D-JRP15-FED-AMR-WP5.1	E. coli strains demonstrated to be suitable for	M26		M38		Deliverables will be made public, but elements of the data included in the	10



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		transformation					deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	
15	D-JRP15-FED-AMR-WP5.2	Optimal growth parameters for cultivating E. coli within the porcine gut model and the time after inoculation at which its concentration is maximal determined	M27		M44		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10
15	D-JRP15-FED-AMR-WP5.3	Pilot experiments using PCR amplicons as ARG donors	M28		M44		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10
15	D-JRP15-FED-AMR-WP5.4	Optimal growth parameters for cultivating the clostridial strains within the gut model determined	M30		M45		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
15	D-JRP15-FED-AMR-WP5.5	Conjugation-mediated HGT between the clostridial donor and recipient strains within the gut model determined	M31		M46		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10
15	D-JRP15-FED-AMR-WP5.6	Clostridial transconjugates characterised by whole-genome sequencing	M33		M47		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10
15	D-JRP15-FED-AMR-WP5.7	Second round of experiments using PCR amplicons as ARG donors	M36		M50		Deliverables will be made public, but elements of the data included in the deliverable may be embargoed or kept confidential, in line with the OHEJP guidelines.	10
15	D-JRP15-FED-AMR-WP6.1	Main code for the mathematical modelling made available in public repository (e.g. GitHub) with associated	M30		M38		This is now a protocol for systematic review (not a code for mathematical modelling) Confidential until publication or	3



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		documentation (which can be used as “Material and Method” section of the forthcoming publications).					registration of the protocol for the systematic review, except for FED-AMR or other One-Health EJP members.	
15	D-JRP15-FED-AMR-WP6.2	Findings presented at one international conference and one national conference.	M36		M45		Due to Covid-19 many conferences have been cancelled. Ideally we would like an in person conference but we will keep an eye on conference opportunities. Findings have been presented internally at University level.	5
15	D-JRP15-FED-AMR-WP6.3	Update of codes and documentations in public repository (e.g. GitHub).	M36		M43		Confidential until full validation of the code or publication (except for FED-AMR or other One Health EJP members).	3

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify); 9. This is supportive to an integrative activity; 10. This is not an integrative activity



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
15	M-JRP15-FED-AMR - 01	Kick off meeting	M26	Yes		
15	M-JRP15-FED-AMR - 02	Database repository active	M27	Yes		
15	M-JRP15-FED-AMR - 03	Webinar forums started	M30	Yes		The consortium initiated the scientific exchange via online



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
						teleconferences and formally installed regular webinars by M31.
15	M-JRP15-FED-AMR-07	List of sampling compartments, points and European test areas available. Harmonized protocols for sample collection + transportation, DNA extraction, qPCR, metagenomics, shotgun sequencing, gene capture and bioinformatics and statistical analysis of sequence data available. Alignment with EFFORT project protocols (T2.1, T2.2)	M26	Yes	M33	The list of sampling compartments, points and European test areas have been defined. Harmonized protocols for sample collection and transportation and DNA extraction protocols are already available for all project members. Protocols for WGS, metagenomics, gene capture and bioinformatics were developed. When possible, sampling protocols where aligned with e.g. EFFORT projects.
15	M-JRP15-FED-AMR-14	Starting preparations for shotgun sequencing (T2.3.1)	M37	Yes		
15	M-JRP15-FED-AMR-25	Completed database with zoonotic types	M36	Yes		
15	M-JRP15-FED-AMR-30	Starting the selection of essential antimicrobials to be quantified in the tested compartments	M25	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
15	M-JRP15-FED-AMR-31	Starting the analysis of antimicrobials in aqueous matrices	M27	Yes	M31	
15	M-JRP15-FED-AMR-32	Starting the analysis of antimicrobials in manure	M31	Yes	M31	
15	M-JRP15-FED-AMR-33	Starting the analysis of antimicrobials in faeces	M29	M35		
15	M-JRP15-FED-AMR-34	Starting the analysis of antimicrobials in soil	M31	Yes		
15	M-JRP15-FED-AMR-35	Starting the quantification of herbicides in agricultural soil	M31	Yes		
15	M-JRP15-FED-AMR-36	Starting the measurement of the concentration of trace elements in environmental samples	M27	Yes		
15	M-JRP15-FED-AMR-37	Bacterial strains supplied to UoS	M25	Yes	M34	Bacterial strains have been supplied to UoS and used successfully.
15	M-JRP15-FED-AMR-38	Porcine gut model set up using faecal samples obtained through WP2, samples stored for trace element analysis (WP4) – experiments can start	M26	No	M39	



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
15	M-JRP15-FED-AMR-39	Samples from gut model experiments set A stored for trace element analysis (WP4)	M30	No	M45	
15	M-JRP15-FED-AMR-40	Samples from gut model experiments set B stored for trace element analysis (WP4)	M31	No	M51	
15	M-JRP15-FED-AMR-41	Samples from gut model experiments set C stored for trace element analysis (WP4)	M33	No	M54	
15	M-JRP15-FED-AMR-42	DNA sent for whole-genome sequencing	M33	No	M53	
15	M-JRP15-FED-AMR-53	Literature review on concept of resilience and modelling in microbial communities.	M27	Yes	M34	Key papers have been reviewed, but of course, we will keep updated with the literature. AS well as review of literature, Garder has successfully reproduced published modelling work.
15	M-JRP15-FED-AMR-54	Identification of relevant available data. Formulation and implementation of the model for the microbiological community within-host. Conditioned to data	M30	Partially	M44	We have discussed this with member at UoS for relevant data, but the format and type of data might not be ideal. Details of ideal format of data have been shared with partners from UoS and we will do the same with the broader FED-AMR community soon.



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
		availability, potential extension of the model to natural environment (e.g. soil).				<p>As a contingency plan, Gardner has identified published data which can be used instead of our novel data if necessary.</p> <p>A model has been formulated and could be implemented as it is, We are trying, however, to improve the model to be able to address additional scientific questions.</p>
15	M-JRP15-FED-AMR-55	Application of the model to address specific questions and dissemination of findings in conferences, preparation of draft papers.	M36	Partially	M44	We have some interesting findings, but we want to address additional questions.

4. Publications and patents

No publications yet.

Additional information:

Abstract describing the FED-AMR project sent to the ASM Annual Meeting, held online on 27th-29th May, 2020 (selected for e-Poster presentation).



Launch of an internal website hosted by AGES that serves as an exchange platform of internal documents. Partners are granted with private access and can download common protocols, minutes from the TCs and other documents.



5. *On-going and planned collaborations with national or European projects or networks*

There are complementarities between the FED-AMR protocols and those available from EFFORT and COMPARE projects, and some from the DTU National Food Institute, the International Organization for Animal Health (OIE), and also needed information from European Food Safety Authority (EFSA). Several partners of FED-AMR participate in other JRP and JIP projects from OHEJP (e.g. AGES participates in the MedVetKlebs, INSA participates in Matrix, etc).

The synergies between these projects could be established at a later stage, once the project partners within FED-AMR share their first results. The same will apply to possible synergies with EFSA, ECDC, the SSB and POC members.

JRP16-TELEVIR

1. *Summary of the work carried out in year 3*

TELEVIR is a 2.5 year Joint Research Project of the One Health EJP that focusses on developing a fast point-of-evidence (poi) toolbox for identification and characterization of emerging virus threats for human and/or domestic and wildlife animals.

In the TELEVIR project we are combining a suitable field-deployable point-of-care approach, and a direct upload of genomic, phenotypic and epidemiological data into a user-friendly bioinformatics toolkit for fast identification and characterization of new emerging virus threats. We are developing and adapting existing point-of care methods and tools and expand these to a harmonized poi protocol for field analysis. The poi protocol will only require a minimum of laboratory equipment and will be designed to be compatible with MinION sequencing technology. Moreover we are combining and intergrate in the poi toolbox phenotypic and epidermiological data to aid risk assessment and management. The poi toolbox will be made available to other interested national and international parties for example shared with established networks.

The worldwide SARS-CoV-2 pandemic has had a great impact on the TELEVIR project. There has been national lockdowns, laboratories have been closed or allocated for SARS-CoV-2 diagnostics. There has been a worldwide shortage of basic laboratory reagents and equipment, which has influenced the ability to perform basic laboratory experiments. Fortunately, in summer the SARS-CoV- crisis was less severe and countries were opening up which meant that the TELEVIR partners could begin to increase their activities. However, at this time point (January 2021) the pandemic situation is very hard affecting again some partners in their daily work.

At the TELEVIR kick-off meeting held at IZSAM, Italy (20th-22nd of January 2020), it was decided to use Coronaviruses and Influenza A virus as model viruses for the proof-of-principle studies. Many of the TELEVIR partners have been involved in SARS-CoV-2 diagnostics and due to the shortage of reagents for NA extraction, the partners have been forced to develop alternative methods for NA extraction which is in line with the development of a field based protocol for MinION sequencing using a minimum of laboratory equipment (the poi tool box). In addition, surveillance programs for the novel coronavirus (SARS-CoV-2) are based on sequencing of the virus, which has resulted in an upgraded version of the INSaFLU software. Further developments are ongoing.

Challenged by the SARS-CoV-2 pandemic, the TELEVIR consortium has shown impressive resourcefulness and adaptability. Deliverables and reports of the first year were reached and submitted. Dissemination included scientific publications and presentations at workshops and webinars. Collaborations with other projects and networks were established.



Overall the COVID-19 pandemic has had also a positive impact on the TELEVIR project and many of the experiences and problems encountered during the crisis can be used or translated to the development of the TELEVIR poi-tool box, which will help in the future to control outbreaks of new emerging viruses at national, regional, European and even global levels.

2. Work carried out in the JRP, scientific results

The work in TELEVIR is organized into three workpackages (WPs) with tasks (T) and some subtasks (sT). The 2.5-years project spans three Annual reports (Y3-Y5, 2020-2022). All the tasks that took place in Y3 (2020) continue to Y4 (2021). Key outputs are available via the project homepage: <https://onehealth.ejp.eu/groups/tele-vir/>



Kick-off Meeting of TELEVIR, January 2020, at Teramo, Italy

WP1: Coordination and impact

TELEVIR_WP1 is responsible for the coordination of the project, the data management and its progress by integrating all results to achieve all the goals. TELEVIR-WP1 ensures that the project adheres to H2020 rules regarding for example ethics, dissemination and publication. To ensure timely submission TELEVIR-WP1 works in close contact with the TELEVIR consortium, to coordinate the compiling of deliverables and reports. TELEVIR WP1 is organizing monthly shorter meetings for the TELEVIR consortium as well as annual meetings to enable teamwork within the TELEVIR project in the best manner. Science-to policy translation and efficient dissemination are emphasized to maximize the impact. Interest Group facilitates targeted dissemination to stakeholders

JRPX-WP1-T1 Management, coordination and communication (M25-M54)

The Kick-off meeting was held January, 2020 in Teramo, Italy with the whole consortium. There the overall structures of the project were presented and agreed on.

The established key structures for management of the project include monthly online meetings with the TELEVIR consortium and the use of the online group for sharing and storage of relevant documents.

The first version of the Data Management Plan (DMP) was drafted, with the participation of the whole TELEVIR consortium and the DMP tool was taken to use.

Dissemination of the outcomes included presentations to relevant audiences case studies and scientific publications.



From October on Maiken Worsøe Rosenstjerne (senior scientist, SSI) left the project as the TELEVIR project coordinator and WP leader, but as a new replacement of this position Katja Spiess (senior scientist, SSI) was hired from October on. Anders Fomsgaard stayed as TELEVIR project leader and made sure that the introduction of the new project coordinator and WP leader took place without a impact on the project. The consortium stayed highly motivated and the general atmosphere is positive and supportive. All Milestones and reports of the the first year were reached and submitted.

WP1: Coordination and data management (M25-M54)

JRP16-WP1-T1: Coordination and project management (M25-M54)

Project management and coordination of the project is proceeding according to the plan. This is ongoing.

JRP16-WP1-T2: Data management (M25-M54)

The OHEJP project management team has postponed the deadline of first DMP. Therefore the first draft of the DMP was submitted in December 2020

JRP16-WP1-T3: Kick-off-meeting at IZSAM, Italy (M25-M26)

The kick off meeting of the TELE-Vir project was held on January 21st – 22nd 2020 at the International Centre for Veterinary Training and Information "F. Gramenzi" (CIFIV) of Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" (Via G. Caporale, Teramo, Italy). All partners were present. This task is completed.

JRP16-WP1-T4: 1st TELE-Vir at Sciensano, Belgium (M37-M38)

The first annual meeting was planned to be held at Sciensano, in Belgium. Due to the SARS-CoV-2 situation, this is not possible. Alternatively, an online meeting will be celebrated the 25th of Januray 2021.

WP2: Development of a Bioinformatics tool-kit for POI data analysis (M25-M54)

JRP16-WP2-T1: Survey and collection of databases for genotype-phenotype associations (M25-M36)

At the kick off meeting it was agreed that the first stage was selection of important phenotypic characteristics for the chosen model virus (influenza and coronaviruses), followed by assessment of the amount and quality of data available. A literature review has been performed to identify coronavirus phenotypes of relevance to tropism, emergence, and clinical disease, and any data available for their prediction based on genotype. A summary has been prepared for circulation to partner institutes and reference laboratories for elicitation of expert opinion. A similar exercise is underway for influenza A virus. These reviews will form the basis for a manuscript, to be submitted for publication in a scientific journal.

A survey for elicitation of expert opinion has also been created and circulated to TELE-Vir partners, other OHEJP partner institutes, and associated virologists. The aims of the survey were two-fold: (1) to obtain the views of virologists (potential end users of the TELE-Vir toolkit) on coronavirus phenotype prediction and variant monitoring activities that they would like to see in a genomic surveillance toolkit; and (2) to obtain test datasets for further development of the toolkit. Analysis of responses is ongoing.

JRP16-WP2-T2: Development of bioinformatics modules for third-generation sequencing analysis and pathogen identification (M25-M39)

In the context of pathogen detection and field genome sequencing, there are multiple advantages in either using online bioinformatics tools or running the platforms locally. As such, a Docker version of the online INSaFLU platform has been built and distributed publicly (<https://github.com/INSaFLU/docker>) in order to facilitate the local installation process. INSaFLU



users, TELEVIR partners and other stakeholders (e.g., ECDC) were notified of this novel feature. INSaFLU has been successfully installed and run locally 'offline' on partner computer servers including at UoS.

As a response to COVID-19 pandemic, both the locally installed INSaFLU version and original website (<https://insaflu.insa.pt/>) were adapted to better accommodate the identification and genome-based analyses of the novel coronavirus (SARS-CoV-2), as follows:

- a new module for rapid assignment of Human Betacoronavirus (BetaCoV), including the novel coronavirus (SARS-CoV-2), has been developed and implemented, and the rationale behind the classification and outputs was documented (https://insaflu.readthedocs.io/en/latest/data_analysis.html#influenza-type-and-sub-type-identification-and-human-betacoronavirus-classification-as-of-march-2020; check more details in the list of current INSaFLU genetic markers used for Influenza type and sub-type identification and Human Betacoronavirus classification);
- the publicly available SARS-CoV-2 reference genome sequence (NCBI accession number MN908947) was inserted as default in the INSaFLU reference database;
- multitasking configurations were changed, considerably speeding up the analyses, and the maximum upload file size was made more flexible;
- a new tab “Settings” was created making software parameterization more flexible and tailored to SARS-CoV-2 NGS analyses, with emphasis on including user-defined parameters for reads quality analysis, mapping and consensus generation.
- automatic masking of low coverage regions was incorporated in the platform, i.e., automatic generation of consensus sequences for incomplete locus, i.e., undefined nucleotides (“N”) are automatically introduced in low coverage regions at a user-selected coverage thresholds

These and other updates were documented and are available at full INSaFLU documentation webpage: (<https://insaflu.readthedocs.io/en/latest/>) and Github (<https://github.com/INSaFLU/>)

Work on this task is ongoing to upgrade the platform for Oxford Nanopore Technologies (from base called data to generation of consensus sequences, SNP/indel identification, etc).

JRP16-WP2-T3: Development of bioinformatics modules for sequence curation and phenotypic association (M25-M42)

This task is highly dependent on the collected databases for genotype-phenotype associations, so the design of the bioinformatics approach cannot be fully drawn at this stage. Still, following the literature review and survey in WP2-T1, data collection has commenced. We anticipate that detection of known genotype-phenotype associations (such as, amino site changes already linked to: antiviral resistance, resistance to neutralizing antibodies, enhanced affinity to host-receptors antibodies or enhanced transmissibility) will be implemented as a priority. Meanwhile, we will investigate the feasibility of inferring biochemical and immunological properties such as antigenic variation, using existing models and machine learning approaches being tested at UoS. Work on this task is ongoing.

JRP16-WP2-T4: Development of bioinformatics modules for genomic and metadata integration towards enhanced surveillance (M25-M54)

Following the kick off meeting a strategic approach has been agreed to achieve this task. First, Nextstrain (<https://nextstrain.org>) tools will be implemented for temporal and phylogeographical analysis. Then, we implement novel functionalities focused on fitting the needs of labs working in different sectors (vet, PH, etc) and that can be handled by users from multidisciplinary fields. In this context, INSaFLU was also upgraded to easily display metadata on phylogenetic trees (through user-defined node colouring and metadata blocks), thus facilitating integration of relevant epidemiological and/or clinical data and pathogen genomic data



(https://insaflu.readthedocs.io/en/latest/change_log.html). Still, further developments are ongoing, dependent on tasks 2 and 3).

JRP16-WP2-T5: Development of a user- and surveillance-oriented web-based interface (M25-M54)

An application for an STM was successful, for UoS and INSA to align bioinformatic approaches and share knowledge of the existing INSAFLU bioinformatics pipeline, in the context of WP2-T2 and WP2-T3 and to facilitate the eventual design of the user interface. The STM has been delayed by current travel restrictions but researchers are coordinating virtually and aim to complete the STM when possible. Work on this task is ongoing. In particular, we are investigating approaches to: i) synchronize local and web instances of the platform, allowing an easier integration and sharing of data, i.e., results of local analysis (“offline”) can be “communicated” to a centralized repository with web access; ii) facilitate data flow and sharing with external resources (GISAID/NCBI/ENA...).

WP3: Development of a protocol for POI MinION sequencing (M25-M48)

JRP16-WP3-T1: Development and validation of a S.O.P for sample handling & pre-treatment (M25-M48)

Testing different lysis buffers for the inactivation of viruses before NA extraction

A detailed research plan for testing of the virucidal activity of MPLB buffer against different animal viruses under various temperature-time conditions was developed. The experiments will meet the requirements of EN 14675: 2015 standard document.

1) Protocol to verify the inactivation of animal viruses using three different lysis buffers.

For propagation of selected animal viruses (myxoma virus, MYXV; canine adenovirus type-2, CAV-2; canine coronavirus, CCoV) representing different virus families, the following cell lines were used: rabbit kidney (RK13), madin-darby canine kidney (MDCK) and A-72. Viruses were grown in cell cultures to the titres of $10^{5.84}$ TCID₅₀ /ml for MYXV, $10^{7.47}$ TCID₅₀ / ml for CAV-2 and $10^{4.20}$ TCID₅₀ /ml for CCoV. Additionally, to increase MYXV titre in cell culture suspension a virus concentration step was performed. The virus stock suspensions were prepared for subsequent inactivation studies. The virucidal activities of MagNA Pure lysis / binding buffer (MPLB, Roche), AL (Qiagen) and AVL (Qiagen) were assessed under various temperature-time conditions. For the virus inactivation studies the following buffer concentrations were tested 40% (MPLB), 50% (AL) and 80% in the case of AVL buffer. The tested virus suspensions were treated with the particular buffer solutions and subjected to heat treatment at 20°C (MPLB, AL, AVL) and 56°C (AL) for 1 and 10 minutes respectively. The inactivation efficiency of viruses was evaluated on the basis of a CPE appearing in the infected cells. The cytotoxicity of the cells caused by buffers used was assessed by a MTT assay. The following reduction of the virus titre was achieved $\geq 6.0 \log_{10}$ (AL, AVL) and $\geq 5.0 \log_{10}$ (MPLB) for CAV-2, $\geq 3.0 \log_{10}$ (AL, AVL, MPLB) for MYXV and $\geq 4 \log_{10}$ for CCoV when MPLB buffer was used. All buffers in the tested concentrations and temperature-time profiles were effective in virus inactivation. This study is ongoing.

2) A protocol to verify the inactivation activity of two buffers for Bovine Beta-CoV (model virus for SARS-CoV-2).

MPLB (Roche) and eNAT™ (Copan) against a Bovine Beta-CoV (Bov-CoV strain 9WBL77) was developed. The eNAT buffer is a Guanidine-thiocyanate based medium that stabilizes the RNA and DNA of Viruses. Bov-CoV strain belongs to the same genus as SARS-CoV-2 but can be propagated in BSL2 facilities and thus was used as a model to validate virus inactivation using different inactivating buffers. The protocol is based on the ultracentrifugation of the virus-inactivating buffer compound in order to remove the toxic effect of the inactivating buffer on cell cultures. For inactivation protocols using MPLB and eNAT buffers, one virus titer (10^5 TCID₅₀/ml) and one contact time (30min) were used. After 30 min of contact, the three samples: 1- Virus control (Virus + MEM), 2- virus + MPLB and 3 -virus + eNAT were ultracentrifuged in sucrose cushion (100.000 g / 2 h) to remove the toxic supernatant. The pellet of samples 1, 2 and 3 was resuspended in the same volume of PBS and then titrated in HRT-18 cells:



Sample 1:10⁵ TCID₅₀/ml, sample 2: < 10 TCID₅₀/ml and sample 3: < 10 TCID₅₀/ml. In conclusion, the inactivating power of the MPLB and eNAT buffers against a beta-CoV under test conditions was highlighted.

3) Heat inactivation protocols for Bov Beta-CoV 9WBL77 as model virus.

One ml of virus suspension with an infectious titer of 10⁵ TCID₅₀/ml was heated at two different temperatures. In the first protocol the virus suspension was heated at 56°C for 30 min and 60 min. In the second it was heated at 60°C for 60 min. After heat treatment, the viral suspensions were inoculated into HRT18 cells for three blind passages and viral growth was tested using a Mab-based virological ELISA. Heat treatment at 56°C was shown not to completely inactivate the virus for both 30 and 60 min as evidenced by viral growth after three passages. The viral suspension was instead completely inactivated at 60°C for 60 min.

4) Developing of a protocol to verify the inactivation of SARS-CoV-2

A protocol for SARS-CoV-2 inactivation using MagNA Pure Lysis Binding buffer (MPLB-buffer), without damaging host cells was developed. The protocol is based on dilution of the buffer in order to remove toxic effect on cell cultures instead of ultracentrifugation. This study is ongoing.

5) Development of a SARS-CoV2 inactivation protocol without the use of lysis buffers.

Development of virus inactivation protocols without the use of lysis buffers. Heat inactivation of SARS-CoV-2 virus has been tested by heating nasopharyngeal swabs positive for SARS-CoV-2 for 5min at 98°C. The heated swabs were filtrated and used for infectivity studies on Vero E6 cells. Preliminary results show that simple heating for 5min. inactivates SARS-CoV-2. This study is ongoing.

JRP16-WP3-T2: Development and validation of a S.O.P for sample NA purification (M25-M48)

Selected nasopharyngeal swabs have been processed with different lysis/inactivation buffers trying to circumvent the NA extraction. The first trials resulted in a lesser viral RNA detection by real-time PCR when compared to the established diagnostic procedure, resulting in the ultimate loss of the weak positive samples. Preliminary assays using samples collected on FTA cards have also led to lower detection of viral RNA. Experiments incorporating simple washing steps of FTA cards, with no need of NA extraction, combined with different RT-PCR reagents allowed to detect positive samples with Ct<30. This study is ongoing.

Heating of nasopharyngeal swabs has also been tested in order to circumvent NA extraction (Fomsgaard and Rosenstjerne, Euro Surveill. 2020 Apr;25(14):2000398. doi: 10.2807/1560-7917). Other alternative methods such as the use of detergents in the RT-PCR mastermix is currently being validated. Whether or not these alternative methods are compatible with library preparation for MinION sequencing will be investigated. This study is ongoing.

As we want to detect RNA and DNA viruses applying the metagenomics approach work is also ongoing on extracting rapidly of nucleic acids from surface liquids (mucus) of fish infected by a DNA virus. Field samples were obtained after an outbreak and tested positive using conventional methods (extraction by columns and test by PCR). Starting with these samples, a simplified extraction procedure with a specific lysis buffer and magnetic beads will be tested in 2021, both in lab. and field.

JRP16-WP3-T3: Development and validation of a S.O.P for NGS library preparation & MinION sequencing (DNA & RNA) (M25-M48)

A SOP for MinION sequencing of Avian influenza virus (AIV) has been tested. Four primer PCR protocol and the PCR Barcoding Kit (SQK-PBK004) from Oxford Nanopore Technologies (ONT, Oxford,UK) was used to sequence and typing of avian influenza virus. Preliminary amplification of RNA was conducted prior to sequencing on MinION utilizing SuperScript IV One-Step RT-PCR System with Platinum Taq (Invitrogen/ThermoFisher Scientific, Waltham, MA) and universal Influenza A primers designed for the conservative ends of all AIV segments (Zhou et al., 2009, J Virol 83:10309-10313). Spot on Flow Cell, R9 version (FLO MIN 106D; ONT) and basecaller Guppy (v3.29; ONT) was used for the real-time



basecalling to produce sequencing data and monitor the run. Sequencing data from two samples were analyzed using CLC Genomics Workbench (Qiagen-CLCBio). Study is ongoing.

A SOP for targeted sequencing of SARS-CoV-2 on MinION has been tested and validated and is used for comparison to a metagenomics approach on the same clinical sample material from humans and mink. The SOP is based on the ARTIC nCoV-2019 amplicon sequencing protocol (<https://artic.network>).

For the metagenomics approach, a sequence independent isothermal amplification step will be included before library preparation and two methods are currently being tested. Sequence-independent single primer amplification (SISPA) and Recombinase polymerase amplification (RPA). Both have the potential to amplify sample NA before sequencing and thereby increase the sensitivity of viral detection. This study is ongoing.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
16	D-JRP#-WP1.1	Kick-off meeting in Italy (IZSAM)	M25	31 st of January 2020			Public https://zenodo.org/record/3734134#.Xwbj0Sgza70 Milestone reached by M25 on time	10
16	D-JRP#-WP1.2	Data Management Plan	M30	M30			Milestone reached by M30 on time	8
16	D-JRP#-WP1.3	1st TELE-Vir meeting (Sciensano, Belgium)	M37		Meeting will take place online on the 25 th of January			
16	D-JRP#-WP3.1	1st version of a poi S.O.P for sample handling & pre-treatment	M36	M36	28/02/2021	Yes		2
16	D-JRP#-WP3.2	1st version of a poi S.O.P for sample NA purification	M36	M36	28/02/2021	Yes		2



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
16	D-JRP#-WP3.3	1st version of a poi S.O.P for NGS library preparation & MinION sequencing	M36	M36	30/06/2021	Yes		2

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
16	M-JRP#-01	Collected and curated databases for genotype-phenotype associations	M36	No	M48	An inventory of phenotypic traits, and the feasibility and value of their association with genotype has been completed through literature review and expert elicitation (see section WP2-T1). The database curation is ongoing due to the dynamic situation caused by the pandemic, with very large amounts of new important data being produced continuously.



4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
An alternative workflow for molecular detection of SARS-CoV-2 – escape from the NA extraction kits shortage, Copenhagen, Denmark, March 2020 https://doi.org/10.2807/1560-7917.ES.2020.25.14.2000398 https://zenodo.org/record/4247188#.X6QLjWhKjcc	NO		GOLD
The One Health European Joint Programme (OHEJP), 2018–2022: an exemplary One Health initiative 10.1099/jmm.0.001228	YES	Yes, (no embargo)	

Additional information:

Following the INSaFLU usability for whole genome sequencing analysis of SARS-CoV-2, INSA's team has been invited to present this platform to broad audiences on behalf of ECDC COVID-19 Laboratory Networks Influenza (20 May 2020) and the WHO Europe Laboratory Workshop (1 June 2020). In Portugal, the INSaFLU platform (<https://insaflu.insa.pt/>) has been used to analyse all SARS-CoV-2 genome sequencing data collected so far on behalf of the nationwide study of SARS-CoV-2 genetic diversity, under coordination of INSA (<https://insaflu.insa.pt/covid19/>). UoS has published a review paper advocating the One Health approach, which highlights the value of projects including Tele-Vir in addressing global zoonotic threats. *The One Health European Joint Programme (OHEJP), 2018–2022: an exemplary One Health initiative*. Doi:10.1099/jmm.0.001228.



5. On-going and planned collaborations with national or European projects or networks

National project RTA2015-00002-C02-01-E. Analysis of new **West Nile virus encephalitis outbreaks** in Spain and its geographical expansion. Field samples obtained in this national project will be used in the WP4 of TELE-VIR project.

Activities as **EU Reference Laboratory for African Swine fever** will allow to compile some field samples to be used in the WP4 of TELE-VIR project.

MEDILABSECURE (CoE Project 037 Ct N° IFS/2013/330 961) (www.medilabsecure.com): INIA coordinates the animal virology network of the «One-Health» based MedilabSecure project, incorporating (in addition to animal virology) human virology, entomology, public health & veterinary services and early warning modelling networks from 22 non-EU countries in the Mediterranean, Sahel and Black Sea regions, aiming at improving diagnostic capacities and integrated surveillance for better response against emerging zoonotic diseases. TELE-VIR can assist in these efforts providing an easy portable tool for a rapid identification and response against viral threats not only for EU but also to our neighbouring non-EU countries.

A collaboration between SSI, Aalborg University and the Danish hospitals have successfully been established during the COVID-19 crisis in order to setup a **national COVID 19 Surveillance system**. The consortium has whole genome sequenced more than 2000 SARS-CoV-2 positive samples using the Nanopore platform and the Artic network protocol. The Danish Ministry of education and science (UFM) and Grundfos Foundation have funded the consortium (1.5 mio. €).

As the project progresses contact to ECDC, EFSA and the Swedish Food Agency as EU-RL for foodborne viruses (<https://www.livsmedelsverket.se/en/production-control-and-trade/eurl-foodborne-viruses?AspxAutoDetectCookieSupport=1>) will be made in order to inform them of our platform.

JRP17-IDEMBRU

1. Summary of the work carried out in year 3

The project has started as planned in January 2020. After the initial kick off meeting held on-line on December 17th 2019, regular on-line meetings have been done on organizing the project work packages (WP), hiring personnel and starting the works on literature data analysis and sample collections. Unfortunately, in March everything had to be stopped due to COVID-19 sanitary crisis. Due to a still ongoing crisis, movement restrictions and partial lockdowns throughout the EU it is still hard to fully restart scientific activities. Sample collection had to be postponed due to a strict movement restrictions. One post-doctoral fellow started working on the project in January, while other hirings were late, mainly due to restricted rules. Other employments had to be postponed. The work on the project interactive data and large file storage platforms as well as data management is finished. It was decided that MTAs between partner institutions will be signed on bilateral basis as needed, based on institutional templates and specific requirements. Data Management Plans (DMPs) were created and have to be validated by each partner. Existing sample collection data have been defined and drafts or an analysis strategy have been prepared in order to detect atypical *Brucella* spp. in different biotopes. Harmonized SOPs for sample collection, identification, and treatment are in various stages of completion and will be tested in several consortium laboratories within following months. Due to all delays and state of emergency still active in all partner countries, the consortium had to postpone all deliverable and milestone deadlines and annual meeting for six months (estimated delay).



2. Work carried out in the JRP, scientific results

WP1: Recording the situation of brucellosis in emergent wild and environmental reservoirs (M25-M44)

Task 1 started on January 2020. Due to all delays, this task was hindered until December 2020. Task 2 was delayed from May to November 2020, and will last until February 2022.

Common database for all samples collected and treated before and/or during the project was created. Each partner will include their sample collection of atypical *Brucella* spp. originating from animal species and environment as well as samples collected during the project. This database will include all results generated during the project from WP-1 to WP-5.

Multiple animal and environmental samples will be represented.

Further animal and environmental sampling was postponed due to currently ongoing sanitary crisis. Therefore, consortium decided to use the existing collections of various previous projects in partner institutions, to generate initial data on which sampling strategy can be based.

JRP17-WP1-T1: Mapping of existing data on emerging *Brucella* spp (M25-M36)

A list of the existing sample collections to be tested was created and shared among the consortium partners. Moreover a list of existing atypical *Brucella* strains within the consortium partners is in preparation. Each partner will collect the data for mapping the emerging *Brucella* species (existing strains) from their respective countries in order to finish this task.

JRP17-WP1-T2: Sampling and analytical strategy according to previous epidemiological information from the different partners (M37-M50)

The list of new target collections is finished. Three biotopes will be targeted: forest, fresh water habitats and coastal regions; animal species to target and number of samples will be defined by each partner. An epidemiological questionnaire and associated SOPs for sample collection and identification were prepared for the forthcoming sampling collection. Moreover, each partner will contribute to the WP with their various collections of tissues and strains. Strains and tissues samples from forest biotope were provided by ANSES, FLI, INIAV and NDRVMI, from freshwater habitats by ANSES, INIAV, BfR, NDRVMI, and from coastal regions by IZASM and INIAV.

The collection of new samples started in Italy (IZSAM), Bulgaria (NDNIVMI) and France (ANSES). In Italy the partner collected 50 turtle tissues found stranded along the Italian shoreline during the period 2019-2020. The tissues comprise brain, thymus, and lung. From Bulgaria in the fall of 2020, samples included muscles, parts of internal organs (as available), and lymph nodes. At the moment 2 samples of wild boar, 1 sample of fallow deer (*Dama dama*), 42 foxes and 32 jackals have been collected in Bulgaria. In France, vaginal or mucosal swabs from various imported animal species have been initially tested by bacteriological and molecular diagnostic methods. Previously isolated DNA samples from wild foxes have been obtained and screening molecular diagnostics performed.

Guidelines for sample treatment are in preparation. The harmonization of serological and bacteriological protocols is still in progress. A time schedule for the sampling period has been produced by all partners.

WP2: Recording the situation of brucellosis in humans (M25-M44)

Due to all delays, task 1 was delayed from January to July 2020, and will last until December 2020. Task 2 was delayed from May to November 2020, and will last until February 2022.

The question was raised about what kind of network the project should produce: Just a *Brucella* mailing list or interactive network.

Future actions:



- Create a survey for different networks to ask how would they diagnose emerging Brucella and/or differentiate classical from atypical species cases.
- Identify the expert point person in ECDC.

Final goal is to develop a diagnostic kit for emerging Brucella in humans.

WP-2 members will test potential antigens on humans, negative for classical Brucella species, first.

Inclusion criteria for other human samples will be: feverish patients and/or potentially exposed to atypical Brucella sources/reservoirs.

Two hundred patient sera have been tested so far, without identifying positive individuals (INSA). Twenty random blood samples have been tested in qPCR and ten individuals have been identified as positive for Brucella spp. These results urge us to analyse more tentatively sera from these individuals and design new serological tests more adapted to atypical and emerging Brucella species.

JRP17-WP2-T1: Creation of a surveillance network dedicated to human brucellosis (M25-M30)

JRP17-WP2-T2: Sampling and analytical strategy according to epidemiological information from the surveillance network (M29-M44)

WP3: Genomic characterisation of Brucella detected from samples and selected isolates (M25-M48)

JRP17-WP3-T1: Optimisation of methods for generating molecular typing data from complex samples (M29-M46)

Due to delays arising from the COVID-19 pandemic, this task has been delayed from M29 (May 2020) to M35 (Nov 2020), and will now be completed by M52 (April 2022).

- Consortium members have contributed to an informal survey describing DNA extraction protocols currently applied to relevant samples in partner laboratories. Through this we have been able to identify where methodological discrepancies exist, and identify areas where further optimisation work is required. This includes optimising DNA extraction methods for relevant novel sample types (e.g. soil and water).
- Optimisation work will be performed by the work-package lead & deputy, with input into relevant areas from other work-package members.
- Preliminary evaluation and standardisation of methods is on-going, initially focusing on methods for DNA extraction from soils using artificially “spiked” samples (due to the limited availability of positive control material for many relevant sample types).
- Methods arising from this work will be disseminated to consortium members for evaluation and implementation (WP3-Deliverable 1).

Validation studies will be carried out in the next year in order to evaluate the extraction protocols from tissue samples.

JRP17-WP3-T2: Harmonisation and standardisation of protocols for whole genome sequencing (M25-M36)

- Due to delays arising from the COVID-19 pandemic, this task has been delayed from M25 (January 2020) to M31 (July 2020), and will now be completed by M42 (June 2021).
- We will harmonise the performance of library preparation and sequencing procedures undertaken in partner institutes. In order to do this a representative panel of Brucella spp. isolates will be identified, and (inactivated) bacterial suspensions will be shared with partner institutes.



- Relevant strains have been identified from existing collections (APHA) and material will be generated from these for distribution.
- In the case of bioinformatics protocols we will benchmark assembly and alignment metrics, to ensure data are comparable between partner institutes, using shared samples and reference strains.
- Harmonised bioinformatics protocols will be shared with all other participating institutes (WP3-Deliverable 2).

JRP17-WP3-T3: Identification of emerging Brucella species: adaptation of molecular tools (M31-M46)

- Due to delays arising from the COVID-19 pandemic, this task has been delayed from M31 (July 2020) to M37 (January 2021), and will now be completed by M52 (April 2022).
- Available genome data from atypical strains has been collated, in order to populate a database with which to design discriminatory assays.
- Using expanded genomic databases (incorporating data generated under this project); we will identify discriminatory SNP typing assay(s) to identify atypical Brucella spp. strains.

WP4: Phenotypic characterisation of Brucella detected from samples and selected isolates (M25-M54)

JRP17-WP4-T1: Phenotyping of novel emerging Brucella spp.

JRP17-WP4-T1-ST1

We are already working on a scheme which will be used to phenotype the novel emerging Brucella spp.. This scheme will be adapted during the course of the project work if necessary.

JRP17-WP4-T1-ST3

All interested project partners will together plan which and how many of the Micronaut™ plates will be ordered to reduce the overall costs for this specific batch.

JRP17-WP4-T3: RNA sequencing of a representative panel of classical and novel Brucella spp.

JRP17-WP4-T3-ST1

To adapt bacterial inactivation to the RNA isolation protocol, we compared different inactivation methods (heat, ethanol etc.).

RNA isolation was performed using the TRIzol Max Bacterial RNA Isolation Kit (Invitrogen). This commercial kit includes an efficient lysis step and isolates total RNA from tissues or cells, including lipid-rich and difficult samples. We performed different quality checks e.g fragment analyzer, Nanodrop and agarose gel-electrophoresis and obtained sufficient high quality, integrity and yield of isolated RNA from different Brucella species, sufficient for RNA sequencing.

JRP17-WP4-T3-ST2

We searched for suitable cDNA library preparation kits and decided to use the Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit (Illumina) for a first sequencing trial. The sequencing run was applied on total RNA extracted from bacteria grown under starvation, and under starvation + low pH stress. We sequenced in biological triplicates and expect a sequencing depth of about 10 M reads per sample. The obtained data will be analyzed soon. Illumina Nextseq instrument was used for to obtain raw data.

In summary, a drafted RNA-seq protocol was successfully established.



WP5: Zoonotic potential and virulence (M35-M54)

Due to all delays, this task had to be delayed from November 2020 to May 2021, and will be finished until December the same year.

JRP17-WP5-T1: Develop an in silico pipeline for preliminary assessment of overall virulence and zoonotic potential.

JRP17-WP5-T2: Develop an in vitro protocol to investigate zoonotic potential based on macrophage cell lines originated from human and animals.

WP7: Coordination, management and communication (M25-M54)

The tasks started on January 2020. Due to all delays, this task was hindered until December 2022. Task 2 started on time, and due to all delays, it will last on December 2022. Tasks 3 and 4 were delayed until July 2020, and will last on December 2022.

The kick-off conference call was done on December 17th 2019, for organization of the project (M-JRP19-M2).

Master data table is finished and an Interactive platform (SharePoint) made by ANSES will be used in this project.

DMPs are done on a LSAM platform and are in the process of validation by all partners on the project.

A large storage file (DNA and RNA seq, and analyses, mapping data, etc.) platform will be available to the consortium from February 2021.

First annual workshop was postponed to second part of 2020 and was organised online on December 11th. The report will be publically available.

JRP17-WP7-T1: Coordination and organisation

Conference calls with all WP leaders and deputies regarding the project organization for WP-1, WP-2, WP-3, WP-4 and DMPs were done on March 5, on April 02, June 09 , July 23 , September 22 2020 (M-JRP19-M4).

Individual meetings with WP-2 and WP-3 members were done on June 17 in order to define WP status, delays and strategies. Individual WP meetings were organised throughout September in order to troubleshoot data and sample collection issues that arouse due to a COVID-19 pandemic.

JRP17-WP7-T2: Data management

DMPs are done on a LSAM platform and are in the process of validation by all partners on the project.

JRP17-WP7-T3: Risk management (M25-M54)

Due to the ongoing situation, on the conference call held on June 09th, consortium reported the delays, which were finally estimated to be six months. During the annual workshop, consortium reevaluated the delays and current estimates, with reprioritization of the tasks, are that six months delays will be sufficient to fulfil the project obligations.

JRP17-WP7-T4: Synthesis and dissemination of recommendations coming from the project outputs (M25-M54)

WP-1 T-1 and WP-2 T-1 will produce the base for the reporting system of emerging atypical *Brucella* spp.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
17	D-JRP17-WP1.Del1	Creation of a common database to share information about emerging Brucella and reservoirs among the consortium partners	M30		M31	No	Public: OHEJP JRP17 IDEMBRU Data management datasheet Zenodo	3
17	D-JRP17-WP2.Del1	Set-up of a human brucellosis network	M30		M36	Yes	Public: OHEJP JRP-17 IDEMBRU Work package 2 Deliverable 1 Zenodo	5
17	D-JRP17-WP3.Del1	Standard operating procedures for the extraction of Brucella DNA from complex matrices	M36		M42	Yes	Confidential	2
17	D-JRP17-WP3.Del2	Harmonised whole genome	M36		M42		Confidential	2



		sequencing and bioinformatic protocols						
17	D-JRP17-WP7.Del1	First draft of data management plan	M30		M36	No	Public	8
17	D-JRP17-WP7.Del2	Creation of a data sharing common platform	M32		M48	Yes	Confidential	4
17	D-JRP17-WP7.Del3	Final draft of data management plan	M34		M40		Public	8
17	D-JRP17-WP7.Del4	Report of the first annual workshop including exchanges between partners and inputs from external stakeholders	M36		M42	No	Public	8
17	D-JRP19-WP4.Del3	Drafted RNASeq protocols to identify regulatory differences between classical species and emerging Brucella strains	M30		M60	Yes	Confidential	2

* Categories of Integrative activities: 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
17	M-JRP17-M1	Mapping of existing human <i>Brucella</i> species (shared database)	M28	Yes		Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M2	Conference call of the steering committee regrouping WP leaders + deputy leaders on data management	M28	Yes		An initial kick-off conference call was organised in December 2019, regrouping all WP's deputy and leaders.
17	M-JRP17-M3	Definition of type of data generated for each WP and structure of the data sharing platform	M28	Yes		Template of database provided to IDEMBRU partners; Data sharing Platform organised by ANSES
17	M-JRP17-M4	Conference call of the steering committee regrouping WP leaders and deputy leaders	M28	Yes		Conference calls with all WP leaders and deputies regarding the project organization for WP-1, WP-2, WP-3, WP-4 and DMPs were done on March 5, on April 02 and June 09 2020 (M-JRP19-M4).
17	M-JRP17-M5	Creation of an epidemiological questionnaire	M29	Yes		Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M6	Information on worldwide emerging <i>Brucellae</i> (shared database)	M30	No	M37	Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
						conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M7	Definition of sampling and testing protocols (molecular, bacteriology and serology)	M30	No	M38	Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M8	Definition of sampling and testing protocols (serology, bacteriology and molecular biology)	M30	No	M37	Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M9	Drafted RNASeq protocols to identify regulatory differences between classical species and emerging <i>Brucella</i> strains	M30	Yes		Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M10	Definition of the programme of the annual workshop	M30	Yes		
17	M-JRP17-M11	Organisation of accommodation and logistic aspects	M32	No		The workshop has been moved online.
17	M-JRP17-M12	Implementation of first annual workshop	M34	Yes		
17	M-JRP17-M13	Definition of criteria to be included in the notification system	M34	No	M40	Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
17	M-JRP17-M14	Choice of the informatic system to implement the notification system	M34	No	M40	Delayed due to Coronavirus crisis (labs overloaded by COVID diagnostic; lockdown conditions; movements restrictions; delayed hiring of collaborators)
17	M-JRP17-M15	Harmonisation of protocols for whole genome sequencing	M35	No	M41	Delayed due to COVID-19 pandemic
17	M-JRP17-M16	Optimised protocols for the extraction of Brucella DNA from complex matrices	M36	No	M42	Delayed due to COVID-19 pandemic

4. Publications and patents

No publications yet.



5. Additional information:

Ponsart C, Al Dahouk S, Ashford R, Daskalov H, De Massis F, Freddi L, Garofolo G, Melzer F., Pelerito A, Umanets A, Whatmore A, Ferreira AC. *“Identification of emerging Brucella species: new threats for human and animals (IDEMBRU)”*. Poster presentation at One Health EJP Annual Scientific Meeting 2020, Prague (CZ), 27-29 May.



6. On-going and planned collaborations with national or European projects or networks

The ongoing EURL (**European Reference Laboratory for Brucellosis**) work program is mainly dedicated to classical *Brucella* species and is restricted to the domestic species, especially ruminants (in relationship with European regulation, including surveillance and control plans). Public results issued from the IDEMBRU project will be disseminated within this lab network in order to improve understanding of new atypical and emerging classical species in reservoirs without any current monitoring.

For the sampling steps, **national stakeholders and policy makers** are currently contacted as the project starts in order to organise the sampling and to establish the list of existing collections. For the human side, INSA is in contact with a national network of hospitals, which will contribute for dissemination of public results. The “BRUCE list” (list of contacts provided by the international society for Brucellosis) will be contacted within the WP2 task in order to interact with a large *Brucella*-focused network.

ECDC and EFSA will be contacted within WP-2 and WP-6 to establish an interactive network and look for experiences in setting up the toolkit. Collaboration with other regional, national and OH EJP projects will be established in order to share experiences and achieve objectives.

JRP18-MEME

1. Summary of the work carried out in year 3

MEME is aiming at filling relevant research gaps highlighted by international agencies for the detection and control of cystic and alveolar echinococcosis. MEME is focusing on standardization, harmonization and validation of existing parasitological and molecular methods, and the development and comparative assessment of innovative molecular tools and biomarkers to detect *Echinococcus multilocularis* (Em) and *Echinococcus granulosus s.l.* (Eg) along the food chain. Production of epidemiological data on the presence of Em/Eg eggs in the food chain is focusing on vegetables for human consumption as well as canine faeces in selected endemic countries. MEME includes integrative activities to harmonize procedures and to improve detection of Eg and Em.

Core activities conducted during the first year of the project:

- Standard Operating Procedures for the sampling of matrices were produced.
- Sampling of different matrices from naturally or experimentally infected definitive and intermediate hosts was started.
- Validation of the established parasitological (Segmental Sedimentation and Counting Technique, SSCT) and novel molecular diagnostic procedures (m-PCRs and MC-RT-PCR assay) was started to detect *E. multilocularis* and *E. granulosus s.l.* in different matrices along the food chain.
- Development and validation of new tools (new molecular markers for *Echinococcus* species from rapid diagnostics to source attribution; new multiplex qPCR for detection and discrimination of Em/Eg and Eg genotypes; sequencing using Region-Specific Extraction (RSE) and NGS for the detection of Em/Eg in complex samples; biomarker discovery in exosomes from sheep plasma for diagnosis of CE infection) was started.
- Start Production of data relevant for epidemiological assessments (contamination of vegetables for human consumption by eggs of Em/Eg; prevalence of Em/Eg in dog faeces;



identification of potential risk factors for human CE/AE infection through questionnaires; molecular epidemiology studies on Eg genotype diversity) was started.

- Dissemination of project results at different levels (general public, populations at risk, biologists, veterinarians, clinicians, health authorities, policy makers and media) was also started.

Scientific papers published on peer-review journals under the framework of MEME:

- Cystic Echinococcosis: Clinical, Immunological, and Biomolecular Evaluation of Patients from Sardinia (Italy). *Pathogens*. 9(11), 907. <https://doi.org/10.3390/pathogens9110907>
- A validated method to identify *Echinococcus granulosus sensu lato* at species level. *Infection, Genetics and Evolution*. 85, 104575. <https://doi.org/10.1016/j.meegid.2020.104575>
- Bayesian Analysis of Three Methods for Diagnosis of Cystic Echinococcosis in Sheep. *Pathogens*. 9(10), 796. <https://doi.org/10.3390/pathogens9100796>
- Species Detection within the *Echinococcus granulosus sensu lato* Complex by Novel Probe-Based Real-Time PCRs. *Pathogens*, 9(10), 791. <https://doi.org/10.3390/pathogens9100791>
- Microsatellite Investigations of Multiple *Echinococcus granulosus* Sensu Stricto Cysts in Single Hosts Reveal Different Patterns of Infection Events between Livestock and Humans. *Pathogens*, 9(6), pp. 444. <https://doi.org/10.3390/pathogens9060444>
- Casulli, A, (2020), Recognising the substantial burden of neglected pandemics cystic and alveolar echinococcosis, *The Lancet Global Health*. 8 (4):PE470-E471. [https://doi.org/10.1016/S2214-109X\(20\)30066-8](https://doi.org/10.1016/S2214-109X(20)30066-8)

2. Work carried out in the JRP, scientific results

WP1: SAMPLING STRATEGY (M25-M48)

The aim of this WP is to collect in the field and in the research facilities and to produce in the laboratory, the biological material needed to implement the activities of MEME. In the reporting period the collection of the samples in the field was started. Participants begin to organize sampling in the field from intermediate and definitive hosts. Part of planned material were collected and preserved for further purposes (red fox intestines and faeces, worms, cysts, dog faeces). Detailed SOPs for sampling of matrices were prepared. In experimental model, *E. multilocularis* strain was maintained in different groups of mice infected at different period in order to have protoscoleces available around every two months. These protoscoleces of *E. multilocularis* were used to infect three red foxes to obtain reference positive faecal material for the validation of methods and the Proficiency Testing schemes of MEME. Moreover, two red foxes were infected by protoscoleces of *E. granulosus* s.s. in order to obtain eggs for the infection of sheep for the proteomic study.

JRP18-WP1-T1. SOPs for sampling in matrices (M25-M28)

The following Standard Operating Procedures (SOPs) were prepared:

- Sampling of faecal material in dog faeces collected from the environment (WP1T2, Responsible ANSES) and their processing for molecular analysis.
- Sampling of *E. granulosus* cysts from naturally infected sheep and pigs at abattoirs (WP1-T2, Responsible PIWET) and experimentally infected sheep (WP1T3) and their processing for molecular analyses.
- Sampling of faeces and small/large intestines from experimentally infected foxes (WP1-T3, Responsible ANSES) and their processing for parasitological and molecular analyses.



- Sampling of vegetables for human consumption (WP3-T6, Responsible ANSES) and their processing for molecular analysis.

JRP18-WP1-T2. Matrices collection in the field from intermediate and definitive hosts (M27-M36)

The aim of this task is to collect different matrices from naturally infected definitive and intermediate hosts for their use in other WPs. Most of participants started to organize sampling from red foxes, dogs, and intermediate hosts. Five hundred and nine intestines of red foxes, 167 intestines of arctic foxes, 100 intestines of raccoon dogs from endemic areas were collected; some of them were examined with SCT/SSCT. From positive intestines *E. multilocularis* worms were isolated. Additionally, faeces from distal part of large intestine were collected. Sixty three red fox intestines from areas supposed to be free of *E. multilocularis* were collected. Moreover, 300 intestines of red foxes from *E. multilocularis* free areas (Ireland) were collected. Other parasites (*Mesocestoides* spp., 5 samples; *Taenia* spp., 4 samples) were isolated from small intestines of red foxes - for specificity controls. Ninety samples of pigs' livers and 100 sheep's livers with suggestive lesions of tapeworm larvae were collected and were prepared for identification. Additionally, few *E. multilocularis* and *Taenia hydatigena* sp. lesions isolated from *Arvicola terrestris* and *E. granulosus* cysts isolated from sheep were collected. Organization of collection of faecal samples from dogs for epidemiological study was started. Participants sent information and collection kits for owners and veterinary cabinet in rural areas of highly endemic regions. Three hundred and eighty faecal samples from dogs were collected in this way. Additionally, hundreds dog faecal samples from environment were collected, all negative for *E. multilocularis* (examined with qPCR) as a potential matrix for validation.

JRP18-WP1-T3. Matrices collection from experimental animal models (M27-M48)

ST1. All the authorizations for animal testing have been obtained at ANSES for the infection of mice and foxes with *Echinococcus*. They are in compliance with the ethical authorizations for animal experimentation (Decree n° 2013-118 of February 1st 2013) and the ethical authorizations for the experimental infection of carnivores by *E. multilocularis* (from mice to foxes) (16-073 N° APAFIS: 20160913348095). Unfortunately, production of matrices (faeces, worm and eggs) from foxes has been delayed for months due to national lock-down consequent to COVID-19 pandemic (absence of workers at the animal facilities). Only mice metacestodes production has been maintained during this period. Metacestodes material (200 µl of protoscoleces) and 200 references DNA/protein samples have been produced. Starting from July 2020, several infected groups of mice were also produced. At the beginning of July, two foxes have been infected with 35,000 protoscoleces from infected mice. Faeces have been daily collected to monitor the infection for 90 days. Collected faeces have been inactivated at -80°C for safety reasons. These faecal samples have been analysed by qPCR and flotation methods. We have obtained reference positive faeces (with or without eggs) for future development of MEME diagnostic protocols. A study about the detection of *E. multilocularis* DNA by copro-qPCR during prepatent and patent infection is in progress in order to obtain a better understanding of *E. multilocularis* DNA detection in fox faeces. At the beginning of October, one more fox have been infected with 50,000 protoscoleces from infected mice. Feces have been daily collected, inactivated at -80°C, and analysed by PCR to monitor the animal infection. At Day 28 we have euthanized the fox and the intestine have been collected and stored at -80°C as reference material for the SSCT Proficiency Testing of MEME.

At the end of October 2020, two red foxes were also infected by *E. granulosus* s.s. protoscoleces from cysts of sheep collected at slaughterhouses in Sardinia (IZSS, Italy). These *E. granulosus* s.s. eggs should be used to infect sheep in Portugal for the proteomic study. The flotations and PCRs at 47 dpi were negative. Analyses of fecal samples by flotation between 60 and 70 dpi will be realized soon to confirm the absence of infection.

ST2. Sheep were selected and kept in animal facilities in Portugal according to animal welfare procedures. Concerning ethics approval for the experimental infection of sheep, this was prepared and submitted to the local ethics committee in Portugal. The ethical authorization for the infection of sheep with *Echinococcus granulosus* eggs have been obtain from the Portuguese Veterinary Authority (DGAV



– Direção Geral de Alimentação e Veterinária) in December 2020, in compliance with the Decree 113/2013 of August 7th 2013. A selection of 20 one year old female sheep has been kept in adapted facilities with increased biosafety in Santarém (Portugal, a INIAV research centre).

WP2: VALIDATION of PARASITOLOGICAL and MOLECULAR ASSAYS (M29-M44)

The harmonization and validation of selected parasitological and molecular procedures have been impacted by national lock-downs due to COVID-19 pandemic. Some task are delayed, time has been used to collect samples. Validation will include estimates of the analytical and diagnostic performance characteristics of tests.

Publications:

Skrzypek K, Karamon J, Samorek-Pieróg M, Dąbrowska J, Kochanowski M, Sroka J, Bilska-Zajac E, Cencek T. Comparison of Two DNA Extraction Methods and Two PCRs for Detection of *Echinococcus multilocularis* in the Stool Samples of Naturally Infected Red Foxes. *Animals (Basel)*. 2020 Dec 11;10(12):2381. doi: 10.3390/ani10122381.

Santucci C, Bonelli P, Peruzzu A, Fancellu A, Marras V, Carta A, Mastrandrea S, Bagella G, Piseddu T, Profili S, Porcu A, Masala G. Cystic Echinococcosis: Clinical, Immunological, and Biomolecular Evaluation of Patients from Sardinia (Italy). *Pathogens*. 2020 Oct 30;9(11):907. doi: 10.3390/pathogens9110907.

Santolamazza F, Santoro A, Possenti A, Cacciò SM, Casulli A. A validated method to identify *Echinococcus granulosus* sensu lato at species level. *Infect Genet Evol*. 2020 Nov;85:104575. doi: 10.1016/j.meegid.2020.104575.

Bonelli P, Loi F, Cancedda MG, Peruzzu A, Antuofermo E, Pintore E, Piseddu T, Garippa G, Masala G. Bayesian Analysis of Three Methods for Diagnosis of Cystic Echinococcosis in Sheep. *Pathogens*. 2020 Sep 27;9(10):796. doi: 10.3390/pathogens9100796.

JRP18-WP2-T1. Segmental Sedimentation and Counting Technique, SSCT (M29-M36)

The SSCT method was chosen due to its high sensitivity (98.3%) compared to reference SCT and above all a significant reduction of time due to the analysis of only two out of five segments of the whole intestines (Umhang et al., 2011). According to the SOP (WP1-T1), each segment of fox intestines collected in the different countries will be independently analysed for the observation and counting of the number of worms. The data obtained will be used for an international validation of the method using the best combination of two segments resulting in the higher sensitivity. Previous results reported from 117 infected fox intestines from France in the context of the original description of the method will be added. According to the protocol, the analyses of the first four segments of intestines has already started in France, Latvia and Poland and will be extended to other participants during the year. Due to national lock-downs consequent to COVID-19 pandemic, the real start of the analyses will be delayed until the end of 2021.

JRP18-WP2-T2. Comparison of multiplex PCRs (M29-M44)

This task focuses on the validation of molecular assays widely used for the detection of *Echinococcus* in definitive and intermediate hosts, targeting mitochondrial and nuclear markers:

Assay 1: Boubaker et al. (PLoS Negl Trop Dis, 2013), a single-tube multiplex PCR allowing discrimination of *Echinococcus* at the level of species/genotypes.

Assay 2: Trachsel et al. (Parasitology, 2007), a single-tube multiplex PCR targeting the definitive host for the identification of eggs belonging to *E. granulosus*, *E. multilocularis* and *Taenia* genus.

The assay 1 was tested resulting in conclusion that it is not possible to validate this method because of the unreliability of the molecular markers used in the multiplex PCR. Next step will be the validation of the assay 2.



JRP18-WP2-T3. Magnetic Capture - Real Time PCR assay (M29-M44)

The validation of the Magnetic Capture-PCR will be conducted according to stage 3 and 4 of the OIE chapter on validation of diagnostic methods, since basic diagnostic characteristics of the test have already been published. Stage 3 validation means testing the assay in different laboratory settings, using equally aliquoted panels of more than 20 samples sent out to each laboratory. To make this possible, the method needs to be set up and verified before this panel is sent out and tested. So far, we have identified the laboratories interested in participating, we have more or less finished SOPs for the methods and a SOP for the validation is in progress. Reference samples from ANSES was received. Next steps are prepare reference material for setting up and verifying the method in the participating labs, and also arrange workshops for the persons in need. Due to COVID-19, measures limiting personnel access to the laboratories and travel restrictions due to quarantine requirements these workshops have been postponed. We are exploring alternative methods to carry out the training and are in dialogue with the project partners to find a viable alternative for early 2021, such as digital training platforms. Once the method is set up and verified in the labs, the stage 3 validation panel can be sent out. This is planned to take place late summer 2021. Not until the data is analyzed, can stage 3 validation be considered finished.

WP3: DEVELOPMENT/VALIDATION of NEW TOOLS and PRODUCTION of DATA RELEVANT for EPIDEMIOLOGICAL ASSESSMENTS (M27-M52)

WP3 aims at generating new innovative tools for rapid detection, differential diagnosis, and tracking of infection, both at large and small-scale settings.

Most tasks of work package 3 are delayed due to the COVID-19 lockdowns as many laboratories were closed. It was therefore not possible to work on several tasks of the work package. In course of the task WP3-T1, samples from various countries worldwide were collected and of these, the mitochondrial genomes of 85 isolates from Turkey (n=47) and Armenia (n=38) were sequenced. Further samples will be collected and analysed by mitogenome sequencing. Four TaqMan® probe-based qPCRs were developed in task WP3-T2. At this stage, they can be used as a single-step genotyping technique for the diagnosis of *E. granulosus* genotypes in four epidemiologically relevant subgroups, i.e. *E. granulosus* s.s. (G1 and G3), *E. equinus* (G4), *E. ortleppi* (G5) and the *E. canadensis* complex (G6, G7, G8 and G10). In task WP3-T4, the study protocol was submitted for approval to the ethics committee in March 2020. Due to the difficulties caused by COVID-19 pandemic, the experimental animal infections for the proteomic study are delayed.

JRP18-WP3-T1. New molecular markers for Em and Eg s.l.: from rapid diagnostics to source attribution (M27-M48)

Following the use of the two published microsatellites EgSca6 and EgSca11 for *E. granulosus* s.s., the presence of two copies in the genome of EgSca11 complicates its interpretation and prevent its use for phylogenetic analyses. There is a need to substitute this microsatellite by one or several other classical microsatellites. A new screening of the *E. granulosus* genome has resulted in the identification of 15 new microsatellites targets, which are currently evaluated for their polymorphism, reproducibility, limit of detection, quality of the electrophoretic profiles and their specificity. The selected profiles will be associated to EgSca6 in order to obtain a panel with a high discriminatory power allowing highly discriminant identification which is necessary for accurate source attribution and for large phylogenetic studies. *E. granulosus* s.s. DNA samples from North Africa (Morocco, Tunisia) and Europe (France, Moldova) are already available and will be completed by others for testing polymorphism of the microsatellites.

Publication:

M'rad S, Oudni-M'rad M, Bastid V, Bournez L, Mosbahi S, Nouri A, Babba H, Grenouillet F, Boué F, Umhang G. Microsatellite Investigations of Multiple *Echinococcus Granulosus Sensu Stricto* Cysts in



Single Hosts Reveal Different Patterns of Infection Events between Livestock and Humans. *Pathogens*. 2020 Jun 5;9(6):444. doi: 10.3390/pathogens9060444.

JRP18-WP3-T2. New multiplex TaqMan qPCR for detection and genotyping of *Eg s.l.* and *Em* (M27-M48)

At this stage, Four TaqMan® probe-based qPCRs can be used as a single-step genotyping technique for the diagnosis of *E. granulosus* genotypes in four epidemiologically relevant species and subgroups, i.e. *E. granulosus sensu stricto* (G1 to G3), *E. equinus* (G4), *E. ortleppi* (G5), the *E. canadensis* complex (G6 to G8 and G10) and a single genotype (G8) of the *E. canadensis* complex. The technique also allows differentiating *E. granulosus* samples from other *Echinococcus* or *Taenia* species in samples derived from cystic or faecal material. The qPCRs show high efficiency (ranging between 99% and 106%), analytical specificity (100%) and sensitivity (ranging between 0.6 and 1.4 copies/μl) when used with DNA obtained from cysts or from cloned PCR products. Therefore, they are suitable for a PCR-based diagnosis of cystic echinococcosis in intermediate hosts, including humans as aberrant intermediate hosts. These qPCRs will now be combined to develop a TaqMan probe-based multiplex Real-Time qPCR as a tool for a simultaneous, rapid diagnosis and typing of *E. granulosus sensu lato* and *E. multilocularis* infections. Moreover, experiments are initiated to develop TaqMan® probe-based qPCR assays that differentiate between the individual members of the *E. canadensis* complex (G6, G7, G8 and G10).

Publication:

Maksimov P, Bergmann H, Wassermann M, Romig T, Gottstein B, Casulli A, Conraths FJ. Species Detection within the *Echinococcus granulosus sensu lato* Complex by Novel Probe-Based Real-Time PCRs. *Pathogens*. 2020 Sep 26;9(10):791. doi: 10.3390/pathogens9100791.

JRP18-WP3-T3. Detection of *Em/Eg* in complex samples: sequencing using Regions Specific Extraction (RSE) and NGS (M27-M48)

Initial RSE with NGS have been performed by FLI in Q1-2 2020. Data analysis still has to be performed. Analysis of *E. multilocularis* DNA positive control material (surveillance extraction method) with RSE and Nanopore using Flongle was planned for summer 2020 at NVI. However, restrictions to laboratory access as a result of preparedness measures for the COVID-19 pandemic, has delayed this work.

JRP18-WP3-T4. Proteomic study on biomarker discovery in exosomes from animal plasma (M27-M52)

The collection of blood samples for the inception of this activity will start one-year after the infection of sheep with *E. granulosus* egg in Portugal. The approval for the experimental infection was obtained from the local ethics committee in Portugal (INIAV). Because of the COVID-19 pandemic, the production of *E. granulosus* eggs for the infection of sheep was delayed.

JRP18-WP3-T6. Contamination of vegetables for human consumption by *Em/Eg* (M29-M48)

The use of a robust and reliable method to detect few *Echinococcus* spp. eggs from vegetables is a prerequisite to estimate the contamination of vegetables for human consumption. The sensitive technique based on sequential sieving coupled with molecular detection developed by the team of Prof. Peter Deplazes at the Zürich Institute of Parasitology (Guggisberg et al., 2020) was chosen as reference for this multicentre epidemiological study. This method using 300 g of lettuce leaves sample was transferred to ANSES. After sieving and DNA extraction, the detection of *E. multilocularis* DNA was done by qPCR (Knapp et al., 2016) which proved to detect one egg (Ct: 30). Using different spiked numbers of *E. multilocularis* eggs obtained from faeces after experimental infection of foxes (WP1-T3-ST1), the limit of detection in 95% of the time (LoD95) was estimated at three *E. multilocularis* eggs (23/24). When spiked with two eggs a positive detection was obtained for 87.5% (21/24) of the lettuce leaves samples and the one for one *E. multilocularis* egg still needs to be estimated. Additionally, the efficiency of washing lettuce leaves under running water and of spinning with salad spinner and on paper towel are also currently under evaluation. One hundred-sixty lettuces were collected from



private kitchen gardens (2 lettuces each) or directly from producers (4 lettuces each) in *E. multilocularis* endemic areas from northern-east France. These analyses detected DNA of *E. multilocularis* in two pools from producers and DNA from *Hydatigera* sp. in six pools from both private kitchen garden and producers. Hundreds of lettuce from others partners are planned to be collected and analysed by ANSES. For each lettuce, the solution obtained at two additional steps (40 µm filter and filtrate from 20 µm) will be also collected in order to later estimate the contamination by other parasites (mainly *Toxocara* spp. and *Toxoplasma gondii*).

JRP18-WP3-T7. Prevalence of Em/Eg in dog (faecal samples) from selected geographical areas (M29-M48)

The study design was discussed among participants. The target group are dogs originating from highly endemic areas (high prevalence of *E. multilocularis* in foxes as a proxy), or from regions with relatively high prevalence of *E. granulosus* in sheep. Dogs included in the epidemiological study will be those owned dogs that may have access on feeding on mice potentially infected by *E. multilocularis*. Specific conditions in individual countries will be taken into consideration (i.e. sled dogs from Svalbard in Norway). Samples must be collected individually (i.e. to be identifiable with individual dog described in the questionnaire). Moreover, organization of collection of faecal samples from dogs was started. Number of samples has been statistically estimated. The questionnaire (to complete by owners/vets) with questions concerning data which will be used in epidemiological analysis was elaborated. The following data were included in questionnaire: data about dog (age, breed and sex), place of living, activity sites and types of activity, deworming (date, frequency and drug), feeding, eating rodents and others. Some participants sent information and/or collection kits to owners and veterinary cabinet in rural areas of highly endemic regions. Additionally, the collection of around 400 hundred of faeces from hunting dogs in France (cooperation with hunting federation) has been launched. Till now, overall, 380 samples from dogs were collected in France, Poland, Latvia and Estonia and preserved for further molecular testing. DNA was extracted from 60 French samples which were preliminary examined with multiplex PCR resulting to the detection of two positive for *Taenia* spp. Next step is to discuss and choose the additional molecular method (qPCR) for the identification of *Echinococcus* spp. in faecal samples.

JRP18-WP3-T8. Potential human risk factors by means of questionnaires (M29-M48)

Semi-structured questionnaires on potential risk factors for human infection with CE and AE will be designed and submitted for ethical approval to the relevant committees of the participating hospitals. Upon written informed consent, questionnaires will be administered to volunteers with CE/AE and matched (by age, sex and country of origin) uninfected controls accessing clinical centres participating to the study. This task delayed because of COVID-19 pandemic.

WP4: TRAINING, DISSEMINATION and PROFICIENCY TESTING SCHEMES (M27-M54)

This WP focuses on establishing the most effective methods for disseminating project results at different levels and training scientists from institutions participating to MEME in the parasitological and molecular identification of *Echinococcus* spp.

Publication:

Casulli A. Recognising the substantial burden of neglected pandemics cystic and alveolar echinococcosis. *Lancet Glob Health*. 2020 Apr;8(4):e470-e471. doi: 10.1016/S2214-109X(20)30066-8.

JRP18-WP4-T1. Trainings (M27-M48)

This task has few months delay because of COVID-19 pandemic. Task WP4-T1 will be achieved in the due time.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
18	D-JRP18-WP1.1	SOPs for sampling in matrices shared with the Consortium	M28	M33		No	Public OHEJP: available Zenodo: available https://zenodo.org/record/4455196#.YAmlsxbSKgo	2
18	D-JRP22-WP1.2	Collection of samples in the field finalized	M36		M40	Yes	Public	8
18	D-JRP22-WP2.1	Validation of SSCT	M36		M48	Yes	Public	8
18	D-JRP22-WP5.1	Data Management Plan	M30	M36		No	Public	8
18	D-JRP18-WP5.2	Periodic technical and financial reports to EJP/Commission	M36	9mth/12mth report	M38	No	Public	8
18	D-JRP18-WP5.5	Kick-off annual meeting in Malzéville (France) by ANSES	M27	M28	/	No	Public OHEJP: available Zenodo: available	8



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted : Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
							https://zenodo.org/record/4455256#.YAmKvxbSKgo	

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
22	M-JRP18-01	Tasks and responsibilities allocation	M25	Yes		Kick off meeting in France used for the allocation of the tasks and responsibilities
2	M-JRP18-02	Organization of Kick-off annual meeting by ANSES	M26	Yes		Nancy, 5-6 February 2020
22	M-JRP18-03	Ethics approval for the use of animal model	M28	Yes for ANSES; Yes for INIAV		<u>COVID-19 pandemic</u> delayed the ethic approval for the sheep model at INIAV, Portugal. This is the most relevant delay with domino effects on proteomic study (WP3-T4). The study is due to start as soon as <i>E. granulosus</i> eggs are obtained after fox infection.
22	M-JRP18-04	SOP for validation of SSCT	M30	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
22	M-JRP18-05	SOP for validation of multiplex PCRs	M30	Yes		
18	M-JRP18-06	SOP for validation of mc-RT-PCR assay	M30	No	M38	SOP preparation is depending on the requirements of the people participating to the training on RT-PCR assay which was delayed by <u>COVID19</u> event
22	M-JRP18-07	Protocols for new molecular methods, NGS included	M30	yes for WP3-T2; draft protocol for the others	M38	Task delayed but achievable before the end of this year
22	M-JRP18-08	Protocol for proteomic analysis in animals plasma	M30	No	Depending on animal infection	Because of <u>COVID19</u> delay: serious concerns on feasibility of this task in due time because of the huge amount of time needed for the growth of parasites after animal infection (>1 year)
22	M-JRP18-09	Protocol for contamination of vegetables by Em/Eg	M30	Yes		
22	M-JRP18-10	Protocol for prevalence of Em/Eg in dog	M30	Yes		
22	M-JRP18-11	Questionnaire scheme for potential human risk factors	M30	No	M38	Task delayed due to national lock-downs.
22	M-JRP18-12	Interim evaluation of collection of samples in the field	M32	Yes		Lockdown delayed the start of sampling but this deadline was achieved in due time.



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
22	M-JRP22-13	Preparation of technical and financial reports to EJP/Commission	M35	Yes		
22	M-JRP22-14	Organization of Training periods	M36	No	48	Due to national lock-downs.
22	M-JRP22-17	Organization of interim annual meeting by FLI	M37	No	48	Due to national lock-downs.

4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Comparison of Two DNA Extraction Methods and Two PCRs for Detection of <i>Echinococcus multilocularis</i> in the Stool Samples of Naturally Infected Red Foxes. https://doi.org/10.3390/ani10122381 https://zenodo.org/record/4384600	YES	Open Access, without embargo period.	Gold Open Access. 1.481,00 €
Cystic Echinococcosis: Clinical, Immunological, and Biomolecular Evaluation of Patients from Sardinia (Italy). https://doi.org/10.3390/pathogens9110907 https://zenodo.org/record/4159681	YES	Open Access, without embargo period.	Gold Open Access. 1.389,79 €



Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
A validated method to identify Echinococcus granulosus sensu lato at species level https://doi.org/10.1016/j.meegid.2020.104575 https://zenodo.org/record/4066416#.X6UQMWhKjcc	YES	Open Access, without embargo period.	Gold Open Access. 2.300,00 €
Bayesian analysis to evaluate three diagnostic methods for Cystic Echinococcosis in sheep. https://doi.org/10.3390/pathogens9100796 https://zenodo.org/record/4061823#.X6PZl2hKjcc	YES	Open Access, without embargo period.	Gold Open Access. 1.389,79 €
Microsatellite Investigations of Multiple Echinococcus Granulosus Sensu Stricto Cysts in Single Hosts Reveal Different Patterns of Infection Events between Livestock and Humans. https://doi.org/10.3390/pathogens9060444 https://zenodo.org/record/3894117#.XvyQ6ygzZM0	YES	Open Access, without embargo period.	Gold Open Access. 654,56 €
Species detection within the Echinococcus granulosus sensu lato complex by novel probe based Real-Time PCRs. https://doi.org/10.3390/pathogens9100791 https://zenodo.org/record/4061718#.X6PY3WhKjcc	YES	Open Access, without embargo period.	Gold Open Access. 1.250,81 €
Recognising the substantial burden of neglected pandemics cystic and alveolar echinococcosis. https://doi.org/10.1016/S2214-109X(20)30066-8 https://zenodo.org/record/3730559#.Xn3FalhKi70	YES	Open Access, without embargo period.	Gold Open Access. Invited by Editor of Lancet GH, no cost.

Additional output:

Presentation of MEME project to:

- Annual online meeting of the European Union Reference Laboratory for Parasites (EURLP). 15 December 2020. Rome, Italy.
- Annual Scientific meeting of One Health EJP project, held online. 27-29 May 2020. Prague, Czech Republic.
- Kick off meeting of MEME. 5-6 February 2020. Nancy, France.



- XXVIII World Congress on Echinococcosis. 29-31 October 2019. Lima, Peru.



5. On-going and planned collaborations with national or European projects or networks

Ongoing and planned collaborations with the following entities:

- the other parasitology-JRPs: PARADISE and TOXOSOURCES;
- the European Reference Laboratory for Parasites (EURLP);
- the WHO Collaborating Centre WHO Collaborating Centre for the Epidemiology, Detection and Control of Cystic and Alveolar Echinococcosis (in humans and animals);
- PERITAS project (molecular ePIDemiological studiEs on pathways of tRansmission and long lasTing cApacity building to prevent cyStic echinococcosis infection). 2018-2021. Funded by the EC under EULAC-Health.

JRP19-PARADISE

1. Summary of the work carried out in year 3

The Kick off meeting (KOM) held at ISS (Rome, 10-11 February 2020) allowed a detailed presentation of the planned activities, followed by ample discussion among the Consortium members. The most important progress for the research-orientated activities are as follows:

- We reached a consensus on the criteria for the selection, preservation and shipment of samples. Despite the restrictions linked to the COVID-19 emergency, many partners were able to collect and ship samples and the first genome sequencing effort has taken place, resulting in the generation of 24 novel *Giardia duodenalis* and 44 novel *Cryptosporidium parvum* genomes. Further samples are being processed and additional genome data will be available January-February 2021. The *in silico* metagenomics approach demonstrated the presence of parasite sequences in metagenomes from various matrices, and a manuscript has been submitted that describes how specificity can be increased. The amplicon-based sequencing approach was more robustly tested, in particular using new primers for the detection of flagellates (including *Giardia*). Reference material (parasute cysts) for spiking experiments has been produced.
- An inventory of the parasite samples (genomic DNA, faeces, and other relevant matrices) available at each partner Institute was completed and judged well suited for the planned tests, which will start at the beginning of Year 4. The first selection of *C. parvum* variable genomic regions was achieved based on a comparative analysis of 70 different genomes. The selection will be evaluated again as additional genome data are generated or became available in public databases. The possibility to include markers typable using fragment analysis was evaluated in consultation with the associated partner CRU. For *G. duodenalis* assemblage B approximately 100 genomic regions suitable for typing marker selection were identified using 18 available genomes and by implementing a newly developed bioinformatic analysis pipeline.
- The animal immunization experiments were performed as planned, the phage library was prepared from cDNA and, using whole cyst and oocyst antigens as prey, an enrichment in potential binders was obtained. For the aptamers, various rounds of SELEX were done for both *Cryptosporidium* and *Giardia* at ANSES and ISS, but due to technical problems, new strategies are being developed. For DNA fishing, two capture systems were designed for *Cryptosporidium* (using as markers the 18S rDNA and the gp60 loci) and one for *Giardia* (using beta-giardin as marker). The results have shown high specificity and sensitivity for *Cryptosporidium*, and are in progress for *Giardia*.



In conclusion, although delay in some activities has occurred, considerable progresses has been made and the project is proceeding as planned.

2. Work carried out in the JRP, scientific results

WP1: Coordination and impact (M25-M54)

JRP19-WP1-T1: Management, coordination and communication (M25-M54)

The Kick Off Meeting of the project was organized at ISS in Rome on February 10-11, 2020. The report (Deliverable D-JRP-PARADISE-WP1.1) was submitted on April 2020. Shortly after the KOM, the Covid-19 pandemics became a serious and widespread issue, and, since after, communication has mostly taken place using virtual platforms. WP and Task leaders met regularly to follow the project's activities. All partners received e-mails containing updates on the project's progress and were consulted before important deadlines (e.g., at the time of reports). The members of the Steering committee have been nominated, while the composition of the Advisory Board needs to be completed.

Due to Covid-19 pandemics, many scheduled meetings, congresses and conferences were cancelled, with a negative impact on the dissemination of the project at both national and international level. In Year3, two manuscripts were published by the Consortium, and another is currently under review process.

WP2: NGS-based genomics and metagenomics (M25-M52)

JRP19-WP2-T1: NGS-based genome study of selected isolates of *C. parvum* and *G. duodenalis* (M25-M36)

The goal of this task is to generate new whole genome data of isolates of *Cryptosporidium parvum* and *Giardia duodenalis*, and to ensure that isolates from different European countries and different hosts are included. The planned sample collection has been only partially achieved, due to restrictions imposed by the COVID-19 epidemics in many countries. Nevertheless, many partner institutes (ANSES, INIAV, ISS, NVI, OKI, PIWET, RKI, SSI and SVA) and associated partners (BIOR) have been able to collect a relevant number of fecal samples positive for *Cryptosporidium* (~ 100) and *Giardia* (~ 50). Most of these samples were shipped to SVA, RKI and ISS for further processing. High quality genomic DNA was prepared, checked for the presence of bacterial contaminants by PCR, submitted (for *Cryptosporidium*) to whole genome amplification (WGA), purified and finally sent to ANSES for NGS-based whole genome sequencing experiments. All NGS experiments were based on Illumina technology (short reads, 2x150 paired ends), and scaled to achieve an average 50X coverage. Up to now, 24 new Assemblage B *G. duodenalis* and 44 new *C. parvum* genomes have been sequenced, but additional experiments are rapidly progressing and the anticipated target (100 genomes) will be reached shortly. At present, isolates of both human and animal (livesock) origin, collected in Denmark, France, Germany, Hungary, Italy, Norway, Poland, Portugal and Sweden, have been sequenced. An ongoing collaboration of one partner (ISS) with Finland will allow to generate more genomic data, and an agreement to use these data for the purpose of marker selection was found. A cloud storage space for data sharing has been set up by SVA, and NGS data of all genomes from the project, as well as data from partners and those from publicly available databases, were uploaded. A password-protected access has been granted to all partners involved in the bioinformatics analyses. Finally, SVA has further implemented the *Cryptosporidium* pipeline developed by a previous EU Horizon 2020 project (COMPARE), whereas RKI has developed another analytical pipeline to process *Giardia* NGS sequence data.

JRP19-WP2-T2: In silico analyses of metagenomes for detection of foodborne parasites (protozoa and helminths) (M25-M52)

The goal of this task is to demonstrate that parasite sequences present in complex metagenomics datasets can be detected with high specificity, and to evaluate the limit of detection, as both specificity



and sensitivity are essential parameters to be taken into account to understand the applicability of metagenomics as a platform for foodborne parasites detection. To this end, publicly available metagenomics data, which were generated from matrices (e.g., gut metagenomes, environmental samples) potentially contaminated with parasites, were downloaded. In parallel, a bioinformatics workflow for detection of parasite sequences was developed at RIVM. Although less extensive than for bacterial and viral pathogens, genome information is available for many protozoa and helminths, and can be collated to create a reference database against which metagenomics reads can be compared. Such reference database is close to be completed, but the deadline for the associated milestone (M-JRP-PARADISE-5, Referenced database of foodborne parasite genomes established) has been moved to M40. The reference database will then be submitted to regular updates (every year).

In a first series of *in silico* experiments, public calf intestinal metagenome datasets were interrogated for the presence of gastrointestinal parasites, and this resulted in the detection of many *Cryptosporidium*-specific reads. Alongside, investigation of a metagenome dataset from irrigation water sampled at the Sao Paulo zoo revealed a complex array of protozoa, demonstrating the suitability of the approach for detection of multiple parasites. Planned experiments include the creation of metagenomics datasets spiked *in silico* with known number of reads from given parasites, in order to explore the detection limit. A more systematic search is planned to identify additional public metagenomes that can be interrogated for the presence of FBPs. A manuscript has been submitted to *Frontiers in Microbiology* and is currently under review. The manuscript presents several test cases and focuses on bioinformatics strategies that may increase the specificity of detection. Due to the specific challenges identified during the test experiments, and limited access to work premises due to the Covid-19 pandemics, the optimization of the pipeline is not completed and the deadline of the associated milestone (M-JRP-PARADISE-6, Pipeline for metagenome data analysis for FBPs optimized) has been moved to M40.

JRP19-WP2-T3: Experimental amplicon-based and shotgun metagenomics for detection of foodborne parasites (M25-M52)

One goal of this task is to optimise a platform based on amplicon-based next-generation sequencing for detecting parasites, with special emphasis on foodborne parasites. The principle of the method relies on amplification of the 18S ribosomal DNA gene (18S rRNA) from eukaryotic organisms. The method is part of a 16S/18S platform developed at SSI. The method has already been used in various studies, and has been applied to genomic DNA extracted from various matrices such as faeces, other clinical sample types (EDTA blood, spinal fluid, skin/cornea biopsies/scrapings, etc), food products and environmental samples. At present, the platform has very low sensitivity for the detection of some flagellates, including *Giardia*, and also microsporidia. Therefore, tests of different samples with a different primer pair are ongoing to address this issue. As such, the deadline for the deliverable D-JRP-PARADISE-WP2.1 (Protocol for 18S rDNA-based amplicon sequencing for detection of relevant FBPs) has been moved to M37.

A second goal is to compare the performance of the amplicon-based next-generation sequencing with that of shotgun metagenomics, an untargeted sequencing approach used to profile the taxonomic composition of communities. To this end, highly purified *Cryptosporidium* oocysts were produced using flow cytometry (SVA), whereas *Giardia* cysts were produced by *in vitro* culture (ISS). This material will be used to spike different food matrices, from which DNA will be extracted and submitted to amplicon-based and shotgun NGS experiments.

WP3: Design, implementation and validation of multi-locus typing schemes (M25-M54)

JRP19-WP3-T1: In silico selection of informative loci from comparative genomics data. (M25-M42)

The goal of this task is to compare whole genome data for the identification of regions of high genetic variability in both *C. parvum* and *G. duodenalis*. To this end, selection criteria were defined, including (i) sufficient physical distance between variable regions to minimize genetic linkage; (ii) coverage of all



chromosomes; (iii) high genetic variability across genomes; (iv) *in silico* specificity for *C. parvum* or *G. duodenalis*. Furthermore, fully automated and comprehensive analysis workflows to process NGS data have been developed for *C. parvum* (SVA) and *G. duodenalis* (RKI).

A first *in silico* selection has been completed for *C. parvum* (SVA), based on a comparison of 75 available genomes, and resulted in the selection of 61 variable regions. Likewise, a first *in silico* selection was completed for *G. duodenalis* Assemblage B (RKI), based on a comparison of 18 available genomes, and resulted in the identification of 104 variable regions. More details on the procedures are given in the report on the deliverable D-JRP-PARADISE-WP3.1 (Report on the *in silico* selection of highly polymorphic sequences in *C. parvum* and *G. duodenalis* genomes).

JRP19-WP3-T2: Development of MLST schemes for *C. parvum* and *G. duodenalis* (M31-M50)

To develop new typing schemes, it is of paramount importance to test potential markers on a large collection of samples from different hosts and geographical origin. All partner institutes have been asked to confirm availability of samples in view of the test experiments and the feedback has been very positive. Partner institutes are in the process of providing metadata on samples they can provide through completion of a standard form. Sample shipment to the laboratories which will undertake MLST development will take place early in 2021. SVA has discussed with CRU (associate partner) about markers for fragment typing, and CRU has agreed to share a list of the most promising markers they have identified in previous projects.

WP4 : Parasite enrichment strategies (M25-M54)

The objectives of WP4 are the development and evaluation by inter-laboratory comparison of: i) two pre-DNA extraction protocols based on two alternative affinity reagents, nanobodies (single-chain variable fragment antibodies) and aptamers (oligonucleotides that bind to a specific target molecule), and their application for magnetic capture of *C. parvum* and *G. duodenalis* (oo)cysts in different matrices; ii) a protocol for post-DNA enrichment to concentrate parasite DNA based on hybridization of target-specific biotinylated probes.

JRP19-WP4-T1: Development of pre-DNA extraction enrichment strategies (M25-M54)

1. Nanobodies: Two new world camelids have been immunized either with extracts from *Giardia duodenalis* cysts or *Cryptosporidium parvum* oocysts, respectively. ELISA and IFA tests showed that both animals reacted to the respective antigens. The cDNA libraries of variable domains of heavy-chain only antibodies have been prepared for both *Giardia* and *Cryptosporidium*. A phage display approach was established that uses whole cyst and oocyst antigens as prey. After several rounds of panning, enrichment of potential "binders" for each antigen was observed. However, as the completion of the entire selection process is expected for early 2021, the deadline for the associated milestone M-JRP-PARADISE-11 (Highly affine nanobodies selected) has been moved from M36 to M40.
2. Aptamers: ANSES has completed ten rounds of SELEX for five aptamer pools for both *C. parvum* (IOWA strain) and *G. duodenalis* (assemblage B). ISS has successfully produced *in vitro* *G. duodenalis* cysts (assemblage A) and completed six round of SELEX for three pools for both *G. duodenalis* assemblages A and B. Melting curve qPCR assay has been set up independently and used to evaluate aptamer pool diversity decrease. A decrease in pool complexity was evident starting from round six (maximum at round 10). However, technical problems (e.g. pool amplification and ssDNA purification) were encountered at advanced cycles of SELEX, preventing the successful selection of aptamers. Different experimental and technical approaches will now be undertaken to overcome these problems, including an increase of SELEX (oo)cyst target, NGS sequencing of aptamer pools at the latest cycle to verify the occurrence of aptamers families enrichment, and streptavidin affinity purification of ssDNA. These attempts will continue in the first part of 2021 and, consequently, the deadline for the



milestone M-JRP-PARADISE-10 (Aptamers sequences selected) has been moved from M36 to M40.

JRP19-WP4-T2: Development of post-DNA extraction enrichment strategies (M25-M54)

SVA has designed a magnetic capture (MC) system for *Cryptosporidium* (18S ribosomal DNA gene) and one for *Giardia* (beta-giardin gene), whereas RIVM has designed a different capture probe for *Cryptosporidium* (gp60 gene). SVA has tested the sensitivity of the *Cryptosporidium* protocol using a panel of FACS-purified sorted oocysts, and established a sensitivity of approximately 40-50 copies, corresponding to 10 oocysts for the multi copy 18S rDNA gene and to 40-50 oocysts for the single copy gp60 gene. RIVM has focused the experimental work on MC-PCR development for gp60 in fecal samples. Various capture oligos were developed and validated in different volumes of fecal samples from cattle. Preliminary results using qPCR indicate that the method is approximately 100x more sensitive than DNA isolation using a commercial stoolkit, and positive results have been obtained for 2g fecal samples spiked with 125 oocysts. The detection limit needs to be established in subsequent experiments. For final application, the qPCR will be replaced by conventional PCR and sequencing to allow identification of subtypes. Additional validations will be carried out with other *C. parvum* genotypes and an internal amplification control will be added to the PCR reactions before final application. In summary, no delay of the work plan is reported, and the expected milestone (M-JRP-PARADISE-13) is reached.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
19	D-JRP-PARADISE-WP1.1	Report of the kick off meeting	M26	M28			Public	10
19	D-JRP-PARADISE-WP1.2	Report of Annual meeting	M42				The possibility to organize a physical meetings will depend on the Covid-19 situation. A virtual meeting is considered as an alternative option.	
19	D-JRP-PARADISE-WP2.1	Protocol for 18S rDNA-based amplicon sequencing for detection of relevant FBPs	M30		M37	Yes, experimental work at SSI impacted by Covid-19 restrictions	Public	2
19	D-JRP-PARADISE-WP2.2	Report on the FBPs detected in metagenomics datasets by in silico analysis	M48					



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
19	D-JRP-PARADISE-WP3.1	Report on the in silico selection of highly polymorphic sequences in <i>C. parvum</i> and <i>G. duodenalis</i> genomes	M30		M36		Public	10
19	D-JRP-PARADISE-WP3.2	Report on the identification of MLST markers for <i>C. parvum</i> and <i>G. duodenalis</i>	M42					
19	D-JRP-PARADISE-WP3.3	SOPs for <i>C. parvum</i> and <i>G. duodenalis</i> MLST schemes	M48					
19	D-JRP-PARADISE-WP4.1	Report on the cloning and sequencing of aptamers specific for <i>C. parvum</i> and <i>G. duodenalis</i>	M36		M40	Yes, experimental work at ANSES and ISS impacted by Covid-19 restrictions		
19	D-JRP-PARADISE-WP4.2	Report on the selection of highly affine nanobodies specific for <i>C. parvum</i> and <i>G. duodenalis</i>	M36		M40	Yes, experimental work at RKI impacted by Covid-19 restrictions		



* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
19	M-JRP-PARADISE-1	Kick-off Meeting (WP1)	M26	Yes		
19	M-JRP-PARADISE-2	Study visit (ISS-ANSES) for optimizing aptamer selection strategy	M28	No	Not planned	Study visit impossible due to the COVID-19 epidemics
19	M-JRP-PARADISE-3	Key isolates of <i>C. parvum</i> collected	M30	No	M33	Delay in the collection/shipment of samples due to the COVID-19 epidemics
19	M-JRP-PARADISE-4	Key isolates of <i>G. duodenalis</i> collected	M30	No	M33	Delay in the collection/shipment of samples due to the COVID-19 epidemics
19	M-JRP-PARADISE-5	Referenced database of foodborne parasite genomes established	M30	No	M40	Delay due to limited access to working places caused by the COVID-19 epidemics
19	M-JRP-PARADISE-6	Pipeline for metagenome data analysis for FBPs optimized	M30	No	M40	Delay due to limited access to working places caused by the COVID-19 epidemics
19	M-JRP-PARADISE-7	First set of candidate markers for MLST development available	M30	No	M36	Activity slightly impacted by delay in WP2-T1
19	M-JRP-PARADISE-8	Animal immunization and cDNA library of nanobody	M30	No	M33	Animal immunization and cDNA library construction completed



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
		sequences for <i>C. parvum</i> and for <i>G. duodenalis</i> completed				
19	M-JRP-PARADISE-9	Submission of genome sequences of <i>C. parvum</i> and <i>G. duodenalis</i> to public repository	M36		M40	The last round of genome sequencing is about to be completed, and all data will then be submitted to a public repository
19	M-JRP-PARADISE-10	Aptamers sequences selected	M36		M40	Experimental work delayed due to Covid-19 restrictions (at both ANSES and ISS)
19	M-JRP-PARADISE-11	Highly affine nanobodies selected	M36		M40	Enrichment of potential "binders" for both <i>Cryptosporidium</i> and Giardia antigens has been obtained, but a short delay is anticipated
19	M-JRP-PARADISE-12	Pre-evaluation of probes directed towards markers commonly used for <i>C. parvum</i>	M36	Yes		Capture systems for <i>Cryptosporidium</i> have been developed and tested for sensitivity and applicability
19	M-JRP-PARADISE-13	Pre-evaluation of probes directed towards markers commonly used for <i>G. duodenalis</i> .	M36	Yes		A capture system for <i>Giardia</i> has been developed and tested for sensitivity and applicability



4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Veterinary Students Have a Higher Risk of Contracting Cryptosporidiosis when Calves with High Fecal Cryptosporidium Loads Are Used for Fetotomy Exercises 10.1128/AEM.01250-20 https://zenodo.org/record/4129990#.X6PX8WhKjcc	YES		
Molecular Characterization of Giardia duodenalis in Children and Adults Sampled in Algeria doi: 10.3390/microorganisms9010054. https://zenodo.org/record/4422456#.X_ayHsKP670	YES		
Mining public metagenomes for environmental surveillance of parasites. Frits F.J. Franssen, Ingmar Janse, Dennis Janssen, Simone Mario Caccio, Paolo Vatta, Joke W B Van Der Giessen and Mark W.J. Van Passel Submitted to Frontiers in Microbiology and currently under review process	YES		



5. On-going and planned collaborations with national or European projects or networks

Complementarities with the OHEJP research project “Toxosources”. Both projects use genomics as a tool to derive new typing schemes, and try to determine the role of specific food matrices (ready-to-eat salads).

- Complementarities with the OHEJP integrative project “Harmony-CAP”, in which *Cryptosporidium* has been selected as a model organism for evaluation of the current and best practices and the development of harmonised protocols.
- Link with the objectives of the **European Union Reference Laboratory for Parasites** in terms of new typing schemes for FBP that, in perspective, may become official, validated methods.
- Complementarities with the **EFSA-funded research project “IMPACT”** for the definition of optimized protocols for the detection and typing of FBP on selected food matrices.

JRP20-DISCOVER

6. Summary of the work carried out in year 3

In these first 12 months of the project, we have achieved the following:

1. Identified members of the Discover Project Management Team (PMT)
2. Held one face-to-face kick-off/annual meeting at DTU on February 10-11, 2020.
3. Held three web meetings for all project partners.
4. Held five project management team web meeting to discuss plans and progress.
5. Developed data inventories of focus hazards to map existing and available data.
6. Mapped knowledge gaps for source attribution through the completion of a rapid systematic review (D-JRP FBZ-1-WP1.1)
7. Determined for each focus hazard (Salmonella, Campylobacter, VTEC/STEC and antimicrobial resistant (AMR) bacteria with a focus on ESBL/AmpC E. coli) which attribution approaches that will be applied, further developed and compared.
8. Held four hazard-specific web meetings to discuss and decide on improvement of datasets to work with throughout the project.
9. Selected for each focus hazard relevant datasets and proposed how these can be improved by additional sampling of particularly non-animal food reservoirs and sequencing of existing isolates.
10. Held several task-specific meetings organised by the task leaders
11. Made the first draft of the DMP
12. Drafted a MTA to be signed by all partners
13. Organised a stakeholder workshop to be held online on January 22, 2021. Invitees include people from EFSA, ECDC, and the EURLs



7. Work carried out in the JRP, scientific results

WP1: Project coordination and administration (M25-M54)

JRP20-WP1-T1: Project management (M25-M54) - ongoing

Since the start of the project, we have had one face-to-face kick-off meeting hosted by DTU on February 10-12, 2020, and 3 web meetings for all partners and 5 web meetings for the project management team (PMT) consisting of all work package leaders, their deputies and task leaders.

A share-site hosted by DTU has been set up, where project partners can share documents and data, including minutes of meetings and the developed data inventories.

JRP20-WP1-T2: Mapping of existing knowledge gaps and recommendations on how to fill them (M25-M28) - completed

For the mapping of existing knowledge gaps a systematic literature search by means of a rapid review was performed. Based on predefined search queries publications relevant for source attribution of the bacterial species *Salmonella*, *Campylobacter*, VTEC/STEC and for antimicrobial resistant (AMR) bacteria (*Salmonella*, *Campylobacter*, *E. coli*) were identified in the two databases Scopus and Web of Science. Each identified publication was tagged according to categories like the organisms and sources they considered or the methods they employed. Using the data of the tagged publications knowledge maps were created which showed how much publications were found for each method-source combination for each of the above-mentioned bacterial species and AMR. The methodology and results of this mapping together with suggestions how to fill the identified knowledge gaps were put together in a report and submitted as draft for deliverable D-JRP FBZ-1-WP1.1 to the project coordination.

JRP20-WP1-T3: Data Management Plan (DMP) (M25-M30) - ongoing

The first draft of the DMP was prepared in Nov 20, but its development is a continuing process. Considering the structure of the new DMP tool, we plan to upload information about our selected datasets, whenever we have a 'finalised' dataset ready for data analysis.

WP2: Data – Coordination of the collection of genomic data, other microbiological data and epidemiological data (M25-M48)

During this period of the project, several meetings took place with WP2 partners to organize data collection.

JRP20-WP2-T1: Mapping of existing data and establishing a joint data-sharing platform (M25-M36) - ongoing

A data inventory form in Excel was adapted to each pathogen subgroup. The purpose of the inventory was to get a quick overview of strains and sequences available in order to pinpoint areas with limited data availability to i) direct further sampling, and ii) identify for which pathogens and models we can expect to have sufficient data for source attribution. The data inventories have been filled-in by partners and hazard-specific web meetings have been organized to present the data available and agree on further sampling of mainly non-animal food reservoirs and/or isolate characterization (mainly sequencing) and transmission to the other WPs for source attribution analysis. Ideally, when these proposed further sampling and sequencing activities are done, we will have a selection of appropriate datasets for source attribution for the focus hazard and attribution approaches (see WP4 description for the approaches selected for each organism).

We are currently exploring, if we can use sciedata.dk to store and share our project data. You can read more about security and storage systems at [DEIC](https://deic.eu).



JRP20-WP2-T2: Data collection for Salmonella (M25-M48) - ongoing

Overall, nine participants from eight countries (Belgium, Czech Republic, Denmark, France, Netherlands, Poland, Portugal and Spain) delivered data about Salmonella serovars in their collections. Seven institutes showed data from animals, food and environment, four concerning humans.

Overall, the most numerous Salmonella serovars are *S. Enteritidis* (>15100 isolates/> 800 sequences), Typhimurium (>9600 isolates/> 170 sequences), monophasic variant of *S. Typhimurium* (>6500 isolates/> 300 sequences) and *S. Infantis* (>2290 isolates/ 40 sequences). Those serovars occur in all main sources. SE and ST were found as a top 1 and 2 in humans and animals. In food *S. Infantis* and *S. Typhimurium* are dominant. The environment is represented by the least number of isolates (>300 isolates/ 0 sequences). Some countries declared ongoing sampling or being ready to sequence selected isolates if needed.

The data inventory was discussed during a meeting and proposals for improving specific datasets were made. For WGS-based source attribution, it was decided to focus on the following serovars: Typhimurium, monophasic Typhimurium, Enteritidis, Infantis, Derby, Agona, Newport and Kentucky. However, we will also attempt to build a multi-country model based on phenotypic information, as this will increase the amount of available data.

JRP20-WP2-T3: Data collection for Campylobacter (M25-M42) - ongoing

The Campylobacter specific questionnaire was filled-in by 10 institutes from 7 countries. A total of >5000 whole genome sequenced strains were reported to be available for the project. This includes genomes of human clinical strains, food, animal and environmental strains. After discussion with the WP3 task leaders on the data needs for modelling, it will be decided if more isolates should be sequenced and/or if sampling of some of the under-represented sources should be done.

The data inventory was discussed during a meeting and proposals for improving specific datasets were made. Fairly good data sets are available already and the modellers expect to be able to use the data, despite the incompleteness when it comes to full coverage of countries, sources, years. New sampling should primarily focus on environmental samples and other sources that are not well covered already. Focus is on *C. jejuni* and *C. coli*. Sequencing should focus on WGS of isolates from recent years, 2016-2020.

Depending on the budget at each institute, the institute should select a number of isolates for WGS giving priority to categories with low representation in general (e.g. environmental isolates) as well as categories that specifically are low/missing in the country (e.g. human isolates, different foods, ...).

JRP20-WP2-T4: Data collection for Verocytotoxin-producing E. coli (VTEC) (M25-M48) - ongoing

The STEC specific questionnaire was filled in by 13 partners from 11 countries. Five partners from five countries shared information regarding human STEC isolates including more than 2400 isolates whole genome sequenced of different serotypes. Information regarding STEC isolates from animal, food and environmental were shared by 11 partners from 10 countries. More than 2800 STEC isolates from animal, food and environmental sources was described, not all isolates were whole genome sequenced. The number of isolates from sources other than food-producing animals was sparse. The main serogroups in the combined dataset are *E. coli* O157 and O26. For now no data has been shared, only information on what is available.

The need for further sampling of other sources than food-producing animals has been highlighted and sampling has been planned. However, due to Covid-19 the sampling has been postponed for many partners.

As data from different sources are sparse a preliminary comparison between human data reported to ECDC (Tessy) and our dataset have been performed to see it it's possible to merge data from several countries with similar distribution of human cases to make the source attribution at regional levels.



JRP20-WP2-T5: Data collection for AMR (M25-M48) - ongoing

Overall, seven countries (Czech Republic, Denmark, Ireland, Netherlands, Poland, Portugal and Spain) have in their strain collections clinical/indicator *E. coli* (including ESBL-*E. coli*) for task 4. Number of available isolates/sequences ranges from less than 100 (Ireland) to 500-700 (Czech Republic, Poland) to > 1.000 (remaining countries). Most of the isolates however come from animals/food products, so that only Denmark and the Netherlands report a large number (>100) of isolates of human origin.

The data inventory was discussed during a meeting and proposals for improving specific datasets were made. It was agreed that it was necessary to focus on years with overlapping data from multiple reservoirs, and thus the period 2013-2020 was considered the best period to concentrate on for further analyses/sequencing. It was proposed to collect additional information for countries with available strains/sequences (possibly at the strain level), including: 1) Availability of phenotypic AMR information, and 2) Specifics on the genotypic information available (for non-sequenced strains): gene families/specific genes.

WP3: Methods - Critical assessment/improvement of existing and development of new source attribution models. (M25-M48)

Four web meeting has been held and monthly meetings are scheduled for after the summer break.

It is planned to use the systematic literature review from BfR (D-JRP FBZ-1-WP1.1) to identify existing models/methods and pinpoint any methodological gaps.

JRP20-WP3-T1: Assessing and developing source attribution methods based on microbial subtyping (M25-M48) - ongoing

A Danish dataset of *Campylobacter* sequences collected from humans, animals incl. pets, foods and environments from 2015-2017 is currently being processed through different bioinformatics pipelines to obtain cgMLST, wgMLST, SNPs and Kmer data. These different types of output data will be explored in different source attribution models using machine learning to identify any host-associated genetic (groups of) markers.

JRP20-WP3-T2: Assessing and developing source attribution methods based on phylogenetic data (M25-M48) - ongoing

A workgroup has been assembled and the research objectives has been defined: Using phylogeny of surveillance data to apply weights in source attribution models to move from reservoir attribution towards source attribution that is directly actionable by public health and food authorities, by better reflecting exposure evidence. Focus will be on *Campylobacter* and *Salmonella*.

JRP20-WP3-T3: Evaluation of microbial subtyping source attribution by infectious disease modelling (M25-M48) - ongoing

This task sets out to develop a method for measuring the quality of source attribution based on subtyping. The initial idea was to simulate infections from different sources using an infectious disease modelling approach, but we are now looking to an approach based on simulating bacterial population using the software: Bacmita <https://doi.org/10.1093/bioinformatics/bty093>

JRP20-WP3-T4: Assessing and developing approaches for source attribution of antimicrobial resistance based on metagenomics (M31-M48) - ongoing

Nothing to report.



JRP20-WP3-T5: Assessing and developing source attribution approaches based on case-control study results (M30-M36) - ongoing

A Bayesian evidence synthesis model that is able to combine the percentage attribution estimates from different studies has been developed. The next step is to extend the model to also combining population attributable fractions and odds ratios, which are the typical association measure used on case-control studies.

JRP20-WP3-T6: Assessing and developing source attribution approaches based on data from reported outbreak investigations (M28-M36) - ongoing

It has been decided to use outbreak data reported by EFSA or this task. Data have been requested from EFSA.

JRP20-WP3-T7: Assessing and developing source attribution approaches based on Risk-assessment (M30-M38) - ongoing

A review of existing comparative assessment models is ongoing. Plan to finish by February.

WP4: Results – Quantifying the contribution of various sources of foodborne zoonoses and AMR (M30-M49)

In WP4, data collected in WP2 and the methods assessed/developed in WP3 will be used to quantify the contributions of the main sources of the three focus pathogens and AMR. Results will be presented per pathogen, attribution method, type of data and, when/if applicable, geographical region/country. Particular attention will be given to environmental and non-livestock (pets and wildlife) sources besides the ‘traditional’ livestock/food sources. The results of applied methods for each pathogen will be compared in the light of data availability and robustness, underlying uncertainties, the point in the food production chain where source attribution takes place, and the usefulness of different methods to answer different One Health questions. Before performing any attribution, it has become evident in the past months that it is necessary to compare the typing data between countries using, e.g. PCA and/or similarity metrics like PSI. In this way, geographical regions could be identified as the “epidemiological units” for the attribution analyses. Moreover, such an analysis would already be very informative in itself as it would provide information on the distribution of relevant subtypes among the DISCOVER partner countries and it would also offer the opportunity to identify potential sources of surrogate data for the attribution analysis. Regarding the exposure assessment, data for all DISCOVER partner countries are incomplete or non-existent. A comparative exposure assessment, to be “comparative” needs to include different sources or to be performed in different regions/countries, which would also be in line with the spirit of OHEJP projects. It was therefore decided that a reasonable approach would be to perform an exposure assessment for a specific source for which we expect to have few typing data, i.e. pets like dogs and cats, for each of the 3 target pathogens and AMR in different countries that have the necessary data, and to compare the exposure estimates for this same source between the countries, as well as to check whether possible differences are also reflected in the attributions based on the other methods.

JRP20-WP4-T2: Salmonella source attribution and comparison of results from different approaches (M30-M40) - ongoing

In the past months, the specific activities of this task have been critically re-assessed and structured as follows: 1) attributions based on microbial subtyping; 2) analysis of outbreak data; 3) meta-analysis case control data; and 4) exposure assessment. People/partners contributing to each activity have also been identified. As an activity of interest for all tasks within WP4 and in collaboration with WP2, we worked together to define a minimum set of (meta)data to be collected to perform source attribution in a meaningful way. A document has been prepared and shared with the consortium. This document will be integrated in the upcoming milestones M-JRP FBZ-1-07 (Format for results presentation



(standard structure) for all pathogens and AMR) and M-JRP FBZ-1-08 (Protocol for presentation of results for Salmonella, Campy, VTEC and AMR).

JRP20-WP4-T3: Campylobacter source attribution and comparison of results from different approaches (M30-M42) - ongoing

Alike the previous task, also for this one on Campylobacter, 3 activities have been defined: 1) attributions based on microbial subtyping; 2) meta-analysis case control data; 3) exposure assessment. This is because the scarcity of documented campylobacteriosis outbreaks would make an analysis of outbreak data not very useful. The document with the minimum set of metadata mentioned in the previous task also include specific indication for Campylobacter data.

JRP20-WP4-T3: VTEC source attribution and comparison of results from different approaches (M30-M49) - ongoing

Also for this task on VTEC, activities have been more clearly defined as: 1) attributions based on microbial subtyping; and 2) exposure assessment. This is because there are already recent works on the analysis of outbreak data and meta-analysis of case-control studies available performed by Sara Pires (deputy leader of WP4), among others. The document with the minimum set of metadata mentioned in the previous task also include specific indication for VTEC data.

JRP20-WP4-T4: AMR source attribution results presented regionally and by region/country, for each applied method and integrated (M30-M49) - ongoing

Nothing to report.

WP5: Conclusions, recommendations and policy translation. (M33-M54)

JRP20-WP5-T1: Translating source attribution estimates into options for control policies (M33-M50) – not started yet

An online stakeholder workshop is being organized for 22. January, 2021 hr. 10-12. Invitees include EFSA, ECDC, and the relevant EURLs.

A Master student enrolled at DTU will in collaboration with ISS address the deliverable D-JRPFZ-1-WP5.1: Map of the current existing control programme and intervention strategies to mitigate the risk of transmission of Salmonella, Campylobacter, VTEC, and antimicrobial resistance to human at the EU and national level. The MSc student will start her project 25 of January 2021.



8. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
20	D-JRP FBZ-1-WP1.1	Mapping of knowledge gaps and recommendations for new data generation and method development	M28	M32			Confidential until it has been published as a scientific article.	10
20	D-JRP FBZ-1-WP1.2	Data Management Plan	M30	M35			The DMP is public	8
20	D-JRPFbz-1 WP2.5	Database/sharing platform solution established	M36		M38		We are currently looking into the possibility of using sciencedata.dk to store and share our project data.	3

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
20	M-JRP FBZ-1-01	Identification of Project Management Team	M25	Yes		
20	M-JRP FBZ-1-02	First annual project meeting	M27	Yes		
20	M-JRP FBZ-1-03	Completion of mapping of knowledge gaps and recommendations for new data generation and method development	M28	Yes		
20	M-JRP FBZ-1-04	Identification of types of samples to investigate and for which species to include in the sampling	M29	Yes	M32	
20	M-JRP FBZ-1-05	Framework for evaluation of Microbial subtyping methods	M33	Yes		
20	M-JRP FBZ-1-06	Methods for source attribution based on outbreak data evaluated	M33	Yes		
20	M-JRP FBZ-1-07	Format for results presentation (standard structure) for all pathogens and AMR	M33	Yes		
20	M-JRPFZ-1-08	Mapping of data available for Salmonella	M34	Yes		
20	M-JRPFZ-1-09	Mapping of data available for Campylobacter	M34	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
20	M-JRPFZ-1-10	Mapping of data available for VTEC	M34	Yes		
20	M-JRPFZ-1-11	Mapping of data available for AMR	M34	Yes		
20	M-JRPFZ-1-12	Protocol for presentation of results for Salmonella, Campylobacter, VTEC and AMR	M34	Yes		
20	M-JRPFZ-1-13	Framework for using phylogenetic data for source attribution	M36	no	M38	In progress
20	M-JRPFZ-1-14	Comparison of data and methods for each pathogen and AMR	M36	no	M38	In progress

9. Publications and patents

No publications yet.



10. On-going and planned collaborations with national or European projects or networks

As part of WP5, we have organized a stakeholder webmeeting on January 22, 2021 inviting EFSA, ECDC and the relevant EURLs. In addition, one of our partners is also part of OHEJP WP5, and she will inform about DiSCoVeR's activities in relevant fora (Stakeholder committee, Scientific Steering Board (SSB) and Program Owner Committee (POC)), and potentially asking DiSCoVeR to give a presentation at a later stage.

JRP21-BIPIGEE

1. Summary of the work carried out in year 3

The BIOPIGEE project is successfully running since January 1st 2020.

In WP1, a communication infrastructure was set up among partners (email-lists, online conferences, shared documents and the website). The kick-off-meeting in Berlin brought all actors together. A BIOPIGEE leaflet and a poster were published. A first draft of the data management plan was developed and entered to CDP. A survey tool for T2.2 was implemented and user support is provided. Plans are being developed how to cope with the consequences of the CoVID-19 outbreak for the BIOPIGEE project. First deliverables incl. the 9 months report were provided. The project lead changed.

In WP2 T2.1, a biosecurity questionnaire for European pig farms was developed to assess the relevance of measures on the prevalence of *Salmonella* and hepatitis E virus. The questions were transferred into a software, translated into the languages needed and tested in the participating countries. In T2.2, the questionnaire is being applied. Protocols for farm recruitment, sampling and laboratory testing were designed. Farm recruitment started and 59 farm visits (status 17.12.2020) have taken place among all participating countries so far. The visits were disrupted by COVID-19 restrictions. Relevant data from a previous study was collected from ANSES (90 farms). T2.3 started to list existing biosecurity protocols for slaughterhouses from partner countries. In T2.4, the three longitudinal studies were designed. They are coordinated to ensure any synergies and are utilised to assist with making the results comparable.

In WP3, discussions are in progress to choose the panel of *Salmonella* isolates and to compare the methods for disinfectant effectivity testing, and to define the methods that will be used to screen the isolates for biofilm forming abilities. Furthermore, work is continued on the further development of an HEV infectivity assay.

WP4 successfully developed a questionnaire covering around 24 questions about farm performance and provided this input to WP2. Information needed from WP5 (T5.2 systematic review/meta-analysis) for the modelling in WP4 is being specified.

WP5 T5.1 created an online catalogue of effective biosecurity measures on the prevalence of HEV and *Salmonella* and first data from WP2-5 were integrated. In T5.2, a systematic literature review was conducted between partners. Literature was screened, assessed, and extracted information compiled in a shared document. Data were prepared for meta-analyses. A first expert panel (scientists) was built and rated biosecurity measures during the questionnaire development for T2.1. This panel has been extended with more types of experts (e.g. advisors, controllers; T5.4) in order to identify weights for the benchmarking in T5.5.

WP6 T6.1 initiated the collection of pictures of good biosecurity practice (collected in T2.2), shared in a protected space at the BIOPIGEE website. A shared file containing a list of relevant websites, where results and compiled information of the project can be disseminated, was developed. A BIOPIGEE flyer with brief information about the project was produced (T6.4). The planned workshop series is



discontinued due to the CoVID-19 outbreak. Instead, we are planning for national/regional information events at the end of the project.

2. Work carried out in the JRP, scientific results

WP1: Project coordination and integration of results (M25-M54)

The project coordination, link between Tasks and WPs, and integration of results is ongoing until the end of the project. Tele-/webconferences have been organised, conducted and followed up (minutes). Email lists for all WPs and Tasks have been created. Shared documents have been set up and uploaded to a protected cloud server, hosted by BfR. Links to all relevant online tables and documents have been listed in the BIOPIGEE private groupsite. Here, a file system for different tasks of the project is developing for the data exchange. A first draft of the data management plan (DMP) has been prepared. After the OHEJP DMP group provided the new tool CDP, a training session on this system was attended and the DMP was entered into and downloaded from this system. A publication plan for the project, listing intended publications for WP2-5 have been set up as a shared document which is currently listing.

Technical set up and user support for a survey tool applied in T2.2 has been managed. Survey material including data protection forms, tutorials for the technical use of the survey and an invitation letter to farmers were prepared and, together with the survey app, made available to T2.2 participants via a download link. Incoming survey data from T2.2 get regularly and continuously checked (eased with a little KNIME tool) and reported as well as provided monthly to T2.2 after a first data cleaning step. Thereby interviewers in T2.2 can follow and check the data income.

First deliverables and the 9 months report were provided.

Overarching problems have been solved on a higher level (e.g. invitation of expert opinions, instructions for literature review). Pressing questions in subtasks have been solved/forwarded.

Plans are being developed how to cope with the consequences of the CoVID-19 outbreak for the BIOPIGEE project.

The project lead changed. Chris Kollas left the project at the end of June 2020, Elke Burow changed from deputy to lead and Veit Zoche-Golob entered the project as new deputy in autumn 2020.

JRP21-WP1-T1: Project management and meeting organisation (M25-M54)

The BIOPIGEE Kick-off meeting was organised and successfully conducted at BfR in Berlin/Germany on 29th-30th of January 2020. All participating organisations were represented, of these 41 BIOPIGEE members took part. Participants stated a good forthcoming of the project and a good organisation of the event. Minutes of the meeting were written, re-worked with all participants and finally published internally.

The BIOPIGEE OHEJP website has been filled with content, participants were invited to join, organised as all having allowance to administrate in the group, which is currently the only technical way to enable participants using the space "Projects" for managing tasks. It is suboptimal that all need to be administrators and e.g. administration information, besides the website is quite slow in opening and displaying content. However, the offered structure and private space for the group is generally very helpful for the group organisation. Therefore, participants were repeatedly encouraged to use that website for exchange within the project. All general, important and finalised documents are getting uploaded to the page and status updates have been written.

Shared documents have been set up and are uploaded to the BfR cloud server. Links and passwords to open these documents are stored at the BIOPIGEE website. Thereby, data protection as well as access of only project people is ensured.



Production of a BIOPIGEE flyer in WP6/ OHEJP Communication Team was supported with text modules and images.

JRP21-WP1-T2: Development of data management plan (M25-36, M43-48)

First, the elaboration of the data management plan was started. All WP-leaders were invited to fill the “old” xls-sheet regarding their data. Later, we were informed that the former tool DMPonline will not be supported by OHEJP any further and a search for a better tool is under way. The deadline for the first version of the DMP was delayed by the OHEJP-PMT. Hence, work on the DMP was put on hold until September. Introduction to the new tool CDP for the DMP took place on September 9th 2020. Afterwards, we filled the DMP in CDP and are waiting for further instructions from OHEJP-PMT (was announced for January 2021).

JRP21-WP1-T3: Provision of project deliverables and reports (M28-M54)

First deliverables (questionnaire, flyer, data management plan, HEV infectivity assay) and the 9M report have been provided, via the private group and/or Zenodo.

WP2: Biosecurity effectiveness studies (M25-M50)

JRP21-WP2-T1: Development of biosecurity protocol (M25-M28)

A biosecurity protocol for European pig farms was developed in order to assess the relevance of measures on the prevalence of *Salmonella* and hepatitis E virus. As first step, existing protocols from Europe and North America were assessed but it was decided to design a new protocol specific to the needs of the project. Evidence from a literature review and from expert opinion (scientists) was used to inform the content of this protocol. The biosecurity measures were reworded as questions (55 biosecurity questions for indoor, 56 for outdoor situation), and questions on general farm characteristics (10) were added.

JRP21-WP2-T1-ST1: Transfer of the questionnaire into an electronic version (M28-30)

The questionnaire was transferred into an electronic survey tool and translated into the necessary languages of the countries participating in T2.2. An app for this survey was produced, set up on devices of interviewers in each of the participating countries and the function of the app and data transfer to a central server were tested, with support of WP1. This system is enabling standardised data collection, and facilitates data income and evaluation.

JRP21-WP2-T2: Application of the biosecurity protocol (M27-M42)

The developed biosecurity questionnaire is getting applied in pig farms in the participating countries. Farm recruitment has started and 59 farm visits have taken place so far from nine different countries. Incoming survey data are being reported to the interviewers quarterly to check and prepare for later data evaluation. Appropriate data imputation methods will be used to correct missing values that impact upon the use of records in our planned analysis. Photos are also being collected of examples of good biosecurity practice, to add to project documentation in work package 6. The task is delayed due to different restrictions in EU member states concerning epidemiological situation related to worldwide pandemic of coronavirus disease COVID-19. NL decided not to collect fecal samples that they had planned in this task for *Salmonella* testing but instead will attempt to use results from routine meat juice sample surveillance to identify high and low risk farms. There have been difficulties in all countries to visit farms and sample as expected. The current situation in the countries is collected by WP1 and WP2 and updated in monthly catch-up conferences between partners. Although most countries started farm visits in autumn 2020, these have continued to be disrupted by COVID-19 lockdowns, as well as African Swine Fever concerns, restricting access to farms and the willingness of farmers to allow access. The partners will hurry to fulfil the planned design yet. The technical set up of the survey and conditions for entering data, is leading to mostly edited and standardised data which will reduce the effort of data cleaning, saving time before data analyses in the later process. However,



reduction in the number of farm visits, sample size and contributing partner countries could occur unless the deadlines for the project can be extended. The team will consider contingency plans, such as using monitoring data to predict disease risk or data from previous studies that might provide some of the necessary biosecurity data. Additionally, biosecurity data from 90 pig farms from a previous ANSES study have been collected and aligned to questions within the biosecurity protocol to allow their usage in this study.

The sampling, laboratory testing and recruitment protocols have been designed. Details of the HEV-testing methods have been discussed between partner labs and participants of T2.2 and T2.5 in order to harmonise methods as much as possible and to reach comparability of results that can also supplement the HEVnet database.

JRP21-WP2-T3: Slaughterhouse biosecurity practices (M31-42)

This Task was planned to start at the beginning of next year (M37). We pulled it forward and have already started to collect existing national and local assessment protocols on biosecurity measures in slaughterhouses. A first virtual meeting took place in September 2020.

JRP21-WP2-T4: Field studies (M25-M48)

The study plans for the three proposed studies have been designed and the teams have been in contact to assess synergies and harmonisation of techniques to improve comparison of potential findings. The protocol has been uploaded to the BIOPIGEE page and is thereby accessible to the whole consortium. The task is ongoing.

WP3: Impact of disinfection on persistence of pathogens in biofilm (M25-M54)

JRP21-WP3-T1: Comparison of methods for testing the effect of disinfectants (M25-M40)

This task is ongoing. Discussions and planning of methods and reference strains to be used by all participating laboratories are ongoing.

JRP21-WP3-T2: Effect of disinfectants on biofilm-associated wild type Salmonella (M25-M50)

This task is ongoing. It is dependent on the outcome of JRP21WP3-T1. Discussions and planning are ongoing among the participants in JRP21WP3 participants. Method establishment has started.

JRP21-WP3-T2-ST1: Selection of wild type Salmonella isolates (M25-M36)

Discussions are in progress to choose the panel of *Salmonella* isolates, and finalise the method that we will use to screen the isolates for biofilm forming abilities. The sub-task is going ahead as planned and will be completed by the deadline.

JRP21-WP3-T2-ST2: Assessing the effect of disinfectants (M37-M42)

Task 2 depends on results of Task1. Labwork has been started, initial testing ongoing. It is on schedule.

JRP21-WP3-T3: Study of HEV stability in relation to disinfection approaches (M25-M54)

HEV stability in microfilms will be studied using appropriate HEV infectivity assays which partly have been developed in JRP21-WP3-T3-ST1, and will be further implemented in year 2 (and 3) of the project.

JRP21-WP3-T3-ST1: Implementation of HEV infectivity assay for testing biofilms (M25-M36)

In the first year of the BIOPIGEE project, a first version of the HEV infectivity assay has been completed. In this assay primary hepatocytes are isolated from liver tissue of (HEV free) piglets using a collagenase treatment. The obtained primary hepatocytes are aliquoted and stored at -180 Celsius until use. For the actual assay the hepatocytes are seeded onto plates and inoculated with different concentrations of HEV. First HEV replication plots have been established.



A second option for testing of HEV infectivity, precision-cut liver slices (PCLS) are used and further study is ongoing. PCLS cuts are directly transferred to an ice-cold organ preservation solution and inoculated with different concentrations of HEV and tested daily using qRT-PCR for increasing levels of HEV RNA. Single replication rounds can be observed but the method still needs optimisation.

WP4: Modelling of the cost and effectiveness of biosecurity measures (M25-M50)

Task 4.1 is finished, and T4.2 and T4.3 have just started.

JRP21-WP4-T1: Development of questionnaire on biosecurity costs (M25-M27)

A questionnaire with respect to health and performance data of pigs located in farms was developed which covers 24 questions. The questionnaire was delivered to the partners of WP2.1, who prepared a protocol on biosecurity practice and who will collect these economic data during their empirical survey at the farm level. Questions about the costs of biosecurity were not included. The reason is that the costs of the selected biosecurity measures will be difficult and very time-consuming to answer for the farmers. Thus, the costs of the biosecurity measures will be estimated using usual country prices such as disinfection costs per litter and via monetary values from the scientific literature.

JRP21-WP4-T2: Stochastic simulations on the effectiveness of biosecurity measures (M33-M49)

Task 4.2. has started in September 2020. Three online meetings were performed to coordinate and harmonise the different task of 4.2 and 4.3 between the partners. In these meetings we have discussed the adaption of currently available transmission models such as QMRA for *Salmonella* and SimInF model for HEV and/or *Salmonella* of the consortium partners in order to analyse the impact of biosecurity and other mitigation measures (e.g. at the slaughterhouse) on prevalence reduction of the zoonotic pathogens. During the meetings, the data requirements (e.g. type of transmission data, meta-data on the animal population e.g. movements data of pigs, and necessary data inputs of the other WPs 2, 3 and 5 about the effectiveness of biosecurity measures and/or mitigation measures on the reduction of prevalence values etc.) was discussed for the transmission model as well as the In- and Outputs of the models.

JRP21-WP4-T2-ST1: Data collection for the transmission models (M33-M36)

Task 4.2.1 has started in September 2020 and in the first two meetings the data requirements for the transmission model was discussed between the partners as well as the outputs of the single models considering different type and aggregation of input data from the other WPs. Additionally, the data requirements and their availability were discussed. In the third meeting, the functionality of the R code and the transmission were presented for each member involved in WP4. The latter procedure was necessary so that everyone has the same level of knowledge about the R Code, functionality of the transmission model and associated strength and limitation of the simulation models.

JRP21-WP4-T2-ST2: Adaption of the models based on the available (M37-M42)

The simulation model will run for three countries for which all data needs are available. The adoption of the models already started e.g. incorporation of the effect of biosecurity measures on the spread within and between herds in the R Code.

JRP21-WP4-T2-ST3: Simulation runs for the identified effective biosecurity measures (M37-M49)

Not started yet.

JRP21-WP4-T3: Merge of models into one QMRA (M34-M50)

Just started

JRP21-WP4-T3-ST1: Different transmission models will be matched to one QMRA zoonotic pathogen model (M34-M47)

Just started



JRP21-WP4-T4: Economic model of biosecurity measures across (M37-M50)

Not started yet.

JRP21-WP4-T4-ST1: Performing economic assessments of best practice biosecurity (M37-M50)

Not started yet.

WP5: Benchmark of biosecurity practice (M27-M52)

JRP21-WP5-T1: Data integration from WP2-4 in catalogue of biosecurity measures (M27-M50)

With support of WP1, an online table (“BIOPIGEE: Biosecurity measures *Salmonella* and HEV”) was set up starting the WP5 catalogue of effective biosecurity measures for HEV and *Salmonella* prevalence. Data from WP2-5, especially from a brief review in T2.1, were integrated into the catalogue. All BIOPIGEE participants were invited to supplement the BIOPIGEE catalogue of biosecurity measures. The catalogue is getting updated continuously with identified biosecurity measures and estimates of their effectiveness. At the end of the project, this catalogue will be a very valuable resource for the project’s stakeholders working on food safety and the reduction of *Salmonella* and HEV in pork products.

JRP21-WP5-T2: Literature review/meta-analysis (M27-M50)

A systematic literature review about the effectiveness of biosecurity measures specifically against *Salmonella* and HEV in pigs farms is nearly finished. The search question, terms, period, and in- and exclusion criteria have been defined in the group with the help of a shared online document. The articles found in literature databases have been evaluated and the extraction of the relevant data is expected to be finished in M36. Afterwards the data will be categorised and prepared for the meta-analysis. The meta-analyses will be performed for all biosecurity measures for which sufficient studies were published in order to estimate their effectiveness to reduce *Salmonella*- and HEV-prevalence in pig herds. As additional data, e.g. about the way of pathogen detection, productions stages and farm types, were extracted from the literature, stratified meta-analyses might be possible which will provide more targeted effect estimates. Besides providing information for the catalogue of biosecurity measures (T5.1), the results of this task will be used in WP4 to parametrise the simulation models. Although it won’t be possible to offer first estimates to WP4 at the end of M36, the data processing and meta-analyses are done in close consultation with WP4 to support WP4 in defining dummy variables for the simulation based on preliminary results until estimates will be available. Thus, WP4 can advance in coding and testing their simulations and the time lost due to the delay in task 5.2 is minimised.

This task is delayed because some partners, who contributed intensively, were additionally charged with covid-19 related tasks and had to leave the work group. This was solved on a higher level, but delays occurred due shortage of personnel for 4 months at BfR after the initial project lead had left. Besides, technical issues around conference tools strained communication between partners until we found solutions. Thanks to personnel reinforcement at BfR since November, we could increase support and contribution, so that this task can be finalised with the help of the group, but with some delay. The task was anyway planned to be carried out in steps and to run until 2022.

In the review, we wish to concentrate on biosecurity measures included in the questionnaire in T2.1 and for which no references on proving evidence of effectiveness has been identified in T2.1. Therefore, knowledge gaps in information from T2.1 is being analysed.

JRP21-WP5-T3: Machine learning approaches (M47-52)

This task has not started yet. However, we are discussing to drop or change it in order to support the data analysis / imputation of missing values in task 2.2 because that task might need additional time and/or finish the data acquisition with much less data than planned due to the Covid-19-restrictions.



JRP21-WP51-T4: Expert panel to add estimations on effectiveness/ weights (M33-M51)

This task will build on and expand the expert panel set up and surveyed in T2.1 (scientists) to additionally incorporate knowledge of other stakeholders like practitioners, advisors, etc.. We are currently précising the list of types of experts to recruit in an online table. There was a comprehensive discussion which kind of experts to invite and how to categorise them. This fruitful discussion showed that there are marked differences between the veterinary and consulting systems within the pig sectors of the different partner states. These differences in the systems considerably influence the understanding of the roles within the pig sector of different professions. A shared spreadsheet with predefined types of experts was created and a first selection of experts from several countries agreed to support the panel. Further experts will be contacted. The procedure of the expert interviews is going to be defined and the expert survey prepared in the next weeks. For this, the questionnaire of T2.2 needs to be reworded to statements and possible need of translations will be identified between partners. Score categories for organising response of experts will be discussed and developed.

JRP21-WP5-T5: Benchmark system for effectiveness of biosecurity practice (M45-52)

This task has not started yet.

WP6: Dissemination (M25-M54)

JRP21-WP6-T1: Assembly and development of biosecurity information (M25-M54)

This task is dependent on data provision from the other WPs and results have not yet been obtained. Collection of best biosecurity illustrations (pictures) during farm visits of WP2 T2.2 has been initialised and is described in the Sampling protocol of T2.2. Due to the CoVID-19 outbreak, there has been a delay in the initiation of farm visits for some partners with subsequent delays in collection of illustrations. This task is ongoing.

JRP21-WP6-T1-ST1 Identification of appropriate websites or other online channels (M25-M54)

A shared file at the BIOPIGEE website has been developed to list appropriate channels for dissemination in partner countries. So far, 21 web sites in 6 countries have already been identified for dissemination. This task is ongoing.

JRP21-WP6-T1-ST2b Provision of slaughterhouse protocol to slaughter industry/related associations (M43-M52)

This task has not started yet and is postponed from M31 to start in M43 when effective biosecurity measures for slaughterhouses have been analysed in T2.3.

JRP21-WP6-T3: Organisation of a workshop-series (M25-M54)

WP5 (expert panel) and WP6 (workshops) are working on the description of stakeholder groups to consider, include and address in each of the two work packages. However, due to the current CoVID-19 situation, workshops have not taken place as planned. It is being discussed to disseminate results in national or regional events like information days of consultant services in 2022. At the moment, it is not possible to make any concrete plans for the workshops based on physical meeting as it is very much depending on how the pandemic situation evolves. Online digital meetings with experts (T5.4) are being considered

JRP21-WP6-T3-ST1 Identification of relevant experts (M25-M52)

As we plan to change from stakeholder to expert discussions, the shared file on listing willing participants for an expert panel (link listed at the BIOPIGEE website) will not only be developed in T5.4 but also in WP6 as experts will also be contacted from WP6.

WP5 and WP6 are working on detailed description of expert groups to consider, include and address.



JRP21-WP6-T3-ST2 Identification of relevant conferences (M25-M26)

Relevant conferences are currently been listed in our publication plan (link to shared document at our website)

JRP21-WP6-T3-ST3 Organisation of Workshop 1 (M25-M30)

This task had to been changed due to the current CoVID-19 situation. Regional or national workshops are intended to use for dissemination of results. It's also been discussed to hold a workshop as digital meeting. If possible, we prefer physical national or physical regional workshops.

Additional Sub-Task JRP21- WP6-T4 Production of BIOPIGEE flyer (M27-M29)

WP6 (supported by WP1 and the OHEJP Communication Team) produced a BIOPIGEE flyer with brief information material about the project for handing out to farmers (T2.2), external collaboration partners, at conferences, workshops (T6.3) and when recruiting experts for the panel (T5.4).



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
21	D-JRP21-WP1.2	First draft of data management plan finished	M30 – postponed by OHEJP-PMT	Uploaded on private space: 18.10.2020	M34	No	Entered DMP to new tool CDP on 18 th Oct. 2020 and submitted download to OHEJP WP3 and Comms Team on 18 th Oct. 2020. We are waiting for feedback (announced by DMP group for January 2021)	8
21	D-JRP21-WP1.4	Project report 1st year submitted	M36	17.12.2020			Public OHEJP: available	8
21	D-JRP21-WP2.1	Biosecurity protocol (addressing Salmonella and HEV) designed for data collection in the field	M28	Uploaded on private space: 01.05.2020			Confidential until publication (A scientific paper is foreseen in 2022)	7
	D-JRP21-WP3.5	Method for testing persistence of infectious HEV in surface microlayers	M36	D-JRP21-WP3.5	M42	Yes	This deliverable is delayed due to conflicting obligations on Covid19. It is proposed to postpone	2



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID -19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
							this deliverable to M42	
	D-JRP21-WP3.6	HEV infectivity assay available	M36	09.12.2020			Delivered but not public yet. A scientific paper is foreseen in 2021.	2
21	D-JRP21-WP6.3	Workshop 1 completed	M30	M54	M54	Yes	WP-leader decision: The series of workshops is cancelled due to covid-19-outbreak, instead an online panel discussion is planned for 2021 and national information events in 2022	8
21	D-JRP21-WP6.4	BIOPIGEE Flyer	Additional deliverable	Uploaded in private space: 24.08.2020	M32		Brief information when contacting potential new collaborators, experts for our panel, recruiting farmers, and as general dissemination to public) https://zenodo.org/record/4009015	8

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
21	M-JRP21-01	Kick-off meeting successfully organised	M26	Yes		
21	M-JRP21-02	Questionnaire on biosecurity costs	M26	Yes	M27	Farm performance questions included. Biosecurity cost question excluded; data will be gathered from other sources.
21	M-JRP21-03	Relevant conferences for workshops to be held are identified	M26	No	Not possible under the current CoVID-19 circumstances	The CoVID-19 situation has put a stop to conferences including workshops for now. It is planned to disseminate findings at local/national workshops at the end of 2021/ beginning of 2022 instead
21	M-JRP21-04	Biosecurity protocol designed for <i>Salmonella</i> and HEV	M28	Yes		
21	M-JRP21-05	Relevant stakeholders identified	M28	Yes	M36	Was postponed due to the current CoVID-19 situation; Instead list of experts for a panel has been expanded and developed in an online table (link in BIOPIGEE private webgroup)
21	M-JRP21-06	Appropriate websites or other online channels for dissemination identified	M30	Yes		List of web sites is being filled in an online table (link in BIOPIGEE private webgroup); Content will be continuously updated throughout the project
21	M-JRP21-07	Workshop 1 completed	M30	No	Not possible under the current CoVID-19 circumstances	WP-leader decision: The series of workshops is cancelled due to covid-19-outbreak, instead an online panel discussion is planned for 2021 and national information events in 2022
21	M-JRP21-08	Design of field study protocols	M32	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
21	M-JRP#-09	First part of meta-analysis finished	M36	No	M38	A literature review was done, information is extracted and data are getting prepared, meta-analysis is delayed due to partners involved in covid-testing and missing personnel for 4 months at BfR
21	M-JRP21-10	<i>Salmonella</i> strains for testing are collected	M36	Yes		
21	M-JRP21-11	HEV infectivity assay available	M36	Yes		

4. Publications and patents

There are no publications yet.

Additional output

Burow E., Prigge C., Smith R., Meester M., Santucci G., Young B., Rose N., Käsbohrer A., Kollas C. (2020): Questionnaire on best biosecurity practices to limit *Salmonella* & HEV occurrence in European pig farms. Poster at OHEJP ASM 2020, web conference, 27.-29.05.2020.

A BIOPIGEE Flyer has been produced to inform about the project. It was finalised on 22.06.2020 (see deliverables).



5. On-going and planned collaborations with national or European projects or networks

Collaboration was planned with Ghent University (developed BioCheck®). We invited Jeroen Dewulf to evaluate our farm survey and to participate in the slaughterhouse study. Unfortunately, he was not funded. Hence, he indicated that his input may be limited.

A cooperation with HEVnet is ongoing. Agnetha Hofhius was our contact until August 2020, she also participated in our Kick-off-Meeting in January 2020. As she is now working on Covid-19 fulltime, our new contact persons are Annelies Kroneman and Claudia Swart-Coipan. Annelies Kroneman participates in our group (covering HEV-test-experts from participating institutes) which is working on the harmonisation of the HEV test protocol to ensure that information from samples collected and tested on HEV in BIOPIGEE (T2.2) can be included in the European harmonised database HEVnet. We are working on expanding the HEVnet data base with some animal/farm/biosecurity related variables.

Frank Boelaert (EFSA) has been invited to support the expert panel (T5.4) informing our benchmarking system. He was also asked for information and contacts related to current projects and new information on biosecurity measures related to *Salmonella*/HEV prevalence.

We plan to inform DG HEALTH, DG AGRI and EFSA, if our findings suggest recommendations to improve existing surveillance programmes.

National collaborations with animal health services/veterinary services and practicing veterinarians, which partly have already existed before and partly are being built during the project, are of high importance and support for the project. These services/vets can recruit farms based on their client pool and are less restricted in accessing farms for sample collection/survey in the Covid-19 situation. They are also involved in our expert panel and will play an important role in the dissemination part as having a special interest in our findings and to disseminate them. In the expert panel, also staff with agricultural and teaching background (chambers of agriculture), scientists from different national (research) institutes (e.g. FLI in Germany) and universities (e.g. Vetmeduni Vienna/Leipzig, University of Rostock, Utrecht University), quality controllers of the pig production chain are included. Thanks to these collaborations, we can build on a strong network of varied experts between practice and science in Europe.

Collaborations with universities in several partner countries are initiated. This gives the opportunity to find support of students to carry out systematic literature reviews on BIOPIGEE relevant questions. For instance, a diploma thesis is currently being prepared in WP4, dealing with the topic "Financial impact of biosecurity and vaccination measures to minimize the use of antimicrobials in pig farms". Additionally, the cooperation with universities also makes it possible to obtain data and information from on-going national projects to fill any data gaps in our WPs. For instance cooperation with Austrian swine clinics enables to recruit farms to participate in questionnaires and to obtain information about existing data sources.

Through these contacts, we may also increase dissemination of findings.

JRP22-TOXOSOURCES

1. Summary of the work carried out in year 3

TOXOSOURCES is a 2.5-year Joint Research Project of the One Health EJP that focuses on *Toxoplasma gondii* at the interface between humans, animals, food, and the environment.

The protozoan parasite *Toxoplasma gondii* is a highly prioritized foodborne parasite that causes a high disease burden. The infection can be acquired by ingesting oocysts (in food, water, or the environment



contaminated with feces of infected, shedding felids; environmental pathway) or tissue cysts (in meat of infected animals that are raised or hunted for human consumption; meatborne pathway). The relative contributions of the different transmissible stages, sources and transmission pathways to the infection and disease in humans remain unknown, partly due to lack of appropriate methods and as a consequence, systematic control of this zoonotic foodborne pathogen is lacking in Europe and globally.

TOXOSOURCES Consortium comprises 20 One Health EJP partners and several external partners. The TOXOSOURCES research question: **What are the relative contributions of the different sources of *Toxoplasma gondii* infection?** is addressed using several multidisciplinary approaches and novel and improved methods, to yield robust estimates that can inform risk managers and policy makers.

TOXOSOURCES started efficiently and adapted well to the challenges related to the COVID-19 pandemic. The Kick-off Meeting was held in Copenhagen, Denmark, in February 2020. The collection of input data and building of a quantitative microbiological risk assessment model were started. A questionnaire was developed for collecting food consumption data from a selection of countries across Europe. An extensive literature review was performed, and complemented by a survey of expert opinions, current practices and experiences, to select the most suitable molecular method for *T. gondii* oocyst detection in fresh produce. Data collection was started for designing a sampling strategy for a multicentre survey of *T. gondii* oocysts in fresh produce. Bioinformatic selection of promising protein candidates for a novel serology method was finalized, and the work on recombinant expression of selected proteins was started. Availability of suitable sera for assessing the proteins of interest was confirmed, and decisions were made regarding technical details of the method to be developed. The retrieval of key *T. gondii* isolates and DNAs from across Europe for Whole Genome Sequencing was successful. Using the DNA sequences, polymorphic marker regions were identified for the establishment of a new typing method to detect within-genotype variation.

Challenged by the COVID-19-pandemic, the TOXOSOURCES Consortium has shown impressive resourcefulness and adaptability, and the careful risks-and-dependencies planning proved useful. All Milestones, Deliverables and reports of the first year were reached and submitted by their planned deadlines. Dissemination included scientific publications and presentations at conferences, workshops and webinars. Collaborations with other projects and networks were established.

The main outcomes of TOXOSOURCES will be quantitative estimates of the contribution of the main sources and transmission routes of *T. gondii* infection based on improved source attribution models covering both meatborne and environmental exposure, new data filling the knowledge gap regarding the role of increasingly popular but unstudied ready-to-eat fresh produce, a novel serological method specifically detecting infections caused by oocysts, and a novel typing method enabling detection of introduction of atypical *T. gondii* strains by import and tracing the infection sources in outbreaks. The results of TOXOSOURCES will contribute to developing efficient interventions at national, regional, European and even global levels.

2. Work carried out in the JRP, scientific results

The work in TOXOSOURCES is organized into five work packages (WPs) with tasks (T) and some subtasks (sT). The 2.5-year project spans three Annual Periods (Y3-Y5, 2020-2022). All the tasks that took place in Y3 (2020) continue to Y4 (2021).

Key outputs are available via the project homepage: <https://onehealthjep.eu/jrp-toxosources/>



Kick-off Meeting of TOXOSOURCES, February 2020, at Statens Serum Institut, Copenhagen, Denmark.

WP1: Coordination and impact (M25-M54)

TOXOSOURCES-WP1 manages the project and is responsible for its progress, and integrates all the results of the project to achieve the goals. TOXOSOURCES-WP1 ensures the project adheres to H2020 rules regarding e.g. ethics, IRP, dissemination and publication. Moreover, TOXOSOURCES-WP1 is responsible for the Data Management Plan of the project. TOXOSOURCES-WP1 coordinates the compiling of deliverables and reports and their timely submission, as well as the organizing of project meetings and communication. Science-to-policy translation and efficient dissemination are emphasized to maximize the impact. Interest Group facilitates targeted dissemination to stakeholders.

The main focus of TOXOSOURCES-WP1 during Y3 was to ensure the project started efficiently, to organize the Kick-off Meeting, the monthly on line WP-leader meetings, and the online consortium meeting, and to draft the Data Management Plan. All these aims were reached.

TOXOSOURCES started efficiently, the first milestone 'Kick-off Meeting held by WP1' was reached, first version of 9M report, the first Deliverable 'Data Management Plan', and the final version of 9M report were all submitted on time. Data Management Plan was established and 12M report was prepared for submission in early 2021. The impact of the COVID-19 situation was followed up closely, and the work was adapted as needed.

JRP22-WP1-T1: Management, coordination and communication (M25-M54)

The Kick-off Meeting was held February 3–4, 2020, at SSI, Copenhagen, Denmark, with a possibility to participate remotely. An online meeting with the whole consortium was held on October 20, 2020.

The established key structures for management of the project include monthly online meeting with TOXOSOURCES WP-Leaders and Co-Leaders, Consortium emails, use of the online group for sharing and storage of relevant documents, and WP-level online meetings.

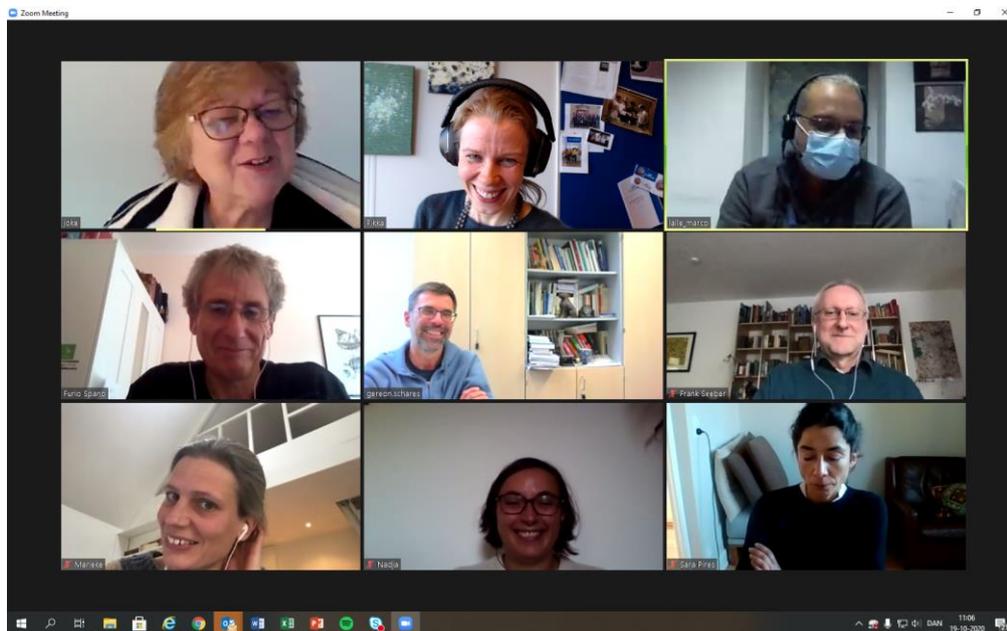


The first version of Data Management Plan (DMP) was drafted, the DMP tool was taken to use, and the PL participated in the work of the DMP committee.

Dissemination of the outcomes included presentations to relevant audiences at conferences, workshops and webinars, and scientific publications.

The collaborations with the Interest Group and other collaborators started by establishing key contacts. Invited by SafeConsume, TOXOSOURCES joined a Horizon Results Booster group. TOXOSOURCES suggested a OHEJP Cogwheel Workshop with SafeConsume, and it was organized on November 25, 2020.

Challenged by the COVID-19-pandemic, the TOXOSOURCES Consortium showed impressive resourcefulness and adaptability, and the careful risks-and-dependencies planning and proved useful. All Milestones, Deliverables and reports of the first year were reached and submitted by their planned deadlines. The consortium is highly motivated and the general atmosphere is positive and supportive.



Screenshot from an online meeting of TOXOSOURCES-WP-Leaders and WP-Deputy-Leaders.

WP2: Multicentre quantitative microbiological risk assessment for *T. gondii* infections (M25-M54)

TOXOSOURCES-WP2 aims to quantify the relative contribution of sources of *T. gondii* infection, including meat products, fresh produce and environmental pathways, in all EU regions by quantitative microbiological risk assessment (QMRA). TOXOSOURCES-WP2 develops QMRA models for infection via tissue cysts (meat) and oocysts (environmental pathways), and applies the models in a multi-country study covering all four EU regions. Input data for the QMRA will be collected by all partners and TOXOSOURCES-WP3. An overview of the prevalence of *T. gondii* infection in humans and animals used for human consumption as well as cats will be obtained by review of available literature, including grey literature. Exposure data will be collected in a harmonised way using a survey specifically designed for QMRA purposes. Region- or country-specific products, dishes or eating habits will be identified and the associated processing parameters collected by the partners. The literature review of human infections also covers risk factor studies, to compare QMRA outcomes and epidemiological data.

The main focus of TOXOSOURCES-WP2 during Y3 was on building the QMRA model and collecting input data. The outlined work started well. All Milestones of the first year were reached by their planned deadlines.



JRP22-WP2-T1: QMRA modelling for human *T. gondii* infections (M25-M54)

The Consortium members involved in QMRA modelling met at RIVM, The Netherlands, to discuss plans. The development of the structure for the QMRA model for environmental transmission of *T. gondii* was finished, and expansion of both the meatborne and environmental QMRA models to include multiple countries was started. The work builds on previous work, in particular on an existing meatborne QMRA model, as well as on simultaneously ongoing work in a PhD project of Huifang Deng, which was successfully defended on December 08, 2020.

JRP22-WP2-T2: Review of prevalence of *T. gondii* infection in animals (M25-M54)

A list of European countries and a list of key animal species raised or hunted for human consumption in Europe were collated. These were used to develop a search strategy for data on prevalence of *T. gondii* in animals. Experiences from systematic reviews performed by EFSA and in the Baltic-Nordic region were taken into account in the process.

The search, screening of retrieved records, and data extraction were finished. This work included collaboration with early-career colleagues.

JRP22-WP2-T3: Quantitative exposure survey (M25-M48)

A general questionnaire was developed by expanding an existing questionnaire and taking into account experiences from the Dutch National Food Consumption Survey, risk factor information from a prospective case-control study in the Netherlands, and applying categorisation of EFSA FoodEx system, which enables comparison with existing surveys.

The questions were adapted to the different countries by including region/country-specific products. Decisions on sample size (respondents per each country) were finalized.

JRP22-WP2-T4: Overview of processing parameters for relevant meat products (M25-M45)

Information on food consumption in the different countries was collected from the EFSA FoodEx2 database. Information on region/country-specific relevant products was also collected from consortium members.

A list of products that need to be included in the exposure survey was provided to TOXOSOURCES-WP2-T3.

JRP22-WP2-T5: Review of prevalence and risk factors for human *T. gondii* infection (M25-M54)

Results from the literature review on *T. gondii* source attribution (including risk factor analyses) for COST-action Euro-FBP were presented at OHEJPASM2020, emphasizing the continuation of the work in TOXOSOURCES.

This task was among those most affected by COVID-19 pandemic due to involvement of several key consortium members in the COVID-19 response. Efficient replacements and synergy with TOXOSOURCES-WP2-T2 ensured that also this task progressed well. The experiences from TOXOSOURCES-WP2-T2 work were incorporated in the planning of the systematic review protocol under this task, and preliminary searches were made.

WP3: Multicentre survey to fill the key existing gap: role of fresh produce (i.e. Ready-to-Eat salads) (M25-M54)

TOXOSOURCES-WP3 aims to fill the knowledge gap concerning the relevance of fresh produce contamination by *T. gondii* oocysts as an infection source for humans. TOXOSOURCES-WP3 selects the most reliable methods for the molecular detection of *T. gondii* oocysts in fresh produce using a literature review, expert experiences, experimental evaluation and inter-laboratory comparison. Harmonised detection is implemented among the partners. TOXOSOURCES-WP3 collects existing data on *T. gondii* oocyst prevalence in fresh produce and the environment, together with information on



fresh produce (e.g. RTE production, trading and consumption) in Europe. The data are used to design a risk based sampling strategy for a multicentre pilot survey to detect *T. gondii* in fresh produce, applying the defined SOP. The multicentre pilot study, spanning all four European regions, will be the first of its kind and will deliver valuable input for the TOXOSOURCES-WP2 and future QMRAs.

The main focus of TOXOSOURCES-WP3 during Y3 was on collection of existing data on prevalence of *T. gondii* oocysts in fresh produce and environment, as well as of information on fresh produce (e.g. RTE production, trading and consumption) in Europe.

All Milestones and Deliverables of the first year were reached and submitted by their planned deadlines.

The collection of data on molecular detection methods for *T. gondii* oocysts and on *T. gondii* oocyst prevalence in fresh produce and environment was completed, and collection of information on fresh produce production, trading and consumption was started.

JRP22-WP3-T1: Selection, evaluation and implementation of detection procedure for *T. gondii* oocysts in fresh produce (M25-M39)

An extensive literature review and multi-attribute assessment of the different steps (oocysts recovery, DNA extraction and DNA amplification) was performed and complemented by a survey on expert opinions, current practices and experiences on molecular detection of *T. gondii* (DNA), to select the most suitable molecular method for *T. gondii* oocyst detection in fresh produce. The Deliverable D-JRP-TOXOSOURCES-WP3.1 summarized this process, which provided a good starting point for developing a standard operating procedure (SOP) for the multicentre survey of *T. gondii* oocysts in fresh produce. The comparative experimental work was completed. Instead of the planned physical technical workshops, video tutorials were produced. The work towards the SOP was reported in Deliverable D-JRP-TOXOSOURCES-WP3.2.

Details are provided in the Deliverables.

JRP22-WP3-T2: Design of a risk-based sampling strategy (M26-M48)

An extensive review of peer-reviewed literature on prevalence of *T. gondii* oocysts in fresh produce was performed, as well as a review of literature on *T. gondii* prevalence in environment (soil and water) and bivalves. These were complemented by an online questionnaire to consortium partners to collate grey literature on the topic. Data summary on literature-based prevalence of *T. gondii* oocysts in fresh produce was provided to TOXOSOURCES-WP2.

The design of a risk-based sampling strategy for the multicentre survey of *T. gondii* oocysts in fresh produce was started by preparing an online questionnaire to gather relevant information on trade and consumption of ready-to-eat salads from local and international industry.

WP4: Serology method based on novel antigens to discriminate *T. gondii* infections acquired from oocysts (M25-M54)

TOXOSOURCES-WP4 aims to develop a source-attributing serological method. TOXOSOURCES-WP4 identifies novel oocyst/sporozoite-specific antigens of *T. gondii* that have source-attributing potential and explores serological methods able to discriminate between oocyst- versus tissue cyst-driven infections. Finally, the methodology is applied to estimate the proportion of oocyst-driven infections in humans and animals used for human consumption.

The main focus of TOXOSOURCES-WP4 during Y3 was on the bioinformatic selection and recombinant expression of sporozoite-specific proteins.

All Milestones of the first year were reached by their planned deadlines.

The bioinformatic selection of promising protein candidates was finalized. The recombinant expression of proteins of interest was started, and the availability of suitable sera for assessing them was secured.



The key sera were characterized using a selection of widely employed serological tests. The screening of the proteins started.

JRP22-WP4-T1: Identification and production of *T. gondii* stage-specific antigens for source attribution (M25-M48)

The predicted proteome of the *T. gondii* oocyst/sporozoite was analysed bioinformatically to identify the best stage-specific and antigenically relevant protein candidates. A list of 96 proteins with source-attributing potential was defined. Main selection criteria were exclusive expression in oocysts, evidence for secretion, and a high score in linear epitope prediction.

The recombinant expression of proteins of interest was started. The set of known stage-specific proteins to be tested in parallel to the novel candidates was selected. First set of purified recombinant proteins were provided to TOXOSOURCES-WP4-T2.

JRP22-WP4-T2: Development of a novel stage-specific antigen-based ELISA to diagnose oocyst- and bradyzoite-driven *T. gondii* infections (M29-M48)

Availability of suitable sera for assessing the proteins of interest from WP4-T1 was determined. The key sera were characterized using a selection of widely employed serological tests. This work yielded interesting comparative data.

The plan and experimental design for the development work were finalized, and first set of purified recombinant proteins were received for assessment from TOXOSOURCES-WP4-T1. The screening of the proteins started.

JRP22-WP4-T2-ST1: Standardization of a POI-based ELISA to diagnose oocyst- and/or bradyzoite driven *T. gondii* infections using reference pig sera

Availability of suitable panel of sera from pigs experimentally infected with either oocysts or tissue cysts was confirmed. The sera were characterized using commercially available and routinely used serological tests and the selection of the best secondary antibody for the novel stage-specific antigen-based ELISA was started.

JRP22-WP4-T2-ST2: Validation of a novel stage-specific antigen based ELISA to diagnose oocysts- and/or bradyzoite-driven *T. gondii* infections using reference sera from several relevant host species including humans

Availability of suitable sera from sheep experimentally infected with oocysts was confirmed. The sera were characterized using commercially available and routinely used serological tests and the best secondary antibody for the novel stage-specific antigen-based ELISA was selected.

WP5: Novel *T. gondii* typing method to detect within-genotype variation (M25-M54)

TOXOSOURCES-WP5 aims at the identification of highly polymorphic regions in genomes of very closely related *T. gondii* strains across Europe, which are made available by partners and collaborators. Preliminary NGS data on European clonal type II *T. gondii* isolates has revealed substantial variation between isolates and relative to reference strains. Using the panel of strains from various parts of Europe, regions in the genome with an optimal SNP density are identified and used to establish a novel typing method.

The main focus of TOXOSOURCES-WP5 during Y3 was on retrieval of relevant isolates and their Whole Genome Sequencing (WGS).

The Milestone of the first year was reached ahead its planned deadline.

The retrieval of key *T. gondii* isolates or DNAs was successful and the aims set were achieved. The work progressed following the timeline, with positive exceptions: the milestone 'Key isolates summarized in



WP5' was reached 2 months ahead of its deadline and one subtask could start earlier than anticipated. Moreover, plans were already made for JRP22-WP5-T3, which starts in Y4.

JRP22-WP5-T1: Retrieval of relevant *T. gondii* isolates or NGS-quality DNAs for NGS and NGS-MST (M25-M44)

T. gondii isolates, WGS-quality DNAs or WGS-data on isolates were collected from across Europe. Isolates were expanded in-vitro, and DNA was extracted for WGS. The focus was on *T. gondii* Type II isolates, while other isolates were included as well. This retrieval of key *T. gondii* isolates or DNAs was successful and the aims were reached. The Milestone was reached 2 months before its deadline, and the total number of isolates retrieved for the work already during Y3 is markedly higher than the original target.

Isolates of northern and eastern European regions were first slightly underrepresented on the list, but further efforts were successful in gathering more isolates from these regions.

Several isolates will be included from a selection of regions/locations to investigate the resolution of typing to trace differences of local isolates.

All DNAs included in the panel were also characterised based on polymorphism of fewer markers using existing standard techniques (PCR-RFLP and microsatellite (MS) typing).

JRP22-WP5-T2: Novel, standardized high-throughput direct NGS-MLST *T. gondii* genotyping method (M25-M48)

Whole genomic sequences were generated. This was successfully done using a batch-approach, to ensure that the first set of sequences were available for TOXOSOURCES-WP5-T2-sT2 in the planned timeframe.

JRP22-WP5-T2-ST1 Whole Genome Sequencing (WGS) of key *T. gondii* isolates and WGS-quality DNAs (M25-M36)

Due to the COVID-19 situation, sending samples was challenging, and we decided to apply a batch-approach to sequencing. The first batch, n=22 isolates, were WGS-sequenced de-novo and together with already existing data, DNA sequences from n=41 isolates were available already by M30. The already existing sequences were from ongoing EURLP-FLI and UCM-CVI (Craig-Venter-Institute) collaborations, and included also sequences of a few Type III and recombinant isolates already available at FLI and UCM. Results from further batches supplemented the data.

The sequences of key *T. gondii* isolates were provided to TOXOSOURCES-WP5-T2-sT2.

JRP22-WP5-T2-ST2 Establishment, validation and refinement of a novel, standardized high-throughput targeted NGS-MLST *T. gondii* genotyping method (M31-M44)

Based on the sequences from WP5-T2-sT1, highly polymorphic marker regions (partially focusing on introns and/or particular gene regions of e.g. virulence associated genes) were identified and are evaluated for suitability for the establishment of a new typing method. This subtask was started ahead of time. The higher number of sequences than originally planned was concluded to be highly beneficial for the work.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
22	D-JRP22-WP1.1	Data Management Plan	M30	M30			Public. 10.5281/zenodo.3924450 OHEJP: available	8
22	D-JRP22-WP3.1	Report on available analytical procedures for detection of <i>T. gondii</i> in fresh produce and list of promising analytical procedures	M28	M28			Public. 10.5281/zenodo.3778719 OHEJP: available	2
22	D-JRP-TOXOSOURCES-WP3.2	SOP on detection of <i>T. gondii</i> in selected fresh produce matrix	M36	M36			Public. 10.5281/zenodo.4405242 OHEJP: available	2

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);



Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
22	M-JRP22-TOXOSOURCES -01	Kick-off Meeting held by WP1	M26	Yes		Kick-off Meeting held on 3.-4.2.2020 at SSI, Copenhagen, Denmark, with possibility to attend online. Milestone reached by M26 (on time).
22	M-JRP22- TOXOSOURCES -02	Bioinformatic selection of oocyst/sporozoite-specific antigens completed in WP4	M28	Yes		Selection done. Milestone reached by M28 (on time).
22	M-JRP22-TOXOSOURCES-03	Key isolates summarized in WP5	M30	Yes		Summary list of key isolates compiled. Milestone reached by M28 (2 months ahead of time).
22	M-JRP-TOXOSOURCES-04	List of meat products or dishes that need to be included in exposure survey is provided by WP2-T4 to WP2-T3	M34	Yes		List provided. Milestone reached by M34 (on time).
22	M-JRP-TOXOSOURCES-05	Experimental selection of the appropriate methods for samples analysis in WP3	M36	Yes		Experimental selection done. Milestone reached by M36 (on time).
22	M-JRP-TOXOSOURCES-06	Delivery of data summary on literature-based prevalence of <i>T. gondii</i> oocysts in fresh produce from WP3 to WP2	M36	Yes		Data summary delivered. Milestone reached by M36 (on time).



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
22	M-JRP-TOXOSOURCES-07	Production of the first sets of purified soluble recombinant proteins (up to 100) for WP4-T2 serological assays	M36	Yes		Production started. Milestone reached by M36 (on time).
22	M-JRP-TOXOSOURCES-08	Evaluation of the source attributing ability of the first sets of stage-specific antigens produced in WP4-T1	M36	Yes		Evaluation started. Milestone reached by M36 (on time).



4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Fluorescent bead-based serological detection of <i>Toxoplasma gondii</i> infection in chickens. https://doi.org/10.1186/s13071-020-04244-6 https://zenodo.org/record/3974390#.X6PV0WhKjcc	YES		Gold, 1990 €
Isolation and genetic characterization of <i>Toxoplasma gondii</i> in Spanish sheep flocks. https://doi.org/10.1186/s13071-020-04275-z https://zenodo.org/record/3974417#.X6PWsWhKjcc	YES		Gold, 1990 €
Expression of in vivo biotinylated recombinant antigens SAG1 and SAG2A from <i>Toxoplasma gondii</i> for improved seroepidemiological bead-based multiplex assays. https://doi.org/10.1186/s12896-020-00646-7 https://zenodo.org/record/4129949#.X6Ldr4hKjcc	YES		Gold, 1890 €

Additional outputs:

Invited talk at Mandagsmøde, Department of Infectious Disease Epidemiology & Prevention, SSI, March 2, 2020:

How and why to prevent *Toxoplasma gondii* infections

Pikka Jokelainen, SSI, Denmark

Oral presentation at OHEJPASM2020, May 27-29, 2020:

Source attribution for *Toxoplasma gondii* infections in Europe



Marieke Opsteegh (1), Hannah Morgan (1), Huifang Deng (1), Gereon Schares (2), Sandra Stelzer (2) Sara Monteiro Pires (3), Helga Waap (5), Jacek Sroka (6), Heidi Enemark (7), Jelena Srblijanovic (8), Olgica Djurkovic-Djakovic (8), Chiara Trevisan (9), Agnetha Hofhuis (1), Lasse S. Vestergaard (4), Pikka Jokelainen (4), Joke van der Giessen (1), Euro-FBP (COST Action FA1408), TOXOSOURCES Consortium

RIVM, The Netherlands (1); FLI, Germany (2); DTU, Denmark (3); SSI, Denmark (4); INIAV, Portugal (5); PIWET, Poland (6); NVI, Norway (7); UoB, Serbia (8); ITG, Belgium (9)

Poster at OHEJPASM2020, May 27-29, 2020:

TOXOSOURCES – *Toxoplasma gondii* sources quantified

Pikka Jokelainen (1), Marieke Opsteegh (2), Marco Lalle (3), Furio Spano (3), Gereon Schares (4), Sara Monteiro Pires (5), Anne Mayer-Scholl (6), Frank Seeber (7), Simone M. Cacciò (3), Joke van der Giessen (2), TOXOSOURCES Consortium (Joint Research Project of the One Health European Joint Programme)

SSI, Denmark (1); RIVM, The Netherlands (2); ISS, Italy (3); FLI, Germany (4); DTU Food, Denmark (5); BfR, Germany (6); RKI, Germany (7)

[10.5281/zenodo.3924467](https://doi.org/10.5281/zenodo.3924467)

Poster and talk at 3-Minute-Thesis competition at OHEJPASM2020, May 27-29, 2020:

Tropism and persistence of *Toxoplasma gondii*: from pork carcass to sausage and dry ham, a quantitative risk assessment

Filip DAMEK¹, Bastien FREMAUX², Dominique AUBERT³, Marieke OPSTEEGH⁴, Sandra VUILLERMET¹, Pikka JOKELAINEN⁵, Joke VAN DER GIESSEN⁴, Pascal BOIREAU¹, Isabelle VILLENA³, Radu BLAGA¹

1 UMR BIPAR, Ecole Nationale Vétérinaire d'Alfort, ANSES, France 2 IFIP - Institut du Porc, France 3 National Reference Center on Toxoplasmosis, Toxoplasma Biological Resources Center, CHU Reims and EA7510, SFR CAP-Santé, University of Reims Champagne-Ardenne, USC EpiToxo ANSES, France 4 National Institute for Public Health and the Environment, The Netherlands 5 Statens Serum Institut, Denmark

Short oral presentation at One Health EJP Cogwheel workshop with JPIAMR, April 28, 2020:

#TOXOSOURCES *Toxoplasma gondii* sources quantified

Pikka Jokelainen, SSI, Denmark



Poster and short oral presentation in a webinar 'Toxoplasma gondii e toxoplasmosis in una prorspettiva One Health' organized by Italian Society of Parasitology (SOIPA), June 30, 2020:

TOXOSOURCES – TOXOplasma gondii SOURCES quantified

P. JOKELAINEN¹, M. OPSTEEGH², M. LALLE³, F. SPANO³, G. SCHARES⁴, S. MONTEIRO PIRES⁵, A. MAYER-SCHOLL⁶, F. SEEBER⁷, S. M. CACCIÒ³, J. VAN DER GIESSEN², TOXOSOURCES CONSORTIUM (JOINT RESEARCH PROJECT OF THE ONE HEALTH EUROPEAN JOINT PROGRAMME)

1 Statens Serum Institut, Copenhagen, Denmark, 2 National Institute for Public Health and the Environment, Bilthoven, The Netherlands, 3 Istituto Superiore di Sanità, Rome, Italy, 4 Friedrich Loeffler Institute, Insel Riems, Germany, 5 Technical University of Denmark, Kongens Lyngby, Denmark, 6 German Federal Institute for Risk Assessment, Berlin, Germany, 7 Robert Koch Institute, Berlin, Germany

Two lectures at One Health EJP Summer School August 17-28, 2020:

Parasites in the food chain: global One Health risks

Wildlife and Public Health

Joke van der Giessen, RIVM, The Netherlands

Oral presentation at PhDay, October 14, 2020:

Desarrollo de un nuevo ELISA para la detección de anticuerpos frente a *Toxoplasma gondii* en el ganado porcino

Nadia María López Ureña, UCM, Spain

Invited talk at International One Health Webinar Series of School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, India, November 5, 2020:

Relative contributions of the different sources of *Toxoplasma gondii*, a globally important pathogen of major public health concern

Pikka Jokelainen, SSI, Denmark

Oral presentation at International Pathology Day, November 11, 2020:

Endemic pathogens and international research projects during a pandemic: *Toxoplasma gondii* and international research project TOXOSOURCES as an example



Pikka Jokelainen, SSI, Denmark and Martha Betson, UoS, UK

Roundtable, International Pathology Day, November 11, 2020:

Discussion topic: Why international knowledge sharing is a winner

Pikka Jokelainen, SSI, Denmark

Oral presentation at ApicoWplexa virtual meeting series, November 12, 2020:

Toxoplasma gondii in Spanish farm animals: opening new avenues from genotype to phenotype

Mercedes Fernández-Escobar, UCM, Spain

Short oral presentation at One Health EJP Cogwheel workshop with SafeConsume, November 25, 2020:

TOXOSOURCES - *Toxoplasma gondii* sources quantified

Pikka Jokelainen, SSI, Denmark

Short oral presentation at 15th Workshop of the National Reference Laboratories for Parasites, December 15, 2020:

#TOXOSOURCES *Toxoplasma gondii* sources quantified

Pikka Jokelainen, SSI, Denmark

Other:

#TOXOSOURCES has been used on social media:

<https://twitter.com/hashtag/toxosources?f=live>



TOXOSOURCES partner institutes have mentioned TOXOSOURCES in their communications:

<https://www.ssi.dk/aktuelt/nyhedsbreve/epi-nyt/2020/uge-4---2020>

https://www.rki.de/DE/Content/Forsch/EJP_OH2020.html

<https://www.sva.se/forskning/internationellt-samarbete/europeisk-samverkan-kring-livsmedelsburna-smittor/toxosources-ett-one-health-ejp-projekt/>

https://www.bfr.bund.de/en/toxoplasma_gondii_sources_quantified_ejp_toxosource_-249310.html



5. On-going and planned collaborations with national or European projects or networks

TOXOSOURCES builds largely on previous work and is actively looking for collaborations and synergies.

- An example of building on previous work, which was emphasized in the oral presentation at OHEJPASM2020, is the building on the work performed within **COST-Action Euro-FBP**. A literature review of source attribution for *T. gondii* infections in Europe was performed within the COST-Action Euro-FBP; TOXOSOURCES will address the identified data gaps. The literature review also covered risk factor analysis; within TOXOSOURCES this will be updated and extended with prevalence studies.
- Another example of building on previous work is that the TOXOSOURCES-WP3-T1 survey questionnaire was developed based on outlines of the **project IMPACT** (Standardising molecular detection methods to IMprove risk assessment capacity for foodborne protozoan Parasites, using Cryptosporidium in ready-to-eat salad as a model organism”; Partnering Grant Project Grant Agreement Number GP/EFSA/ENCO/2018/03 – GA03). Moreover, in TOXOSOURCES-WP3 development of ‘SOP on detection of *T. gondii* in selected fresh produce matrix’ was built on work done in IMPACT.
- TOXOSOURCES established a collaboration with **SafeConsume project** (<http://safeconsume.eu/>), and representatives of TOXOSOURCES were invited to participate in SafeConsume Multi-Actor Workshop, which was unfortunately cancelled due to the COVID-19 situation. Both projects are interested in the relevance of *Toxoplasma gondii* contamination in fresh produce, with different focus: SafeConsume focuses on consumer behaviour on safe handling of fresh produce at home, whereas TOXOSOURCES WP3 focuses on fresh produce from harvest to packaging. TOXOSOURCES suggested a OHEJP Cogwheel Workshop with SafeConsume, and it was organized on November 25, 2020. Several persons from TOXOSOURCES participated. SafeConsume invited TOXOSOURCES to join a Horizon Results Booster group. Via this collaboration, TOXOSOURCES also established links with projects **Stance4Health** and **EAT2beNICE**.
- Collaboration with **International network for environmental Toxoplasma studies (INETS)** is another important established collaboration. INETS is a global network that organizes e.g. workshops.
- TOXOSOURCES builds on the PhD work by Dr. Huifang Deng ‘ Source attribution of human toxoplasmosis, A quantitative microbiological risk assessment approach.
- **One Health EJP PhD project ToxSauQMRA** (PhD candidate Filip Damek) is closely linked to TOXOSOURCES.
- There are discussions about collaborations with **other One Health EJP projects**, including **COHESIVE, ORION, MEmE, PARADISE and OH-Harmony-CAP**. Synergies and complementary approaches have been identified.
- To enable and encourage collaborations, the QMRA models will be made available via a repository (<https://foodrisklabs.bfr.bund.de/rakip/>)
- **European Reference Laboratory of Parasites** and network of **National Reference Laboratories** are well represented in the consortium.
- The German EFSA Focal Point encouraged TOXOSOURCES to reach out to the **Focal Point network** to distribute the link to the survey gathering information on production and trade of fresh produce in Europe (TOXOSOURCES-WP3). Consumption of fresh produce is one of the possible routes of *Toxoplasma gondii* transmission to humans, which has been little explored



to date. Thus, one aspect of the project is to investigate the presence of *T. gondii* in ready-to-eat fresh produce. The data will be used to evaluate the possible role of fresh produce as a source of *T. gondii* infection (quantitative microbiological risk assessment), and to design a sampling strategy for a multi-centre study investigating selected fresh produce for presence of *T. gondii* oocysts. Action on this was taken in early 2021 (Y4), and the EFSA-contact of OHEJP was informed via OHEJP-WP5.

- TOXOSOURCES collaborates with and builds on the results of several national and regional projects.

JRP23-ADONIS

1. Summary of the work carried out in year 3

Although challenging due to the COVID19 situation the project is running so far without major/critical problems and delays. Detailed work plans have been made following the kick-off meeting in January 2020 and are monitored regarding their progress with project management video calls. During this first period the project especially focussed on preparation for data analysis and data gathering. For WP2 this includes the gathering of *Salmonella* National Control Programs (NCP) audit reports, the preparation of a survey to collect information on the main characteristics of NCP for *Salmonella* in laying hens at the country level, and the design of a study protocol for primary production on-field investigations (WP2). For WP3 this includes the selection of countries for evaluation of human surveillance systems and detailed epidemiological trend analysis. For WP4 this includes the preparation of an inventory of available sequence data and a pilot GWAS experiment (finding DNA markers for specific phenotypes). For WP5 this included the identification of determinants possibly associated with the reversal of the decreasing trend in *Salmonella* incidence and the possible interventions options.

2. Work carried out in the JRP, scientific results

WP1: Project management (M25-M54)

JRP23-WP1-T1: Coordination

Ongoing.

Although challenging due to the COVID19 situation the project is running so far without major problems. The project has its kick-off meeting at 15 and 16 January 2020 which was very successful. The meeting produced detailed planning for each WP (see D-JRP23-WP1.1). Regular TCs within the project management team and within WPs are conducted.

JRP23-WP1-T2: Aligning and communication

Ongoing.

JRP23-WP1-T2-ST1: Reporting

Draft 9M report is being made (this document) and 12M report is prepared.

JRP23-WP1-T2-ST2: Alignment and communication. Ongoing.

Efforts are undertaken to align the EJP projects of ADONIS, DISCOVER and BEONE in the area of *Salmonella*. The RIVM appointed a PhD that will work primarily on ADONIS but will also do work regarding source attribution of *Salmonella* (DISCOVER) and genomics-epi integration (BeONE). In this way we maximize the cross-fertilization of the different projects.



WP2: Salmonella controls at the primary production level (M25-M54)

This WP covers comparative analysis and management measures between MS in the EU based on current NCP and on a questionnaire to collect data on farms. On-field investigations are also expected in two MS (France and the UK) to collect data which will contribute to the comparative analysis performed in this WP and to the analysis of S Enteritidis increase within the project.

JRP23-WP2-T1: Comparative analysis of Salmonella controls and management measures at MSs level (M25-M48). Ongoing.

Analysis of *Salmonella* National Control Programs (NCP) audit reports is ongoing. 232 recommendations were identified in 38 publicly available reports from 24 countries. Most (143) recommendations concern not full compliance on implemented programs with EU regulations on *Salmonella* control. The others refer mostly to legislation on official controls and food hygiene. The ongoing work focuses on identification of specific issues raised in recommendations.

A questionnaire has been developed to document control and management measures for *Salmonella* in laying hens at the country level in several participating MSs. The questionnaire is presently circulating in MSs among the veterinary agencies. The survey contains questions directed at the population and scope of the program as well as some specific questions on the type of controls performed by the Food Business Operators and Competent Authorities.

The first preliminary review of historic data from controls performed by competent authorities and food business operators has been delayed due to the COVID-19 crisis but is expected to be performed in the upcoming months.

JRP23-WP2-T2: Comparative analysis of Salmonella controls and management measures at farm level (M25-M48). Ongoing.

JRP23-WP2-T2-ST1 Field studies during outbreaks and sensitivity testing

The study protocol of on-field investigations was designed by APHA and Anses. In France, this protocol was presented and agreed by the competent authority. Recruitment of flocks presenting *Salmonella* cases started by September 2020 with the help of local authorities.

JRP23-WP2-T2-ST2 On farm surveys

Currently, intensive preparatory work is underway regarding the developing of the questionnaire and guidelines for completing it. The questionnaire will be a kind of survey that will be distributed / carried out on poultry farms in which the presence of *Salmonella* infection was found as well as on farms where such infection has never occurred. The questionnaire contains detailed questions about the herds on which the survey will be conducted / implemented, on the welfare of herds, breeding conditions, their feeding, detailed therapy if were carried out, if preventive vaccinations were carried out from the moment the chickens were introduced to the farm until the chickens were sent to the slaughterhouse.

After completing the questionnaire and its guidelines, it will be sent to WP2 partners to disseminate prepared surveys in other EU countries.

WP3: Surveillance, epidemiology and source attribution (M25-M48)

Determinants of the recent increase in *S. Enteritidis* incidence could be related to changes in the performance of the human surveillance systems/diagnostic standards in place, as well as changes in the epidemiological and exposure patterns of *S. Enteritidis* in the population at large.

JRP23-WP3-T1 Evaluation of surveillance systems in humans (M26-M48). Ongoing.

Trend analyses were performed (Tau parameter) of the increasing/decreasing incidence of *S. Enteritidis* of multiple countries in the period 2013-2018 based on ECDC data



(<https://www.ecdc.europa.eu/en/surveillance-atlas-infectious-diseases>). Countries with low *S. Enteritidis* numbers or without *S. Enteritidis* data were excluded. Countries should have experienced a decreasing trend observed in the 2009-2012 period to meet the research question of ADONIS. The non-parametric Mann-Kendall Test was used to detect monotonic trends. Kendall's Tau permits a comparison of the strength. To select countries with a decreasing/increasing trend in 2013-2018 period, they were ranked according to the Tau value (strength of the trend). Stable trends in the 2013-2018 period were selected based on a Tau value close to 0. The Pettitt's test was used to identify a point at which the values in the data change over time (breakpoint). A document was prepared, showing the trends of each of the countries, as well as the breakpoints. This was further discussed during a teleconference meeting with all partners of WP3. Breakpoint analyses showed that the breakpoint (i.e. change in trend) for most countries was in 2013. Therefore, it was decided to compare the time periods 2007-2012 and 2013-2019. Concerning the selection of countries, it was decided to focus on Spain and UK as countries with an increasing trend (WP2 also focusses on these countries), Netherlands and Belgium as countries with a stable trend, and Norway as country with a decreasing trend. Next steps are to approach the relevant persons in Spain, UK, Netherlands, Belgium, and Norway to assess the feasibility of obtaining the data required to complete WP3.

Key elements for description and key attributes and indicators for evaluation were defined using an adapted version of the 2014 ECDC evaluation framework of public health surveillance systems, focusing only on those surveillance attributes that could potentially affect the number of captured human *S. Enteritidis* cases. A detailed list with these key elements for description and key attributes and indicators for evaluation was composed. Next step is to describe and evaluate the surveillance systems of the selected countries within the selected time periods once the surveillance data from these countries have been obtained. Eventually it will be assessed whether identified changes in the surveillance systems over time have affected the number of captured human salmonellosis cases over time.

Due to the COVID-19 pandemic, resources to finalize the description of key elements of surveillance systems have been limited. Therefore, only key elements of the surveillance systems of the Netherlands have been described partly (surveillance objectives, surveillance category, population under surveillance, type of surveillance). However, we plan to allocate resources to finalize it for the Netherlands in month 37 (1 month delay), and for Spain and the UK in month 38 and month 39, respectively.

JRP23-WP3-T2: Assessment of changes in the epidemiology of human *S. Enteritidis* cases and other relevant serovars (M29-M48). Ongoing.

So far, data on human *S. Enteritidis* cases in the period of interest have been obtained from Belgium, Spain and the Netherlands. Data from the United Kingdom will follow but data delivery has been delayed due to lack of resources as a result of the COVID-19 pandemic. Syntaxes for data cleaning, description of data and time series analyses have been developed based on data from the Netherlands. Description of the epidemiological characteristics will follow in month 39 for Belgium, the Netherlands and Spain. Depending on data availability from the United Kingdom, it will follow shortly. Although this deliverable has been delayed, we have full confidence that we will be able to successfully complete this task (WP3-T2) because major parts of the next deliverable in this task have already been done (i.e. scripts to prepare data to be entered in times series models). Also, the time series models have been thoroughly tested and adapted based on data from the Netherlands and are almost ready-to-use.

JRP23-WP3-T3: Assessment of human exposure to *S. Enteritidis* (M32-M48)

To be started.



WP4: Salmonella Genomics (M25-M54)

JRP23-WP4-T1: Collection overview (M25-M30). Ongoing.

All partners are involved in WP4, especially in the collection of sequences. During the Kick-off meeting, we agreed on the creation of an inventory to get an overview of the available strains and sequences at each partner institution. The inventories were collated and presented at a teleconference where we then discussed the available strains and decided what to sequence. At present we are awaiting the final lists of strains to be sequenced from all partners and are also awaiting the sequences to be finalized, both are influenced by the COVID-19 pandemic.

D-WP4.1 : Sequence Inventory Report (July 2020): Ongoing.

The lists of strains to be sequenced and already available sequences have been provided by most partners. Report will be written once all partners have provided this information.

M-7: Sequence collection to be shared amongst partners (July 2020): Ongoing.

Certain partners have experienced delays in the sequencing due to COVID-19 reorganisation in their institutions. A data sharing site (data hub) has been ordered at European Nucleotide Archive (ENA), however they are also experiencing delays.

M-8: Phylogenetic trees describing the sequence collection (August 2020): Not yet started.

Once all sequences will have been collected, the analysis will start. Expected one month after all sequences are finalized.

JRP23-WP4-T2: Population structure and comparative genomics (M25-M48). Ongoing.

This task is awaiting the sequence inventory in order to really begin. Small pilot studies on limited datasets have been initiated on Mobile Genetic Element (MGEs) detection and analysis.

JRP23-WP4-T3: Phylodynamics and Phylogeography (M31-M51). Ongoing.

This task is just beginning and is also awaiting the sequenced genomes to be collected and shared at ENA.

JRP23-WP4-T4: Mutant creation and testing including GWAS studies (M31-M54). Ongoing.

A pilot GWAS study was performed on a limited dataset of 120 genomes. The study detected 6 plasmid related genes slightly associated with the S. Enteritidis increase since 2014. More analysis is needed to confirm this pilot study.

WP5: MCDA model to support priority setting (M28-M52)

As it is possible that no single factor is able to explain the observed changes in S. Enteritidis incidence in humans and poultry, but that the reasons for these changes is multifactorial and interconnected in nature, WP5 will conduct a MCDA to determine: 1) what are the main determinants that may explain the reversal of the decreasing trend in the incidence of human S. Enteritidis infections in the ADONIS partner countries; 2) which interventions are expected to have the largest impact on stopping the reversal of the decreasing trend in incidence of human S. Enteritidis infections in the ADONIS partner countries.

JRP23-WP5-T1: Framework building (M25-M48). Ongoing.

The MCDA framework has been defined. The determinants possibly associated with the reversal of the decreasing trend and the possible interventions (i.e. options) are being identified based on the (hierarchical) structure of the transmission chain and all possible interrelation between primary production, exposure and pathogen characteristics: a literature review helps with this process. The MCDA framework is being based on the Analytic Hierarchy Process (AHP) approach, which also requires independent evaluation criteria and sub-criteria for both the determinants and interventions: these



have also been defined. A list of experts to conduct the MCDA has also been compiled, including 2 to 5 experts per ADONIS partner country that cover all three domains of the project (i.e. poultry primary production, Salmonella genomics, salmonellosis epidemiology) and a balanced representation of the medical and veterinary fields. The next step will be to organize a first round of elicitations to define the weights for the criteria.

JRP23-WP5-T2: MCDA modelling (M36-M52).

Not yet started.

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3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
23	D-JRP23-WP1.1	Detailed work plan developed (Kick-off meeting minutes)	M28	M28	-		Public Detailed project management plans with goals and actions (in x-matrix format) OHEJP: available https://zenodo.org/record/4478368#.YBPPIfZFw2w	10
23	D-JRP23-WP2.1	Report on the evaluation of the questionnaires	M30		M43		Public Following the preparation of the detailed work plan we noticed that a deadline in M30 is a mistake made in the proposal. It should be M43.	10
23	D-JRP26-WP3.1	Description of surveillance systems regarding their key elements	M36		M39	Yes		
23	D-JRP26-WP3.2	Description of basic epidemiological characteristics	M36		M39			



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
		of human S. Enteritidis cases and other relevant serovars						
23	D-JRP23-WP4.1	Sequence inventory report	M31		M33		The COVID-19 pandemic has had an impact on laboratory performance.	10

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
23	M-JRP23-1	Kick-off meeting organised	M26	M25		
23	M-JRP23-2	Communication partners mapped	M28	No	M32	Delayed due to COVID
23	M-JRP23-3	Inventory of documents available for the comparative analyses	M30	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
23	M-JRP23-4	Farm survey planed and questionnaires developed	M30	Yes		The questionnaires are developed; they are circulating among partners to be addressed.
23	M-JRP23-5	Selection of three countries of which their national S. Enteritidis surveillance systems will be evaluated	M27	Yes	M28	Trend analyses were performed of the increasing/decreasing incidence of S. Enteritidis of multiple countries in the period 2013-2018 based on ECDC data
23	M-JRP23-6	Obtained national surveillance data on S. Enteritidis and other relevant serovars	M30	Yes		For NL; the methodology and data needs (incl gaps) will be used for the other selected countries
23	M-JRP26-7	Sequence collection to be shared amongst partners	M31	yes		
23	M-JRP26-13	List of candidate strains for phenotype testing	M37			

4. Publications and patents

No publications yet.



5. On-going and planned collaborations with national or European projects or networks

Within the EJP consortium the ADONIS project strongly liaises with the EJP projects DISCOVER regarding source attribution and BeONE regarding the genomics and bioinformatics. We have strengthened this at RIVM with a PhD that is involved in all three projects.

Outside the EJP consortium the ADONIS project maintains strong interactions with ECDC and EFSA. The project leader especially has intensive connections within the ECDC Food- and Waterborne Disease network and acts as a member of the FWD steering committee. In that role updates were given by E. Franz on the ADONIS and DISCOVER projects on the ECDC FWD steering committee meeting (online) in October 2020.

The Project Leader has contacts with local authorities (stakeholders) as described in the WP.



JRP24-BEONE

1. Summary of the work carried out in year 3

The main research and development takes place in WorkPackages (WPs) 1-4. WPs 3 is largely progressing as planned. WPs 1, 2, 3 and 4 have experienced some delays due to the Covid-19 situation, additionally WP4 has been affected by personnel changes. Especially critical is the delay in dataset collection, since this blocks dependent WP 1 tasks as well as WP2-T2 and subtasks, as well as the testing of the outputs of WPs 3 and 4 (WP5-T2, M-BeONE.5.3).

The kick-off meeting (KOM) and subsequent teleconferences (TCs) successfully aligned the partners with regard to a common goal, and provided insights into the different realities of institutes from the animal health, food safety and public health sectors.

The goal to develop a decentralized system for collaborative outbreak surveillance and investigation has crystallized into an architecture for the platform and a model for data exchange (M-BeONE.4.2). This will be implemented in a prototype for testing and continued development. The prototype will feature automated basic strain characterization (WP4-T2), user driven searching and subsetting of sequences, and display of certain data categories like geospatial data and phylogeny (WP4-T1, M-BeONE.4.3). The prototype will submit compute tasks to the Norwegian High Performance Computing (HPC) infrastructure metacenter.no.

We have evaluated the data sharing practices in member states. Building on these insights, we have defined a meta-data schema and a preliminary ontology implementation plan (WP3-T2+3) for samples leading to a data model implemented in the database.

A literature review of factors impacting outbreak detection has been undertaken and a manuscript containing the main findings has been drafted; the results are being implemented in a model for farm-to-fork tracing of bacterial pathogens. (WP2-T1)

2. Work carried out in the JRP, scientific results

WP1: Typing comparability and nomenclature (M25-M48)

The goal of WP1 is to provide a solid foundation for the remaining WPs to build on. WP1 has faced some difficulties both with hiring additional staff at INSA (due to changed hiring rules in Portugal as discussed with the OHEJP-PMT), and especially during the ongoing Covid-19 crisis. Following a contingency plan, it was agreed that if the recruitment was not possible until month 9, some sub-tasks (specially Tasks 2) would require a higher involvement of other partners. The recruitment process could only be finished during month 12, so, as predicted, it is anticipated a high contribution of other partners to accomplish WP1 task 2. Also, the WP leader (Vitor Borges) has been almost entirely occupied by the public health effort during the crisis. Impacts will be stated under the specific tasks. During the KOM and subsequent Coordination team monthly meetings, the WP1 workflow was amply discussed, leading to an agreement on the approaches to be applied for each task (see details below). The discussion benefited from data collected before and during the KOM through both surveys and parallel sessions.

JRP24-WP1-T1: Establishment of state of the art (M25-M30)

Status: ongoing

At the KOM, it was discussed the need of this task, considering the huge amount of available literature on this subject and/or the outputs of other ongoing EJP projects (namely ORION). This prioritization was done both to avoid duplication of work and to decrease the workload at INSA due to expected hiring difficulties. It was agreed that BeONE would not focus on delivering an exhaustive state of the



art of methods and existing public platforms for WGS-based typing. Instead, BeONE will build-on the ORION project report by adding not-covered topics related to the BeONE project, namely a discussion around the advantages and disadvantages of centralized versus decentralized approaches. It was also decided to assemble documentation on the confluence site, about the state of the art of different currently available surveillance platforms to regard to 1) software, 2) Data Management and 3) Analysis Pipelines. The deliverable D-BeONE.1.1 was delayed by the COVID-19 response.

JRP24-WP1-T2: Dataset selection and curation (M25-M32)

Status: Ongoing

JRP24-WP1-T1-ST1: Dataset selection and collection

After the KOM, a survey was conducted within the BeONE project partners to define their WGS and metadata contribution. However, presumably due to the Covid-19 crisis not many participants responded. Following a discussion on the needs of each WP among Coordination team members, guidelines for WGS data and metadata collection were built. For metadata collection and harmonization, the strategy was developed within “WP3 Task 2 - Metadata acquisition and standardisation” activities (see below). This task and the accompanying milestones are currently pending by decisions on what metadata is needed on the samples. Since the dataset is to be used for testing, and the needed data is quite sensitive, a fully anonymised dataset is required. Because of the anonymization it is not possible to request additional data once the data have been collected, without recreating the dataset anew. Apart from these constraints, it is expected that at M33 / M36, a curated template along with guidelines for data anonymization is already sent to each partner and all data is filled out and uploaded to the HPC platform (metacenter.no, see below). As agreed, this strategy allows for the efficient collection of data required for WP1, WP2 and WP3 to be performed in a single instance. In summary, this task should have finished in M30, but is still ongoing due to delays caused by the COVID-19 pandemic.

JRP24-WP1-T1-ST2: Quality check and assurance, and genome assembly

During the KOM, this sub-task was redefined and inserted within WP1-T3 activities (see next section).

JRP24-WP1-T3: Clustering congruence and thresholds (M25-M48)

Status: Ongoing

JRP24-WP1-T3-ST1: Selection of WGS-based typing methods to be evaluated

Status: Completed

Due to the high heterogeneity of methods and parameters used for WGS-based typing, at the kick off meeting, it was decided that the full independent pipelines used by each BeONE partner would be run on the collected dataset. Thus, once the dataset is fully collected and stored, partners that volunteered to test their own pipeline will install it and run it up to the clustering steps (which will be handled by INSA in WP1-T3-ST2). As such, this will bypass the evaluation of the impact of variations in any given parameter within each step of a pipeline while providing a more grounded and realistic approach to the comparison of different WGS-typing methodologies currently being used for foodborne pathogen surveillance. This task was completed before M30 as planned.

JRP24-WP1-T3-ST2: Assessing clustering congruence between different methods at different hierarchical levels

This task is not planned to start until M33 as it is dependent on the completion of the dataset collection that is currently ongoing. Still, it was decided that the different hierarchical levels of clustering would be defined for each method/pipeline used by the partners in task WP1-T3-ST1 (by determining cluster stability threshold ranges and cluster thresholds in association with known outbreak data), which then would be used to correlate cluster congruence.



JRP24-WP1-T3-ST3: Correlating clustering congruence with existing nomenclature schemes

Not planned to start until M39.

WP2: Joining molecular and epidemiological methods (M25-M48)

WP2 will make the link between the genomics and epidemiology by building knowledge and algorithms on outbreak detection. The first task of the package will summarize the existing knowledge on epidemiology of the targeted pathogens, while the second will implement elements of that knowledge in an algorithm for detection of outbreak episodes. The already incurred or expected to incur delays, and the reasons for it are detailed for each task.

JRP24-WP2-T1: Conceptualization of epidemiological and biological factors impacting on fine resolution clustering and outbreak detection (M25-M34)

Status: complete

In order to provide a basis for a better informed integration of WGS in epidemiological surveillance, we attempted to formalize the cluster detection problem and outline a conceptual model for the biological and epidemiological factors impacting on cluster detection. We propose a conceptual model of the common practice steps performed towards food-borne pathogens' cluster detection. We underline the complex relations between the biological and ecological factors at play in the evolution of some of the most common food-borne pathogens, the standards chosen and the ways how different conceptual sources can be used for improving integrative algorithms of cluster detection. The proposed model is a schematic representation of the reality – in this particular case it aims to describe the biological and ecological factors associated with the chain of thoughts and actions used in defining a food-borne infection cluster. We have identified 11 factors that are directly or indirectly impacting on the observed genetic distances between bacterial isolates, and thus on the definition of a cluster in view of outbreak detection. The most important factor is time, as it is one of the main components in the evolutionary path of any microorganism.

The proposed conceptual model could serve as a first step in developing and evaluating new algorithms of cluster definition/detection. Design-oriented conceptual modelling would be a later step, just before the quantitative model development, where the conceptual model could be revised based on the availability of the data and potential inconsistencies identified, and it will be the subject of BeONE-WP2-T2.

In relation to novelty of research and self-plagiarism issues in the process of publication, the full version will be available only upon submission to a peer-reviewed scientific journal (estimated to be achieved by M39).

JRP24-WP2-T2: Integrating genomics with epidemiology (M33-M46)

Status: ongoing.

Although the development of pathogen-specific algorithms is expected to be delayed, as it would be partly based on the outcome of tasks BeONE-WP1-T2 and BeONE-WP2-T1, we are trying to make a head start with a proof of principle, generalizable algorithm, on a smaller dataset of *Salmonella* Enteritidis; this will be updated at a later stage.

JRP24-WP2-T2-ST1: Comparison of the epidemiologic clusters with the phylogenetic tree of the *Campylobacter jejuni* isolates

Dataset has been identified and an initial meeting for the *BeONE-WP2-T2-ST1* work group has been held.



JRP24-WP2-T2-ST2: Comparison of the epidemiologic clusters with the phylogenetic tree of the Shiga toxin producing Escherichia coli (STEC) isolates

Dataset has been identified and an initial meeting for the *BeONE-WP2-T2-ST2* work group has been held.

JRP24-WP2-T2-ST3: Comparison of the epidemiologic clusters with the phylogenetic tree of the Salmonella enterica isolates

Dataset has been identified and an initial meeting for the *BeONE-WP2-T2-ST3* work group has been held.

JRP24-WP2-T2-ST4: Comparison of the epidemiologic clusters with the phylogenetic tree of the Listeria monocytogenes isolates

Dataset has been identified and an initial meeting for the *BeONE-WP2-T2-ST4* work group has been held.

WP3: Storage, management, and sharing for meta- and molecular data (M1-M30)

In WP3 we have made substantial progress regarding the conceptualization and implementation of the (meta)data management within the BeONE framework. For all tasks in this work package we aimed to harmonize our developments with existing approaches and projects via communication with scientists within BeONE but also from other projects. We were able to decide on a database system to build on and design compatible data structures which capture the complexity of intersectional surveillance data while keeping a strict focus on usability. The concrete proceedings are described within the belonging tasks and subtasks.

JRP24-WP3-T1: Evaluate national level data sharing (M25-M34)

Status: Complete

As discussed at the KOM and described in Task *BeONE-WP1-T1 Establishment of state of the art*, we decided to base the output of this task on existing literature and bilateral communication. The key hurdles described by project partners at the KOM focused on both technical restrictions and data privacy concerns.

A preliminary state of the art has been taken by the questionnaire conducted in COHESIVE: ["Questionnaire on available databases and Information Systems for WGS DATA MANAGEMENT"](#).

The COHESIVE deliverable D-4.1.2 and D-4.1.3 provides the status of the feasibility studies for sharing, integrating and harmonizing WGS data and related epi/metadata between human and veterinary organizations at member state level. The involved member states are Italy, the Netherlands and Norway.

JRP24-WP3-T2: Metadata acquisition and standardisation (M25-M48)

Status: Ongoing

As part of *BeONE-WP1-ST1* (Dataset selection and collection), a survey was conducted by WP1 among BeONE partners to estimate their data contribution and to inquire minimal-to-optimal sample attributes that each participating institute is allowed to share publicly (s. *Sub-Task: BeONE-WP1-T2-ST1 Dataset selection and collection*). These covered historical, geographical and source attributes acquired during sample collection as well as provisional analysis results. Strikingly, varying institutional privacy restrictions impose a heterogeneous depth of metadata information.

JRP24-WP3-T2-ST1: Metadata acquisition

Status: Delayed



To relieve the constraints of a least common denominator approach at the level of information, we opted for gathering metadata information encoded by hierarchical controlled vocabularies (CVs) to an individual granularity each submitter is able to publish.

The European Food Safety Authority (EFSA) provides controlled vocabulary in their Data Collection Framework (DCF) catalogues (doi.org/10.5281/zenodo.3243215) for many aspects relevant to surveillance and with excellent coverage of European market products and culture. The geographic catalogues for European (NUTS) and world regions (GAUL) as well as the consumer-exposed matrix catalogues (FOODEX2 - subsections food, feed, non-food matrices) were integrated into the BeONE sample submission table for drop-down-menu query in Microsoft Excel. Multiple attributes per sample may be stated by concatenating catalogue codes. This simple yet powerful approach is computer-readable, complies with strict institutional IT security policies that prohibit executable code in shared documents, e.g. VBA macros, and is approved by WP1-4 collaborators. The final decision if changes to the metadata collection system are necessary had to be postponed as the data collection is delayed (s. Sub-Task: BeONE-WP1-T2-ST1).

JRP24-WP3-T2-ST2: Standardisation using ontologies

Status: Ongoing

As described in *Sub-Task: BeONE-WP3-T2-ST1 Metadata acquisition*, we have selected controlled vocabularies to enable the implementation of ontology systems when after seeing the metadata that will be provided by data submitters in *BeONE-WP1-T2-ST1 Dataset selection and collection*. We are in close contact to an ontology expert group (s. *Task: BeONE-WP6-T2 Communication*) to enable compatibility with existing ontology systems. Beyond the planned use of ontologies for acquired metadata, we have assessed the possibility to assign ontologies to technical metadata such as results from tools, used references, tool versions etc. to enable easier development of APIs for different tools and analysis pipelines. As planned for Milestone M-BeONE.3.5, we designed a way to add ontologies to metadata as proposed by OBO Foundry (using e.g. GenEpiO and FoodOn for the respective terms) due to their reach in the field proven by projects such as COHESIVE, ORION and IRIDA. A final, project wide decision on the concept has been delayed until the exact needs of the database structure are defined in BeONE-WP3-T3-ST1.

JRP24-WP3-T3: Database design and implementation (M25-M54)

Status: Ongoing

Database design was looked at to incorporate a diverse set of data. Structure will be put in but an expectation that regions can put additional fields for their own needs is taken into consideration. Another key part of the design is that it needs to fit our planned data sharing model where each member controls their own data and what others can see. This follows the decentralized approach so that data is not stored in a master database. The database needs to be designed to handle WGS and epi data and should function as the source of information for the dashboard.

The data structure for the database has been determined to be based on Bifrost (<https://github.com/ssi-dk/bifrost>), a platform developed and in use internally at SSI. The bifrost platform uses a flexible mongoDB which will enable a decentralized sharing model and storage of varied data and data types.

JRP24-WP3-T3-ST1: Determine and implement data structure for database

Status: Delayed (due by M33)

Database schemas have been established for WGS data though some details need to be finalized. Database design regarding epi data has also begun but still needs to be established. This section has been impacted due to work priorities related to covid-19 from related developers at SSI.



JRP24-WP3-T3-ST2: Implement API for import of data

Status: Delayed (by M33)

As we have chosen to implement the Bifrost system from SSI we have access to their processed data and can port data from their existing pipelines. The Bifrost codebase has been adapted for the use of a APIs. However, the implementation of the API itself had to be delayed due to work priorities from related staff at BfR.

JRP24-WP3-T3-ST3: Data porting from an existing pipeline

Status: ongoing - not due to start until M35.

The SSI-bifrost pipeline was set up at BfR to analyze its native mongoDB data structure. The results of the NGS quality control pipeline AQUAMIS, developed and used by BfR, were restructured to match the mongoDB collections "runs", "samples" and "sample-components". This requires a mapping of AQUAMIS result values to the appropriate bifrost stores or to extend the documents with AQUAMIS-specific attributes and is ongoing. Ontologies will guide this result mapping procedure in a later phase according to the ontology designed in Milestone M-BeONE.3.5.

JRP24-WP3-T3-ST4: Data porting from further pipelines

Status: Not starting until M41.

The data structures and schemas are being designed with compatibility to a number of different pipelines in mind. The data porting frameworks are not yet in development.

JRP24-WP3-T3-ST5: Expansion of the API for referencing/exporting of entries in other reference databases

Status: Not starting until M41.

JRP24-WP3-T3-ST6: Expansion of the API for queries from the dashboard WP4

Status: Not starting until M47.

WP4: Development of a user-oriented interface for analysis and sharing of epi and molecular data (M25-M54)

In WP4 we have been severely affected with personnel changes. The previous WP lead and developer associated with the the dashboard has left SSI. Other developers associated to this part of the project have been brought in to work on covid-related tasks as their top priority. A new person has been hired after multiple months delay and work regarding the dashboard has been re-started. Additional assistance will also be brought in to assist on work related to the dashboard. Due to the changes a new tech-stack will be used for the implementation of the dashboard. These changes have impacted our timeline and development as we are starting over on the technical aspects of the dashboard. We believe however, this change will allow us to still deliver the dashboard on time, though the ongoing covid pandemic may complicate this more.

JRP24-WP4-T1: Dashboard (M25-M42)

Status: Ongoing

Tools used to build the web application come as JavaScript based web components created through the React JavaScript library (<https://reactjs.org/>).

JRP24-WP4-T1-ST1: Core display components

JRP24-WP4-T1-ST2: Component integration

JRP24-WP4-T2: Back end analysis implementation (M25-M36)

Status: Ongoing



Back end analysis draws significantly from the Bifrost platform being developed at SSI (see above). This framework employs Dockerized Snakemake workflow management pipelines, the non-relational database MongoDB (<https://www.mongodb.com/>) and a Python-based set of tools to handle data flow from bioinformatic tools to database storage.

JRP24-WP4-T4: Data sharing front end (M29-M54)

Status: Ongoing

Significant investigation has been carried out with regard to defining the best implementation of user-user data sharing of sensitive and non-sensitive data. This has been described extensively elsewhere.

JRP24-WP4-T4-ST1: Web-based input system

Status: Ongoing

The dashboard has support to select specific databases and type of data to have access to and share among users. The dashboard will have an entry point and database drop function for these data types:

- Sequence data in .fasta format
- Read data in .fastq format
- Allelic profiles in tabular format
- Tree graphs in Newick format
- The implementation is ongoing.

JRP24-WP4-T4-ST2: Import/Export front end for reference databases

We have at this stage defined the import/export of core genome MLST reference schemas to be used for cgMLST typing.

JRP24-WP4-T4-ST3: BeONE data exchange system

Status: Ongoing

We are currently investigating a database-centric implementation of data exchange based on the idea of a two public-private databases system. The front end dashboard will contain functionalities that enable a user to select sets of data and make them available to collaborators, for example by copying them to a public database.

WP5: Dissemination, Testing, Evaluation and Sustainability (M25-M54)

JRP24-WP5-T1: Dissemination (M25-M54)

Status: Ongoing

The base code is kept and shared in the form of Github repositories, publicly available. These repositories have support for thorough documentation, which is used to disseminate the scope, functionalities and technical aspects, such as installation, of the BeONE platform.

JRP24-WP5-T2: Continuous Testing and Feedback (M25-M54)

Status: ongoing

The initially designed BeONE platform prototype including the dashboard web application and computational layer, has been significantly restructured as the main developer has left the team (see WP4). The new approach will keep the same objectives but will make use of an updated view on implementation and required resources. The secure server will be hosted at the norwegian Metacenter infrastructure (<https://www.metacenter.no/>).



The strategy and planning for the initial and continuous testing of the dashboard were compiled in an Initial Test Plan (M5.3.). The main goal of the testing is to ensure that the needs of the future users (from a wide range of professional specializations, such as microbiologists, epidemiologists, technicians) are met. Briefly, the testing will be done on two main levels: functional testing and user-friendliness testing. The functional testing will be performed by members of the WP5 at the RIVM. The features to be tested will include evaluating that each function in the dashboard works as expected, that there is enough and clear documentation and that the data sharing complies with the necessary (GDPR) regulations. The user-friendliness testing will be performed by a group of “users” that will give their feedback (by answering a survey) about their experience with the dashboard. We have put in place a reporting system on our project documentation system for gathering feedback from users, which will then be translated in a prioritized list of features and improvements to be implemented within each release cycle.

JRP24-WP5-T4: Sustainability (M25-M54)

Status: ongoing.

As described in the project proposal, sustainability is at the forefront of this project’s priorities. The issues of sustainability are identified and documented in close collaboration with each WP leader. The most pertinent sustainability material will be compiled in a yearly sustainability document, alongside with recommendations for future work. Due to a delay in WP4 that stalled the development of the dashboard, no testing has been performed so far. The testing and the associated sustainability document will be performed/completed as soon as there is a workable and testable dashboard.

WP6: Management (M25-M54)

JRP24-WP6-T1: Management (M25-M54)

Status: Ongoing

The coordination team has been set up. Project management tool Jira has been set up for task management. Further details are covered below in *BeONE-WP6-T2*.

JRP24-WP6-T2: Communication (M25-M54)

Status: ongoing

This task comprises Internal and external communication

Internal communication:

- Monthly meetings have been held by the coordination team except in April.
- An active slack group has been made for rapid informal communication.
- A digital oceans server has been set up to host various web content.
- A confluence site has been set up on the digital oceans server as a more structured repository for documentation and decisions.
- Frequent web meetings have been held in the WP3+4 group.
- The project has applied for and been granted HPC resources at the norwegian national infrastructure for computational science (Metacenter) to set up the computational back end for a test implementation of the BeONE system.

External communication. Communication has been established with EFSA contact Mirko Rossi, especially with the following focus:

- aligning and avoiding duplication of work on the EFSA cgMLST pipelines
- aligning metadata terms and ontology use



Contact has been established with IRIDA contact Aaron Petkau, this was an informal talk focusing on:

- Broad decision process on development of Irida and how they compare to Bifrost
- Possibility of sharing data between the systems
- Differences between the systems that came from both technology used and requirements of stakeholders

Communication with COHESIVE on data structure

Initiation of an ontology expert group, including Emma Griffiths (BCCDC), Damian Dooley (BCCDC), Fernanda Dorea (SVA, ORION), Mirko Rossi (EFSA)

Communication with Mario Ramirez (INNUENDO) on their software on microbial surveillance.

JRP24-WP6-T2-ST1: Kickoff meeting

Status: Completed

Kick-off meeting was held at RIVM.

JRP24-WP6-T3: Data Management (M25-M54)

Status: Ongoing

Data management plan has been created using the new DMP tool.



3. Progress of the research project: milestones and deliverables

Deliverables

JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
24	D-BeONE.1.1	Report on the state-of-art	M30		M39	Yes	Public. Scope changed to avoid duplication of work with ORION, and mitigate delays due to COVID-19. Delivery date changed to wait for ORION delivery fall 2020.	10
24	D-BeONE.6.1	Initial data management plan	M30	M33			Public	8
24	D-BeONE.1.2	Finalized BeONE dataset	M32	M33	M39	Yes	Public	3
24	D-BeONE.2.1	Draft manuscript on the conceptual model	M34	M37			Public – the summary only. In relation to novelty of research and self-plagiarism issues in the process of publication, the full version will be available only upon submission to a peer-reviewed scientific journal (estimated to be achieved by M39). https://zenodo.org/record/4476394	8



JRP /JIP code	Project deliverable number (Original number, if different from the actual one)	Deliverable name (Original name, if different from the actual one)	Delivery date from AWP 2020	Date delivered on Project Group website	If deliverable not submitted: Forecast delivery date	Is this delay due to COVID-19?	Comments (Please mention: public or confidential, the Zenodo reference and other comments)	Proposed category* (1 to 8) (several categories may be applicable)
24	D-BeONE.4.1	Back-end analysis pipeline	M36		M43	Yes		4?
24	D-BeONE.4.2	Web input system	M36		M44	Yes		4?

* Categories of Integrative activities : 1. Design and implementation of surveillance and control activities; 2. Harmonised protocols and applied best practice; 3. Databases of reference materials and data, incl. metadata; 4. Standardised data formats, aligned data analysis for interpretation of surveillance data; 5. Sharing and communication of surveillance data; 6. Sharing of best intervention activities ; 7. Prevention: aligned use of facilities and models; 8. Other (please specify);

Milestones

JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
24	M-BeONE.4.1	Initial requirement list from workshop at kick-off meeting	M26	Yes		
24	M-BeONE.3.1	Agreement on minimal and optimal set of metadata	M28	Yes		
24	M-BeONE.4.2	Implementation plan	M28	Yes		



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
24	M-BeONE.5.3	Initial test plan	M28	Yes	M31	Delayed but achieved by M31
24	M-BeONE.1.1	State-of-the-art completed	M30	No	M39	Delayed due to COVID-19 crisis and constraints in hiring personnel
24	M-BeONE.1.2	Collected dataset	M30	No	M39	Delayed due to COVID-19 crisis and constraints in hiring personnel
24	M-BeONE.1.4	Agreement on the methods/solutions to evaluate	M30	Yes		
24	M-BeONE.1.3	Curated WGS dataset completed	M32	No	M39	Delayed due to COVID-19 crisis and constraints in hiring personnel
24	M-BeONE.2.1	The critical factors for outbreak detection have been identified	M32	Yes	M34	
24	M-BeONE.4.3	Prototype dashboard	M32	No	M40	Being redone due to personnel changes
24	M-BeONE.3.2	Finalized evaluation of data sharing experiences	M34	Yes		
24	M-BeONE.3.3	Implementation of data structure	M34	No	M48	Final structure should kept flexible as long as possible, basic structure exists
24	M-BeONE.4.4	Plan for data sharing system	M34	No	M38	
24	M-BeONE.3.4	Finished implementation of controlled data entry	M36	No	M38	Final remarks for data upload were missing
24	M-BeONE.3.5	Plan for ontology implementation	M36	No	M40	Two concept exist, decision pending



JRP/JIPCode	Milestone number	Milestone name	Delivery date from AWP 2020	Achieved (Yes/No)	If not achieved: Forecast achievement date	Comments
24	M-BeONE.3.6	Implementation of API for import of data	M36	No	M39	Delayed due to covid related personell priorities
24	M-BeONE.4.5	Basic system for display component integration	M36	No	M40	
24	M-BeONE.5.2	Yearly sustainability document	M36	No	M40	Will be available once the dashboard and database will be available

4. Publications and patents

Publication title and DOI reference	Is OHEJP acknowledged?	Is it a Green Open Access? If yes please provide the embargo length and the manuscript release date	Is it a Gold Open Access? If yes please provide the processing fees (in €)
Prediction of antimicrobial resistance in clinical <i>Campylobacter jejuni</i> isolates from whole-genome sequencing data 10.1007/s10096-020-04043-y https://zenodo.org/record/4249355#.X6UnVWhKjcc	YES		Gold Open Access; 3,825.00 EUR



5. On-going and planned collaborations with national or European projects or networks

Collaboration on-going with OHEJP projects ORION and COHESIVE, and to a smaller extent with CARE, MATRIX and OH-HARMONY-CAP.

Collaboration on-going with Danish FVST, on cross sector sequence analysis platform for investigation of foodborne outbreaks.

Collaboration with EFSA as described in BeONE-WP6-T2.

Collaboration with ontology expert group, including Emma Griffiths (BCCDC), Damian Dooley (BCCDC), Fernanda Dorea (SVA, ORION), Mirko Rosso (EFSA)