D6.18: Thesis Reports of up to 17 PhD studentships

Work Package 6

Responsible Partner: University of Surrey, UK (P23)

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Thesis Reports of up to 17 PhD studentships

Introduction

Sixteen PhD projects have been co-funded (44% by the EU; 56% by home institute) through the OHEJP Work Package 6 (Education and Training). The research focus of the individual PhD projects falls within at least one of the three domains of the OHEJP: foodborne zoonosis, emerging threats, and antimicrobial resistance, and all align with the Strategic Research Agenda. Each PhD project has at least two OHEJP consortium partners collaborating on the project, our consortium partners can be found here.

Additionally, one PhD project (SUSTAIN) was co-funded through Work Package 7 (Sustainability). This PhD project lies in the field of social sciences and public health. The progress of this PhD was monitored and assessed differently, and further details can be found in the report.

The PhD projects provided opportunities for the PhD students to explore and share skills, expertise, and knowledge from within the OHEJP consortium and key stakeholders (e.g., European Centre for Disease Prevention and Control, European Environment Agency, European Food Safety Authority), therefore accelerating both the rate and quality of research in addition to developing the One Health scientific leaders of the future.

There is significant scope for inter-disciplinary networking amongst OHEJP partners in addition to the interaction with the Joint Research Projects (JRPs) and Joint Integrative Projects (JIPs). The JRPs and JIPs have expertise that can support the PhD students and provide opportunities to explore and share skills and knowledge, accelerating both the rate and quality of the research. These interactions help to bring the physical, biological, and social sciences together and allow greater flexibility in the PhD projects ensuring innovative hypothesis-driven research. Multi-country and inter-disciplinary approaches help inform decisions on market viability and EU policy relevance of project outputs.

The PhD projects provided excellent added value to the OHEJP, including improved integration (both geographical and interdisciplinary), and an opportunity to develop the next generation of One Health scientific leaders, contributing to the sustainability and longevity of the One Health approach.

Each year, the PhD projects reported on their research activities, progress, scientific results, and outcomes, risks, ethics, and impact for the 12-month reporting period. However, in this public deliverable, each PhD project has provided a detailed overview of the entire PhD project, including key scientific results, a PhD project self-evaluation, progress of the project (measured by milestones and deliverables), interactions with JRPs, JIPs and OHEJP stakeholders, transferrable skills and training learnt, One Health Impact of the project and the added value and benefits during the PhD resulting from being part of the OHEJP doctoral programme and consortium. The purpose of this deliverable was to monitor, report, and disseminate the progress and results that can be shared publicly.

In 2023, five PhD projects have been completed, these are LIN-RES (M48), ECO-HEN (M58), PEMbo (M58), Codes4strains (M59), and ToxoSauQMRA (M62). A further 12 PhD projects are expected to be completed at various stages throughout 2023 – 2024: there are HME-AMR (M74), KENTUCKY (M72), METAPRO (M69), MACE (M72), DESIRE (M72), UDOFRIC (M69), WILBR (M72), EnvDis (M69), AptaTrich (M69), VIMOGUT (M72), TRACE (M69), and SUSTAIN (M66; please refer to timeline on page 8).

The PhD projects delivered many outputs that are now publicly available according to the Open Access policy of the Horizon 2020 framework programme. Collectively, the PhD projects have produced more than 34 publications that are available through Zenodo, which was chosen as the official repository of the One Health EJP. Valuable outputs were achieved; to mention a few, ECO-HEN identified different sets of Russian dolls for AMR gene dissemination in commercial layer farms that scarcely use antibiotics, which suggest that reduced antimicrobial use still contributes to the accumulation of resistance; LIN-RES PhD project identified that linezolid resistant determinants were present in a wide diversity of Gram positive isolates circulating in the agricultural sector (i.e., pigs and veal calves), which may pose a health risk to humans, despite not being licenced for use in the agricultural sector; Codes4strains PhD project created a strain nomenclature from assembled genomes. The public availability of the tool (https://gitlab.pasteur.fr/BEBP/diphtoscan) and its ease of use may advance the genomic epidemiology of the diphtheria agent, the clinical management of patients, and knowledge of the links between animal and human diphtheria cases; PEMbo identified a persistent genotypes of *M. bovis*, that is circulating and persists in France, contain multiple copies of IS6110 (> 10); and SUSTAIN provided insight into the challenges posed by the implementation of a cross-sectoral One Health approach, which identified opportunities that can be used to facilitate One Health institutionalisation, creating networks, and bridging disciplinary silos.

All details can be found in the current document section on the One Health EJP website.

In the final months of the OHEJP, the most impactful and promising of these outputs will be further disseminated across targeted stakeholders, i.e., relevant national and international authorities, research and clinical laboratories, risk assessors, and risk managers (i.e., ECDC, EEA, EFSA). In addition to the publications and deliverables, the OHEJP has created a unique network across public health, animal health and food institutes throughout Europe. The added value of this is described in the *'One Health Impact'* sections in the final thesis reports.

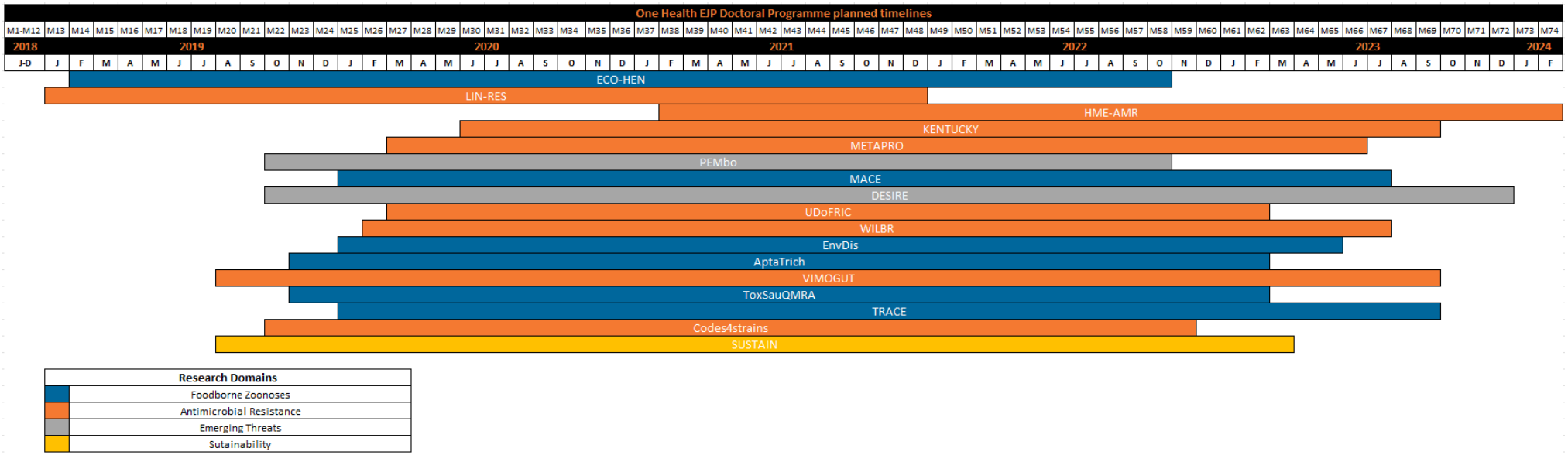
For each PhD project, the PhD student, in conjunction with their supervision team, produced a Final PhD Thesis Report, which was evaluated following the 'Final Thesis Report Evaluation Criteria' (These criteria are available as an annex at the end of the report). It is worth mentioning that the following PhD projects did not undergo an evaluation as the PhD students completed their studies prior to the commencement of the evaluation period and have since been awarded their doctorates; these projects were: ECO-HEN, LIN-RES, PEMbo, ToxSauQMRA, and Codes4strains. However, each PhD project provided a PhD Final Thesis Report, which is included in this deliverable. Therefore, each PhD Final Thesis Report which was subject to evaluation, was evaluated by three independent evaluators as follows:

- An external scientific expert who has already reviewed and evaluated a PhD project in full before.
- An additional external scientific expert.
- A member of the Project Management Team of the One Health EJP (PMT member)

The external scientific evaluators provided comments for each of the evaluation criteria, with positive and constructive suggestions. As requested in the guidelines, the evaluators have assessed the alignment of the PhD projects with the initial objectives as described in the full proposal, the scientific and innovative approach of the PhD projects, whether the PhD project delivered its expected outcomes, whether the PhD students interacted and collaborated with OHEJP JRPs and JIPs, the impact of the training and education resources offered by the OHEJP (i.e., Summer Schools/Continual Professional Development Modules), dissemination of research findings (i.e., publications, oral and/or poster presentations) and the impact of the research on policy at a national and international level. Most of the comments were valuable, and the scores provide an idea of the efforts carried out by the PhD students to produce impactful research.

Although all evaluators received detailed instructions with an extensive description of the criteria and guidance for scoring, the final scores should be interpreted with caution and should not be used to compare PhD projects and their general performance. Furthermore, not all external reviewers provided comments to support the scores provided and this has been highlighted. In general, the PhD Final Thesis Reports were given above average scores.

The anonymised evaluation reports were shared with the PhD students and their respective supervision teams to support their future work and for reasonable adjustments to the PhD Final Thesis Report to be performed (i.e., providing additionally clarity on questions raised during the review process). The PhD students then re-submitted their report for an evaluation by a member of the OHEJP PMT. The PMT members focused on ensuring that the PhD student and supervision team have sufficiently addressed the comments/feedback from the external scientific reviewers before the deliverable could be finalised; and that the comments/feedback from the external scientifical evaluators were fair and reflective of the work produced.



The planned timeline for the 17 PhD projects co-funded by the One Health EJP. The timeline begins at M1-12 and ends at M74. The completion of the consortium is expected at M69. The colour of the projects corresponds to a domain of the One Health EJP outlined in the bottom left table.

Projects final reports and evaluations

In this document, every PhD Final Thesis Report is followed by its evaluation, except for the completed PhDs mentioned above. The names of the evaluators were removed from the report to guarantee the confidentiality of the process.

PhD1-AMR2-ECO-HEN

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Irene Aldea Ramos	PhD Student	Complutense University of Madrid	Spain
Miguel Ángel Moreno Romo	PhD Lead Supervisor	Complutense University of Madrid	Spain
Pimlapas Leekitcharoenphon	PhD Secondary Supervisor	Technical University of Denmark	Denmark

Summary of the work carried out in the PhD project

The ECO-HEN PhD project began in M14, in 2019 and finished in M58, in 2022. A nine-month extension was provided due to the SARS-CoV-2 pandemic. ECO-HEN focused on the dynamics of antimicrobial resistance (AMR) in *Escherichia coli* (*E. coli*) from a commercial layer farm where antibiotics were sparsely used. The farm environment explored was studied over 29 months, encompassing five animal batches (B1 – B5). Batches B1 – B4 were checked eight times from day-old chicks to laying hens (~ 82 – 85 weeks), while B5 was checked only four times from pullets (~ 14 weeks) to laying hens (~ 52 weeks). Three different MacConkey agar plates were used for recovery of *E. coli* isolates from transport-boxes, these were with cefotaxime, with ciprofloxacin and without antibiotics. Isolates were collected from first sampling B1 – B4 and litter.

The project collected 687 *E. coli* isolates, from which 272 were characterised by whole genome sequencing (WGS). To note, 10 isolates were collected per culture media and sampling, therefore, a preliminary phylogenetic analysis served to detect 53 putative duplicated isolates, which were omitted from further study. Consequently, only 218 isolates were included in the final WGS analysis. < 32 AMR determinants were detected, and 115 isolates harboured at least one of these identified determinants. At isolate level, both integrons and multi-resistance (MR) regions were identified, proving that AMR determinants were frequently linked.

Further analysis detected at least one plasmid replicon (PR) in 196 isolates. A total of 11 different PRs were identified, only eight harboured AMR determinants in at least one isolate, which were *IncF*, *IncI*, *IncX*, *IncK*/B/O/Z, IncA/C2, *IncQ*, *IncH* and *IncN*. Finally, 30 clones, comprising between two and eight isolates were detected. The research identified integrons, MR regions, plasmids, and clones in the context of AMR, therefore, the focus of the PhD

moved to identify different Russian Doll sets for AMR determinants. The spread of these Russian Doll sets in this commercial farm were identified, with plasmids being the most common clone set involving both single and linked (intergon and MR regions) AMR determinants and single patterns, when only a plasmid harbouring an AMR determinant or clones (AMR determinants into the chromosome) were involved. Finally, events related to gain or loss of AMR determinants by plasmids or the gain or loss of AMR-containing plasmids in detected isolates were recorded.

Dissemination activities of this ECO-HEN PhD project were enshrined in a peer-reviewed publication in the Journal of Veterinary Microbiology, four oral and poster presentations at the national (i.e., XXVII Microbiology Spanish Society National Congress of Microbiology) and international level (i.e., One Health EJP ASM 2022).

Work carried out on the PhD, scientific results, and outcomes

FIRST: *E. coli* MLST profile data allow for the better estimation of bacterial diversity compared to phenotypic antibiotic resistance profile data, as the same resistance profile is found in different MLST profiles.

SECOND: The high values of the diversity index used (Simpson's index) indicate that the number of *E. coli* isolates analysed per sample (between eight and ten) is sufficient to estimate bacterial diversity in the type of samples analysed.

THIRD: The detection of AMR genes in *E. coli* in day-old chicks indicates that hatcheries are sources of entry of antibiotic resistance genes into egg production farms, but not the only source, as the finding of other resistance genes in subsequent sampling indicates that other sources exist.

FOURTH: The detection of AMR *E. coli* isolates in eggshells marks a pathway for their entry into the food chain.

FIFTH: The detection and genetic characterisation of a wide range of resistance profiles in *E. coli* throughout the commercial egg production cycle points to the desirability of adding this indicator bacterial-animal species binomial to surveillance programmes for AMR in animals.

SIXTH: Transmission of AMR genes in *E. coli* in commercial production farms is mediated primarily by plasmids and, to a lesser extent, by clones.

SEVENTH: Although isolates resistant to cephalosporins, antibiotics declared to be of clinical importance, do not seem to be predominant in laying hens in this type of farms, their presence and dissemination in clones and in mobile genetic elements makes it necessary to monitor them.

The PhD thesis is currently under embargo and more information on the PhD project can be found here.

The objectives that were set at the beginning of the project have mostly been achieved. Deliverables D-E14-5.1 (List of plasmids putatively present on *E. coli* isolated) and D-E14-6.1 (List of *E. coli* from animals isolates to be sequenced) were delayed because of the SARS-CoV-2 pandemic. Deliverable 6.1, antimicrobial susceptibility studies were performed to obtain phenotypic profiles of interest for sequencing. The impossibility of accessing the laboratory during the prolong lockdown due to the SARS-CoV-2 pandemic delayed the completion of this deliverable. For the same reason, deliverable D-E14-6.2 was delayed from September 2020 (M33) to July 2021 (M43). This published manuscript, contained data on cefotaxime-resistant isolates, for which we had to perform antibiotic susceptibility testing and subsequent sequencing. The postponement in laboratory work and, therefore, in obtaining the list of isolates to be sequenced, meant that the manuscript had to be delayed. In addition, the WGS of isolates was carried out in another institute that was also affected by the SARS-CoV-2 pandemic. Deliverable D-E14-7.1 (A list of *E. coli* from eggs to be sequenced) was produced one month ahead of schedule. Deliverable D-E14-8.1 (Manuscript) was delayed from June 2021 (M42) to February 2022 (M50), yet it has not yet been delivered. And thesis draft (D-E14-9.1) was scheduled for December 2021 (M48) but is being delivered in October 2022 (M58). The reason that these two deliverables have been delayed so much is because of the backlog of previous deliverables. In addition, due to the end of the grant, the student started working in another research centre, so she did not have enough time to finish the draft of the thesis in the scheduled month. Derivable 8.1 will be supplied after the completion of the draft thesis document.

As far as milestones are concerned, all of them have been achieved. Milestone 1 (Updated list of phenotypic features of the *E. coli* isolates collection) was achieved on time. Milestone 2 (Training of the PhD-student on bioinformatic analysis of raw sequences) was achieved in September 2019 (M21), when the student carried out the short-term mission in Denmark. Milestone 3 (New sequenced *E. coli* isolates from animals) was delayed from September 2020 (M33) to April 2021 (M40). As indicated above, the deliverable associated with this milestone was delayed due to the impossibility of accessing the laboratory. In addition, the external institute carrying out the sequencing was affected by the pandemic and accumulated a heavy workload. The last milestone (4) was also delayed: although the list of isolates to be sequenced was delivered ahead of schedule, sequencing was postponed due to the reason discussed above.

Overall, and albeit with some delay, all deliverables, and milestones (except deliverable 8.1) have been successfully achieved.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
PhD1-AMR2-ECO-HEN	D-E14-5.1.	A list of plasmids putatively present on *E. coli* isolates	M27 (March 2020)	M31 (May 2020)		
	D-E14-6.1	A list of *E. coli* from animals isolates to be sequenced	M28 (April 2020)	M33 (July 2020)		
	D-E14-6.2.	Manuscript	M33 (September 2020)	M43 (July 2021)		
	D-E14-7.1.	A list of *E. coli* from eggs to be sequenced	M37 (January 2021)	M36 (December 2020)		
	D-E14-8.1.	Manuscript	42 (June 2021)	-		
	D-E14-9.1.	Doctoral thesis draft	48 (December 2021)	M58 (October 2022)	Be aware that this is the first draft of the PhD thesis and that this document must be fully revised, corrected and improved before be public available.	

* 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD1-AMR2-ECO-HEN	M-E14-1	Updated list of phenotypic features of the *E. coli* isolates collection	M15 (March 2019)		Yes	
	M-E14-2	Training of the PhD-student on bioinformatic analysis of raw sequences	M17 (May 2019)		Yes	10.5281/zenodo.5708230
	M-E14-3	New sequenced *E. coli* isolates from animals	M33 (September 2020)		Yes	
	M-E14-4	New sequenced *E. coli* isolates from eggs	M37 (January 2021)		Yes	

Publications and additional outputs

Publications

The ECO-HEN PhD project has produced one peer reviewed publication entitled 'Clonal and plasmid-mediated flow of ESBL/AmpC genes in *Escherichia coli* in a commercial laying hen farm' published in July 2022 in the journal of Veterinary Microbiology (DOI - https://doi.org/10.1016/j.vetmic.2022.109453).

The publication has been uploaded to Zendo, which is gold standard open access, and can be found, here.

Additional outputs (i.e., poster/oral presentations)

The ECO-HEN PhD project further disseminated these works through oral and poster presentations at the following events:

- 'Putative APEC isolates from healthy animals and eggs in a laying-hen commercial farm' Poster presentation at One Health EJP ASM 2022, Orvieto, Italy. 11-13th April 2022. Abstract available here.
- 'blaCMY-2 gene dynamics in a commercial egg production farm' Oral presentation at XXVII Microbiology Spanish Society National Congress of Microbiology. 28th June to 2nd July 2021.
- 'Dynamics of extended spectrum β-lactamase resistance gene blaSHV−12 in a laying hen commercial farm' Poster presentation at One Health EJP ASM 2021, hybrid event. 9-11th June 2021. Abstract available here.
- 'First report of trimethoprim resistance gene dfrA36 on an IncF-plasmid in *Escherichia coli* isolated from day-old chicks' Poster presentation at One Health EJP ASM 2020, online. 27-29th May 2020. Abstract available here.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Whole bacterial genome sequencing. Tools and applications	Bioinformatics	February 2019	DTU
EURL-Training course on antimicrobial resistance	Antimicrobial resistance	24/09/2019-27/09/2019	EURL-AR Network
Specialisation diploma of in bioinformatics analysis	Bioinformatics	07/10/2019-22/06/2020	Universidad Pablo Olavide de Sevilla (UPO)
Spreadsheets with EXCEL I	Informatics	01/01/2020-01/05/2020	Universidad Complutense de Madrid (UCM)
Epidemiological and statistical aspects of a research	Epidemiology	03/03/2020-09/06/2020	UCM
Biosecurity seminar	Biosecutiry	15/06/2021	UCM
Digital innovations for One Health Practitioners	Digital innovations	15/02/2021-18/02/2021	OHEJP, BfR
Research career conference	Research	14/01/2021, 18/01/2021	UCM
One Health EJP Communications and Media Workshop	Communications	05/10/2020-06/10/2020	OHEJP
ASM Satellite Workshop	Digital Innovation and Data Management	21/05/2019	OHEJP
Python data visualization	Informatic	14/09/2020-15/12/2020	UCM
Summer school	Environmental Issues in One Health: from risk assessment to surveillance	26/07/2021-06/08/2021	OHEJP, Instituto Superiore di Sanitá
Advanced workshop on bioinformatics and omics data analysis in R	Bioinformatics	24/04/2022-29/04/2022	UCM
Spreadsheets with EXCEL II	Informatics	11/06/2021-31/10/2021	UCM
Visavet journal Club	Scientific articles discussion		Visavet, UCM

One Health impact

The study of antibiotic resistance on a layer farm and its impact on the One Health perspective for national and international policy makers is of the utmost importance because of the implications for public health. The findings of this study strongly reveal that resistant bacteria persist on the farm even in the absence of antibiotics and have the ability to be transmitted through eggs. This transmission of resistant bacteria through poultry products can lead to serious health problems both locally and globally.

In this context, the One Health approach becomes central to addressing the problem of antibiotic resistance in animal husbandry, including laying hens. The One Health approach recognises the interconnection between human, animal and environmental health, and

highlights the need to address these problems in a holistic and collaborative manner. National and international policy makers play a crucial role in implementing effective policies and regulations to address antibiotic resistance in poultry production.

It is essential that policy makers promote measures that restrict the unnecessary use of antibiotics in animal husbandry and encourage responsible management practices. Robust surveillance and monitoring systems must be put in place to detect and control the presence of resistant bacteria on poultry farms as well as in poultry products for human consumption. In addition, research, and development of alternatives to antibiotics in animal husbandry, such as improved hygienic conditions and the use of probiotics, should be promoted.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

Doing her PhD thesis within the OHEJP project has allowed the student to get to know the work of other people working in the same and related research fields. It also allowed her to do a short-term mission in which she was able to acquire knowledge to use in her own work. She has also been able to share her project with other colleagues in the 3-minute thesis competitions, and to present the project at the conferences organised by the OHEJP that she was able to attend. In addition, she has attended several workshops and seminars given by members of the OHEJP project.

Interactions with JRPs/JIPs or with external (EU or national) relevant projects or initiatives such as national action plans

No interactions with JRPS/JIPs, OHEJP stakeholders or external collaborations were noted.

Interactions with OHEJP stakeholders, national and international surveillance programmes

No interactions with OHEJP stakeholders or external collaborations were noted.

Evaluation of the Final Thesis Report

Not applicable as the PhD student submitted their thesis manuscript.

PhD2-AMR2/3/6-PhD LIN-RES

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Timmermans Michaël	PhD Student	Université Libre de Bruxelles (ULB)	Belgium
Boland Cécile	PhD Lead Supervisor	Sciensano	Belgium
Denis Olivier	PhD Secondary Supervisor	Sciensano and Université Libre de Bruxelles	Belgium

Summary of the work carried out in the PhD project

The LIN-RES PhD project began in M13, in 2019 and finished in M48, in 2021. LIN-RES focused on the investigation of the molecular basis, origin, transferability, and risk factors associated with antimicrobial resistance (AMR) in bacteria of human and animal origin. The One Health vision agrees that humans, animals, and the environment are intimately linked in terms of health. This particularly involves AMR, which today represents one of the greatest threats to public health according to the World Health Organisation (WHO) and the World Organisation for Animal Health (OIE). One of the pillars of the fight against AMR is AMR monitoring organised regularly and at different scales by and in different countries. The aim of this thesis was to develop molecular tests and to show their added value for AMR monitoring.

To do this, a tool for detecting antibiotic resistance determinants was developed, the AMR-ARRAY. This molecular biology tool, which targets a large number of resistance genes and point mutations, is competitive in terms of time and cost compared to various existing techniques. This technology is quickly and easily adaptable and easy to use. Finally, it offers very good performance with selectivity and specificity of 99.3% and 100%. In parallel, a retrospective study was carried out on colistin-resistant *Escherichia coli* strains isolated from food-producing animals (PDA) in Belgium between 2012 and 2016, integrating phenotypic and genotypic analyses. This study showed that colistin resistance was related to the presence of one or more *mcr* genes and revealed the first observation, to our knowledge, of an isolate carrying three different *mcr* genes.

Finally, linezolid resistance surveillance (LIN-RES) was carried out in 2019. This revealed a large reservoir of enterococci carrying linezolid resistance genes in food producing animals as well as a large genetic variability. These various studies have revealed a reservoir of AMR genes of critical importance in human medicine. The development of the AMR-ARRAY and the LIN-RES project are part of a One Health perspective, integrating bacteria isolated from different origins: animal, food intended for humans or human samples.

The contribution of monitoring dedicated to certain antibiotics and of a genetic component in the monitoring of AMR from a One Health perspective would be significant assets for better use of antibiotics and in the fight against AMR.

LIN-RES research work highlights the importance of monitoring antibiotic resistance in farm animals, particularly for critical antibiotics in human medicine from a One Health perspective. Molecular diagnostic tools make it possible, at a lower cost, to determine important resistance mechanisms whose prevalence could be monitored as already established in human surveillance.

Dissemination activities of this LIN-RES PhD project were enshrined in a peer-reviewed publication in the Journal of Antimicrobial Chemotherapy, and three oral and poster presentations at the international level (i.e., One Health EJP ASM 2019, Ireland).

Work carried out on the PhD, scientific results and outcomes

AMR-ARRAY development

The AMR-ARRAY is composed of a set of 53 Padlock shaped Probes (PLPs) all present in a single mix. It is able to detect resistance determinants to the following antibiotic families: β-lactams, (fluoro)quinolones, colistin, macrolides and aminoglycosides. *bla*TEM and *bla*SHV ESBLs are discriminated from non-ESBL *bla* genes by targeting mutations involved in amino acids modification at Ambler positions 104 and 238 for *bla*TEM and 238 and 240 for *bla*SHV. *bla*CTX-M genes from groups 1, 2, 9 and 8/25 are differentially identified as well as the *bla*OXA-1, *bla*OXA-2 and *bla*OXA-10 like genes. Specific mutations were targeted in the promotor region of the chromosomic *amp*C gene at position -42 and -18. These mutations are responsible for the overexpression of the *amp*C cephalosporinase (Caroff *et al*., 2000; Peter-Getzlaff *et al*., 2011; Guérin *et al*., 2021; Jacoby, 2009; Tracz *et al*., 2007). Additional probes were designed to identify the *amp*C phenotype-causing variants *bla*CMY-1, *bla*CMY-2 and *bla*ACC. The AMR-ARRAY probe set is adaptable, and probes can be removed or added depending on the purpose of the analysis. The all-inclusive reagent cost per sample is currently €18 for 94 samples for the full set of 53 PLPs.

Comparison of susceptibility profiles versus AMR-ARRAY results

Two hundred fifty-one food-producing animal *E. coli* isolates expressing AMR phenotypically documented in preliminary studies were analysed with the AMR-ARRAY. ESBL and *amp*C phenotypes but also resistance to quinolones, colistin, gentamicin and azithromycin were independently compared with AMR-ARRAY results. The aim of this comparison was to assess whether the AMR-ARRAY detected genetic resistance determinants in most of the isolates expressing a resistance profile for the corresponding antibiotics. *amp*C phenotype comparison with AMR-ARRAY results showed the weakest concordance of 58.5% (24/41) while concordance with azithromycin resistance was higher (88.2%; 90/102). All other concordances were above 95% with 96.2% (178/185) of concordance for quinolones resistance and 98.2% (111/113) for gentamicin resistance. Finally, the highest scores were observed for colistin resistance (n = 41) and for the ESBL phenotype (n = 171) with 100% concordance for both.

One hundred twenty-four food indicator *E. coli* isolates with antibiotic resistance demonstrated phenotypically were also analysed with the AMR-ARRAY. The isolates were analysed for their ESBL phenotype, quinolones resistance and/or colistin resistance. Resistance to quinolones showed a concordance of 93.3% (111/119) and a concordance of 98.3% (117/119) was

observed for the ESBL profile while 100% of concordance was obtained for colistin resistance (5/5).

Finally, 9 isolates resistant to meropenem provided by the European Union Reference Laboratory (EURL) were also analysed with the AMR-ARRAY. For each isolate, the AMR-ARRAY detected a carbapenemase-encoding gene.

Considered globally, AMR-ARRAY results were 94.7% concordant (856/904) with susceptibility profiles irrespective of the origin of the isolates.

Comparison of genetic resistance profiles versus AMR-ARRAY results

One hundred twelve food *E. coli* isolates, 64 clinical *Salmonella* isolates and 97 clinical *Shigella* isolates were analysed with the AMR-ARRAY to assess genetic results obtained through PCR and Sanger sequencing or other validated Luminex® xTAG® assays. The AMR-ARRAY correctly detected all genes/SNPs responsible for ESBL or BL phenotypes and all, but one SNPs involved in resistance to quinolones. For all isolates taken together, 351/352 (99.7%) expected determinants were correctly identified by the AMR-ARRAY. The discrepant quinolone resistance result was probably due to a mutation causing an amino acid modification in the *par*C gene at codon position 80 (Ser to Ile) as described in the 'comparison of WGS data versus AMR-ARRAY results' section. This results in a variant *par*C allele unable to anneal with the AMR-ARRAY probes PARC-80-WT and PARC-80-ILE. As a result, for this discrepant isolate, the two *par*C PLPs tested negatively.

Comparison of WGS data versus AMR-ARRAY results

A subset (*n* = 139) of the 251 animal *E. coli* isolate collection was sequenced through WGS (identified by prefix "VAR"). Selection was made to cover a various panel of susceptibility profiles and included isolates displaying discrepant profiles (experimentally demonstrated resistance not detected with the AMR-ARRAY and vice versa). Resfinder and Pointfinder algorithms (Bortolaia *et al*., 2020; Zankari *et al*., 2017) were used to map antimicrobial resistance genes/ mutations in WGS sequence data and to infer predictive resistance profiles. When assessed with the AMR-ARRAY, 697/702 determinants (SNPs and genes) expected from WGS analysis were detected by the AMR-ARRAY (Table 1). Failure to detect the expected determinants was limited to 5 quinolone-resistant isolates harbouring a *par*C variant characterised by an identical point mutation in the *par*C gene sequence at codon position 80 (Ser to Ile), resulting in a variant *par*C allele unable to anneal with the AMR-ARRAY probes PARC-80-WT and PARC-80-ILE. The *par*C allelic variant in scope was neither detected by the WT nor by the mutant PLP probe. These AMR-ARRAY results do not allow to draw any conclusion with regards to fluoroquinolone resistance.

Table 1. Genes and mutations observed in 139 isolates detected by WGS and AMR-ARRAY.

Antibiotics	Resistance genes	Detected number by WGS	Detected number by AMR-ARRAY
Beta-lactams	*bla*$_{TEM-1}$	80	80
	bla$_{TEM-52}$	6	6
	bla$_{TEM-199}$	1	1
	bla$_{TEM-135}$	1	1
	bla$_{TEM}$*	7	7
	bla$_{SHV-12}$	10	10
	bla$_{SHV-2}$	1	1
	bla$_{CTX-M-1}$	19	19
	bla$_{CTX-M-2}$	6	6
	bla$_{CTX-M-3}$	2	2
	bla$_{CTX-M-14}$	11	11
	bla$_{CTX-M-15}$	12	12
	bla$_{CTX-M-27}$	1	1
	bla$_{CTX-M-32}$	5	5
	bla$_{CTX-M-55}$	4	4
	bla$_{OXA-1}$	8	8
	bla$_{OXA-10}$	2	2
	bla$_{CMY-2}$	3	3
FQs	*qnrB19*	7	7
	qnrS1	18	18
Colistin	*mcr-1*	29	29
	mcr-2	1	1
	mcr-3	1	1
	mcr-4	8	8
	mcr-5	1	1
Aminoglycosides	*aac(3)-II*	34	34
	aac(3)-IV	12	12
	aac(3)-VI	6	6
	aac(6')-Ib all	13	13
	ant(2'')-Ia	6	6
Macrolides	*ermB*	8	8
	Mph(A)	37	37
SNPs	*parC*** and *gyrA***	278	273[A]
	ampC n-18	49	49
	ampC n-42	15	15
Total		702	697
Percentage detected		99.3%	

*Abbreviations: FQs=fluoroquinolones *Unidentified allele through WGS. **targeted SNPs, both WT and mutated.*

[A] *Discrepancies are caused by an untargeted mutation next to the targeted one (see text for details).*

Finally, WGS analysis resolved discrepant results noticed when assessing AMR-ARRAY performance on isolates susceptible to azithromycin. mph(A), known to confer azithromycin resistance (Gomes *et al*., 2017), was found through both WGS and AMR-ARRAY analysis in such isolates considered susceptible according to current epidemiological cut-off (>16 mg/L^{-1}).

The PhD thesis and associated publications can be found here.

PhD self-assessment

In the beginning of the project, the aim was to collect resistant isolates from the Belgian antimicrobial resistance official monitoring. As the previous Belgian data showed few linezolid resistant bacteria through this non-selective monitoring, it was planned to compare WGS results between all isolates recovered from the different geographical origins.

At the end of the 2019 selective monitoring, 139 samples were positive for linezolid resistant bacteria, more than estimated. Giving these results, it was decided to not investigate concerned farms for linezolid resistant bacteria as it was planned because a lot of isolates were already analysed and because the number of concerned farms was too many. Moreover, the time and budget necessary to investigate all farms would have been too high.

All linezolid resistant isolates recovered from the Belgian selective monitoring (*n* = 147) and from Sciensano collection (*n* = 3) or given by collaborators of the project (*n* = 5) were sequenced by NGS to look for resistance determinants and determine the genetic context surrounding resistance genes. To do that, we developed a pipeline to analyse the NGS data. Bacterial species was determined both by MALDI-TOF and Kraken and it revealed no correlation between bacteria and carried linezolid resistance determinants, except for staphylococci in which only the *cfr* gene was found. The genetic context analysis revealed several genetic organisations surrounding linezolid resistance genes, especially for the *optrA* gene. Finally, phylogenomic analyses were carried out, showing no correlation between phylogeny and linezolid resistance determinants.

The NGS data showed that several incompatibility group markers were present among the isolates. Unfortunately, NGS data couldn't allow us to determine for all isolates which marker was associated with which gene. Additional work proceeded to the transferability experiments. Unfortunately, no transconjugant was obtained while several experiments have been conducted. Based on our findings and the observed limitations of the design of our experiments, we cannot conclude whether or not the linezolid resistance genes of the selected donor candidates could be transferred. However, the results of this deliverable are useful to improve the design of future conjugation experiments.

Finally, a risk factors analysis based on epidemiological and consumption data has been conducted. This study explored putative risk factors for the occurrence of linezolid resistant bacteria (commensal *Enterococci* and *Staphylococci*) in faeces from veal calves, broilers, laying hens and pigs and in nasal swabs samples from pigs collected in 2019 in Belgium. The results of this study are available and will be published after this final reporting. As a conclusion, all main project objectives have been met and the expected deliverables have been sent.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 2.1~~ D-PhD02-2.1	Genetic resistance profiles and subtypes of strains sequenced during M13-M24	M24	M35	*public:Zenodo reference: 10.5281/zenodo.6721422*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 2.2~~ D-PhD02-2.2	Poster presentation at an international conference of the first results obtained from NGS analysis of linezolid-resistant strains.	M24	M35	*public:Zenodo reference: 10.5281/zenodo.6723515*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 2.3~~ D-PhD02-2.3	Genetic resistance profiles and subtypes of strains sequenced until M36	M39	M39	*public:Zenodo reference: 10.5281/zenodo.6723790*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 2.4~~ D-PhD02-2.4	Results of in silico analysis of genetic scars of horizontal transfer or recombination events	M36 (delay to M48 agreed)	M46	*public:Zenodo reference: 10.5281/zenodo.6724194. A delay was requested and agreed until M48 to submit it with D3-3.*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 3.1~~ D-PhD02-3.1	Results of in silico analysis of transferability	M39	M39	*public :Zenodo reference :10.5281/zenodo.6724449*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 3.2~~ D-PhD02-3.2	Poster or oral presentation at an international conference of the results of the linezolid selective monitoring during 2019 in Belgium	M36	M35	*Public: Zenodo reference: 10.5281/zenodo.6724563. The topic of the poster was changed with the agreement of WP6 team. This poster has been presented in the ASM2020..*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 3.3~~ D-PhD02-3.3	Results of laboratory experiments to demonstrate transferability of linezolid resistance genes	M48	M48	*public : Zenodo reference: 10.5281/zenodo.6778115*	*If applicable**
PhD02-AMR2/3/6-PhD LIN-RES	~~D E27 4.1~~ D-PhD02-4.1	Summarize of the risk factors analysis (epidemiological	M48å delay to M54 agreed	M54	*CONFIDENTIAL, to ensure publication of original research results in the future paper.*	*If applicable**

		and consumption data)				
PhD02-AMR2/3/6-PhD LIN-RES	~~D-E27-5.1~~ D-PhD02-5.1	Final synthesis and reporting	M48à delay to M54 agreed	M54	*Public in Zenodo: it is actually this Word document.*	*If applicable**

Milestones

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD2-AMR2/3/6-PhD LIN-RES	M-E27-1	Synthesis of genetic resistance profiles and subtypes of strains sequenced during M13-M24	M24	M35	Yes	
	M-E27-2	Poster presentation at an international conference of the first results obtained from NGS analysis of linezolid-resistant strains.	M24	M24	Yes	
	M-E27-3	Synthesis of genetic resistance, subtyping and transferability markers analysis of the linezolid resistant bacteria collected.	M39	M39	Yes	
	M-E27-4	Poster or oral presentation at an international conference of the results of the linezolid selective monitoring during 2019 in Belgium	M36	M35	Yes	The topic of the poster was changed with the agreement of WP6 team. This poster has been presented in the ASM2020.
	M-E27-5	Final synthesis and reporting	M48→delay to M54 agreed	M54	Yes	

Publications and additional outputs

Publications

The LIN-RES PhD project has produced the following peer reviewed publication:

- Timmermans M, Wattiau P, Denis O, & Boland C. (2021). Colistin resistance genes mcr-1 to mcr-5, including a case of triple occurrence (mcr-1, -3 and -5), in Escherichia coli isolates from faeces of healthy pigs, cattle and poultry in Belgium, 2012-2016. International journal of antimicrobial agents. 57(6), 106350. DOI: https://doi.org/10.1016/j.ijantimicag.2021.106350
- Timmermans M, Bogaerts B, Vanneste K, De Keersmaecker S C J, Roosens N H C, Kowalewicz C, Simon G, Argudín M A, Deplano A, Hallin M, Wattiau P, Fretin D, Denis O and Boland C. (2021) Large diversity of linezolid-resistant isolates discovered in food-producing animals through linezolid selective monitoring in Belgium in 2019.

Journal of Antimicrobial Chemotherapy, dkab376. DOI: https://doi.org/10.1093/jac/dkab376

The publications have been uploaded to Zendo, which is gold standard open access, and can be found, here.

Additional outputs (i.e., posters/oral presentations)

In addition to the posters already listed in the deliverables, several oral presentations were given during the project at the ASMs: in 2019 a short oral presentation was given by Michaël Timmermans and in 2022 Cécile Boland presented the project through an oral presentation.

Moreover, the project was presented in 2020 and 2021 during the 3MT EJP competition. Another poster, in addition to the ones presented in the deliverables, was presented at the ASM 2021. It can be found in Zenodo, here.

Thus, the LIN-RES project was presented at each of the OHEJP ASM during the 2019 – 2022 period, either by a poster and/or an oral communication.

The PhD thesis itself was publicly defended with success on the 9th of March 2022.

Transferrable skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Vulgariser sa recherche à l'écrit	Scientific vulgarisation	14 Decembre 2018	ULB
Vulgariser sa recherche à l'oral	Scientific vulgarisation	14 Decembre 2018	ULB
Galaxy live training 2019	NGS data analysis	4 April 2019	Sciensano
Formation sur l'encadrement d'équipe	Management	24-25-26 June 2019	ULB
Midi Cross-Experience - Infographie & data-visualisation	Data visualisation	13 Decembre 2019	ULB

One Health Impact

The project highlighted the potential risk associated to linezolid resistance among animal bacteria. Indeed, the Belgian antimicrobial resistance official monitoring in food-producing animals showed a low level of linezolid resistant bacteria. The LIN-RES project, through a selective monitoring, revealed a much more important presence of linezolid resistance determinants carried by bacteria isolated from farm animals. Given that farms are environments propitious to contacts between animals and humans, this represents a potential risk of transfer of linezolid resistance genes from animals to humans. It is important to follow, not only resistance to linezolid but the resistance to the most important antimicrobials.

As Sciensano is the Antimicrobial Resistance National Reference Laboratory, authorities are already aware about our work and discussions with AMCRA, responsible for strategies about antimicrobial uses in the veterinary field, are already on the table. The creation of a technical

working group in Belgium on the question of the use of florfenicol in animals and the impact on the linezolid resistance is planned.

An interesting fact is that our results presented during Sciensano meetings challenged scientists in the human clinical field. They were surprised by the proportion of isolates resistant to linezolid found in the study because it is unusual to observe this kind of proportion. The apparition of linezolid resistant bacteria is an issue because: (i) linezolid is a last resort antibiotic for human health; (ii) the genes responsible for linezolid resistance give resistance to other antibiotics and cross-selection through the use of non-critical antibiotics (i.e., phenicols) will maintain these genes; and (iii) new antibiotics are rare.

Geographically, related isolates were spread out across the whole country and even across international borders. Based on a LIN-RES paper such studies could be conducted in other countries to assess the situation in other European countries. The EURL-AR will launch soon a questionnaire for the member states to assess the interest and feasibility of conducting such linezolid selective monitoring in their country. The genomes of the study are published and could be compared with future studies to broader assess the clonality/diversity of linezolid-resistant isolates.

The results and the related One Health impacts were already shared with Belgian Institutions (FASFC, FAMHP, AMCRA) and European institutions (EURL-AR/EFSA/EC) and with the One Health EJP stakeholders (i.e., during the SSB meeting of the 24th of March 2022). Such scientific research is important to be continued to ensure a good surveillance and understanding of the mechanisms and spread of resistance and to promote a prudent use of all antibiotics in a One Health perspective.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

The annual scientific meetings were each time good opportunities to meet other researchers including other PhDs. It was also good opportunities to share the results of the project with the One Health community. It gave a large visibility to the project. Both the ASMs and the annual meeting with Belgian stakeholders organized by Sciensano enabled to share the results with people using linezolid to treat patients at hospitals and raise awareness about the issue related to the emergence of linezolid resistance and about cross-selection of critical resistance through the use of non-critical antibiotics.

Specific outcomes to highlight

During the project, a pipeline was developed to analyse NGS data for *Enterococci* and *Staphylococci*. These tools allow for the automatic analysis of NGS data for a large number of samples simultaneously.

Based on the publication of the research paper, Norway has started in 2022 a linezolid selective monitoring.

The key results were shared with Belgian institutions (FASFC, FAMHP, AMCRA) & European institutions EURL-AR/EFSA/EC and presented at the 16th EURL-AR workshop in June 2022. Following this latest workshop, the EURL-AR will make a survey, with the help of Sciensano,

to assess the interest and feasibility of conducting such linezolid selective monitoring in the other member states.

The awareness of the human sector has also been raised through the oral communications at the ASMs and at the Belgian stakeholder annual meetings.

AMCRA starts discussions in Belgium about the classification of phenicol antibiotics (in particular the florfenicol) in the veterinary field as these antibiotics are potential selectors of linezolid resistance genes.

Interactions with JRPs/JIPs or with external (EU or national) relevant projects or initiatives such as national action plans

Not applicable.

Interactions with OHEJP stakeholders, national and international surveillance programmes

Samples analysed during this project were provided by the Federal Agency for the Safety of the Food Chain for the Belgian antimicrobial resistance official monitoring. The results of the LIN-RES project published in the Journal of Antimicrobial Chemotherapy (https://doi.org/10.1093/jac/dkab376) were shared with the EFSA through personal communications and through an oral communication given at the 16th EURL-AR workshop in June 2022 and previous communications at the different ASM of the OH-EJP.

Evaluation of the Final Thesis Report

Not applicable as the PhD student submitted their thesis manuscript, which can be found, here.

PhD3-AMR2.1-HME-AMR

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Elena Anedda	PhD Student	University of Galway	Republic of Ireland
Dr Kaye Burgess	Lead PhD Supervisor	Teagasc Food Research Centre	Republic of Ireland
Prof. Dearbháile Morris	Second PhD Supervisor	University of Galway	Republic of Ireland
Dr Gro Johannessen	Other Supervisor	Norwegian Veterinary Institute	Norway

Summary of the work carried out in the PhD project.

The HME-AMR PhD project began in M38, in 2021 and is expected to finish in M74, in 2024. The HME-AMR project aims to investigate the role of heavy metals as a selective pressure on AMR in the primary food production environment. A scoping literature review was initially conducted to identify the knowledge gaps in the existing literature to summarise the evidence regarding the impact of heavy metals on AMR in the primary food production environment. The results obtained show a clear link between heavy metals and AMR in this sector. Specifically, the review reported that heavy metals can influence the abundance and spread of mobile genetic elements (MGEs), which can consequently facilitate consequently facilitate the dissemination of antimicrobial resistance genes (ARGs). It also demonstrated that a wide variety of methods are used to assess relationships between AMR and heavy metals. A recommendation arising from the review is that harmonised approaches are needed to facilitate comparisons between different studies. Moreover, a limitation in the geographical distribution of the studies was observed; therefore, further research on AMR and heavy metals in the primary food production environment is needed to provide a more complete picture.

In an effort to address this the role of heavy metals on AMR distribution in the primary food production environment was also studied examined through a number of field based studies. The first study focused on the isolation and characterisation of antimicrobial resistant Enterobacterales from soil and spinach samples collected from areas with zinc amendments and without. The second study also focused on the detection and characterisation of antimicrobial resistant Enterobacterales in soil and bovine milk filters collected from areas representing production areas with high and low zinc concentrations in across Ireland. The results of both studies demonstrate that primary food production environments can harbour clinically relevant antimicrobial resistant Enterobacterales. These studies will be complemented by metagenomic analysis of selected sample sets to provide a further analysis of the resistome structure in areas of high and low zinc concentration. Although the studies were being conducted in Ireland, the results are broadly applicable and relevant to all the European countries. Findings of this work will contribute to meeting the objectives of Ireland's Second National Action Plan on Antimicrobial Resistance, providing evidence of antimicrobial

resistance in the food production environment and will be of relevance to the European Antimicrobial Resistance Surveillance System, considering that Ireland is a member of the EU AMR One Health Network.

Dissemination activities of this HME-AMR PhD project were enshrined in a peer-reviewed publication, in the Journal of Environmental Pollution, and four oral and poster presentations at the national (i.e., Environ Conference 2023, Ireland) and international level (i.e., One Health EJP Annual Scientific Meeting 2021).

Work carried out on the PhD, scientific results, and outcomes.

Chapter 1: Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review.

The literature review shows a rapid increase in the number of relevant studies since 2016 regarding the role of the environment in the development and transmission of AMR. The studies included in the scoping review considered different types of environmental samples: soil, water and manure principally; but also examined the characteristics of the environment where they were collected from, for example the vicinity to mines or industries, or exposure to a specific treatment. Through the analysis of the included studies, an association between AMR and the presence of heavy metals was generally observed. In some cases, a direct correlation was established, such as promotion of ARGs dissemination induced by heavy metals; while in other cases, the role of MGEs on the spread of ARGs and MRGs was demonstrated. Specifically, a limitation in the geographical representativeness of studies included, heterogeneity of methods applied to detect AMR and HMR, and the effect of heavy metals on MGEs, were discussed in the review. Full details can be found in the published study

More information on this chapter can be found, here.

Chapter 2: Occurrence and characterisation of antimicrobial resistant Enterobacterales in soil and spinach in the presence and absence of zinc amendment

The work undertaken for this chapter has been drafted for submission for publication. To ensure this publication process is not compromised detailed results have not been included, with a brief synopsis provided of the results instead.

A number of Enterobacterales isolates representing five different species were obtained from both the soil and spinach samples in both locations tested, including multi drug resistant isolates. Whole genome sequencing (WGS) analysis of the isolates complemented the phenotypic analysis, facilitating genotypic confirmation of the antimicrobial resistance phenotypes observed.

Chapter 3: Occurrence and characterisation of antimicrobial resistant Enterobacterales in dairy production environments of differing zinc concentrations

This work is still in progress and due for completion by September 2023. To ensure the publication process is not compromised, detailed results to date are not included.

This work is ongoing but to date antimicrobial resistant isolates of seven different Enterobacterales species have been identified from both soil and milk filter samples, with a diverse range of AMR profiles characterised. Analysis is ongoing to compare the phenotypic and genotypic profiles of the isolates via antimicrobial susceptibility testing and WGS. They will also be compared with relevant isolates from other One Health domains, through the interrogation of public databases and collaboration with other OHEJP project partners, including those involved in ARDiG and DiSCoVeR.

Chapter 4: Metagenomic analysis of soil and bovine milk filters

This work is still in progress and due for completion by January 2024. As this work is not scheduled for completion until early 2024 further details cannot be provided at this time.

The previous two chapters focus on a group of antimicrobial resistant organisms of particular public health concern. However, in order to gain a more complete understanding of the differences which may exist in AMR profiles in association with heavy metal content it is important to look at the wider microbial community, including unculturable species. To facilitate this DNA has been extracted from a subset of samples collected as part of Chapter 3 and these are currently undergoing metagenomic analysis. Analysis will predominantly focus on the characterisation of the resistome through the detection of ARGS, as well as MGEs and heavy metal and biocide resistance genes.

PhD self-assessment

As outlined above the project objectives indicated in the proposal have been achieved through a number of research based chapters which are complete, or well on their way to completion. All necessary sampling plans were prepared through collaboration with Geological Survey Ireland and the Teagasc advisory service, broadening the awareness of the project and its objectives. All necessary samples have been collected and the isolation and characterisation of antimicrobial resistant Enterobacterales in low and high metal containing environments, as well as in bovine milk filters from cattle grazing on grass in low and high metal areas, have been completed. Complementary metagenomics analysis which will form the content of Chapter 4 is currently under way. Together these chapters will provide an overview of the presence of antimicrobial resistant organisms and ARGs of public health concern in the production of both plant and animal derived products and whether the presence or addition of zinc may influence the AMR patterns observed.

There was one necessary deviation from the project proposal. From 2022, high level zinc oxide supplementation in pig diets is no longer permitted in the EU, which impacted on one *HME-AMR* objective. Specifically, one objective in the original proposal was the analysis of the resistome on sites following manure application from pigs with heavy metal included in feed and from pigs with no/minimal heavy metal in feed. However, with the removal of the use of high levels of ZnO in pig production there was an associated expected decrease in Zn levels in slurry being land spread and also greater difficulty in identifying farms to fulfil the requirements for meeting the project objective. This study was therefore replaced with the spinach focused study. The revised study objective was to assess the AMR profile in spinach and soil in the presence and absence of zinc amendment. In this case zinc sulphate was directly applied to the soil, with unamended control plots also available. This enabled direct

comparison of the impact of zinc amendment on the AMR profile. This project amendment has been reported and approved previously.

As part of completing this PhD a range of microbiological and molecular skills have been learnt by the student, as well as bioinformatics analysis. They have also had the opportunity to improve their presentation and communication skills, in both oral and written formats, which led to being awarded a 'best oral presentation' prize at the recent ENVIRON conference.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
PhD03-AMR2.1-HME-AMR	D-PhD03-1.1	Report on AMR bacteria present in soils of differing heavy metal content	M60	M72	Delay in student starting project due to COVID-19 and rearrangement of study commencement order will delay the first deliverable being achieved.	
PhD03-AMR2.1-HME-AMR	D-PhD03-1.2	Report on the impact of the application of heavy metal containing amendments to AMR bacteria in soil	M54	M69	The study has been completed and is currently being written up for publication.	
PhD03-AMR2.1-HME-AMR	D-PhD03-1.3	Report on AMR bacteria present in milk filters from animals grazing in areas of differing heavy metal content	M66	M72	Delay in student starting project due to COVID-19 will delay the achievement of this deliverable.	

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD03-AMR2.1-HME-AMR	1	SOP in place for sampling culture based analysis	M27	M40	Yes	Delayed due to the late recruitment of student but the SOPs for culture based analysis are now in place and have been utilised in the first sampling study undertaken.
PhD03-AMR2.1-HME-AMR	2	Sequences of sufficient quality obtained from metagenomic analysis	M35	M68	No	Delayed due to late recruitment of PhD student

Publications and additional outputs

Publications

At the time this deliverable was submitted, the HME-AMR PhD project has published one peer-reviewed publication:

- Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review. (2023). *Environmental Pollution*. https://doi.org/10.1016/j.envpol.2023.121035.

The publication has also been uploaded to the Zenodo repository here, and is gold open access.

Additional outputs (i.e., poster/oral presentations)

The HME-AMR PhD project disseminated these works through oral and poster presentations at the following events:

- Anedda, E., Burgess, C., Morris, D. (2021). A scoping review to evaluate the impact of heavy metals in the agri-food environment as selective pressure for the mobilisation of antimicrobial resistance. Poster presentation. ONE HEALTH EJP Annual Scientific Meeting, Copenhagen, Denmark, 09-11 June 2021. More information here.
- Anedda, E.; Madigan, G.; Morris, D.; Burgess, C. (2022). Assessment of the presence of antimicrobial resistant bacteria in spinach and its production environment after zinc application. Poster presentation. ONE HEALTH EJP Annual Scientific Meeting, Orvieto, Italy, 11-13 April 2022. More information here.
- Anedda, E.; Madigan, G.; Morris, D.; Burgess, C. (2022). Evaluating the impact of zinc application on the presence of antimicrobial resistance in spinach and its production environment. Poster presentation. ONE Conference 2022, Brussels, Belgium, 21-24 June 2022. More information here.
- Anedda, E., Ekhlas, D., Alexa, E., Gaffney, M., Madigan, G., Morris, D.1, Burgess, C.M. (2023). Occurrence of AMR Enterobacterales in soil and spinach in the presence and absence of zinc amendment. Oral presentation. Environ Conference 2023, Donegal, Ireland, 3-5 April 2023.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Biosafety Awareness	Health and Safety	23/04/21	Teagasc
Lab risk assessment	Health and Safety	29/04/21	Teagasc
Lab chemical safety awareness	Health and Safety	19/04/21	Teagasc
Lab safety basics	Health and Safety	17/03/21	Teagasc
Manual handling	Health and Safety	08/02/21	Teagasc
Antimicrobial resistance –theory and methods	Relevant to PhD topic	04/02/21	Coursera
Metagenomics applied to surveillance of pathogens and antimicrobial resistance	Relevant to PhD topic	19/02/21	Coursera
Scopus workshop	Database interrogation	24/03/21	University of Galway
Present your research with confidence	Presentation skills	24/05/21	Environ 2021
Statistical analysis with R	Statistics	14-17-18/06/21	Teagasc
The Impostor Syndrome	Mental Health	06/07/21	Walsh Scholarships Programme
Health and Safety Briefings	Health and Safety	26/08/21	University of Galway
Academic Writing Centre summer workshop	Writing skills	27/08/21	University of Galway
Understanding AMR in Water-Webinar	Relevant to PhD topic	28/08/21	Centers for Disease Control and Prevention (CDC)
General Data Protection Regulation	Data protection online	30/08/21	Teagasc (E-Learning)
Safety Responsibilities for Principal Investigators	Laboratory Safety	30/08/21	University of Galway
Safety Responsibilities for Heads of Units	Laboratory Safety	31/08/21	University of Galway
Chemical safety training	Laboratory Safety	31/08/21	University of Galway
Academic English Writing	Writing skills	21/09 - 23/11 (10 lessons)	University of Galway -English Language Centre
Plain English Training	Writing skills	27/09/21	Teagasc-NALA
Mental Health Workshop	Mental Health	28/09/21	Spectrum Life
Gas Cylinders	Health and Safety	15/10/21	Teagasc

AMR Surveillance in The Netherlands (and beyond) from a One Health perspective	Relevant to PhD topic	18/10/21	MetVetNet Association
One Health Annual Conference	Relevant to PhD topic	03-04/10/21	Ryan Institute Centre
Online writing workshop	Writing skills	01/12/21	University of Galway -Academic Writing Centre
CMNHS English Language Workshop	Writing skills	Feb-Mar-Apr (30 hours)	University of Galway -English Language Centre
Research Integrity	Training to conduct research	April 2022	Epigeum
Bioinformatic Workshop	Data analysis	20/05/2022 27/05/2022 13/06/2022	Teagasc
JPIAMR Workshop-New perspectives on bacterial drug resistance	Antimicrobial resistance	09/06/2022	JPIAMR
UCD One Health Conference 2022	One Health	15/06/2022	UCD
ONE – Health, Environment, Society – Conference 2022	Antimicrobial resistance	21-24/06/2022	EFSA
VIBE-Conference	Bioinformatic	24/06/2022	University of Limerick
DGSA-Training	Shipment of research specimens	07/07/2022	Teagasc
The Antimicrobial Resistance (AMR) and Alternatives to Antibiotics (ATA) Research Webinar Series	Antimicrobial resistance	14/06/2022 19/07/2022 20/09/2022 18/10/2022	U.S Department of Agriculture
Antimicrobial Resistance and Use in the Farming Sector: Negotiating Behaviour Change under New Legislations-Seminar	Antimicrobial resistance	28/09/2022	Safefood &Teagasc
ESCMID/ASM Conference on Drug Development to Meet the Challenge of Antimicrobial Resistance	Antimicrobial resistance	04-07/10/2022	European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Centre for One Health Annual Conference 2022	One Health and Antimicrobial resistance	03-04/11/2022	Ryan Institute
Teagasc Festival of Farming and Food (Science Week)	Oral presentation skill	15/11/2022	Teagasc
BTYSTE-BT Young Scientist & Technology Exhibition	Oral presentation skill	12/01/2023	BTYSTE
Environmental DNA sequencing with Oxford Nanopore: in the lab and in the field	Relevant to PhD topic	26/01/2023	Oxford Nanopore Technologies
Lab Chemical Safety Awareness	Health and Safety	8/03/2023	Teagasc
Chemical Spill Awareness Training	Health and Safety	22/03/2023	Teagasc
ASM training (Next Generation sequencing)	Relevant to PhD topic	March/April/June 2023	American Society for Microbiology

One Health Impact

Although the project was conducted in Ireland, the results are broadly applicable and relevant to all the European countries. The provided data on AMR will contribute to meeting the objectives of *Ireland's Second National Action Plan on Antimicrobial Resistance* and to the European Antimicrobial Resistance Surveillance System, considering that Ireland is a member of the EU AMR One Health Network. As identified in the scoping literature review there are limited studies on heavy metal and AMR linkages in food production, particularly in Europe, and the outcomes of this project will help address that gap. Such data will inform more targeted risk management of AMR in food production.

The project team are members of a national One Health AMR Thematic Network which will provide an ideal forum for sharing project outcomes with a diverse range of stakeholders and its activities have also been reported through the national OHEJP mirror group.

Dr Burgess and Prof Morris collaborated on the Irish EPA funded project AREST which examined antimicrobial resistance in the environment and contributed to One Health orientated AMR research and policy making at a national and international level. This project is highly complementary to those activities and will facilitate an increasing collaborative relationship between the research groups. Dr Burgess has worked previously with Dr Johannessen of the Norwegian Veterinary Institute as part of the *HUPLANT Control* COST action focusing on microbial food safety issues and this project will further develop this relationship. This project has also led to Dr Burgess forging collaborative linkages with Geological Survey Ireland in relation to heavy metal mapping and with the SFI funded *VISTA MILK* research centre. This work has contributed to Dr Burgess and Prof Morris submitting an application for national research funding on AMR mitigation measures in the environment which has recently been funded.

Ms Anedda has actively participated in OHEJP conference and training activities and has had the opportunity to share and discuss her work with colleagues in different disciplines and One Health domains, ensuring that the role of food production in AMR transmission is more fully understood, as well as the factors which may influence it. Once complete, the work of *HME-AMR* will provide complementary culture dependent and independent analysis of antimicrobial resistance of public health concern in horticultural crops and dairy production, as well as the environment in which they are produced. This will provide a valuable link between One Health domains.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

Being part of the One Health EJP has given me the opportunity to create a wide network with colleagues who work on different topics, but who share the same One Health principles. Attending conferences, including OHEJP ASM 2021 and OHEJP ASM 2022, enabled the sharing of ideas among participants, which helped me to evaluate and see my own work from a different point of view, bringing innovation and motivation. Also, being part of the OHEJP community on social media, such as LinkedIn, led me to interact with multiple people and be informed about the latest news. Moreover, training, such as the ASM Satellite Workshops, broadened my general knowledge.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The HME-AMR PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- A number of meetings have taken place with Geological Survey Ireland and through collaboration with the Tellus survey. This enabled the selection of suitable high and low metal content areas across Ireland for resistome analysis in soil and bovine milk filters.
- Dr Burgess and Prof Morris both contribute to Ireland's Second National Action Plan for Antimicrobial Resistance and the results of this project will contribute to achieving the objectives of that plan.
- Dr Burgess and Dr Johannessen were both participants of the OHEJP DISCoVeR project which focused on source attribution of a range of pathogens and AMR E. coli. Isolates from this study will be of relevance for the One Health strain database built as part of the DISCoVeR project and the linkages forged through the DISCoVeR project will enable access for Elena to suitable strain data for comparative purposes.
- This project is complementary to the EPA funded *AREST* project which is examining antimicrobial resistance in the environment. Prof Morris is the coordinator of this project and Dr Burgess is a participant. The culture-based methodologies being employed in AREST will be particularly relevant for HME-AMR. The student will have the opportunity to collaborate with these colleagues for methodologies and isolate characterisation.
- HME-AMR is complementary to an ongoing PhD project (*ZnO*) in Dr Burgess' group focusing on the impact of zinc oxide supplementation on the porcine resistome which will facilitate a smooth transition for the HME-AMR student in relation to methodologies and analysis.
- HME-AMR is complementary to a project led by Dr Orla O'Sullivan as part of the SFI funded VistaMilk project which is examining the soil resistome in different sites across Ireland. Dr Burgess and Dr O'Sullivan will collaborate to ensure synergy of the projects and avoid duplication.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	*n/a*	*n/a*
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	5	3
Were all the milestones and deliverables completed?	4	2
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	4	2
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	2
Did the PhD student actively engage in Education and Training activities?	5	4
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	4	2
Was the PhD managed and implemented in accordance with the DMP?	5	1
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	3	3
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	*n/a*	*n/a*
Is there any direct or indirect impact of the project for national or international stakeholders?	4	2
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	5	2
Does the project outcomes have policy implications?	*n/a*	*n/a*
TOTAL	43/50	23/50

AVERAGE: 33/50

NB. The HME-AMR PhD project is not scheduled to be completed until September 2024 at the earliest; therefore three questions were not assessed based on this timeline.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

Only the results of the scoping review are presented in some detail in the report (due to confidentiality issues related with the publication process of the more "research oriented" chapters) and therefore not many specifics are presented in the report. Still, the PhD student mentions some important points that should be the focus of future research.

Reviewer 2: External Scientific reviewer

Project not yet finished, conclusions are vague and future work limited to the direct project-related work, no vision presented.

Reviewer 3: PMT member

For various (understandable) reasons, no details nor results are presented in chapter 6, which makes the evaluation challenging. Either no score, or a figure that refers to neutral score (3?) seems most appropriate.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Though a significant change in the plan was introduced, this change was well justified (and had been already communicated). Other than that, only delays (due to SARS-CoV-2-related issues) are mentioned.

Reviewer 2: External Scientific reviewer

Mostly focused on the individual skills acquired.

Reviewer 3: PMT member

The text describes and justifies the modification of the work. Since the project has not yet come to completion, a neutral score is proposed.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Most deliverables and milestones are set as achieved, with only one milestone being the exception (due to SARS-CoV-2-related issues).

Reviewer 2: External Scientific reviewer

Difficult to assess as no data from field studies presented. Rationale that this could prevent publication is weak.

Reviewer 3: PMT member

Unfortunately, no text is added under 8, but the table with deliverable and milestones indicate the progress (and delays). Care should be taken to finalize the work before the planned end date.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Connections with OHEJP JRPs/JIPs are not mentioned in the report, which is a pity given the important topic addressed in the project and the potential complementariness with some of the other projects conducted in the frame of the OHEJP. Relevant contacts with other important stakeholders in the frame of AMR surveillance are nevertheless mentioned.

Reviewer 2: External Scientific reviewer

Some interaction with Irish surveys, none reported for JIPs/JRPs.

Reviewer 3: PMT member

The linkage with EJP project DiSCoVeR is explained, as well as with external projects.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

The student mentions several benefits of being part of the OHEJP, mostly in terms of the networking opportunities but also training through OHEJP-related workshops.

Reviewer 2: External Scientific reviewer

Good added value with other projects of supervisors, but not outside of that.

Reviewer 3: PMT member

The chapter focuses on scientific collaborations, less on interaction with stakeholders, who may be the end users of the PhD outcome.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

The report includes an impressive record of courses and training conducted over the duration of the project, spanning different fields and aspects of the training, and thus demonstrating the development of multiple skills during her PhD.

Reviewer 2: External Scientific reviewer

Good training and skills development.

Reviewer 3: PMT member

Numerous training opportunities.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

One paper derived from the PhD project has been already published but at least two other papers related with the remaining objectives are planned and, given the importance of the research conducted, no major problems publishing them are foreseen.

Reviewer 2: External Scientific reviewer

1 review, two papers promised (results not shown at all with excuse of publications.

Reviewer 3: PMT member

Good to see that the review was published, and that a planning for more publications is available.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

No deviations from the DMP (publication in open access) regarding the scientific publications are observed).

Reviewer 2: External Scientific reviewer

Not specifically reported. No data shown in report.

Reviewer 3: PMT member

Not mentioned.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

Results from the research conducted so far have been further disseminated through poster presentations and one oral presentation at European conferences.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Good dissemination efforts.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

Because the final results of two of the three objectives are not described in the report it is difficult to assess the relevance of the finalised project outputs (furthermore, one of the objectives is still being pursued and it is only planned to be finalised by the end of 2024). Nevertheless, given the expected outcomes (and the results presented so far), results in terms of the assessment of the impact that heavy metals can have in the epidemiology of AMR should be highly relevant.

Reviewer 2: External Scientific reviewer

The review is of use. No other data presented, not even in abstracted form. Report is incomplete and difficult to assess.

Reviewer 3: PMT member

Chapter 17 focuses on collaborations, but not really on cross-sector work and the added value of it. It might be useful to discuss what One Health means in the context of this PhD project, and how public health, animal health and the food safety sector can profit from the PhD's outcome.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

There is an expected impact in terms of AMR surveillance, as exemplified by the link between the research conducted and the (Ireland) national AMR action plan, which could be easily translated into other settings. This impact is however expected but has not occurred yet since the research is still ongoing.

Reviewer 2: External Scientific reviewer

Difficult to say as no information provided.

Reviewer 3: PMT member

No information provided. Difficult to assess.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

The project has helped to build/strengthen the network of the PhD supervisor and has supported/complemented other research conducted in the neglected field of study of AMR in the environment, and as such has been highly beneficial in the development of the research conducted in the institution hosting the PhD student.

Reviewer 2: External Scientific reviewer

At most strengthened existing links.

Reviewer 3: PMT member

See chapter 9.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

Though the results could be used to inform future surveillance and control activities, it is difficult to foresee how these may look like without having specific results to consider.

Reviewer 2: External Scientific reviewer

Not clear at this stage as no information provided.

Reviewer 3: PMT member

Difficult to discuss, as the PhD has not made sufficient progress and details are not available.

The full Final Thesis Report for the HME-AMR PhD project can be found of here.

PhD4-AMR2.1-KENTUCKY

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Alaa Albasiony	PhD Student	Sciensano	Belgium
Abram Aertsen	PhD Lead Supervisor	KU LEUVEN	Belgium
Pieter-Jan Ceyssens	PhD Second Supervisor	Sciensano	Belgium
Benoit Doublet	Other Supervisor	INRAE	France

Summary of the work carried out in the PhD project.

The KENTUCKY PhD Project began in M30, in 2020 and is expected to finish in M69, in 2023. An 11-month extension was provided due to the SARS-CoV-2 pandemic. KENTUCKY focused on various genetic engineering techniques to investigate the increase that conjugation and transposition efficiency play in order to maximise the likelihood of visualising the transfer dynamics of the IncHI-archetype plasmid pR27 and the carried ISEcp1 in real time using microfluidics and fluorescence microscopy. Furthermore, the dynamics of H-NS, a silencer of horizontally acquired genes, were investigated in real-time upon plasmid acquisition. pR27 also carries its own copy of H-NS, which likely reduces its acquisition cost. Our results showed conservation of the DNA-binding domain among plasmid-derived (i.e., R27) and chromosomally encoded H-NS. Moreover, the plasmid-borne H-NS copy (i.e., R27) could partially complement a chromosomal H-NS deficiency in a gene-dependent manner. Finally, using live microscopy we have timed and quantified the expression of plasmid-derived H-NS at the very moment of plasmid acquisition.

Dissemination activities of this KENTUCKY PhD project were disseminated through, oral and poster presentations at the international level (i.e., Annual Scientific Meeting OHEJP 2022 & International Symposium Salmonella and Salmonellosis I3S2022).

Work carried out on the PhD, scientific results, and outcomes.

Chapter 1. Full-length sequencing of S. Kentucky isolates

Short read sequencing libraries of isolates were prepared with an Illumina Nextera XT DNA Library Preparation Kit and sequenced on an Illumina MiSeq instrument with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Trimming of the short reads was performed with Trimmomatic (version 0.32). First the Illuminaclip option was used to remove the Nextera adapter sequences. Then a sliding window approach of four bases and trimming when the Phred score dropped below 30 was employed. Lastly, the leading and trailing bases of a read were removed when the Phred score dropped below 3. Lastly, all reads that were smaller than 50 bp were removed.

The MinION long read sequencing library was prepared by using the 1D ligation sequencing kit (SQK-LSK108, Oxford Nanopore) according to the manufacturer's protocol for genomic DNA without barcoding. In total there were two MinION flowcells used, and libraries with 8 and 12 barcodes were loaded on them respectively (EXP-NBD103, Oxford Nanopore). For the Flongle long read sequencing libraries, the adapted 1D ligation protocol for Flongles was used with the SQK-LSK109 sequencing kit. From each isolate 500 ng of DNA was used at the start of the protocol. DNA repair was no longer optional in SQK-LSK109 and therefore this was performed for the Flongle runs. In the SQK-LSK109 there are two washing buffers the SFB and LFB of which the latter enriches for DNA fragments >3,000 bp. Both these washing buffers and the inclusion or exclusion of the shearing step were used on separate Flongle flowcells. Moreover, on the Flongles no barcoding was performed. Basecalling and demultiplexing of the Nanopore sequences was performed with Guppy. Then all Nanopore reads with a quality score lower than 7 or a length lower than 1000 were removed with NanoFilt. The statistics of the Illumina reads was determined with FastQC and of the Nanopore reads was determined with NanoStat. Raw sequencing data and the de novo assemblies were submitted to NCBI Sequence Read Archive (SRA) and NCBI Genbank.

The chromosomes of isolates S16BD08730, S18BD03394 and S18BD05011 share the AMR genes *aac(3)-Id*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *sul1, tet(A)*, and *aac(6')-Iaa*. All these AMR genes except for *aac(6')-Iaa* were localised very close to each other and by aligning this region to the NCBI nucleotide database it was determined that these genes were part of a *Salmonella* genetic island 1 K (SGI1-K). Two isolates (S16BD08730 and S18BD03394) carried the ESBL gene $bla_{CTX\text{-}M\text{-}14b}$ in the chromosome, while one isolate (S18BD00684) contained another ESBL gene, $bla_{TEM\text{-}1B}$, in the chromosome. Moreover, the latter isolate also contained the ESBL gene $bla_{CMY\text{-}2}$ on a plasmid. Isolate S18BD05011 contained no ESBL genes on its chromosome, but $bla_{CTX\text{-}M\text{-}104}$ and $bla_{TEM\text{-}1B}$ were localised on two different plasmids, contigs 2 and 3, respectively. Initially, ResFinder assigned $bla_{CTX\text{-}M\text{-}14b}$ to isolate S18BD05011 with an identity of 99.89%, but with the CARD database 2 it was determined that a point mutation at position 824 corresponds to the $bla_{CTX\text{-}M\text{-}104}$ gene. Upon further inspection, a region of 2850 bp including the ESBL gene was found to be similar in the chromosome of S16BD08730 and S18BD03394 and in the plasmid (contig 2) of S18BD05011. In these regions, there was only a 1 bp difference, resulting in either the $bla_{CTX\text{-}M\text{-}14b}$ (S16BD08730 and S18BD03394 on the chromosome) or $bla_{CTX\text{-}M\text{-}104}$ (S18BD05011, on a plasmid) variants. The ISEcp1B transposase, which is part of the IS1380 family, was detected in this region adjacent to the ESBL gene. In the NCBI database there were no exact matches, but with a literature search, a description of this 2850 bp fragment was found in Lei *et al*. 2020 in the chromosome of a *S.* Kentucky isolated from Chinese poultry.

Chapter 2. Abundance of insertion sequences and AMR genes

A dataset of *Klebsiella pneumoniae* assemblies at the contig level has been downloaded from the public database 'NCBI Assemblies'. To investigate the presence of associations between IS elements and AMR genes in the dataset, detection of antimicrobial resistance has been carried out through ResFinder, detection of IS elements through ISEScan, detection of replicons through PlasmidFinder and prediction of the origin for each contig with the machine learning program RFPlasmid. By comparing the coordinates on the contigs of the detected elements, distances between IS and AMR genes have been calculated as the number of bp between the end of one element and the start of the other, and stored in python data frames.

This process has been done by measuring the number of associations that would be present in the dataset for each of the most common IS families when considering different thresholds. The value chosen was 10,000 bp, as it would allow to capture a good number of IS6 associations without overestimating the number of associations for any of the other relevant IS families.

The number of associations in the dataset was thus approximately 30,000 on 4,325 samples, the large majority of which were located on a contig that was predicted to be plasmid derived. The IS family that presented the highest number of associations with different AMR types was IS6, which was particularly associated with aminoglycosides and beta-lactams, followed by IS91, IS1380 and IS5.

Considering specifically beta-lactamases, the IS family that is most often associated with genes of this class of resistance was IS1182, followed by IS21, IS1380 and IS6. In particular, the genes $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{KPC.3}$ seem to be often found close to many of the IS families present in the dataset.

The IS1380 family can be found close to the following beta-lactam resistance genes, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}132}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}36}$, and $bla_{KPC\text{-}2}$. Among these, the CTX-M-15 gene is by far the most relevant in terms of number of associations, most of which are found on plasmid-derived contigs.

The ISEcp1 element belongs to the IS1380 family of insertion sequence and indeed is one of the most important elements that mobilise $bla_{CTX\text{-}M\text{-}15}$ genes (Hamamoto *et al*., 2020). In this context, ISEcp1 is included between a IRL and an IRR sequence and is found upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene. It can mobilise its downstream region with variable lengths, creating a transposition unit through the recognition of the IRL and the IRR, or an alternative IRR (IRRalt) (Hamamoto *et al*., 2020). This transposition is called a one-ended transposition (Poirel, Decousser and Nordmann, 2005). Interestingly, ISecp1 is known to also provide a promoter sequence to different AMR genes in its right side (Poirel, Decousser, and Nordmann, 2003).

Chapter 3. Insertion of P1-parS and lactis-parS labelled transposition unit into pR27

pKD46, a plasmid with temperature-sensitive replicon, encoding lambda Red recombinases was electroporated into MA8508ΔpSLT strain. Next, pR27 was conjugated by filter mating to MA8508ΔpSLT containing pKD46. In short, cultures of the donor (*E. coli*, pR27) and recipient strains (MA8508ΔpSLT containing pKD46) were grown in LB supplemented with the required antibiotics at 25°C in static conditions for 16 h. Cells were washed with 0.1M MgSO4 to eliminate the antibiotics. The recipient strain suspension (0.4 ml) and donor strain suspension (0.1 ml) were mixed on a 0.22 µm membrane and incubated at 25°C for 2 h. Serial dilutions were plated inappropriate media to select for trans-conjugants (MA8508ΔpSLT containing pKD46 and pR27).

P1-parS was then PCR amplified as P1-parS- frt-cat-frt fragment and electroporated into MA8508ΔpSLT containing pKD46 and pR27 to be inserted in an intergenic region on pR27 backbone via lambda red recombineering (Datsenko *et al*., 2000). Next, we flipped the chloramphenicol cassette using pCP20, a plasmid encoding Flp recombinase. To label the ISEcp1-bla CTX-M-14b unit with Lactococcus lactis-derived parS, we PCR amplified the unit as two fragments with the 18bp parS sequence as an overhang

(GGGGCTAAATTTAGCCCC). The two fragments were then assembled *in vitro* by gibsson assembly and inserted into pR27 backbone by homologous recombination (Datsenko *et al*., 2000). The 18bp parS sequence was inserted downstream the terminator region of the ISEcp1 gene and upstream the promoter region of $bla_{CTX\text{-}M\text{-}14b}$ gene. We then confirmed the insertion of P1 and *Lactococcus lactis*-derived parS together with the conservation of the ISEcp1,$bla_{CTX\text{-}M\text{-}14b}$ genes by sequencing.

Chapter 4. Optimising P1-ParB and Lactis-ParB Proteins

First, we separately inserted by recombineering the two parS sequences into the chromosomes of MA8508ΔpSLT to serve as a positive control for fluorescent microscopy. Next, we transformed the MA8508ΔpSLT labelled with P1-parS and lactis-parS with pALA2705 and pMK17-01 respectively. pALA2705 plasmid encodes P1-parB N-terminally fused to mCherry whereas pMK17-01 encodes lactis-parB C-terminally fused to msfGFP. Time-lapse microscopy experiments showed only localized foci with P1-parB-mCherry, indicating that lactis-parB failed to bind its cognate parS sequence. Thus, it was reasoned to codon optimise the lactis-parB protein to a Gram-negative host using the genescriptTM platform. Then the codon-optimised lactis-parB protein was then cloned into a variant of pALA2705 encoding GFP to obtain pALA2706 (lactis-parB N-terminally fused to GFP). pALA2706 was then transformed into MA8508ΔpSLT labelled with lactis-parS. The microscopy experiment showed again diffused fluorescence. We thought to change the fusion type into C- terminal fusion and try different linkers. In short, pALA2706 was PCR linearized to remove GFP- lactis-ParB. Then msfGFP and lactis-ParB were PCR amplified, assembled *in vitro* to produce lactis-parB C-terminally fused to msfGFP with two different linkers (SSSRGSGGEAAAKAGS, (SGGGG)4), and ligated back to PCR-linearized pALA2706 by Gibson assembly. The two variants of the plasmid were then separately transformed to MA8508ΔpSLT labelled with lactis-parS. yet we failed to obtain localised foci. The inability of lactis-ParB to bind its cognate parS sequence *in vivo* may be attributed to improper folding in Gram-negative host despite being codon-optimised. It is also possible that parB fails to oligomerise upon binding, so we don't observe the localised focus over the background diffused fluorescence. Moreover, in *E. coli*, the integration host factors (IHF) bind specific sequences on P1-parS and facilitate the binding of P1-parB to P1-parS (Barbara Funnel, 1991) and since lactis-parS does not carry an IHF-binding sequence it might have failed to attract the partitioning machinery.

Chapter 5. Construction of a reporter plasmid based on P1, pMT parS/ ParB partition system

pR27 displays a conjugation efficiency of 10-5 at 25°C and 10-7 at 37°C. Since conjugation of the wild-type R27 plasmid is only a one in a 10-5 event, three genetic engineering approaches were instigated to increase the conjugation efficiency of pR27 to maximise the likelihood of observing the conjugation in Real-time. (i) The first approach was knocking out the conjugation repressor HtdA which is involved in the repression of four tra operons and has a pivotal role in the growth phase dependency of R27 conjugation. In short, pMT parS was PCR amplified as pMT-parS- frt-cat-frt fragment, and the fragment was electroporated into MA8508ΔpSLT containing pKD46 and pR27 to replace htdA gene. *In vitro* conjugation assay has shown a 2-3 log increase in the conjugation efficiency of pR27, htdA mutant as compared to the wild type. We then labelled the transposition unit with P1-parS as previously described followed by electroporation into MA8508ΔpSLT containing pKD46 and pR27(pMT-labelled, ΔhtdA),

yielding double parS labelled pR27 (R27 [ΔhtdA::frt-Cm-frt- pMTparS -ISEcp1-P1parS-blaCTX-M-14b]). (ii) Next, we opted for overexpressing the conjugation regulators TrhR and TrhY which are known to activate the expression of four different Tra operons (Gibert *et al*., 2016). The trhR and trhY genes were PCR amplified and assembled *in vitro* (i.e., Gibson assembly) under a strong constitutive promoter (J23119). The assembled construct was electroporated (MA8058 pKD46 ΔpSLT), to be inserted into an intergenic region, flanked by two terminators to prevent read-through. The transformants were PCR confirmed, however, the sequencing data showed multiple mutations resulting in an early stop codon in trhY. In a second attempt, we tried to over-express trhR and trhY in cis on pR27 (R27 [ΔhtdA::frt-Cm-frt-pMTparS]). The native promoter of trhR and trhY was replaced by the strong constitutive promoter (J23119). After the exchange of the native promoter, the construct was PCR confirmed, however, frameshift mutations were found in the trhR rendering the construct not functional. Previous work by Forns *et al*. (2005), and Gibert *et al*. (2014) suggests that conjugation of R27 can be increased by either knocking out the hns gene of the chromosome of the donor cells or the plasmid itself. (iii) The third approach to increase the conjugation efficiency was thus based on the deletion of hns in the chromosome of the donor strain (MA8058 pKD46 ΔpSLT). In short, The ORF of hns was replaced by frt-Km-frt except for the first 11 amino acids (MA8058 pKD46 ΔpSLT Δhns::frt-Km-frt). Next, The double parS labelled plasmid (R27 [ΔhtdA::frt-Cm-frt- pMTparS -ISEcp1-P1parS-$_{blaCTX-M-14b}$]) was conjugated from a Δhns strain *S.* Enterica strain (MA8058 ΔpSLT). The respective conjugation frequencies were calculated as the transconjugant over recipient ratio (T/R) and the transconjugant over donor ratio (R/D). At 25°C the T/R conjugation efficiency is ca. 1.12x10- 1 and the T/D conjugation efficiency is ca. 7.70x10-2. Hence, Conjugation efficiency has increased from ca. 10-5 (pR27 wild type) to ca. one in 10 by knocking out both HtdA and H-NS.

Chapter 6. Construction of the fluorescent recipient strain to visualise the transfer of pR27 in real-time

Next, a recipient strain, which encodes orthogonal ParB proteins (Nielsen *et al*., 2006) fused to fluorescent reporters (MA8058 ΔpSLT Δara::tetR-Km-CFP:P1parB-YGFP:pMTparB), was assembled, to visualise the double parS tagged R27 plasmid. These proteins are under the control of an inducible, non-leaky promoter PLtetO-1 which is repressed by TetR. The system can be induced with 200 ng/mL anhydrotetracycline (ATC). We reasoned to assemble cognate parB proteins fused to fluorescent markers into a chromosomal operon under the control of an inducible promoter to avoid ectopic expression from the plasmid. The strain was constructed through 3 consecutive steps. First, the arabinose operon was deleted from MA8058 ΔpSLT strain and replaced by tetA-sacB cassette by positive selective on tetA. Second, the cognate parB proteins translationally fused to fluorescent reporters (CFP:P1parB-YGFP:pMTparB) were PCR amplified and electroporated into MA8058 ΔpSLT, pkd46, Δara::tetA-sacB to replace tetA-sacB under the native arabinose promoter by negative selection against sacB/tetA (Li *et al*., 2013). Finally, the native arabinose promoter was crossed out and replaced by an inducible, molecularly evolved PLtetO-1.

Next, To validate both the reporter plasmid and the recipient strain, pR27 plasmid labelled with pMTparS and P1parS (R27 [ΔhtdA::frt-Cm-frt-pMTparS ISEcp1-P1parS-$_{blaCTX-M-14b}$]), was conjugated to the recipient strain (MA8058 ΔpSLT Δara::tetR-Km-CFP:P1parB-YGFP:pMTparB) and the resulting transconjugants were tracked in real-time with fluorescence microscopy (AB-glycerol at 30°C; ATC at final conc. 200 ng/ml). This results in overlapping

foci between the green (YGFP; the plasmid backbone) and blue (CFP)(the transposon) channel. The foci are shown in the mid-cell at 0 min,18 min, and 36 min (after cell division). At 90 minutes two foci are segregated from the mid-cell before cell division (Lawley *et al*., 2003). Recipient cells that carry WT pR27 showed only diffused fluorescence (data not shown) indicating that the localised green and blue foci are likely due to the pMT-parS (plasmid backbone) and P1-parS (transposon) respectively.

Chapter 7. Tracking the transfer dynamics of pR27 in real-time via microfluidics

The next step was to track the conjugation of the R27 plasmid using time-lapse fluorescence microscopy. This was performed numerous times but did not yield any conjugation events. Recent research performed by Carranza *et al*. (2021) also did not observe any conjugation events with time-lapse fluorescence microscopy. They hypothesise that the agar pad might be inhibiting the conjugation. Nolivos *et al*. (2019) were able to capture conjugation events in real-time only via a microfluidic platform. Hence, we reasoned to use a microfluidic platform to track the conjugation in real-time. Donor cells (MA8058 ΔpSLT; Δhns; pR27 [ΔhtdA::frt-Cm-frt- pMTparS -ISEcp1-P1parS-$_{blaCTX-M-14b}$]), were mixed with recipient cells (MA8058 ΔpSLT Δara::tetR-Km-CFP:P1parB-YGFP:pMTparB) in 1:4 ratio respectively and loaded into a chamber in a microfluidic chip. Time-lapse fluorescence microscopy was performed at 25°C with an inverted Ti-Eclipse, Nikon microscope.

GFP filter (FITC single emission filter and quad-edge dichroic filter-395/470/550/640 nm) and CFP filter (Emission filter Em475/20+triple excitation filter) were used. Snapshots of the cells were made every 15 for a total of 6 h. After 90 min foci started to emerge indicating a transfer event.

Chapter 8. In Vivo dynamics of host H-NS: Construction of host- H-NS translational fusions and Δhns

H-NS was translationally fused to a fast-maturing fluorescent protein, SYFP2, with a maturation time of 4.1 ± 0.3 min (t50) at 37°C. A C-terminal translational fusion was constructed using a flexible linker GSAGSAAGSGEF as previously reported by Gao *et al*. (2017) (MA8058 pKD46 ΔpSLT hns:SYFP2-frt-Km-frt,). The construct was confirmed by both PCR and sequencing. Since SYFP2 might be blocking the native function of H-NS, a second version of the translational fusion was made. Different fluorescent proteins might have different physical properties in terms of phototoxicity and photostability, that's why a second translational fusion protein was made by fusing H-NS to mCerulean3 as described before (MA8058 pKD46 ΔpSLT hns:mCer3-frt-Cm-frt, the construct was PCR and sequence confirmed.

Next, hns knock-out mutant was made to phenotypically compare the impact of the H-NS translational fusions. The ORF of hns was replaced by frt-Km-frt except for the first 11 amino acids (MA8058 pKD46 ΔpSLT Δhns::frt-Km-frt. Knocking-out hns is known to decrease swarming because H-NS is normally regulating gene expression of flagellar genes. Furthermore, the deletion of H-NS is kown to cause a growth deficiency in *S.* Enterica. The phenotypic comparison was thus based on a growth assay and swarming motility assay. Since fusing H-NS with mCerulean3 or SYFP2 might lead to a knock-down (or even knock-out) of H-NS, the swarming motility of the strains was tested and compared to the wild-type (MA8058

pKD46 ΔpSLT) and a Δhns strain (MA8058 ΔpSLT Δhns::frt- Km-frt). The hns:SYFP2 shows no significant difference in swarming and growth compared to wild type.

Chapter 9. pR27 partially complements a Δhns swarming and growth phenotype

Forns *et al*. (2005) showed that pR27 partially complements Δhns phenotypes like growth (at 25°C) and hemolysin production in *E. coli*. To test this complementation in *S.* Enterica, the double parS labelled pR27 plasmid (R27 [ΔhtdA::frt-Cm-frt- pMTparS ISEcp1-P1parS-$_{blaCTX\text{-}M\text{-}14b}$]) was conjugated to the Δhns strain (MA8058 pKD46 ΔpSLT Δhns::frt-Km-frt). The resulting transconjugant was used for a swarming motility assay and compared to the Δhns and wild-type strain. The transconjugant strain shows a significant difference from both the Δhns strain ($p = 1.1 \times 10\text{-}4$), and the wild-type strain (***p*** = 0.024,), meaning that a partial recovery to the wild-type phenotype occurred. Next, growth experiment was also performed for (A) wild-type cells (MA8058 pKD46 ΔpSLT), (B) Δhns (MA8058 pKD46 ΔpSLT Δhns::frt-Km-frt) and (C) Δhns complemented with double parS labelled pR27 (R27 [ΔhtdA::frt-Cm-frt- pMTparS ISEcp1-P1parS-blaCTX-M-14b]). Almost no cell divisions were observed for the Δhns strain, while Δhns cells complemented with the pR27 plasmid (C) were able to partially recover the growth-deficient phenotype.

To test the interchangeability of plasmid-borne H-NS and host-borne H-NS copies, multiple sequence alignments were performed for distinct groups of H-NS proteins, including native H-NS, StpA, IncHI and IncF plasmid-derived H-NS. Dorman *et al*. (1999) showed that the H-NS protein family has a conserved DNA- binding domain (TWTXGRXP). Our multiple sequence alignments data suggested the conservation of the 'TWTXGRXP' DNA-binding domain among native H-NS, IncHI, and IncF. The similarities between the DNA-binding domains of native H-NS, and IncHI-derived H-NS might explain the partial complementation of the growth phenotype and the swarming assay of an hns knock-out strain since they might be binding to similar DNA sequences. Moreover, Deighan *et al*. (2003) observed that Sfh (H-NS protein from pSF-R27 from IncHI), and native H-NS can interact with each other. This is supported by Fitzgerald *et al*. (2020) who observed that the residues for dimerization are conserved between the native H-NS and IncHI-derived H-NS. Yet, The exact function of plasmid-derived HNS is largely obscure thus we opted to C-terminally fuse pR27-H-NS to mScarlet to be able to track in real-time the cross-talk between the host-HNS (hns: SYFP2) and the pR27-born H-NS(pR27 hns:mScarlet; figure 5). In short, pR27 H-NS was translationally fused to a fast-maturing fluorescent protein, mScarlet-I, with a maturation time of 36 min. The C-terminal translational fusion was constructed using a flexible linker GSAGSAAGSGEF as previously reported by Gao *et al*. (2017).

Chapter 10. Investigating and visualising the interplay between R27-H-NS and host H-NS in real-time

We opted to visualise in real-time the conjugation of donor cells carrying pR27-hns:mscarlet-I and recipient cells with fluorescently labelled HNS (hns:SYFP2) in order to analyse the timing and level of expression of pR27 H-NS and also to assess the crosstalk between plasmid born and host born H-NS copies. In short donor cells were mixed with recipient cells in 1:4 ratio respectively and loaded into a chamber in a microfluidic chip. Time-lapse fluorescence microscopy was performed at 25°C with an inverted Eclipse Ti2-E microscope (Nikon), using NIS software for image acquisition. Acquisitions were performed using 50% power of a Fluo

LED Spectra X light source at 488 and 560 nm excitation wavelengths. Exposure settings were 100 ms for sYFP2 and mscarlet-I and 50 ms for phase contrast. Image acquisition of the cells were made every 5 mins for a total of 6 h. The timelapse primary observations indicated that host and plasmid-born H-NS are overlapping and nucleoid bound and expression of R-27 H-NS in the newly formed transconjugants gradually increased over time and reached donor level expression after 20 min of plasmid acquisition. Next, we are planning to perform ChIP-seq analysis to probe for host's candidate genes targeted by plasmid-born H-NS.

Finally, to track in real-time conjugation dynamics of pR27 and the subsequent transposition of ISECP1-$_{blaCTX-M-14B}$ from the pR27 to the chromosome, we have constructed an over-expression pBAD33 plasmid carrying the transposon (ISEcp1), under strong inducible promoter aiming to increase the transposition frequency. Yet the effect of overexpressing the transposon on the transposition frequency needs to be further evaluated through optical microscopy *in vivo* and GFP hopper assay (Saito *et al*., 2010) *in vitro*. In short, pBAD- ISECP1 overexpression plasmid could theoretically modify the transposition frequency of a promoter-less variant of GFP (regular green fluorescent protein (GFP) which lacks transcriptional and translational start sites) flanked by the canonical inverted repeats (IRR) sequences recognised by the ISEcp1 transposase enzyme. Upon transposition and chromosomal integration, GFP will be expressed and can be detected and sorted by using Fluorescence assisted cell sorting (FACS) and hence we could potentially have a quantitative estimation of the transposition rate *in vitro*.

PhD self-assessment

As all PhDs pursued during the pandemic, it was an extraordinary time for research. Directly due to consequences of the SARS-CoV-2 crisis, the original candidate (Jasper Van der Peet) had to return permanently to The Netherlands in M28. He was replaced by Alaa Albasiony who picked up activities in M34. This caused an inevitable delay in lab progress, and a switch from a more bioinformatic profile to a strong cell biology profile – which is evident from the lab report described in section 2.

The focus through the PhD remained on the transfer dynamics of IncHI type plasmids, abundant in Enterobacterales and very often associated with drug resistance. Through findings in mainly the second year of the project, research focus was led to H-NS type proteins. These complexes are silencing gene expression in bacterial cells, and we showed their role in conjugation and transfer of mobile genetic elements. As this research particularly required real-time microscopy (which was not defined in such detail in the original project application).

A second deviation was the inclusion of microfluidics. To confirm our findings and to quantify the observed plasmid transfers, a research visit to the world-renown laboratory of Prof. Christian Lesterlin (CNRS) was performed in Q1 of 2023. This visit allowed to generate conclusive data on the role of plasmid- and chromosomal-encoded H-NS proteins and will undoubtedly lead to high-end publications by the end of the year.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
KENTUCKY	D-PhD04-1.1	Genomic Characterization of clinical Salmonella Kentucky strains	M32	M35		3
KENTUCKY	D-PhD04-1.2	Construction of reporter strain, able to show transfer of ESBL gene between plasmid and chromosome	M42	M47		8
KENTUCKY	D-PhD04-1.3	Paper: (Tentative title). Characterization of factors influencing the transfer of ESBL genes from plasmid to chromosome.	M60	M70	Given the results explained in section 2, we aim for publication in Q4 of 2023	8
KENTUCKY	D-PhD04-1.4	Paper 3 (Tentative title). The prevalence of ISecp-1 mediated transfer of ESBL genes to the chromosome among clinically isolated Enterobacteriacea	M60	M71	Given the results explained in section 2, we aim for publication in Q4 of 2023	8
KENTUCKY	D-PhD04-1.5	Thesis dissertation	M60	M72	The thesis will be defended before the end of 2023	8

Milestones

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
KENTUCKY	M-PhD04-1	Quality training completed at Sciensano	M23	M36	Yes	Needed to be repeated for second PhD candidate
KENTUCKY	M-PhD04-2	S. Kentucky strain and genome collection completed	M30	M34	Yes	
KENTUCKY	M-PhD04-3	Completion of hybrid assemblies of four S. Kentucky strain	M36	M38	Yes	
KENTUCKY	M-PhD04-4	Finalization of characterization of MGEs and selection of elements for further study at KULeuven	M46	M50	Yes	
KENTUCKY	M-PhD04-5	Research visit to INRAE	M52	No	Yes	

Publications and additional outputs

Publications

At the time this deliverable was submitted, the KENTUCKY PhD project has not published any scientific publications.

Additional outputs (i.e., poster/oral presentations)

The KENTUCKY PhD project disseminated these works through oral and poster presentations at the following events:

- Albasiony, A., Ceyssens, PJ., Aertsen, A. (2021) Exploring the ISECP-1 mediated chromosomal integration of blaCTX-M-14 in *Salmonella* Kentucky. Annual Scientific Meeting OHEJP, June 9-11th, 2021. Poster presentation.
- Albasiony, A., Ceyssens, PJ., Aertsen, A. (2022) Exploring the evolutionary success of the antibiotic resistant *Salmonella* Kentucky ST198. Annual Scientific Meeting OHEJP, April 11-13th, 2022. Poster presentation. More information can be found here.
- Albasiony, A., Ceyssens, PJ., Aertsen, A. (2022) Exploring the evolutionary success of the antibiotic resistant *Salmonella* Kentucky ST198. International Symposium Salmonella and Salmonellosis I3S2022. June 20-22, Saint-Malo, France. Oral presentation.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Supervised Master Student thesis (Bioscience Engineering, Gene Technology)	Supervision skills	01/09/2021-30/06/2022	KU Leuven
Technology transfer and exploitation of research.	Research valorization	25/10/2022-10/04/2023	KU Leuven
Supervising Master Student thesis (Bioscience Engineering, Gene Technology)	Supervision skills	01/09/2022- 30/06/2022	KU Leuven
Advanced biological data analysis	Data analysis	29/09/2021- 31/12/2021	KU Leuven
Presentation and seminar skills	Soft skills	01/03/2022- 30/06/2022	KU Leuven

One Health impact

As reflected above, this PhD focused strongly on the molecular mechanisms behind the transfer of antimicrobial resistance genes in and between bacteria. A very large part of the work expanded on the molecular mechanisms behind this transfer, are quite fundamental and cannot be directly translated to policy measures.

However, we observed that a particular mobile genetic element which is highly prevalent in bacteria, ISECp1, is frequently associated with genes provoking resistance to third generation cephalosporins. Our data shows that this element enables the transfer of these AMR genes from plasmids to chromosomes, leading to fixation of the drug resistance in the genome and vertical heritage of resistance.

Although the net result stays the same (i.e., a drug resistant strain), the public health impact of plasmid vs. chromosomal carriage of drug resistance is different. Once transferred to the chromosome, resistance is stable and can for example no more be reduced by reducing selective pressure. Therefore, results from this thesis strongly argue for expanding surveillance to the genetic regions flanking AMR genes, to infer their mobility and potential spread within and between bacterial species.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

A great advantage of working in collaboration with public health institutes was the availability of contemporarily collections of Salmonella isolates. As shown above, it was by studying these clinical strains that the transfer of the mobile AMR genes has been identified. Another great advantage was the wide availability and easy access to very specific expertise in the field of plasmid biology. By reaching out and discussing with scientist of the OHEJP network, novel experiments were designed and performed.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The KENTUCKY PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- Long-read sequencing was performed according to methodology derived from the Full Force project (JRP-14). Short-term travel grant to CNRS (France) was obtained through MVNA and performed in Q1 of 2023.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	3	4
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	4	3
Were all the milestones and deliverables completed?	3	3
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	4	5
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	5
Did the PhD student actively engage in Education and Training activities?	5	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	2	3
Was the PhD managed and implemented in accordance with the DMP?	-	4
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	4	4
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	4	3
Is there any direct or indirect impact of the project for national or international stakeholders?	2	2
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	3	4
Does the project outcomes have policy implications?	4	2
TOTAL	42/65	47/65

AVERAGE: 44.5/65

NB. Note that reviewer 1 did not provide scores for one question, which may affect the overall score.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The subsequent focus of the project in the future is mentioned in the report.

Reviewer 3: PMT member

Agree (3).

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Deviations were justified.

Reviewer 2: External Scientific reviewer

A most research, this project had to deal with the consequences of the pandemic, just as delay but also change of PhD candidate. In relation to the findings, the focus of the project was adapted towards H-NS proteins. This however left some of the original objectives less investigated.

Reviewer 3: PMT member

Agree (4) - The project was complex, but the results obtained here intersect with the objectives, and these results were very good and above average, taking into account, as the report states, that "research was quite fundamental and mainly focused on academic work to understand the basics of transfer of AMR genes".

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Milestones completed; deliverables are foreseen to be completed.

Reviewer 2: External Scientific reviewer

The research visit didn't take place but was replaced by another. Goals for the redaction of papers are set at Q4 of 2023, although from the report I would deviate that still some research is needed (according to the tentative titles).

Reviewer 3: PMT member

Agree (3).

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

JRP-14 & CNRS (France)

Reviewer 2: External Scientific reviewer

Interaction with JRP and externals has taken place.

Reviewer 3: PMT member

Agree (5).

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The interaction with the network made it possible for the student to work with experts in different fields and brainstorm on the project.

Reviewer 3: PMT member

Agree (5).

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The student was enrolled in trainings and surprised Master students.

Reviewer 3: PMT member

Agree (5).

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

Publications are foreseen for Q4 2023

Reviewer 2: External Scientific reviewer

Publications are planned in Q4 of 2023

Reviewer 3: PMT member

Agree (2) - Publications are only planned, and it is not possible to be writing 4 papers at the same time.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree (non applicable).

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

Conferences were attended.

Reviewer 2: External Scientific reviewer

The student performed 2 poster presentations and 1 oral presentation.

Reviewer 3: PMT member

Agree (4).

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The focus of the project was fundamental but highlights the importance of monitoring AMR genes/mobile elements in the view of the One Health EJP objectives.

Reviewer 3: PMT member

Agree (4).

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

This was not the focus of the project, which is on unravelling the molecular mechanisms of AMR genes transfer. However, it suggests the importance of the surveillance of these mobile elements.

Reviewer 3: PMT member

Disagree with (2), but would agree with (4), namely reading the justification of Reviewer 2. ("… However, it suggests the importance of the surveillance of these mobile elements….").

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Interactions with other partners was leading to the current outcome of the project. Experts in different fields were collaborating with the student.

Reviewer 3: PMT member

Agree (4).

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

Although not directly translated to policy measures, recommendation to expand surveillance to the genetic regions flanking AMR genes, to infer their mobility and potential spread within and between bacterial species.

Reviewer 2: External Scientific reviewer

This was not the focus of the project, which is on unravelling the molecular mechanisms of AMR genes transfer. However, it suggests the importance of the surveillance of these mobile elements.

Reviewer 3: PMT member

I agree (4), as Reviewer 1 so well justifies. This is in line with the evaluation of "Is there any direct or indirect impact of the project for national or international stakeholders?".

The full Final Thesis Report for the KENTUCKY PhD project can be found on Zenodo.

PhD5-AMR2/6.1/ET5-METAPRO

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Bosco Matamoros Rodríguez	PhD Student	Complutense University of Madrid	Spain
Bruno González Zorn	PhD Lead Supervisor	Complutense University of Madrid	Spain
Roberto La Ragione	PhD Second Supervisor	University of Surrey	United Kingdom
Phillipe Glaser	Other Supervisor	Institut Pasteur	France

Summary of the work carried out in the PhD project

The METAPRO PhD project began in M27, in 2020 and is expected to finish in M67, in 2023. A seven-month extension was provided due to the SARS-CoV-2 pandemic. METAPRO focused on the application of a metagenomic approach for the study of the resistance determinants to next generation aminoglycosides in ecological niches from human, animal, and environmental origins, and the investigation of non-pathogenic bacteria in the acquisition and maintenance of these genes. More specifically, the aims of the project were to:

1. Evaluate the prevalence of next-generation aminoglycoside resistance genes in different ecological settings.
2. Elucidate the bacterial and ecological reservoirs of resistance determinants to next generation aminoglycosides, with a special focus on the low abundant acquired 16S rRNA methyltransferases.
3. Predict the potential dissemination routes and describe the clones and mobile genetic elements that entail a major risk of aminoglycoside inefficiency.
4. Analyse the effect of the use of antibiotics in the promotion of selection of next-generation aminoglycoside resistance genes.

The results show the prevalence of acquired 16S rRNA methyltransferases was higher than expected, because instead of detecting the highly reported enzymes *armA* or *rmtB*, lower reported enzymes like *rmtD*, *rmtF*, *rmtG* and *npmA*. The focus of the work was on the enzyme *npmA*, as it is found in both humans and animals in high prevalence, and it has been described as the enzyme with the broader aminoglycoside-resistance spectrum. It was found in different genetic environments, plasmatic, and chromosomic, and seem to be different in the different settings where it has been recovered.

In a more comprehensive study of the distribution of the gene it was observed that it is already spread worldwide and that up to nine different variants of the gene can be found. However, current metagenomic analysis did not allow for the detection of the specific reservoirs of the

gene in the different sources. Therefore, the project tested the possibility of detecting the gene using a combination of fluorescence *in situ* hybridisation (FISH) with fluorescence activated cell sorting (FACS) to be able to isolate single cells that throw some light on the bacterial species and genetic elements that maintain *npmA* in the different environments. The preliminary results on a control model evidenced promising results as ~50% of the *npmA*-containing cells can be sorted effectively. Finally, we evaluated the effect of the aminoglycoside apramycin, an antibiotic in the development pipeline to be the next one released for its use in humans, pending FDA approval. We observed that apramycin application has no effect on the bacterial composition, but strongly selected for apramycin resistance genes aac(3)-IV and *npmA*, that seem to be a common component of the gut microbiome.

Overall, the present works exhibits that 16S rRNA methyltransferases, and specifically *npmA*, might be a great concern once old and next-generation aminoglycosides start to be used more widely as their prevalence might be underestimated. Understanding which bacteria and genetic elements act as reservoirs can help us design strategies to limit their spread to pathogenic bacteria and prevent the inefficacy of all the aminoglycosides yet to come. A great example of the actual problematic is the use of the next-generation aminoglycoside apramycin, that greatly selects for apramycin resistance genes and promotes their mobilisation, so it can become ineffective in a short time after its approval for human use.

Dissemination activities of this METAPRO PhD project were presented at the national (i.e., XXVIII Congreso de la Sociedad Española de Microbiología) and the international level (i.e., European Congress of Clinical Microbiology and Infectious Diseases).

Work carried out on the PhD, scientific results, and outcomes

Chapter 1. Acquired 16S rRNA methyltransferases in Spain

A total of 884 Spanish metagenomic samples from 13 different projects were found in the SRA database (816 Human, 28 pig and poultry, 20 of animal origin, 13 waste water, 5 freshwater and 2 of environmental origins). All the samples were screened mapping the reads of the different metagenomic samples to the Resfinder database to see if they contained any acquired 16S rRNA methyltransferase. Of the 884 samples surveyed, 61 presented one or more methyltransferases. In terms of origin, no 16S rRNA methyltransferases were detected in environmental samples. However, a 3.1% of human samples, a 15.4% of wastewater samples, a 50% of poultry samples and an 85.7% of the pig samples showed the presence of methyltransferases. The enzymes detected is *rmtD*, *rmtF*, *rmtG*, *rmtH* and *npmA*. *rmtF* is the most prevalent, been found in 33 samples, closely followed by *npmA*, present in 26 samples. Breaking it down per sample type, *rmtD* is the most prevalent methyltransferase in humans, being detected in 10 samples, followed by *rmtF* and *npmA*, present in 8 samples each, and *rmtH*, in 3. In pig samples, 23 out of the 28 samples investigated showed the presence of *rmtF*, and 8 out of the 28 presented *npmA*. Interestingly, *rmtG* and *rmtD* were also detected in pig samples once and twice, respectively. In poultry samples the only methyltransferase detected was *npmA*, present in half of the metagenomes analysed. Finally, in wastewater only *rmtF* was found in 2 out of the 13 samples investigated.

The relative abundance of the different genes in the different environments was evaluated in reads per kilo base per million mapped reads (RPKM). In general, the relative abundance of all the acquired 16S rRNA methyltransferases was low. Most of the samples with independence of the resistance gene detected or the origin show RPKM values between 0.1 and 1. The highest abundance values are observed in *npmA*, that show a peak of abundance in 16.5 RPKM. However, in some samples it is also detected a very low relative abundance (0.08 RPKM). Similar values are shown by *rmtD2* in two human samples (4.67 and 8.12 RPKM), but since these are the two projects with the less amount of reads the abundance can be overestimated. Again, in some samples *rmtD2* is barely detected, with a 0.05 RPKM value.

Nevertheless, differences in the abundance per origin of the sample can be detected for some of the methyltransferases. For example, *npmA* in humans and pigs show a similar abundance, but in poultry is much higher (Tukey HSD, $\boldsymbol{p} < 0.01$). On the other hand, *rmtF* levels in humans and pigs show a similar trend, while in wastewater is lower, but no statistical difference can be detected (ANOVA, $\boldsymbol{p} = 0.192$).

Since mapping approaches are known for the high possibility of false positives, confirmation of the presence of the 16S rRNA methyltransferases was attempted with an assembly approach. Due to the low abundance of the methyltransferases detected, *rmtD*, *rmtF*, *rmtG* and *rmtH* were not detected in any of the assemblies. On the other hand, *npmA* was assembled in a contig in 9 out of the 26 samples where the gene was detected by the mapping approach. All the contigs correspond to poultry samples. The *npmA* gene, corresponding to the *npmA1* variant, can be detected in two different genetic contexts. The first genetic environment presented with high nucleotide identity with the original context where the gene was firstly described in 2007, as well as with the *pTRACA22* plasmid, a plasmid that has not been linked to any bacterial species yet. However, all the open reading frames (ORFs) surrounding *npmA* in these contigs have more than a 90 percent of identity at the protein level with different bacteria from the order Eubacteriales. The second environment found is completely new and presents as well high-level identity at a nucleotide level with different genera from the Eubacteriales order. At the protein level, most of the ORFs have > 99% of identity with proteins from *Clostridioides difficile*.

Further, we wanted to see to what extent these genetic contexts can be associated with a plasmid. Based on the previous results of the machine learning classifier PlasX, the first environment had a high probability of being of plasmatic origin (0.96), while the second one is most likely not a plasmid (0.04). These results correlate with the ORF study, as in the first context we can see functions normally associated with plasmids, like toxin-antitoxin systems or a plasmid recombination enzyme, as well as being detected this similar context in the unknown plasmid pTRACA22 of the human gut microbiome. On the other hand, the second context observed present a relaxed, which could suggest a plasmatic origin. However, the rest of the ORFs seem to form part of the normal chromosome of different Eubacteriales.

Binning of the contigs containing *npmA* was performed with three different binning tools. 16 different bins from 6 of the *npmA* positive samples were produced. Except for one, all the bins were highly uncomplete, highly contaminated or both. However, running a cleaning tool on the bins removed the *npmA* contigs from them, as they may have been included incorrectly.

Targeted metagenomics allow for the identification of specific genes in a complex metagenome background. A sequencing capture platform for antimicrobial resistance genes has already been designed and implemented in the project PRJNA371649 (Lanza *et al*., 2018), that forms part of the dataset of the present study. The same 8 pig faecal samples were analysed both by conventional shotgun metagenomics and using this targeted metagenomics method. Interestingly, when with conventional metagenomics only half of the samples presented an acquired 16S rRNA methyltransferase, after the application of the capturing platform all samples presented both *rmtF* and *npmA*. In addition, 5 samples harboured *rmtG* and 2 samples presented *rmtD*.

From the assembly approach we were able to retrieve three out of the four methyltransferases but not in all the samples that contained them. Thanks to the capturing of more reads, *npmA* was assembled in all the samples, but only in 6 of them the gene was complete, as one contig was falling short and in other the *npmA* gene was interrupted. In four of the contigs the *npmA2* variant was found, while two of them showed a potential novel *npmA* variant not found to date. In this new variant, the C124T nucleotide mutation with respect to both *npmA1* and *npmA2* produce an aminoacidic change of a leucine for a phenylalanine at position 42. In addition, the *npmA3* variant also shows the same mutations in positions 330, 393 and 450 that *npmA2* presents against *npmA1*. All six contigs with the gene complete show a similar genetic environment, that differs completely from the genetic contexts where *npmA1* and *npmA2* have been found so far, as well as the genetic context of *npmA* in poultry. The *npmA* gene seems to be also associated with an IS30 like the *npmA2* gene, but in this research's case, it seems that is from the same family, but it is not the same insertion sequence.

With the same approach, *rmtF* was assembled in 7 out of the 8 samples, but 3 of them did not cover the whole length of the gene. The other 4 present various polymorphisms with respect to *rmtF1* and *rmtF2*. All of them present the A193G characteristic of *rmtF2* in comparison to *rmtF1*, and up to 23 different positions are mutated in the 4 *rmtF* genes, presenting all different mutation profiles. These mutations lead to aminoacidic changes that make our finding to be potentially considered as 4 new *rmtF* variants.

In the contigs produced, only two were long enough to detect an ORF flanking *rmtF* in its upstream region. This upstream ORF present high nucleotide identity with regions found in uncultured bacteria from human, and a protein level has high identity with a tRNA-guanine transglycosylase related to Clostridiales bacteria.

The low abundant 16S rRNA methyltransferase *rmtG* was also assembled in one of the samples. The *rmtG* recovered presented 25 polymorphisms with respect to the canonical *rmtG* sequence reported. All the nucleotide changes produce 11 aminoacidic modifications (95.8% protein identity with *rmtG*), that is at the border of been considered a new methyltransferase. In the *rmtG* contig there is not much information about the genetic context where the gene is located (46 base pairs upstream and 293 base pairs downstream), but it seems to be already detected in uncultured bacteria from human habitats.

Acquired 16S rRNA methyltransferases seem to be more prevalent that the global genomic studies tell mainly due to its maintenance in non-pathogenic bacteria in the gut microbiome of humans and different animal species. It is especially worrisome the case of *npmA*, a low abundant methyltransferase with the widest range of aminoglycoside resistance. In the Spanish dataset it appears to be part of the gut microbiome of humans, pigs, and chickens, but the exact bacterial reservoir in the different environments is still not known. However, due to the great efforts performed by other research groups, we had access to a major screening of antimicrobial resistance genes in shotgun metagenomic samples (Martiny *et al*., 2022).

In the 214,095 sequencing runs analysed, the *npmA* gene was detected in 1437 of them (0.67%). Breaking down the samples by origin, it can be seen that, as expected from the projects previous results, most of the samples (94.5%) come from human, poultry, and pig origins, with 657, 385 and 316 positive runs, respectively. Interestingly, *npmA* was detected in other animal sources; cow (n = 10) or deer (n = 4), but also in environmental samples from wastewater (n = 8) or air origin (n = 14).

An analyse of the percentage of projects positive *npmA* form the total number of sequencing runs analysed, it was confirmed the observations seen in the Spanish dataset. Less than a 1% of the human samples were *npmA* positive, but the prevalence of *npmA* in pig and poultry origins is way higher, being a 9.7% and 26%, respectively.

If we divide the *npmA* positive samples per country of origin, we can observe that this 16S rRNA methyltransferase is globally spread. The *npmA* methyltransferase can be detected from all the continents except for South America, but the low number of metagenomic projects of these countries might have contributed to this result. The country with most positive samples was China with 54.5% of the *npmA* positive projects, followed by Australia with a 15.5% and by the United States of America with 6.5% of all the samples. In Europe, the country with a greater number of samples was Germany, with the 3.3% of the samples, followed by France with 2.6% and Spain with 1.8% of the positive samples.

The mapping approach of the *npmA* positive samples was followed by an assembly-based analysis. Due to limited computational capacity, only samples selected were though mapping, which showed that the *npmA* gene was completely covered. Using this filter, the projects dataset was reduced to 378 samples. From the assembly of these 378 samples, 350 contigs containing *npmA* were produced. The 350 *npmA* positive contigs come from different sources and different countries. As expected, most of the contigs come from the Chinese samples, but the other 12 countries from different continents are also represented. In terms of source of the samples, poultry, human and swine origins are the most represented, but also 2 contigs from hospital surfaces and 1 from cattle form part of the dataset.

The project extracted the *npmA* gene from the 350 contigs produced to evaluate the genetic variants more prevalent in the world. The variant most represented is *npmA1*, encountered a total of 291 times (an 83.1% of the samples). The *npmA2* variant was only detected in 3 samples (0.86%). Interestingly, 15 samples presented different polymorphisms in the *npmA* gene unknown, being potentially novel variants of the gene. In the end, only 11 *npmA* genes

presented aminoacidic substitutions, resulting in the description of 6 potential novel variants, named *npmA4 – npmA9*.

Several residues of *npmA* have been marked as essential for the aminoglycoside resistance function in the literature (Husain *et al*., 2011; Dunkle *et al*., 2014). Interestingly, these critical residues are maintained in all the new variants found from metagenomic samples and, therefore, they should maintain the methylation of the A1408 of the 16S rRNA methyltransferase and the resistance spectra.

Chapter 3. Single cell genomics for the detection of npmA

Based on the results obtained in the first chapter of this section, it was estimated that the abundance of the reservoirs of the *npmA* resistance gene is in the limit of the detection and, since it is frequently associated to mobile genetic elements, conventional metagenomic analysis fall short to identify the bacteria that contribute to its maintenance in the gut metagenome. Here we describe a method that combines fluorescence labelling of the resistance gene with fluorescence activated cell sorting for the isolation and sequencing of single cells harbouring the *npmA* gene.

Following the selection criteria proposed, three probes with the most appropriate characteristics were selected for fluorescence *in situ* hybridisation of the *npmA* gene. Probes with a length of less than 20 base pairs were discarded because of high levels of hybridisation to different bacterial species commonly found in the gut microbiome. A threshold of 80% or less of homology was set to ensure that unspecific binding to other bacterial targets can be avoided. The probes were selected to have different unspecific binding profiles and to be separated at least 50 base pairs from each other to cover different topological positions in the gene. The red fluorescent dye Atto647N was attached to the 5' end of the probes.

To evaluate whether the *in silico* designed probes perform as expected and are suitable for the detection of *npmA* bacterial reservoirs, the resistance gene was inserted in the pCR2.1 plasmid. pCR2.1 plasmid has a pBR322 origin of replication, that makes it have around 20 to 40 copies per cell. Both pCR2.1 and pCR2.1::*npmA* were introduced in *Escherichia coli* K-12 to be used as negative and positive control of probe hybridisation, respectively. Microscopic visualisation after application of a conventional FISH protocol with 2.5 ng/μl of probe npmA_P1 to an overnight culture of positive and negative control strains revealed that a very low proportion of cells stained.

Applying an ethanol dehydration step has been reported to enhance the hybridisation of the probes. Results of hybridisation of 2.5 ng/μl of probe *npmA*_P1 after the ethanol dehydration step showed that more than an 80% of the cells in the positive control stained, while no cells stained in the negative control. Increasing *npmA*_P1 probe concentration to 8 ng/μl improved Atto647N signal, but a similar proportion of cells were stained as with lower concentration. Nevertheless, the signal intensity varies between cells and sometimes the inclusion of a cell as stained or unstained is subjective. Probe *npmA*_P2 was also evaluated with ethanol dehydration and different probe concentrations (ranging from 2.5 – 15 ng/μl). Again, around 80 – 90% of the cells positively stained while in the negative control none of them did. On the other hand, with independence of the *npmA*_P3 probe concentrations tested, no fluorescent signal was captured for any of the cells in the positive control with this FISH protocol. In

addition, combination of *npmA*_P1 (8 ng/µl) and *npmA*_P2 (15 ng/µl) was evaluated to see the performance in terms of signal intensity and percentage of cells stained. Surprisingly, in the visual observation of the microscopic images, a slightly lower proportion of cells stained (~72% of cells).

Hybridisation efficiency of probes *npmA*_P1 and *npmA*_P2 was further analysed via combination of FISH with fluorescence activated cell sorting (FACS). First, the negative control was analysed to set the gates for population and negative fluorescence in the corresponding scattergrams. The study of the positive control followed and based on the gates proposed by the negative control, it can be clearly defined as a subpopulation of cells distinctively marked with the red fluorescence provided by Atto647N dye that can be gated and sorted out for further analysis. If we focus on the numbers, it can be seen that a 46.3% of the cells can be sorted giving margin to avoid potential false positives. This same experiment was performed with mock communities with different ratios of negative and positive cells (50:50 and 90:10) to test whether this method is capable of detect *npmA* positive cells even when the abundance is low. The results confirmed what we saw with the positive control alone, being detected around one cell every two as positive to *npmA*, as a 26% and a 5.2% of cells were detected in the positive gate.

The same methodology was used to evaluate the performance of *npmA*_P2 probe. Interestingly, *npmA*_P2 probe seems to work slightly better than *npmA*_P1, as it detects almost a 4% more of cells as positive to *npmA* leaving also margin to avoid potential false positives. In the mock communities, this better performance is not maintained. A 23.4% of cells can be sorted in the positive gate in the 50:50 mixture, while only a 3.6% of the events could be sorted from the 90:10 community.

Chapter 4. Effect of apramycin use in the selection of apramycin resistance genes in poultry

In the present study, metagenomic sequencing was used to analyse the gut microbiome of poultry to determine the effects that the use of apramycin has on the microbiome composition. For that purpose, the analysis of twenty Spanish poultry farms with antibiotic use known collected within the EFFORT project were used. The twenty sampled farms were divided into two groups based on apramycin use: 1) a treatment group comprised by seven farms that used apramycin at the time of sampling, and 2) a control group that did not use apramycin during that time.

To assess whether apramycin use had any effect on the gut microbiome composition the Shannon-Wiener diversity index was calculated for bacterial composition and antimicrobial resistance gene content from every sample individually. When grouping samples per treatment group it was observed that there was no statistical significance difference in terms of bacterial composition between groups (Mann-Whitney U test, $\boldsymbol{p} = 0.757$). On the other hand, when looking into the diversity of resistance genes in the different farms we observed that those that used apramycin exhibited a higher diversity (Mann-Whitney U test, $\boldsymbol{p} = 0.024$).

To further assess whether apramycin use reshapes the contents of the gut microbiota, the similarity of the bacterial and resistance genes composition of all the samples was compared through the Bray-Curtis dissimilarity index. Plotting the distances between the different bacterial compositions in a Principal Coordinates Analysis (PCoA) shows that all the samples

tend to cluster together with independence of the treatment group. This observation is further confirmed by the statistical analysis performed directly on the distances between samples (PERMANOVA, ***p*** = 0.271). In terms of antimicrobial resistance gene content, using the same methodology, this time a more evident clusterisation based on the treatment group can be observed, being again the statistics confirming the observations (PERMANOVA, ***p*** = 0.037).

Diversity analysis carried out exhibited that apramycin use increased the diversity of antimicrobial resistance genes without having a major effect on the taxonomic composition of the poultry gut microbiome. To determine which features are the main contributors to the differences observed in the antimicrobial resistance gene composition, a Linear discriminant analysis of the Effect Size (LEfSe) was performed. Resistance genes to aminoglycosides (aph(4)-Ia, aph(6)-Id, aac(3)-IV, aph(3'')-Ib, ant(4')-Ib, ant(9)-Ia, *npmA*, *str1*), to chloramphenicol (cmx), to betalactams (bleO) and to tetracycline (tet(O/W)2) are more abundantly found in the farms that used apramycin, while only tetracycline resistance genes tet(O/W)5, tet(O/W)1 and tetK are more frequently found in the control group. Interestingly, many resistance genes to aminoglycosides were more abundant in the apramycin group, but only aac(3)-IV and *npmA* are known to confer resistance to apramycin.

Based on these results, the presence of two apramycin resistance genes and their relative abundance in the different samples were explored. aac(3)-IV gene was detected in all the sample with independence of the treatment group, with abundances ranging from 9.95 – 137.14 RPKM (median 77.41 RPKM) in the farms that used apramycin, and abundances of 0.17 – 116.43 RPKM (median 0.59 RPKM) in the control farms. In contrast, *npmA* does not appear as abundant as aac(3)-IV. *npmA* was detected in all the farms that used apramycin (with abundance ranging from 1.35 – 16.52 RPKM, median 5.08 RPKM), while it was only detected in three out of the thirteen control farms sampled (with abundances of 0.54, 4,82 and 0.13 RPKM). Since *npmA* has been largely studied in the previous sections of this report, here we decided to focus our study on aac(3)-IV resistance gene.

To understand how the apramycin resistance genes became more abundant after apramycin use, an assembly-based approach was used to know the close genetic context of the genes. In the assemblies performed from all the samples, aac(3)-IV gene was recovered from sixteen out of the twenty farms sampled, while *npmA* gene was recovered only in nine of them. Length of aac(3)-IV contigs range from 2528 – 10505 base pairs.

The close genetic context of this gene reveals a high association with other aminoglycoside resistance gene, aph(4)-Ia, an insertion sequence, ISEc59, and a truncated form of a Tn3-family transposase, as in all the contigs obtained these four genes were found together. Four of the contigs were long enough to show the genetic context where this association unit is embedded, showing that it can be found in very different environments. It seems that different insertion sequences can facilitate the mobilisation of the association unit, as an IS26 and an ISL3 family transposase, as well as truncated forms of an IS6100 and an IS5564 are found in the near context. ISL3 is commonly found in different families of the Micrococcales order, IS5564 in *Pseudomonas aeruginosa*, Micrococcales, as well as different species of *Corynebacterium*, and IS26 and IS6100 are highly associated to plasmids belonging to the Enterobacteriaceae family, suggesting that they could be actively mobilising the unit between different bacterial species in the gut microbiome. In addition, the rest of open reading frames present in the contigs confirm the different origins of the aac(3)-IV gene. The partial CDS of a

recombinase found flanking the IS26 is identical to one found in different Enterobacteriaceae plasmids, and the two CDSs encoding a stress protein and a transporter associated to the truncated form of IS5564 show high identity nucleotide levels with different Micrococcales species.

Since aac(3)-IV seems to be highly prevalent in chicken farms, and the project wanted to evaluate the overall prevalence of aac(3)-IV in the world. To do that, we used the NCBI NDARO database (Accessed September 2022) as the designated reference. At the date of consultation, the NDARO database presented 17809 organisms carrying aac(3)-IV out of the 1188814 total organisms included (1.19% of the organisms). From all the positive isolates, a 99.8% correspond to different species from the Enterobacteriaceae family. Based on the origin of the sample where the isolate was obtained, it was observed that human, animal, and environment were represented. Most of the isolates with an origin known come from poultry (4767), followed by human (2968), pigs (1454), and turkey (685). In addition, 422 isolates are labelled as from water or environmental samples. Nevertheless, a 30 percent of the samples did not specify the origin.

In terms of location where the isolates were taken, aac(3)-IV seems to be already worldwide spread. The country that presents the greatest number of isolates is the United States of America, with 8729 (49% of them), followed by China with 3696 (20.75%). In Europe, the countries that accounted for the most isolates were the United Kingdom and Northern Island and Germany, with 631 and 178 respectively (3.54% & 1%).

PhD self-assessment.

Despite a slow start due to pandemic restrictions, we believe that METAPRO has met the two major objectives that we considered in our proposal. In our work we have showed that plazomicin resistance determinants are more prevalent as genomic studies and databases show and we have explored new methodologies for the detection of the reservoirs of such resistance mechanisms. However, to reach this point we needed to do a few adjustments during the project.

Initially focused on plazomicin, we believed that the aminoglycoside class has more to offer than just this compound. We found that there are many projects focused on obtaining semi-synthetic derivatives from all aminoglycoside subclasses, as well as clinical trials to release apramycin for human use. Therefore, we decided to target in METAPRO the resistance mechanism that can prevent the action of the old and the next generation aminoglycosides, the acquired 16S rRNA, methyltransferases, as well as deepening the understanding of the effect of the use of apramycin, as it may be the next antibiotic released to the market.

The project was initially based on collecting samples, but SARS-CoV-2 made this approach unpractical for a long time. Therefore, we shifted our focus to the metagenomic projects already available. We limited ourselves to Spain, to mimic the sampling that was initially report. Rapidly, we saw that 16S rRNA methyltransferases are more prevalent than it is thought and that the normal carriers of this enzymes are not species form the Enterobacteriaceae family but rather commensal non-pathogenic bacteria from the gut of humans and animals. Here we decided to change another task proposed, that was the isolation of enterobacteria carrying plazomicin resistance genes to isolating commensal non-pathogenic bacteria from samples

where the resistance genes of interest were spotted. In addition, at this point we were able to gather some samples from different animal origins that confirmed the *in-silico* results.

At this point, since conventional culture approaches were not suitable for our purpose, we asked for a Short-Term Mission to try to achieve this using a combination of fluorescence *in situ* hybridisation and single-cell genomics. These experiments are still ongoing, and we expect to have results during this year.

We also proposed a sampling in the United Kingdom, but we believe that the information obtained from there will be limited and, therefore, not compensate the money and the effort. Then, we exchange the task for a global analysis of one of the resistance genes found in our first metagenomic study, as there is a lot of information in the databases that could has been helpful than a random sampling.

We are still working on analyses and experiments that complement what it has been presented in this report and we believe that more projects can come after this one to fill the gaps that the time limitation has not let us explore.

Progress of the project: milestones and deliverable

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
METAPRO	D1	Questionnaire on farm management, biosecurity, health and antibiotic use in livestock farming	M37	M40		
METAPRO	D2	Thesis manuscript	M63	-	Delayed. There is still some work that needs to be performed to complete the PhD project	

** 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

PhD Reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
METAPRO	M1	Spanish sampling desing	M32	M65	Yes	Due to COVID, access to samples was denied for a long time, so we focused on the metagenomic projects already accessible through databases.
METAPRO	M2	Spanish sampling execution	M35	M65	Yes	Spanish metagenomic projects were gathered and analysed. In addition, some animal samples were collected.
METAPRO	M3	Metagenome sequencing of the Spanish sampling	M39	M65	Yes	Some samples were sequenced but they did not offer more information than the one provided by the sequences already deposited in the databases.
METAPRO	M4	Bacterial isolation and WGS from Spanish sampling	M39	-	Ongoing	Several attempts have been made to isolate the bacteria harbouring the resistance genes of interest, but we have not succed yet. We are exploring new methodologies (FISH + SCG) to isolate the reservoirs.
METAPRO	M5	Design of United Kingdom sampling	M42	-	No	We decided not to invest more time on a second sampling. This task was modified for an analysis of one plazomicin resistance gene in all the metagenomic projects in the world.
METAPRO	M6	United Kingdom sampling execution	M45	-	No	Refer to M5
METAPRO	M7	Metagenomic sequencing of the United Kingdom sampling	M48	-	No	Refer to M5
METAPRO	M8	Bacterial isolation and WGS from United Kingdom sampling	M48	-	No	Refer to M5
METAPRO	M9	Analysis of the genomic and metagenomic data from both samplings	M48	-	Ongoing	There is a lot of information gathered and we are still trying to add a few more analysis and experiments that complement all the work done.
METAPRO	M10	Guideline for early detection of plazomicin resistance determinants to preserve plazomicin for human clinical use	M57	-	No	In the end will not be performed.
METAPRO	M11	Thesis manuscript	M63	-	No	PhD project will be continued for another year to finish all the experiments and analyses that are planned.
METAPRO	M12	Thesis defence	M66	-	No	Refer to M11

Publications and additional outputs

Publications

At the time this deliverable was submitted, the METAPRO PhD project has not published any scientific publications.

Additional outputs (i.e., poster/oral presentations)

The METAPRO PhD project disseminated these works through oral and poster presentations at the following events:

- Interbacterial mobilisation of aac(3)-IV in the gut microbiome. Oral presentation. XIII Reunión del.
- Grupo de Microbiología Molecular of the Spanish Society of Microbiology, Granada, Spain. 7-9th September 2022.
- Metagenomic analysis of the effects of apramycin on the microbiome. Oral presentation. VIII VETINDOC – PhDay Complutense 2022, Madrid, Spain. June 2022.
- Metagenomic sequencing analysis of the effects of apramycin in the poultry gut microbiome. Oral presentation. One Health EJP Annual Scientific Meeting 2022, Orvieto, Italy. 11-13th April 2022.

- Matamoros, B. R., Serna, C., Moyano, G., Wedel, E., Pulido-Vadillo, M., Montero, N., & Gonzalez- Zorn, B. (2022). Metagenomic analysis reveals that apramycin restructures the resistome and favors interbacterial spread of aac(3)-IV gene. Poster presentation. 32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Lisbon, Portugal. April 2022. More information can be found, here.
- Matamoros, B. R., Serna, C., Hernández, A., Montero, N., Garcia, M., Blanco, J. L., & Gonzalez-Zorn, B. (2021). First report of the acquired 16S rRNA methyltransferase armA in an Enterobacter hormaechei ST171 isolated from a horse. Poster presentation. 31st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). 9th July 2021. More information can be found, here.
- Matamoros, B. R., Serna, C., Hernández, A., Montero, N., Garcia, M., Blanco, J. L., & Gonzalez-Zorn, B. (2021). One Health: *armA* and Enterobacter hormaechei ST171. Oral presentation. XXVIII Congreso de la Sociedad Española de Microbiología (SEM). 28th June 2021. More information can be found, here.
- Matamoros, B. R., Serna, C., Delgado-Blas, J. F., Wedel, E., Montero, N., Garcia, M. E., Blanco, J. L., & Gonzalez-Zorn, B. (2021). Acquired 16 rRNA methyltransferase *armA* maintained for a decade in a veterinary hospital via an *IncR* plasmid. Poster presentation. One Health EJP Annual Scientific Meeting 2021, Copenhagen Denmark & online. 9-11th June 2021. More information can be found, here.
- Matamoros, B. R., Glaser, P., La Ragione, R. M., & Gonzalez-Zorn, B. (2020). METAPRO: Metagenomics and genomic approaches for the prevention of the spread of plazomicin resistance in humans, animals, and the environment. Poster presentation. One Health EJP Annual Scientific Meeting 2020, online. 27-29th May 2020. More information can be found, here.
- OHEJP blog post in June 2022 for the “One Health EJP PhD Life” campaign: A Day in the life of Bosco Rodríguez Matamoros, can be found here.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Workshop "Aspectos epidemiológicos y estadísticos de un trabajo de investigación"	Epidemiology and Statistics	03-04/03/2020, 02/06/2020, 09/06/2020	Faculty of Veterinary Medicine, Universidad Complutense de Madrid
qPCR technician and analyst at Lab UCM COVID-19	COVID-19, qPCR	15/03/2020 – 01/06/2020	Universidad Complutense de Madrid
Course "Textos científicos con LaTeX"	LaTeX software	27/05/2020 – 16/09/2020	Universidad Complutense de Madrid
Biosafety seminar	Microbiology and Biosafety	14/07/2020	Faculty of Veterinary Medicine, Universidad Complutense de Madrid
One Health EJP Summer School 2020	One Health	17-28/08/2020	Wageningen University
Teaching of Practical classes in the Veterinary Medicine Degree	Microbiology and Immunology	Academic Course 2019/2020	Faculty of Veterinary Medicine, Universidad Complutense de Madrid
VISAVET Journal Club	Microbiology	Academic Course 2019/2020	VISAVET
Resistomap Webinar Series	Antibiotic Resistance and Environment	25/08/2020 – 24/11/2020	Resistomap
Course "Análisis de datos con Phyton"	Phyton	14/09/2020 – 11/12/2020	Universidad Complutense de Madrid
OHEJP FARMED Meeting	Metagenomics	29/10/2020	Sciensano
K-mer alignment training workshop	Metagenomics	6/11/2020	Technical University of Denmark
Webinar "Jornada Complutense sobre Resistencia a Antibióticos"	Antibiotic Resistance	23/11/2020	Universidad Complutense de Madrid
MicroMundo Symposium	AMR	27-28th/04/2021	Universidad Complutense de Madrid
Webinar Series 'Jornadas sobre la Carrera Investigadora'	Scientific Research	14/01/2021 – 29/04/2021	Universidad Complutense de Madrid
Course 'Hojas de Cálculo con Excel I'	Excel	15/03/2021 – 30/06/2021	Universidad Complutense de Madrid
Course 'Hojas de Cálculo con Excel II'	Excel	14/06/2021 – 29/10/2021	Universidad Complutense de Madrid
Workshop 'Bioinformatics tools and methods for antimicrobial resistance'	AMR	15/10/2021	PH4GE, JPIAMR and CLIMB-BIG-DATA
BioinfoCAM meeting	Bioinformatics	21/10/2021	SEBiBC
VI Jornadas de Bioinformática	Bioinformatics	24-25/02/2022	Universidad de Granada
Jornada "One Health, Salud Humana, Animal y Medioambiental. Impacto económico y social"	One Health	24/03/2022	Universidad Complutense de Madrid
Course "Aprende a manejar R y RStudio"	R language	30/05/2022 – 03/06/2022	Universidad Complutense de Madrid
Course "Introducción a la estadística como herramienta metodológica para la investigación"	Statistics	07-15/06/2022	Universidad Complutense de Madrid
Course "Visualización de datos con Python"	Python language	13/06/2022 – 30/09/2022	Universidad Complutense de Madrid
One Health EJP Final School 2022	One Health	05-07/12/2022	University of Surrey
One Health EJP Short Term Mission	AMR	01/03/23 – 31/12/23	Karlsruhe Institute of Technology

One Health impact

Antimicrobial resistance is one of the fields where the importance of One Health can be easily seen. In this doctoral project, we have been focused on the resistance to an important antibiotic family, the aminoglycosides. Both World Health Organisation (WHO) and World Organisation for Animal Health (WOAH) have categorised this class as critically necessary. Nevertheless, there are some resistance mechanisms that already compromise the application of the aminoglycosides, even the next-generation ones. The acquired 16S rRNA methyltransferases modify the target of the aminoglycosides, so even in the case of the new aminoglycoside modifications that are in progress to be released in a close future, the resistance is conferred to the bacteria that expresses them.

Since the prevalence of these resistance enzymes is thought to be low, in this project we have evaluated their abundance in complex environments and in non-clinical settings, where the role of non-pathogenic bacteria in the maintenance and dissemination of antimicrobial resistance genes can be assessed. There we have found a high prevalence of 16S rRNA methyltransferases in the gut microbiota of human, pigs, and chickens. From our data seems that unknown commensal bacteria may be the carriers, but association to mobile genetic elements is already seen, so it is not illogical to think that these resistances genes that are maintained in the gut of potentially every pig or chicken could be mobilised to a pathogenic bacterium and jeopardise the application of this antibiotic class. We have the perfect opportunity for the monitoring of these resistance genes before we cannot control them anymore.

In this work we propose that complex microbial communities are the potential origin of some of the resistance genes that we see in our clinics. Surveillances through metagenomics and single cell genomics are still not in a point where implementation can be completely feasible, but investing in this direction may be a good approach to tackle the next widespread resistance genes before they even enter our clinical settings. For example, the One Health EJP project FARMED aimed to do that on site and some promising results were obtained from it.

Finally, we have evaluated the role of apramycin in the selection of resistance genes. Since apramycin has gained a lot of attention lately and a great effort is being made to release it for human use, we believe that more studies are required from an antimicrobial resistance perspective before the final decisions are taken. Apramycin is approved in animals, which offered us a perfect setting for this study. In the farms that we analysed we saw that the use of apramycin heavily selects for apramycin genes. These genes seem already be widespread in every One Health setting that you can think of, so, apramycin application in humans could lead to a quick inefficacy of the compound. More studies are needed to evaluate this selection phenomenon before apramycin is approved, because there is a lot of effort and money in the table that could have little return.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

Apart from the obvious, that is funding the PhD project, being part of the One Health EJP has offered a lot of alternatives for the best development of the research that we were carrying out. One Health EJP Annual Scientific Meetings have been an excellent opportunity to present the work done and discuss it with many different top level European researchers not always from our field, that have enlarged our point of view. Having the possibility to present posters and oral talks in the ASM has contributed enormously to the development of the communication skills of the PhD student, quality that is necessary in science. In addition, being part of the consortium has given the possibility to participate in numerous activities organised by the One Health EJP, like summer schools, webinars, or a Short-Term Mission. Especially important has been the latter, that has resulted in the collaboration with another European group outside of the consortium, where the exchange of ideas has taken place and where we have taken our research to a whole new level.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The METAPRO PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- FARMED: Fast Antimicrobial Resistance and Mobile-Element Detection using metagenomics for animal and human on-site tests. Since the objectives and the techniques of both projects are similar, the candidate took part actively in the tasks performed at UCM associated to this project, performing long read metagenomics from a set of samples from environmental origin.
- AVANT: Alternatives to Veterinary Antimicrobials. The student has been also involved in some of the analysis performed in the project focused on the development and test of alternatives to antimicrobials.
- DAMR-Una Europa: “Disseminate antimicrobial resistance knowledge and the use of whole genome sequencing on relevant bacterial pathogens during SARS-CoV-2 world emergency. The student took part in this project teaching a webinar on metagenomics and collaborating on the sequencing of bacterial pathogens and their genomic analysis.
- EFFORT: Ecology from Farm to Fork Of microbial drug Resistance and Transmission. The group participated actively in the EFFORT.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	3	5
Were all the milestones and deliverables completed?	2	4
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	3	5
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	3	5
Did the PhD student actively engage in Education and Training activities?	5	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	3	1
Was the PhD managed and implemented in accordance with the DMP?	1	4
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	1	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	4	5
Is there any direct or indirect impact of the project for national or international stakeholders?	-	5
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	-	5
Does the project outcomes have policy implications?	2	1
TOTAL	31/65	55/65

AVERAGE: 43/65

NB. Note that reviewer 1 did not provide scores for two questions, which may affect the overall score.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer
The student mentioned several unfinished aspects of his work and suggested future directions for the research to complete gaps in several areas. The first focused on time limitations of the project for FISH, the latter three were more on recommendations for future research studies.

Reviewer 2: External Scientific reviewer
In addition of cultivation approaches a collaboration project with KIT has been started, to specifically target, label sort and sequence the microorganism of interest.

Reviewer 3: PMT member
n/a

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer
Like most research conducted during the past 2-3 years, this one was heavily impacted by SARS-CoV-2 pandemic. This mostly applied to shifts away from new field sampling to relying instead on existing metagenomic and other datasets. This allowed for both Spanish only, as well as global investigations; albeit, often lacking appropriate denominators. Forays into the UK were thwarted, while other new avenues (FISH) were pursued. Some work remains incomplete.

Reviewer 2: External Scientific reviewer
Goals were met and justification for SCG was provided.

Reviewer 3: PMT member
n/a

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer
Much work from original plan was delayed or diverted due to SARS-CoV-2 pandemic. Some projects were completely discarded like UK sampling components. Much was redirected to existing databases or else to new and novel approaches or else new aminoglycoside targets. The apramycin studies were very interesting and well directed in my view. Thesis is delayed as student is yet to defend and graduate.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
It has been rated 2 out of 5 despite accepting the justification of the SARS-CoV-2 pandemic, which has delayed some of the work and necessitated modifications to the proposed plan. I consider the score to be too low, as the doctoral student justifies the proposed changes and

the pending work. However, it is true that the expected completion date of the thesis is not specified, something that has not been achieved to date, as indicated by Reviewer 1.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer
Student interacted with three other projects. Details were lacking, but these included FARMED, AVANT, and DAMR-Una Europa. I expect these were limited by SARS-CoV-2 pandemic as well, though the student does not mention this explicitly.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
The student has provided additional information about the interactions with the mentioned projects, although it is not specified whether the SARS-CoV-2 pandemic has influenced these interactions, as suggested by Reviewer 1.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer
This is listed as interaction with stakeholders in student report which mentions the EFFORT project, though that ended in 2019 I believe. Added benefits were described by student as the scientific meetings, working towards the PhD, summer schools, webinars, and other groups outside the European consortium.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
The student mentions the completed EFFORT project, which ended before the thesis period, as indicated by Reviewer 1. However, the student reasonably details the benefits of being part of the OHEJP project, so I consider the reviewer's rating of 3 out of 5 to be excessive.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer
The student provided an extensive tabulation of courses in various courses and short courses with transferrable skills. This suggests the student was very actively pursuing a marketable set of skills in a broad cross-section of techniques suited to his project and beyond.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
n/a

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer
Because of the lack of field work sampling there were no ethical issues encountered.

Reviewer 2: External Scientific reviewer
There are no publications listed, but in preparation. In addition, there is one co-author publication from 2020: J Antimicrob Chemother. 2020 Nov 1;75(11):3173-3180. doi: 10.1093/jac/dkaa311. Lorena Rodríguez-Rubio 1 , Carlos Serna 2 , Manuel Ares-Arroyo 2 , Bosco R Matamoros 2 , Jose F Delgado-Blas 2 , Natalia Montero 2 , Cristina Bernabe-Balas 2 , Emilia F Wedel 2 , Irene S Mendez 2 , Maite Muniesa 1 , Bruno Gonzalez-Zorn.

Reviewer 3: PMT member
n/a

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer
No scientific publications are provided in the report, other than this report itself.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
Reviewer 1 rates this section with 1 out of 5, while Reviewer 2 rates it with 4 out of 5. I understand that this point may not apply as no scientific publications have been produced. Furthermore, I believe that Reviewer 1's comment does not apply to this point.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer
Nor are there any mentions of outcomes to highlight in communications or to be implemented in policy. Section 18 (later in this report) seems to be in a tabular checklist format.

Reviewer 2: External Scientific reviewer
Extensive antimicrobial resistance mobilization via multicopy plasmid encapsulation mediated by temperate phage.

Reviewer 3: PMT member
It is true that no specific outcomes related to policy implications are mentioned, but the dissemination of project results is detailed. I believe that Reviewer 1's rating is not in line with the presented work, and their comment on the format of section 18 is also inaccurate, as the table from the document has been used. Reviewer 2's comment is not understood.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer
This succinct section hits major points of importance regarding aminoglycoside resistance in a One Health context. Apramycin has been used for a very long time in veterinary medicine and may soon be used in human medicine. This makes the topic a prototypical 'One Health' issue. It will be great to see peer-reviewed publications arising from this work to allow for its more assured dissemination to scientific community and policy makers.

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
n/a

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
Reviewer 1 did not rate this point. Reviewer 2's comment is not understood.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
Reviewer 1 did not rate this point. Although the collaborations conducted are not detailed, a general mention is made in point 11. Reviewer 2 rated this point with a 5, although no comments are provided regarding it.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
n/a

Reviewer 3: PMT member
Reviewer 2 rated it with 1, although no comment is provided. There is no mention of this point in the document submitted by the student, indicating that the project has had no implications in this regard.

The full Final Thesis Report for the METAPRO PhD project can be found here.

PhD6-ET2.1-PEMbo

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
CHARLES Ciriac	PhD Student	University of Paris Est and ANSES	France
BOSCHIROLI Maria-Laura	PhD Primary Supervisor	University of Paris Est and ANSES	France
BIET Franck	PhD Secondary Supervisor	INRAE Val de Loire	France
MICHELET Lorraine	Other Supervisor	ANSES	France

Summary of the work carried out in the PhD project

The PEMbo PhD project began in M22, October 2019, and finished in M58, in 2022. PEMbo focused on the patho-evolution of *Mycobacterium bovis* strains in France, studying both their genotype and phenotype. *Mycobacterium bovis* (*M. bovis*) is the etiological agent of bovine tuberculosis (bTB), a zoonotic disease with a high socioeconomic impact. After having reduced the prevalence of this disease below 0.1% for 6 years, France was recognised bTB free in 2001 which is a very advantageous situation for trading. However, this status is threatened in the recent years by the re-emergence of bTB in some French regions. bTB outbreaks are present in very localised areas and are due to persistent *M. bovis* strains with specific genotypes (defined by spoligotyping and MLVA typing). It is therefore important to understand if among the reasons for bTB increase, genetic factors linked to these dominant genotypes could be included. Furthermore, the transmission link between infected animals (which can involve those of different cattle herds and different wildlife species) remains difficult to establish in regions sharing the same *M. bovis* genotype. Analysis of whole-genome single nucleotide polymorphisms compared with appropriate reference genomes can accurately differentiate field strains. However, new whole genomes closer to the French field strains are needed to perform these studies.

Ten new complete genomes were sequenced using MinION and Illumina technologies. Despite the high stability of *M. bovis* genome, their characterisation and comparison allowed a better description of genetic lineages by defining specific genetic criteria for some of them. The use of complete genomes allows a better genomes annotation which improves our knowledge of the genetic diversity of the *M. bovis* genomes. The copy number of an insertion sequence, IS*6110*, was found to be variable between the different complete genomes. This

sequence plays an important role in genome plasticity of the human pathogen *Mycobacterium tuberculosis* and was found in high copy numbers in representative genomes of highly prevalent genotypes whereas *M. bovis* is considered to have one or very few copies.

The abundance and location of IS*6110* were therefore studied in a larger panel of *M. bovis* genomes representative of the French diversity. These results confirmed that the most persistent genotypes were those with multiple copies of IS*6110* and that two of the most common genotypes found in France in recent years present a very high copy number (more than 10). Further studies on 3 important genotypes, SB0120-DHV, SB0120-CO and a sub-group of Cluster A/F4 family, which are respectively found in Dordogne and Haute-Vienne, Côte d'Or and Atlantic Pyrenees (French regions), showed a quite high stability of IS*6110* number and their insertion site between host species over 6-to-17-year periods. IS*6110*-related genetic changes did not appear to occur for host adaptation as suggested for highly prevalent *Mycobacterium tuberculosis* strains.

This work has improved our knowledge on the *M. bovis* lineages circulating in France and worldwide by establishing a list of genetic characteristics specific to them. Some of these, such as IS*6110*, seem to be correlated with the epidemiological success of certain genotypes. The new genomes will help to better understand the bTB transmission dynamics in multi-host systems and to implement more effective control measures to eradicate the disease in these areas.

Work carried out on the PhD, scientific results and outcomes

The full PhD Thesis can be found here. The thesis outlines in totality the scientific results and outcomes of the PEMbo PhD project.

PhD self-assessment

Task 1: Establishment of reference sequences from the main French clonal groups

This task has been achieved successfully. Ciriac has developed a bacterial genomic DNA extraction protocol which has enabled him it to obtain DNA of sufficient quantity and quality to perform 3rd generation sequencing of 10 *Mycobacterium bovis* (*M. bovis*) strains with the MinION (Nanopore) technology. Using long read -MinION's data- *de novo* assembly and short reads -Illumina's data- for genome correction, Ciriac obtained new reference genomes. He developed a pipeline for assemblies. The 10 complete genomes have been submitted to National center for biotechnology information (NCBI) under BioProject accession number PRJNA832544.

More information can be found, here.

Task 2: Genomic analysis

For this task, Ciriac first worked on several panels of strains already available: the first of 87 genomes representative of French genetic diversity, then three panels of sympatric strains belonging to the current major genotypes. He focused on the IS*6110* sequence, an insertion sequence of the *Mycobacterium tuberculosis* complex –to which *M. bovis* belongs-, with an important role in genome plasticity and in the bacterium, evolution caused by IS*6110*

transposition. This work was the subject of his first publication in which he showed that IS*6110* is present in multiple copies in endemic French *M. bovis* groups and could lead to phenotypic changes that explain their epidemiologic success. Some IS*6110* interrupt or could regulate (with their strong promoter) important genes implied in virulence, host persistence or environmental stress resistance.

Thanks to the new reference genomes, Ciriac was able to carry out further genomic analyses on *M. bovis*. In a second article, a pangenomic study has been performed on the 10 genomes and showed a high similarity between them. Whole genome SNP (single nucleotide polymorphism) was performed on short read sequencing of the 10 strains in comparison to one of the current *M. bovis* reference genome AF2122, BCG and Mb3601. Pangenome analysis revealed a “closed” pangenome composed of 3900 core genes and only 96 accessory genes. Whole genomes-based alignment using progressive Mauve showed remarkable conservation of the genomic synteny except that the genomes have a variable number of copies of IS6110. Characteristic genomic traits of each lineage were identified through the discovery of specific indels. Altogether, these results provide new genetic features that improve the description of *M. bovis* lineages.

More information can be found here and here.

Task 3: Analysis of the antigenic variability: biochemical and lipidomic studies

This task could not be completed. This was partly due to the SARS-CoV-2 crisis and to the prolonged closure of the level 3 biosafety laboratory which delayed the completion of the first two tasks. However, the pangenomic analyse realised by Ciriac allowed the identification of genomic events (insertion / deletion or broad sequence polymorphism (LSP)) between the new reference genomes. Possible mutation, deletion or overexpression of a gene have been identified and which will be good molecular targets to be explored. A confirmation of first *in silico* result will be reinforced with an appropriate biochemical or microbiological approach.

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
PhD06-ET5-PEMbo	D-E5-1	First steering committee report	M26	M32	Delay due to the COVID crisis. https://zenodo.org/record/4305724	None*
PhD06-ET5-PEMbo	D-E5-2	Second steering committee report	M39	M39	https://zenodo.org/record/4772558	None
PhD06-ET5-PEMbo	D-E5-5	Final steering committee	M42	None	This document is not requested by Ciriac's doctoral school.	None
PhD06-ET5-PEMbo	D-E5-6	Oral communication at a congress	M46	M52	Oral communication on new *M. bovis* complete genomes study in ABIES doctoral days 2022 congress.	None
PhD06-ET5-PEMbo	D-E5-7	Publication in an international journal	M46	M53	This deliverable was delayed because writing/correction time and result improvement delayed by COVID-19 crisis. The article is published in Frontiers in Microbiology. https://zenodo.org/record/6821763	None
PhD06-ET5-PEMbo	D-E5-8	PhD manuscript	M48	M58	French and English version of PhD manuscript have been achieved.	None
PhD06-ET5-PEMbo	D-E5-9	SHH2020 oral communication	M27	M53	New deliverables add. Oral communication (20min) in French at Maisons-Alfort (France). Published in Zenodo in public access: https://zenodo.org/record/6581493	None
PhD06-ET5-PEMbo	D-E5-3.1	ASMOHEJP2020 oral communication	M29	M53	New deliverables add. Oral communication (3min) in English at Prague (Czech republic). Published in Zenodo in public access: https://zenodo.org/record/6581483	None
PhD06-ET5-PEMbo	D-E5-10	LABIO AU LABO 2021 event	M37	M53	New deliverables add. Internet event. Published in Zenodo in public access: https://zenodo.org/record/6581428	None
PhD06-ET5-PEMbo	D-E5-11	MT180 2021 oral communication	M38	M53	New deliverables add. 3 minutes thesis competition in French. Published in Zenodo in public access: https://zenodo.org/record/6581480	None
PhD06-ET5-PEMbo	D-E5-12	ASMOHEJP2021 poster 1	M42	M53	New deliverables add. Poster presentation at Copenhagen (Danemark). Published in Zenodo in public access: https://zenodo.org/record/6581523	None
PhD06-ET5-PEMbo	D-E5-12.1	ASMOHEJP2021 poster 2	M42	M53	New deliverables add. Poster presentation at Copenhagen (Danemark). Published in Zenodo in embargo: https://zenodo.org/record/6581535	None
PhD06-ET5-PEMbo	D-E5-12.2	ASMOHEJP2021 oral communication	M42	M53	New deliverables add. Oral communication (3min) at Copenhagen (Danemark). Published in Zenodo in public access: https://zenodo.org/record/6581468	None
PhD06-ET5-PEMbo	D-E5-13	SHH 2021 oral communication	M45	M53	New deliverables add. Oral communication (20min) in French at Maisons-Alfort (France). Published in Zenodo in public access: https://zenodo.org/record/6581474	None

PhD06-ET5-PEMbo	D-E5-14	JSDA2021 poster	M45	M53	New deliverables add. Poster presentation at Maisons-Alfort (France). Awarded "best poster presentation". Published in Zenodo in public access: https://zenodo.org/record/6581509	None
PhD06-ET5-PEMbo	D-E5-17	ANSES INTERVIEW 2021	M47	M53	New deliverables add. Internet event. Published in Zenodo in public access: https://zenodo.org/record/6581392	None
PhD06-ET5-PEMbo	D-E5-15	SFM poster 2021	M45	M53	New deliverables add. Poster presentation at Montpellier (France). Published in Zenodo in public access: https://zenodo.org/record/6581542	None
PhD06-ET5-PEMbo	D-E5-16	Doc Avenir 2021 oral communication	M45	M53	New deliverables add. Oral communication (10min) in French. Awarded 1st public price and 2nd jury price. Published in Zenodo in public access: https://zenodo.org/record/6581466	None
PhD06-ET5-PEMbo	D-E5-18	ASMOHEJP2022 poster	M52	M53	New deliverables add. Poster presentation at Orvieto (Italy). Published in Zenodo in embargo: https://zenodo.org/record/6581531	None
PhD06-ET5-PEMbo	D-E5-18.1	ASMOHEJP2022 oral communication 1	M52	M53	New deliverables add. Oral communication (3min) in English at Orvieto (Italy). Published in Zenodo in public access: https://zenodo.org/record/6581461	None
PhD06-ET5-PEMbo	D-E5-18.2	ASMOHEJP2022 oral communication 2	M52	M53	New deliverables add. Oral communication (5min) in English at Orvieto (Italy). Published in Zenodo in public access: https://zenodo.org/record/6581451	None
PhD06-ET5-PEMbo	D-E5-19	M.bovis2022 poster 1	M54	M53	New deliverables add. Poster presentation at Galway (Irlande). Published in Zenodo in public access: https://zenodo.org/record/6581495	None
PhD06-ET5-PEMbo	D-E5-19.1	M.bovis2022 poster 2	M54	M53	New deliverables add. Poster presentation at Galway (Irlande). Published in Zenodo in embargo: https://zenodo.org/record/6581503	None
PhD06-ET5-PEMbo	D-E5-20	Second publication in an international journal	M54	M60	https://zenodo.org/record/7533730	None

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD06-ET5-PEMbo	M-E5-1	PacBio + Illumina sequencing for obtaining reference genomes	M30	Yes	M41	Strains are sequenced in MinION technology and two of them are also sequenced in PacBio technology. As planned, all strains are also sequenced in Illumina technology.
PhD06-ET5-PEMbo	M-E5-2	Assembly and annotation of reference genome	M38	Yes	M45	
PhD06-ET5-PEMbo	M-E5-3	Illumina sequencing on supplemental strains	M32	Yes		Three genomes were added to the 7 initial genomes and were sequenced with the same
						sequencing technologies (MinION +Illumina).
PhD06-ET5-PEMbo	M-E5-4	Mapped genome	M36	Yes	M47	
PhD06-ET5-PEMbo	M-E5-5	Strain selection for WP3	M40	Yes	M43	Strains are selected and cultivated to obtain good concentration of them. Moreover the BSL 3 closure put the experiments of WP3 on hold.
PhD06-ET5-PEMbo	M-E5-6	SNP matrix	M44	Yes	M46	SNP matrix was obtain. Complete genome allow to study bacterial genomic structure like the region of difference and broken coding sequence in 10 genomes. These events were also listed and described.
PhD06-ET5-PEMbo	M-E5-7	List of SNP in virulence genes	M44	No	M50	
PhD06-ET5-PEMbo	M-E5-8	Protein profiles	M42	None		This task has not be completed because our Biosafety Level 3 (BSL 3) laboratory was unavailable at the end of the project.
PhD06-ET5-PEMbo	M-E5-9	Lipidomic profiles	M42	None		This task has not be completed because our Biosafety Level 3 (BSL 3) laboratory was unavailable at the end of the project.

Publications and additional outputs

Publications

At the time this deliverable was submitted, the PEMbo PhD project has published two peer-reviewed publication:

- Charles, C., Conde, C., Vorimore, F., Cochard, T., Michelet, L., Boschiroli, M. L., Biet, F. (2023). Features of *Mycobacterium bovis* Complete Genomes Belonging to 5 Different Lineages. *Microorganisms*. 11(1), 177. DOI: https://doi.org/10.3390/microorganisms11010177
- Charles, C., Conde, C., Biet, F., Boschiroli, M-L. & Michelet, L. (2022). IS*6110* Copy Number in Multi-Host *Mycobacterium bovis* Strains Circulating in Bovine Tuberculosis Endemic French Regions. *Frontiers in Microbiology*. 9, 891902. DOI: https://doi.org/10.3389/fmicb.2022.891902

Each of these publications have been uploaded to Zendo, which is gold standard open access, and can be found here and here, respectively.

Additional outputs (i.e., posters/oral presentations)

The PEMbo PhD project disseminated these works through oral and poster presentations at the following events:

- La bio au labo. More information can be found, here.
- A day in the life of Ciriac CHARLES. More information can be found, here.
- Pourquoi la tuberculose bovine persiste dans certaines régions françaises et pas dans d'autres ? » - Portait du doctorant Ciriac Charles. More information can be found: here.
- Tuberculose bovine : pourquoi certaines souches persistent en France?. More information can be found: here.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Journée d'accueil des nouveaux doctorants	Introduction of the doctoral school; explanation of the good progress of the thesis during the next three years.	02/10/2019	ABIES
UZB training for working in the NSB3 lab	To learn how to work in a confined type 3 biosecurity lab	31/10/2019	ANSES
MOOC Bioinformatique: algorithmes et génomes	To develop skills in bioinformatics and genome data analysis.	1/11/2019→9/12/2019	MOOC Fun platform
Linux et script pour la bioinformatique	To develop skills in bioinformatics, especially Python.	19/11/2019→21/11/2019	CNRS Montpellier
Bases en épidémiologie des maladies animales et zoonotiques	To learn basic notions on epidemiology. To learn how to set up an epidemiology study. To learn how to use different statistics tests ...	01/12/2019→19/12/2019	MOOC Fun plateform
UZB training for working in the molecular biology lab	To understand UZB's molecular biology lab organisation and to evaluate my skills	19/12/2019	ANSES
ADOC - Construire et activer son réseau	To develop skills in professional	20/01/2020	Paris Est university
	presentation and elevator pitch. ADOC give tips to improve your networking.		
Developing Fluency in English: Intermediate - Advanced level in English (B1-C1) session 2	To enhance the students' awareness appropriate vocabulary, pronunciation, intonation and improve their overall confidence in oral communications.	4/02/2020→21/04/2020	Paris Est university
Doc 'Avenir 2020	This was ABIES PhD candidates' annual day, organised by doctoral students to discuss about their future career, and to learn different techniques to help them applying and getting a suitable job. This year, the main subject was about networking and developing a good professional profile	12/02/2020	ABIES
Devenir acteur de la science ouverte : ouvrir ses publications et les déposer dans HAL	To understand concepts and practices in relationship to "open science". To learn how to apply your rights for the deposit of publications in an open archives To learn how to use the HAL interface.	08/07/2020	ABIES

research integrity in Scientific professions (EN and FR)	The objective of this training is to disseminate a culture of research integrity within institutions. Rather than passing on knowledge (this is not a learning process), it is a matter of raising awareness of the various issues associated with research integrity and encouraging a critical approach by proposing the basic elements necessary to understand and support the requirements of research integrity.	05/01/2021	MOOC Fun plateform
Ma thèse en 180 secondes	To learn how to explain thesis subject quickly and clearly to a non-scientific people.	11/02/2021	Paris Est University
MinION sequencing training	Presentation of MinION sequencing and how to run it.	22-26/02/2021	ANSES
Bionumerics training	Presentation of Bionumerics	19/04/2021	ANSES
ABIES doctoral days 2021	Scientific meeting on "transition" organised by Ciriac's doctoral school.	6-7/05/2021	Doctoral school ABIES - AgroParisTech
Webinar on bartonella	Knowledge in the detection and treatment of bartonella.	20/05/2021	SFM
Interdisciplinary Seminar 'City and Health: What is the value of the 'One Health' approach for research and action?	Scientific knowledge and exchange on the integration of the Onehealth concept in research and the health system and the need for it to be taken into account at the interdisciplinary and international level.	09/02/2021	Doctoral school ABIES - AgroParisTech
Webinair on legionella	Knowledge in the detection and treatment of legionella.	03/06/2021	SFM
ASMOHEJP2021	Annual scientific meeting of One health European joint programme.	09-10-11/06/2021	One health EJP
Webinair on "useful or useless bacterial serologies "	Webinair on "useful or useless bacterial serologies ".	10/06/2021	SFM
Webinair on measles	Knowledge in the detection and treatment of measles.	24/06/2021	SFM
SARSCoV-2 sequencing with nanopore technology	History of sequencing and bioinformatics analysis. Presentation of the latest tools developed for genome assembly and comparison!	01/07/2021	Doctoral school ABIES - AgroParisTech

Bioinformatic training	One week training at INRAE to confirm genome assembly strategy and refines their analysis.	16-20/08/2021	INRAE
Doc'Avenir 2021	To discover the different possibilities of professional orientation after the thesis.	15/09/2021	Doctoral school ABIES - AgroParisTech
Meeting on « Impact des technologies de rupture : quel rôle des établissements de l'ESR ?»	Knowing the impact of disruptive technologies: what role for reference healthcare institutions.	22-24/09/2021	Doctoral school ABIES - AgroParisTech
Scientific and doctoral days of ANSES 2021	Scientific meeting on: Epidemiology and Surveillance Exposure and toxicology of chemical contaminants Antibiotic resistance Plant health	13,16,20,28 and 30/09/2021	ANSES
	Food safety Animal health and welfare		
Congress of the French Society of Microbiology (SFM) 2021	French scientific meeting on microbiology fields.	22/10/2021	SFM
ASMOHEJP2022	Annual scientific meeting of One health European joint programme of 2022.	11-13/04/2022	One health EJP
Training on Phd thesis writing « Journée de formation à la rédaction du manuscrit – présentiel »	Training on thesis writing and the organisation of the end of the thesis.	20/04/2022	Doctoral school ABIES - AgroParisTech
ABIES doctoral days 2022	Scientific meeting on "one health" organised by Ciriac's doctoral school.	21-22/05/2022	Doctoral school ABIES - AgroParisTech
M.bovis congress	International scientific meeting on *Mycobacterium bovis*.	07-10/06/2022	*M. bovis* 2022 organising committee

One Health impact

The main objectives of this thesis were to obtain ten new complete genomes of different *M. bovis* clusters and to improve our knowledge of the main genotypes circulating in France. Our work allows us to propose, in addition to AF2122/97 and Mb3601, a complete genome for each main French cluster described above but are also representative of the main clusters present in other countries, they can thus be used as references adapted to these groups in genomic epidemiology studies, in order to improve the understanding of *M. bovis* transmission in multi-host systems, among others.

The use of complete genomes during pan-genomic studies allows a better annotation of these and a finer definition of the accessory genome which can be overestimated with the use of fragmented genomes and/or tools that are not adapted.

Despite the high similarity between the different genomes, a number of major genomic events could be identified, some of which are cluster-specific and can be used as their signatures.

These data, in addition to the SNPs already found and defined as specific to certain lineages, make it possible to better describe the *M. bovis* clusters present in France, but also in the world. This work is therefore in line with recent work on the classification of *M. bovis*, which aims to propose an operational nomenclature to help comparative genomic studies between different countries.

The PEMbo project showed a correlation between the high copy number of IS*6110* in certain strains and the fact that they are representative of certain genotypes that have represented 80% of bovine tuberculosis outbreaks in France for the last 15 years (Cluster A, SB0120-CO and SB0120-DHV). This led us to wonder about the role of IS*6110* in the success of these genotypes persisting in very localised regions where bTB is actively circulating. Thus, these multiple insertion sites alone would not explain the persistence of specific genotypes in France or the possibility of adaptation to different animal hosts. Other genetic events, in addition to or alone, could also play a role in the persistence of these strains.

The decrease in the diversity of *M. bovis* strains, described in the literature has drastically reduced the number of genotypes present in France has drastically reduced. Bottlenecks have certainly occurred over time resulting in the fixation of some *M. bovis* genotypes circulating in France today. These strains, persisting today could be due to the national control has not been effective enough to eradicate them locally in regions where they have already existed for a long time (e.g. measures poorly adapted to local epidemiological risk factors). It could also be assumed that they have perpetuated themselves due to improved epidemiological fitness to an environment and their ability to overcome control measures and/or infect their hosts due to phenotypic characteristics acquired after genetic modifications.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

Being part of the OHEJP consortium allowed Ciriac to give an extra dimension to his thesis. It allowed him to contact many other PhD students of the program, opening his network to researchers from other countries but also working on other interesting themes.

The annual scientific meetings organised by the OHEJP also allowed Ciriac to challenge himself and present his results to scientists who are not experts in his field. It was very formative for him.

Evaluation of the Final Thesis Report

Not applicable as the PhD student submitted their thesis manuscript.

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Mahbod Entezami	PhD Student	University of Surrey	United Kingdom
Dr Joaquin M. Prada	PhD Lead Supervisor	University of Surrey	United Kingdom
Dr Giovanni Lo Iacono	PhD Second Supervisor	University of Surrey	United Kingdom
Dr Adriano Casulli	Other Supervisor	Istituto Superiore di Sanità	Italy

Summary of the work carried out in the PhD project.

The MACE PhD project began in M25, in 2020 and finished in M62, in 2023. A seven-month extension was provided due to the SARS-CoV-2 pandemic. The MACE project aimed to address these issues by utilising an interdisciplinary approach that combines spatial analysis, individual-based transmission modelling, and stakeholder elicitation techniques.

A spatial model was designed to investigate the spatial heterogeneity of Cystic echinococcosis (CE) infection in different livestock species across farms and was implemented in central and southern Italy. The model allowed the identification of disease “hot spots” at high spatial resolution between multiple Italian provinces, while also highlighting species-specific infection risk. A total of 3141 animal samples from abattoirs were collected and used to predict the probability of infection in farms. Areas of high infection rates were found in the regions of Sardinia, Sicily, and Salerno province. The resulting maps of infection risk will be valuable for targeted intervention efforts and further surveillance programmes, enabling efficient allocation of resources and optimised disease control strategies. This study was carried out in collaboration with Prof. Rinaldi and her team from the University of Naples, director of the WHO Collaborating Centre for Diagnosis of Intestinal Helminths and Protozoa and works closely with the local ministries.

An individual-based CE transmission model in farms was developed and implemented in the context of a resource constrained setting using the province of Rio Negro, Argentina as a case study. The model captures key aspects of the parasite life cycle and host interactions, allowing for the evaluation of intervention strategies such as anthelmintic treatment and vaccination, while accounting for individual-level heterogeneities. The results contribute to the increase in insights into the effectiveness of these strategies in reducing disease prevalence, assisting policymakers in making evidence-based decisions for CE management. This study was carried out in close collaboration with Ministry of Health officials from Rio Negro. Further analysis of intervention costings will be conducted to allow for economically viable strategies.

A socio-economic study was carried out involving an elicitation survey to assess stakeholders' willingness to invest/disinvest in surveillance sensitivity for infectious diseases in a One Health

context. The survey explored this at different levels of surveillance sensitivity and with different uncertainties as to the outcomes of the decisions being made. Understanding these priorities and financial constraints is key to identify the most acceptable and cost-effective surveillance strategies. The findings will contribute to the development of tailored intervention plans that balance the need for sensitivity and cost effectiveness and was presented at a European One Health conference, attended by key partners from the OHEJP.

The MACE project's integrated approach, combining spatial analysis, transmission modelling, and social-economic elicitations, offers a comprehensive framework for understanding the complex dynamics of CE transmission and control. The project's findings will be instrumental in guiding the development and implementation of effective intervention and surveillance strategies, ultimately contributing to the reduction of disease burden and economic impact of CE on affected communities. The MACE project represents a development in the field of zoonotic disease management, laying the ground to improve both human and animal welfare in regions affected by cystic echinococcosis. Through innovative modelling techniques and stakeholder engagement, the project provides a solid foundation for evidence-based decision-making in the design and implementation of targeted intervention and surveillance strategies.

Dissemination activities of this MACE PhD project were enshrined in five publications in journals including PLoS Tropical Neglected Diseases, and nine oral and poster presentations at national (i.e., British Society for Parasitology) and international conferences (i.e., 4th International Conference on Animal Health Surveillance).

Work carried out on the PhD, scientific results and outcomes.

Chapter 1. Introduction – Literature review and summarising the project objectives (D-PhD07-6.1)

A comprehensive the literature review and objectives was already uploaded as part of deliverable MACE.Y2.A.

The main objectives of the project were as follows:

- Develop an individual-based model for CE that is calibrated for settings in South America, accounting for individual heterogeneity.
- Develop a Geostatistical model to estimate prevalence of CE at fine spatial scales.
- Combine both the individual-based and Geostatistical models to inform nation-wide surveillance programmes at small spatial scales (below state level).
- Conduct an economic analysis to understand the financial burden of CE.
- Use the results of the economic analysis for a cost-effectiveness analysis for a range of possible control interventions.
- Conduct an elicitation questionnaire to assess the value stakeholders place on surveillance sensitivity and detection of incident CE cases.
- Generate realistic control scenarios, accounting for the epidemiology of the disease, the economic viability of the proposed interventions, and their likely acceptance by stakeholders.

More information can be found, here.

Chapter 2. Geospatial analysis of CE in Italy- Investigating the probability of infection in Italian farms using a spatial model (D-PhD07-6.2)

Introduction: Cystic echinococcosis (CE) is a zoonotic parasite caused by the cestode *Echinococcus granulosus sensu lato* (s.l.) which predominantly affects livestock. The disease is endemic in central-southern and insular Italy, with CE particularly infecting sheep, goats, cattle, and water buffalo. The spatial distribution of CE in endemic regions is not widely understood, with surveillance efforts varying across the region.

Methods: In this study, we investigated the spatial distribution of CE in livestock using samples from farms across different livestock species using a Stochastic Partial Differential Equations (SPDE) model. Samples were collected during a survey conducted in the area of central-southern and insular Italy between the years 2019 – 2021.

Results: A total of 3141 animal samples (126 goats, 601 sheep, and 2414 cattle and water buffalo) were inspected for *Echinococcus* s.l. cysts through routine surveillance in abattoirs by post-mortem visual examination, palpation, and incision of target organs. The geographic location of the farm of origin (a total of 2,878) for each sample was recorded. CE prevalence of 46.0% (1,323/2,878) was estimated at the farm level with 78.3% (462/590) of farms with sheep, 28.6% (36/126) of farms with goats, 36.5% (747/2,049) of farms with cattle, and 23.5% (102/434) of farms with water buffalo infected.

Discussion: The spatial model evaluated the probability of infection in farms across the sampled regions, with the distribution of CE showing high clustering of infected cattle farms in Sardinia and Sicily regions, and sheep farms in Salerno province (Campania region). The output of this study can be used to identify CE hot-spots and to improve surveillance and control programs in endemic areas of Italy.

More information on this chapter can be found, here.

Chapter 3. Transmission model – Evaluating the effectiveness of intervention for CE in the setting of South American farms (D-PhD07-6.4)

The chapter presents an individual-based transmission model developed to simulate the transmission of Cystic Echinococcosis (CE) within a farm setting in Rio Negro, Argentina. This comprehensive model encompasses the vital elements of the parasite life cycle and host interactions and assimilates comprehensive information regarding disease dynamics culled from a broad spectrum of literature, which enables the assessment of various intervention strategies, such as anthelmintic treatment and vaccination. As one of the only individual-based models developed for CE, it incorporates disease dynamic attributes such as age dependant cyst fertility, individual host heterogeneity of cysts and worm burden in hosts. To calibrate the model to its aforementioned setting, the farming practices of the region were reproduced, and the model was fitted to reflect the host prevalence in the region, based on field data. This Chapter is being finalised, and the implementation of this model will yield insight into the effectiveness of intervention strategies, revealing their potential to reduce the prevalence of CE. As such, the outcomes of this study will provide substantial evidence that could aid policymakers in making informed decisions regarding the management of CE. The findings underscore the value of utilising such individual-based models in the understanding and control of parasitic diseases.

Chapter 4. Interventions for CE – Extension on the scenarios evaluated using the transmission model considering intervention costs (D-PhD07-6.4)

The chapter introduces an extended version of the previously developed individual-based transmission model, further expanding the scope of evaluated intervention strategies for Cystic Echinococcosis (CE) and incorporating their respective costings using findings from a scoping review that we recently published. This expansion enables a more nuanced and practical exploration of various interventions, considering not only their biological effectiveness in reducing disease prevalence but also their economic feasibility. This Chapter is being finalised, and the results from this expanded model will offer a comprehensive understanding of the cost-effectiveness of each strategy, thus providing valuable insights for policymakers. The discussion suggests that such cost-inclusive models can be instrumental in making evidence-based decisions that balance both the health and financial impacts of different strategies in managing parasitic diseases like CE.

More information can be found, here.

Chapter 5. Elicitation – Investigating the investment preferences of stakeholders for surveillance sensitivity (D-PhD07-6.3)

This pilot study focuses on understanding the valuation of changes in surveillance sensitivity by public-health officials using tools like Willingness to Pay (WTP) and Willingness to Accept (WTA). An online survey was designed to estimate these values for two levels of surveillance sensitivity, considering a societal perspective typically funded by state or local authorities. Participants were asked to provide their perspectives on the cost and value of a surveillance system for non-specific non-zoonotic diseases. The study found that stakeholders were willing to invest a relative increase of 2.48% to 2.77% in their budget for every 1% increase in sensitivity, while for every 1% decrease in sensitivity, they expected a relative reduction of 2.48% to 1.87% in costs. The study revealed a risk aversion towards disinvestment in disease surveillance programs. Despite certain limitations, like a small sample size and a cognitively demanding survey, the study emphasises the importance of understanding stakeholder biases and preferences in disease surveillance investment decisions, especially in the context of One Health disease surveillance systems. The research underscores the necessity of further exploration of economic evaluations of these systems and the determination of appropriate investment/disinvestment thresholds. A manuscript has been prepared based on this chapter and is currently under review by collaborators.

Chapter 6. Discussion - Assessing the impact of the project and further works.

The MACE project, through its comprehensive approach involving transmission modelling, spatial analysis, and socio-economic evaluations, provides an evidence-based framework to understand and control the complex dynamics of Cystic Echinococcosis (CE) transmission. The findings of this PhD thesis offer valuable insights that can impact the development and implementation of effective intervention and surveillance strategies, thus reducing the disease burden and economic effects of CE on affected communities. The project makes use of disease dynamics data from prior studies and introduces innovative methodologies to improve human and animal welfare in regions suffering from CE. However, the thesis acknowledges the need for additional research to coalesce the components of the study for optimal results. The methodology introduced in Chapter 2 can be used to map disease prevalence in any

country with data containing multiple samples from single farms. Prevalence data can be used in conjuncture with the mathematical model for spatially localised evaluations of different intervention strategies. The model is versatile and can be modified to fit different contexts, enabling region-specific assessments, however, this requires further analysis using field data from the specified region. For a comprehensive understanding of stakeholder investment preferences, the method discussed in Chapter 5 will need to be applied to disease-specific scenarios, considering different surveillance and intervention programs. An audience familiar with budget management and the disease of interest would need to be identified, with only minor adaptation to the questionnaire, investment preferences for surveillance of the specific disease can be obtained. This approach will provide insights into the acceptable cost thresholds for various surveillance and intervention strategies.

PhD self-assessment

The initial objectives of my PhD project were to develop an advanced mathematical model to capture the transmission of CE in South America. This model was to be informed by studies addressing various gaps in the existing literature related to CE, including the fine spatial distribution of the disease, the costings of CE surveillance and control, and the investment preferences of stakeholders in intervention and surveillance. To meet these objectives, training in coding, mathematical modelling, spatial modelling, cost-effective analysis, and survey development was required.

The original plan underwent some adjustments in the first year, primarily driven by the opportunity to attend the 4th ICAHS conference. This conference was seen as a potential venue to develop a survey to investigate the investment preferences of key stakeholders. Thus, my focus in the first year gravitated towards the literature review and the development of the elicitation survey, causing the model development to be pushed to the second year. However, the cancellation of the 4th ICAHS conference due to the SARS-CoV-2 pandemic led to a delay in conducting the survey.

I was able to establish a collaboration with the Commission of Zoonosis in Uruguay, initially aimed at analysing data from that year's surveillance programme. Unfortunately, the surveillance programme was also halted due to the SARS-CoV-2 pandemic, leading us to pivot our collaboration towards planning for the subsequent year's surveillance programme. A spatial model and an efficient data collection methodology were developed in preparation for this, but a lack of funding post-SARS-CoV-2 put the project on hold. Despite these challenges, I was able to adapt the methodology and redesign it for a new collaboration with colleagues in Italy.

In addition to these collaborations, my leadership and contribution to several peer-reviewed articles presenting novel research in the field are notable achievements. My research was presented at various conferences, in both poster and oral forms, with one presentation winning the "Best Oral Presentation" award. I also developed new skills in programming (R, Python, Java) and mathematical, spatial, and statistical modelling.

The most prominent challenge faced was the impact of the SARS-CoV-2 pandemic, which led to cancellations and delays in several planned activities. Despite these circumstances, I was able to adapt and adjust the project's direction multiple times, demonstrating resilience and resourcefulness. The ability to establish productive collaborations and contribute meaningfully

to the field, even amidst such adversities, is a testament to the project's resilience and my personal growth.

Overall, I believe that this PhD project has catalysed a significant development in my skills and capabilities as an academic researcher. The project has yielded novel research contributions to the field, and despite the adversities brought about by the SARS-CoV-2 pandemic, has persevered, and adapted to continue moving forward. I am eager to continue contributing to the field and further developing my skills and expertise.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
D-PhD07-6.1		Draft of review of surveillance and control tools for CE	M37	M37		
D-PhD07-6.2		Spatial-Temporal Model of CE validated in Italy and Iran	M49	M60		
D-PhD07-6.3		Questionnaire to Elicit WTP/WTA of One Health Surveillance Activities	M52	M66	*The methodology and structure of the questionnaire has been fully developed. The questionnaire has been conducted within the 4th ICAHS conference. A short communication was written from the results and its subsequent analysis, It is currently being expanded to be published as a research article.*	
D-PhD07-6.4		Final draft of publication with the model and control scenarios	M50	M65	*The model transmission has been developed and parameterised. Fitting and incorporating control scenarios is also been completed. The article currently being written as a research article.*	

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
	MACE.Y2.A	Relevant literature on surveillance and control of CE identified	M23	M23	YES	Relevant literature was identified. Draft document included in student's Confirmation report (due M39)
	MACE.Y2.B	Training in Mathematical modelling	M36	M36	YES	Some training activities delayed due to COVID
	MACE.Y3.A	Fitted model of CE to data from Uruguay	M37	M37	YES	First iteration of statistical model fitted to Uruguay historical data. This work could not continue further due to COVID. Pivoted to Fitting transmission model in Argentina and statistical model in Italy.
	MACE.Y3.B	Simulations of different control scenarios	M44	M64	YES	Simulations to be run once transmission model is fitted
	MACE.Y4.B	Eastern European data	M40	M48	YES	Due to COVID, engagement with stakeholders has been challenging. Different data was identified and data from Italy and Argentina was used.
	MACE.Y5.A	Online polls with stakeholders completed	M36	M44	YES	Poll finalised. Deployment pending.

Publications and additional outputs

Publications

The MACE PhD project has produced five peer reviewed publication, these are:

- The spatial distribution of cystic echinococcosis in Italian ruminant farms from routine surveillance data. (2022). *Frontiers Tropical Disease*. doi. 10.3389/fitd.2022.1034572
- Investigating Seasonality and Spatial Clustering of Dog-Mediated Rabies in Nigeria. (2022). *Social Science Research Network*. doi. 10.2139/ssrn.4003084
- Quantifying spillover risk with an integrated bat-rabies dynamic modelling framework. (2022). *Authorea*. doi. 10.22541/au.165402909.96757900/v1
- The economic evaluation of Cystic echinococcosis control strategies focused on zoonotic hosts: A scoping review. (2022). *PLOS Neglected Tropical Diseases*. doi. 10.1371/journal.pntd.0010568
- Records of Human Deaths from Echinococcosis in Brazil, 1995–2016. (2022). *Veterinary Sciences*. doi. 10.3390/vetsci9080436

NB. Not all publications from the MACE project have been uploaded to Zenodo at the time this deliverable was submitted. Those publications that have been uploaded to Zenodo as gold standard open access can be found here, here and here, respectively.

Additional outputs (i.e., poster/oral presentations)

The MACE PhD project disseminated these works through oral and poster presentations at the following events:

- Oral presentation at British Association for Veterinary Parasitology Annual Meeting (BAVP2022), Belfast, UK. 8-9th September 2022.
- Prize winning oral presentation at Research celebration event, University of Surrey, UK. 16th June 2022.
- Poster presentation at 4th International Conference on Animal Health Surveillance (ICAHS4), Copenhagen Denmark. 3-5th May 2022.
- Poster presentation, 3 minute thesis presentation & roundtable discussion at OHEJP ASM 2022, Orvieto, Italy. 11-13th April 2022.
- Poster presentation at British Society for Parasitology (BSP) Annual Meeting, York, UK. 21-25th March 2022.
- Poster presentation at British Society for Parasitology (BSP) Annual Meeting, virtual. 21-25th June 2021.
- Poster presentation, 3 minute thesis presentation & quiz organisation at OHEJP ASM 2021, hybrid event. 9-10th June 2021.
- Oral presentation at University of Surrey Symposium, UK. 7-8th September 2020.
- Poster presentation & 3 minute thesis presentation at OHEJP ASM 2020, online. 27-29th May 2020.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
OHEJP Summer School 2020	One Health	17 August 2020 - 28 August 2020	OHEJP
Writing Coherently	Writing techniques	12 February 2020	University of Surrey Doctoral College
Driving your Doctorate	How to get the most out of your PhD	12 February 2020	University of Surrey Doctoral College
Engaging with you Literature: Finding Literature	Finding Literature for your projects	6 February 2020	University of Surrey Doctoral College
Welcome to your Doctorate	Introduction to your PhD	7 February 2020	University of Surrey Doctoral College
Python for Data Science and Machine Learning Bootcamp	Basic Python coding for Data analysis and visualization	27 January 2020	Udemy
Judgement & Decision-making Lecture	Decision making from a Psychological view	9 March 2020	University of Surrey
Stage 3 Spanish	The Spanish Language	September 2020 – August 2021	Global Graduates Award
Writing a Confirmation Report	Confirmation process	12 January 2021	University of Surrey Doctoral College
Confirmation process - virtual	Confirmation process	07 December 2020	University of Surrey Doctoral College
Writing A Confirmation Report	Advice on writing the confirmation report	12/Jan/2021	University of Surrey - Doctoral College
GGA Spanish Stage 3	Spanish Language	29/Sept/2020 – 5/May/2021	Universtiy of Surrey - Global graduate award
OHEJP Summer School 2021	One Health	26th July – 6th August 2021	OHEJP

One Health impact

Cystic echinococcosis (CE) is a One Health issue and requires a One health approach to effectively control. Interventions have to consider infection in animal hosts and the environmental contamination to be able to reduce infections in humans. The MACE project, with its comprehensive approach to understanding and managing CE, offers several direct and indirect impacts. It presents improved methodologies for risk assessment and management through its spatial model and individual-based transmission models. The spatial model, which identifies CE hotspots in livestock species across Italian farms, can be adapted to other zoonotic diseases and regions, providing a valuable tool for surveillance programmes. The individual-based transmission model simulates CE transmission in a farm setting, providing a platform to assess intervention strategies, which can also be adapted for other diseases, enhancing understanding and informing policy decisions.

The MACE project has established collaborations with many professionals working on one health topics. Professor Majid Fasihi Harandi from Kerman University of Medical Sciences in Iran and Professor Laura Rinaldi from the University of Naples. They have also engaged with members of the MATRIX OHEJP project and have been in contact with colleagues from APHA (UK) and PIWET (Poland). In terms of OHEJP stakeholders, this project is a close collaboration between UoS and ISS, Rome. We are working closely with Dr. Adriano Casulli from the Istituto Superiore di Sanità in Italy, who leads the MEmE JRP. The project has also been in touch with Dr. Benadette Abela, the Team leader for Neglected Zoonotic Diseases at the World Health Organisation headquarters in Geneva. Furthermore, extensive engagement has occurred with Ministry of Health officials in Peru, Argentina, Chile, Brazil, and Uruguay, leading to potential future collaborations. These collaborations have allowed the project to expand its studies on cystic echinococcosis, share research articles, and explore activities related to surveillance and management of the disease. Moreover, some of the methods developed during this thesis have supported work in a different disease context (Rabies), another NTD.

The data generated from the MACE project is a valuable addition to existing databases for risk assessment. The robust data from Italy and Argentina, regarding infection rates, host interactions, and intervention effectiveness, can be utilised by these international stakeholders to update their risk assessment protocols and strategies. This evidence-based data can influence international policy and guidelines on CE management, making the MACE project highly relevant to these stakeholders.

The stakeholders' elicitation survey provides insights into the socio-economic factors influencing surveillance and intervention strategies. Understanding these factors can help in the development of more acceptable and cost-effective strategies, especially in resource-limited settings.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

Participation in the OHEJP has significantly enhanced the quality and scope of my data-driven doctoral research. The multitude of educational workshops and conferences provided by the programme have not only facilitated the development of my research competencies, but also offered a wealth of networking opportunities. Many lectures within these OHEJP workshops have directly addressed essential training elements for my PhD, including the acquisition of requisite data for spatial modelling and the foundational principles of Susceptible, Infected, Recovered (SIR) models.

Moreover, my inclusion in such a diverse consortium has immensely broadened my perspective, providing a more nuanced understanding of complex global health issues. The consortium's collective of passionate and inspirational academics and researchers has been a powerful motivator in my scholarly development. As I progress in my career, the extensive network of academics and professionals I've connected with through OHEJP will undoubtedly serve as a valuable resource. These enriching experiences and relationships cultivated within the OHEJP have thus added substantial value and benefits to my PhD journey.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The MACE PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- Collaboration with Professor Majid Fasihi Harandi from the Kerman University of Medical Sciences in Iran, with the studies they are conducting on cystic echinococcosis within the region's dog population. Another collaborator is Prof Laura Rinaldi from the University of Naples who is involved with one of our research articles, as they provided the field data from across central and southern Italy required train the spatial model. More information can be found, here.
- We also engaged informally with members of the MATRIX OHEJP project. As there were some activities around *Echinococcosis Multilocularis* surveillance which our work could be extended to. We have since been in contact with colleagues from APHA (UK) who assisted in conducting the elicitation survey and PIWET (Poland).
- We have also extensively engaged with Ministry of Health officials in Peru, Argentina, Chile, Brazil and Uruguay, with potential future collaborations generated through this project.
- In terms of OHEJP stakeholders, this project is a close collaboration between UoS and ISS, Rome. We are working closely with Dr. Adriano Casulli from the Istituto Superiore di Sanità in Italy, who leads the MEmE JRP.
- We have been in touch with Dr Bernadette Abela, who is the Team leader for Neglected Zoonotic Diseases at the World Health Organisation headquarters in Geneva.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	1	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	4	5
Were all the milestones and deliverables completed?	5	5
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	4	5
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	3	5
Did the PhD student actively engage in Education and Training activities?	5	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	5	5
Was the PhD managed and implemented in accordance with the DMP?	5	5
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	4	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	3	5
Is there any direct or indirect impact of the project for national or international stakeholders?	4	5
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	3	5
Does the project outcomes have policy implications?	5	5
TOTAL	51/65	65/65

AVERAGE: 58/65

NB. The PMT member who reviewed the MACE PhD project provided a global overview of the PhD Final Thesis Report, outlined briefly below:

In my view the first reviewer submitted in a fair report (including scores). The second reviewer sent in mostly fair comments but clearly overscored the project.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

Unfortunately, I don't think future work was demonstrated clearly. A section titled conclusions and future work could signpost readers to a summary paragraph.

Reviewer 2: External Scientific reviewer

The PhD student has recommended future research in their project's conclusions. They identified the need for further research to optimise the study's results, adapt their methodology to different contexts, and understand stakeholder investment preferences. In essence, the student has highlighted specific areas requiring further exploration and provided clear directions for future work.

Reviewer 3: PMT member

See above a global comment from PMT member.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Some restructuring of the self-evaluation could help with this. E.g. list the original objectives; state which were achieved as planned; state which weren't achieved as planned, why and what actions you took to address that. Believe content is all there but could be communicated better.

Reviewer 2: External Scientific reviewer

The student acknowledges that while the initial objectives were partially shifted due to unforeseen circumstances, notably the SARS-CoV-2 pandemic, they adapted their approach and successfully met the project's goals. They established valuable collaborations, contributed to peer-reviewed articles, and presented their research at various conferences, demonstrating resilience and adaptability.

Reviewer 3: PMT member

See above a global comment from PMT member.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Happy that all delivered.

Reviewer 2: External Scientific reviewer

All the milestones and deliverables of the PhD project were completed, albeit with some delays due to the SARS-CoV-2 pandemic. Despite facing challenges, the student effectively

managed to meet the project's goals, showcasing their ability to adapt and persist in their efforts.

Reviewer 3: PMT member

See above a global comment from PMT member.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Not sure if required or not but would like to know more about why these people were relevant to your work. E.g. what past work qualifies them for involvement. What expertise did they bring to the table? What question(s) were they really useful for solving? if not required then 5.

Reviewer 2: External Scientific reviewer

The PhD student has actively interacted with various relevant projects and initiatives. This includes collaborations with international academics, engagements with health officials across South America, and involvement with stakeholders in the One Health EJP consortium. These interactions have fostered potential future collaborations and contributed to the project's success.

Reviewer 3: PMT member

See above a global comment from PMT member.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

Could be greatly enriched by specific examples of where you were facing a problem and seeking training through the program or talking to someone in the consortium helped you solve that problem. Maybe a line on how this will impact onward career.

Reviewer 2: External Scientific reviewer

The PhD student has highlighted the significant benefits gained from being part of the OHEJP doctoral programme and consortium. These include enhanced research skills, valuable networking opportunities, and a broadened understanding of global health issues, all contributing to their professional development. Additionally, the student emphasised the motivational aspect of being part of a diverse consortium of driven academics and researchers. This environment fostered a deeper comprehension of multifaceted health issues and provided a platform for the student's growth and development in the field.

Reviewer 3: PMT member

See above a global comment from PMT member.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

Happy demonstrated.

Reviewer 2: External Scientific reviewer

The PhD student has demonstrated active engagement in a variety of educational and training activities. These include participation in the OHEJP Summer School 2020, writing and doctoral process workshops at the University of Surrey, a Python for Data Science and Machine Learning Bootcamp, a lecture on psychological decision-making, and a Spanish language course. These activities have significantly contributed to the student's skill development, enhancing their research capabilities, writing proficiency, decision-making skills, and language competency.

Reviewer 3: PMT member

See above a global comment from PMT member.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

One publication listed not peer reviewed (still in pre-print), the Janouskova paper, but others are peer-reviewed.

Reviewer 2: External Scientific reviewer

The PhD candidate has one primary authorship in a peer-reviewed publication titled "The spatial distribution of cystic echinococcosis in Italian ruminant farms from routine". Additionally, the candidate has participated as a co-author in several other scholarly works. This information reflects the candidate's active participation in research dissemination within their field.

Reviewer 3: PMT member

See above a global comment from PMT member.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

Happy demonstrated.

Reviewer 2: External Scientific reviewer

The PhD candidate has effectively managed the project data, meeting all milestones despite SARS-CoV-2 related delays. This demonstrates proficient data management skills and adaptability, contributing to the successful execution of the project's objectives.

Reviewer 3: PMT member

See above a global comment from PMT member.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

Doesn't show estimated number of people, a ballpark figure based on the largest conference attended could work here. Annual EJP scientific meeting 2020 doesn't state what kind of event this was (perhaps: participation in activities jointly organised).

Reviewer 2: External Scientific reviewer

The PhD candidate has actively disseminated their research findings. They have presented their work at multiple conferences and participated in various scientific meetings, including the Annual One Health European Joint Project (OHEJP) Scientific Meeting. These activities demonstrate the candidate's commitment to sharing their research with the broader scientific community.

Reviewer 3: PMT member

See above a global comment from PMT member.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

Would like to see more on how you interacted with different institutes/stakeholders in multiple areas of One Health. For e.g., transmission models directly link to animal health, but you also said one of the outcomes was influencing food safety surveillance in Italy - worth raising as a One Health impact. You could highlight the public health benefits of your stakeholder elicitation surveys if there were any. The cost-effectiveness is a good outcome. Did/would working with cross-sectoral outlook increase cost-effectiveness? Or would it integrate new elements of cost-effectiveness to improve depth of analysis *etc*.

Reviewer 2: External Scientific reviewer

The project outputs align with the One Health EJP's objectives. The developed models enhance risk assessment and management methodologies, and the collected data supports international stakeholders in updating their risk assessment protocols. The project's stakeholder survey contributes to the development of cost-effective strategies in resource-limited settings. The data generated from the project is a valuable addition to existing databases for risk assessment. The robust data from Italy and Argentina regarding infection rates, host interactions, and intervention effectiveness can be utilised by international stakeholders to update their risk assessment protocols and strategies. These outputs demonstrate the project's relevance to the One Health EJP's goals.

Reviewer 3: PMT member

See above a global comment from PMT member.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

These are mentioned in the chapters section but would like to see some kind of summary in OH impacts section with direct reference to stakeholders.

Reviewer 2: External Scientific reviewer

The project has direct and indirect impacts on stakeholders. The developed spatial model for CE surveillance can be adapted for other diseases and regions, enhancing surveillance programmes. The project outcomes are under discussion with health officials in Rio Negro, Argentina, for potential implementation.

Reviewer 3: PMT member

See above a global comment from PMT member.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

Peppered throughout the report, but I think would benefit from a summary statement in the OH impacts section.

Reviewer 2: External Scientific reviewer

Collaborations have been established and strengthened within the One Health EJP and beyond, including with academic institutions and health ministries in various countries. This fosters a collaborative approach to addressing health challenges.

Reviewer 3: PMT member

See above a global comment from PMT member.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

Happy that this is addressed, although, again, it might be nice to summarise the policy implications in the OH impacts section.

Reviewer 2: External Scientific reviewer

The project outcomes do have policy implications. The robust data from Italy and Argentina regarding infection rates, host interactions, and intervention effectiveness can be utilised by

international stakeholders to update their risk assessment protocols and strategies. This evidence-based data can influence international policy and guidelines on CE management.

Reviewer 3: PMT member

See above a global comment from PMT member.

The full Final Thesis Report for MACE PhD project can be found, here.

PhD8-ET5.1/4.1/FBZSH3-DESIRE

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Marieke de Cock	PhD Student	Wageningen University and Research, the Netherlands (NL)	The Netherlands
Dr Miriam Maas	Lead PhD Supervisor	National Institute for Public Health and the Environment	The Netherlands
Dr Hein Sprong	Second Supervisor	National Institute for Public Health and the Environment	The Netherlands
Prof Wim H. M. van der Poel	External Supervisors	Wageningen Bioveterinary research	The Netherlands
Dr Rainer G. Ulrich	External Supervisors	Friedrich-Loeffler Institut	Germany

Summary of the work carried out in the PhD project

The DESIRE PhD project began in M29, in 2020 and is expected to finish in M72, in 2024. A four-month extension was provided due to the SARS-CoV-2 pandemic. DESIRE investigated rats and rat-borne zoonotic pathogen surveillance in urban ecosystems, with four core objectives:

1. Compare pathogen detection methods to improve surveillance.
2. Identify points of improvement for the surveillance of wildlife-borne pathogens.
3. Monitor rat populations.
4. Monitor rat-borne zoonotic pathogens.

These objectives were achieved by investigated by looking at different components of surveillance, such as the use of new detection techniques (next-generation sequencing) versus conventional methods (Real-time Polymerase Chain Reaction; q/PCR). In addition, the project created an overview of zoonotic pathogens that have been tested and detected in the most common urban wildlife mammals by performing a systematic literature review, identifying points of improvement for the surveillance of wildlife-borne pathogens.

Furthermore, investigating improvements for surveillance, DESIRE performed a field study in which wild rats were trapped and investigated for zoonotic pathogens in urban areas. The effect of urban greening on both the abundance of rats, and on the prevalence and diversity of rat-borne zoonotic pathogens was carried out. In addition to these works, the effect of urban greening on the presence and occupancy of rats in private gardens using camera trapping data was carried out.

Combining the results from the DESIRE project to date, points of improvement for the surveillance of wildlife-borne zoonotic pathogens have been identified, focused on both the type of pathogens and animal species investigated in urban areas, and the positive and negatives in the use of new detection methods for surveillance purposes. It was observed that the abundance of rats increased in greener urban areas, but the presence or occupancy of rats in private gardens was not affected by the amount of urban greenness. Besides that, the prevalence of mainly vector-borne zoonotic pathogens detected in rats increased in greener urban areas. When combined both the increase in abundance with the increase in pathogen prevalence in greener urban areas, this results in a higher disease hazard and potentially also a higher disease risk for public health in greener urban areas. This highlights the importance of performing more research on the ecology of wildlife and wildlife-borne zoonotic pathogens in the urban ecosystem to prevent increased pathogen transmission to humans.

Dissemination activities of this DESIRE PhD project were enshrined in three peer-reviewed publications in journals such as Transboundary and Emerging Diseases and Viruses, and one interview, one short-term mission, and eight oral and poster presentations at the national (i.e., Wildlife diseases going viral', Utrecht, the Netherlands) and international level (i.e., 4th International Conference on Urban Planning).

Work carried out on the PhD, scientific results, and outcomes

Chapter 1. Microbiome-profiling for detection of zoonotic pathogens in wild rats

From the sequencing data, 14 potentially zoonotic bacterial genera were identified from which the presence of zoonotic *Leptospira spp*. and *Bartonella tribocorum* was confirmed by qPCR or Sanger sequencing. In addition, more than 65% of all samples were dominated (> 50% reads) by one of three bacterial taxa: *Streptococcus* (*n* = 59), *Mycoplasma* (*n* = 39) and *Leptospira* (*n* = 25). These taxa also showed the highest contribution to the observed differences in beta diversity. VirCapSeq sequencing in rat liver samples detected the potentially zoonotic rat hepatitis E virus in three rats. Although 16S rRNA gene amplicon sequencing was limited in its capacity for species level identifications and can be more difficult to interpret due to the influence of contaminating sequences in these low microbial biomass samples, we believe it has potential to be a suitable pre-screening method in the future to get a better overview of potentially zoonotic bacteria that are circulating in wildlife.

Chapter 2. Identifying points of improvement for the surveillance of wildlife-borne pathogens

Confidentially clause: this data still needs to be analysed; therefore, no results and discussion can be shared yet.

Chapter 3. Monitoring of rat populations

Rat trapping paper

The project observed positive relationships between the relative abundance of rats and both greenness (NDVI) and different proxies for food sources (restaurants, waste items and petting zoos). In addition, there were more municipality rat complaints in residential areas compared to parks, while there was a higher rat trap success in parks. These findings corroborate that greenness is associated with a higher abundance of wild rats, and that municipality rat complaints may underestimate the abundance of rats in greener urban areas. This study provides new insights on factors affecting the relative rat abundance in cities and can guide policy makers and city planners how to minimise rat nuisance in cities, for example by using a smart urban greening approach, in which urban greening is designed to optimise its beneficial effects, while structurally reducing the carrying capacity for rats.

Camera trapping paper

These preliminary results showed negative relationships between rat presence and cat presence. The occupancy model showed an additional positive relationship between rat occupancy and water density. In contrast to previous results, these results suggest that the effect of urban greenness is less important for the presence of rats in private gardens compared to public areas. This could be related to the availability of food or the differences in detection methods used.

Chapter 4. Monitoring of rat-borne zoonotic pathogens

13 different zoonotic pathogens were detected. Rats from greener urban areas had a significantly higher prevalence of two vector-borne pathogens, and a significantly lower prevalence of ESBL/AmpC-producing *E. coli* and rat Hepatitis E virus. Rat age was positively correlated with pathogen diversity while greenness was not related to pathogen diversity.

Additionally, *Bartonella spp*. occurrence was positively correlated with that of *Leptospira spp*., *Borrelia spp*. and *Rickettsia spp*., and *Borrelia spp*. occurrence was also positively correlated with that of *Rickettsia spp*. Our results show an increased rat-borne zoonotic disease hazard in greener urban areas, which for most pathogens was driven by the increase in rat abundance rather than pathogen prevalence. This highlights the importance of keeping rat densities low and investigating the effects of urban greening on the exposure to zoonotic pathogens in order to make informed decisions and to take appropriate countermeasures preventing zoonotic diseases.

PhD self-assessment

There were two components of the study in the original project proposal that we were not able to perform, due to unforeseen circumstances. First, we would compare pathogen diversity between brown and black rats to identify associated host-factors. However, since we only trapped 5 black rats and > 400 brown rats, this comparison could not be made. Second, we planned to assess the suitability of a Dutch citizen science-based system for rat surveillance, called the ‘Rattenmonitor’, a mobile application for rat surveillance. However, this surveillance system is still not fully functional yet, as the number of pest controllers that report rat reports

is very low, which makes the data incomplete and unreliable to be used for scientific comparisons.

On the other hand, we also included studies to the PhD project: we included a systematic literature review to give a comprehensive overview of zoonotic pathogens detected in the most common urban wildlife mammals. Furthermore, we included a large camera trapping study to investigate the effects of various environmental, socio-economic, and predator-related variables on the occurrence of wild rats in private gardens.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
DESIRE	D2	Comparative study between cities and neighbourhoods for rat and rat-borne diseases.	September (M45)	September (M57)	Due to the limited number of samples from the field work in 2020, field work was also performed in 2021, which causes a delay.	
DESIRE	D3	An international, peer reviewed publication about the pathobiome of brown and black rats in various living environments	M57	M59		
DESIRE	~~D5~~ D5a	An international, peer reviewed publication about the effect of city greening on rat populations and related public health risks.	M45	M69 (study urban environment and rat populations)	Due to additional fieldwork in 2021, this deadline has shifted. Manuscript under review.	
DESIRE	~~D5~~ D5b	An international, peer reviewed publication about the effect of city greening on rat populations and related public health risks.	M45	M69 (study urban environment and zoonotic rat pathogens)	Due to additional fieldwork in 2021, this deadline has shifted. Manuscript under review.	
DESIRE	D7	Oral or poster presentation on a One Health conference.	M57	M52 M 54	OHEJP ASM & DSWH/BWDS symposium: oral presentation ICUP & NAEM: poster presentation	

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
DESIRE	M1	Pathobiome analysis: list of pathogens and protocols	M27(March)	DEC 2021	Yes	An initial list and protocol was designed for analysis of the bacterial pathobiome early in 2020. However, we decided to complement this with additional analysis of the viral pathobiome. This will be finished in 2021.
DESIRE	M2	Field study set-up	M30 (June)	M29	Yes	
DESIRE	M3	Visit laboratories of partner institutes WBVR and FLI	M42	M51	Yes	5 week STM to FLI; Delay due to COVID-19 restrictions)

Publications and additional outputs

Publications

The DESIRE PhD project has produced three peer reviewed publications, these are:

1. Marieke de Cock, Manoj Fonville, Ankje de Vries, Alex Bossers, Bartholomeus van den Bogert, Renate Hakze- van der Honing, Ad Koets, Hein Sprong, Wim van der Poel, & Miriam Maas. (2022). Screen the unforeseen: Microbiome-profiling for detection of zoonotic pathogens in wild rats. doi. 10.1111/tbed.14759
2. Inge M. Krijger, Max Strating, Marga van Gent-Pelzer, Theo A.J. van der Lee, Sara A. Burt, Fleur H. Schroeten, Robin de Vries, Marieke de Cock, Miriam Maas, Bastiaan G. Meerburg. (2022). Large-scale identification of rodenticide resistance in Rattus norvegicus and Mus musculus in the Netherlands based on Vkorc1 codon 139 mutations. doi. 10.1002/ps.7261
3. Elisa Heuser, Stephan Drewes, Jakob Trimpert, Dusan Kunec, Calvin Mehl , Marieke P. de Cock, Ankje de Vries, Christiane Klier, Martin Oskamp, Peter Tenhaken, Fatima Hashemi, Daniela Heinz, Mariana Nascimento, Marc Boelhauve, Rasa Petraityte-Burneikiene, Dina Raafat, Miriam Maas, Detlev H. Krüger, Andreas Latz, Jörg Hofmann, Gerald Heckel, Johannes Dreesman and Rainer G. Ulrich. (2023). Pet Rats as the Likely Reservoir for Human Seoul Orthohantavirus Infection. doi: 10.3390/v15020467

NB. Not all publications from the DESIRE project have been uploaded to Zenodo at the time this deliverable was submitted. The one publication that have been uploaded to Zenodo as gold standard open access can be found, here.

Additional outputs (i.e., poster/oral presentations)

The DESIRE PhD project disseminated these works through an interview, Short-term mission, and eight oral and poster presentations at the following events:

- Interview by the Netherlands Centre for One Health (NCOH), December 2021 Interview: ‘Catching and analysing rats might unnerve some people, but not me’ – NCOH
- “A day in the life of a OHEJP PhD student”, interview by OHEJP, December 2021 A day in the life of Marieke de Cock – #OHEJPphdlife #DESIRE – One Health EJP
- Short-term mission to the Friedrich-Loeffler Institut in March-April 2022 Short Term Missions 2021 – One Health EJP
- OHEJP ASM Thesis 3MT competition 1st prize in 2021 and 2022
- ‘Zoonoses in the city’ Oral presentation at the Wilde Stadscafé, Utrecht, the Netherlands. 8th November 2022.
- Oral presentation at Belgian Wildlife Disease Society (BWDS) & Dutch Wildlife Disease Society (DWDS) Symposium ‘Wildlife diseases going viral’, Utrecht, the Netherlands. 13th October 2022.
- Poster presentation at Netherlands Annual Ecology Meeting, Lunteren, the Netherlands. 20-21st September 2022.

- Poster presentation at 4th International Conference on Urban Planning (ICUP2022), Barcelona, Spain. 27-29th June 2022.
- Oral presentation at OHEJP Annual Scientific Meeting, Orvieto, Italy. 11-13th April 2022.
- Poster presentation at WIAS Annual Conference 2021, Wageningen University and Research, Lunteren, the Netherlands. 28-29th April 2021.
- Poster presentation at OHEJP Annual Scientific meeting, online. 27-29th May 2020.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Start to supervise BSc and MSc students	Learning basic tips on how to be a good supervisor	26-6-20	Wageningen University
Presenting with impact	Train how to present your research with impact	2-3-20 & 9-3-20 & 16-3-20	Wageningen University
Brain friendly working and writing	Tips and tricks to work more brain-friendly and efficiently	18-3-20	Wageningen University
Laboratory Animal Sciences	Learn the context and regulations of setting up animal experiments. Also an article 9 (permission to design and perform animal experiments) is obtained.	30-11-20 t/m 11-12-20	Utrecht University
Introduction to R	How to work with R	12-01-21 t/m 02-02-21	Wageningen University
Scientific publishing	Scientific publishing	09-03-21	Wageningen University
Scientific writing	Scientific writing skills	29-03-21 t/m 10-05-21	Wageningen In'to languages
Microbiome Data Analysis Workshop (MDAW)	Data analysis of microbiome data	20-04-21 t/m 23-04-21	Hasselt University
Supervising students	How to supervise BSc and MSc students	17-06-21 & 18-06-21	Wageningen University
Principles of Ecological and Evolutionary Genomics	Principles of Ecological and Evolutionary Genomics	28-09-21 & 29-09-21	Wageningen University
Animal Ethics course	Animal Ethics	1st and 2nd of March 2022	Wageningen University
Critical thinking and argumentation course	Critical thinking and argumentation	September 15th	Wageningen University
Effective behaviour in your professional surroundings course	Effective behaviour	October 11th, 18th, 26th and November 1st	Wageningen University

One Health impact

The outcomes of this research can have international implications for wildlife-borne infectious disease risks related to urban greening activities, depending on the outcomes of this study. This study could point out the potential negative effects of urban greening, which could in turn be taken into account when designing or changing urban green spaces in cities by urban planners. It is not only important regarding rats, which we focussed on in this research, but also for other wild animals and vectors (such as ticks) that are able to host and spread zoonotic pathogens to humans. The recent SARS-CoV-2 pandemic highlights the importance of investigating and preventing wildlife-borne zoonoses. Urban areas may pose an additional risk for pathogen spill over, due to the close contact and space use overlap of humans and domestic and wild animals.

For this research, we collaborated with Dutch municipalities. We hope our output will help them to make better informed decisions regarding urban greening and which countermeasures to take to avoid rat nuisance and to reduce the risk of zoonotic pathogen transmission to humans. In addition, a report will be written for the Dutch ministry combining the most important results of this study with advice for future research and actions. Apart from that, we transferred the results of our study to relevant stakeholders (e.g., municipalities and pest control organisations) by giving presentations and writing non-scientific articles in their magazines.

In addition, this project has helped us to strengthen the relationships with partner institutes abroad, especially with the Friedrich-loeffler Institute in Germany, where I went to for several weeks on a short-term mission. They did not only help me with several pathogen analyses, but we also had very valuable discussions about my research results, and we discussed several possible collaborations and sharing of samples between RIVM and FLI, which has already resulted in one joint paper, which can be found here. Next to that, we also collaborated with the Dutch pest and wildlife expertise centre (KAD) in a study on rodenticide resistance in the Dutch rat and house mice population, which also resulted in a joint paper (DOI 10.1002/ps.7261).

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

My PhD being part of the OHEJP doctoral programme enabled me to work with other institutes outside of the Netherlands (e.g., Friedrich-Loeffler Institut) much easier. This collaboration was a really nice and valuable experience for both me and my research. Next to that, OHEJP provides ample opportunity to network with other specialists inside or close to your own field of expertise and offers many activities and trainings to increase your skills and knowledge.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

No interactions with JRPS/JIPs, OHEJP stakeholders or external collaborations were noted.

Evaluation of the Final Thesis Report

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	-
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	5	3
Were all the milestones and deliverables completed?	3	3
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	5	1
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	3
Did the PhD student actively engage in Education and Training activities?	5	3
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	5	3
Was the PhD managed and implemented in accordance with the DMP?	4	5
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	5	4
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	5	4
Is there any direct or indirect impact of the project for national or international stakeholders?	5	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	4	3
Does the project outcomes have policy implications?	3	3
TOTAL	57/65	39/65

AVERAGE: 48/65

NB. Note that reviewer 2 did not provide a score for one question, which may affect the overall score.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer
The student concluded well on the results of the studies but did not describe in detail an outline for future work.

Reviewer 2: External Scientific reviewer
Could not be evaluated due to missing information in the section due to confidentially clause.

Reviewer 3: PMT member
Reviewer 2 score (i.e., 0) was not an option and may thus bias the average scoring.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer
Yes as their good justification for the deviations.

Reviewer 2: External Scientific reviewer
The self-evaluation could have better be matched regarding the original objectives/questions and the objectives/questions actually addressed.

Reviewer 3: PMT member
The average of both scores seems realistic.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer
M1 (milestone 1) the report should have been done in 2021- the report was received in June 2023 and no update on M1 was included into this report.

Reviewer 2: External Scientific reviewer
Some delays for deliverables and milestones. However, considering that natural systems have been studied such delays are to be expected.

Reviewer 3: PMT member
n/a

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer
STM - FLI in the report in the milestones also WBVR is also mentioned but not later on. This should be corrected.

Reviewer 2: External Scientific reviewer
No interactions are listed. However, it is unclear if such interactions were expected at the start of the project. If no interactions were expected, also a "5" would be appropriate.

Reviewer 3: PMT member
Very disparate scorings, maybe due to an interpretation of the question (do reviewers know JIP/JRP)? The PhD report only mentioned 1 JRP (TOXOSOURCES).

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
The added value apart from networking should have been better described. Visits in other labs can be "nice", but what is the added value? Did the PhD student learn lab techniques that for example were not used in the home laboratory? Has the programme and consortium increased collaboration activities? Have benefits already emerged for the post PhD student period?

Reviewer 3: PMT member
The reviewers scores are comparable.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
The skills and training are at the level that one nowadays can expect from PhD students working in international consortia.

Reviewer 3: PMT member
The average of both scores seems realistic.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer
Very good publication outcome, also considering the manuscript that are under review or preparation.

Reviewer 2: External Scientific reviewer
At this stage, there is 1 peer-reviewed scientific publication, which is average in the academic environments I am working in.

Reviewer 3: PMT member
There seems to be a misunderstanding, since three publications are mentioned, although the PhD student is first author of only one paper. The average of the scores however seems realistic.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer
n/a

Reviewer 2: External Scientific reviewer
OHEJP was acknowledged and the publication is published Golden Open Access. The score is however only based on 1 publication.

Reviewer 3: PMT member
DMP was not mentioned in the PhD report. Difficult to assess, and to assess the reviewers comments.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer
Very relevant for the project.

Reviewer 2: External Scientific reviewer
The PhD student participated in multiple dissemination and outreach activities that not only targeted the scientific community but partly also and in one case exclusively the general public.

Reviewer 3: PMT member
Both reviewers scores are aligned.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer
This project is relevant and interesting for the One Health aspect. As climate changes combined with zoonotic events can increase, the surveillance of the rat population as an indirect measure to detect potential zoonotic pathogens is an interesting approach.

Reviewer 2: External Scientific reviewer
The project outputs are of likely of high relevance for the One Health EJP aims and objectives. Unfortunately, and since results are only presented for one of the thesis chapters, it is difficult to better assess the value of the project.

Reviewer 3: PMT member
Both reviewers scores are aligned.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer
For national stakeholders. There are good collaborations with international stakeholders initiated, which should be even increased.

Reviewer 2: External Scientific reviewer
Likely yes, but this has not been highlighted in an appropriate way. Rats are an increasing challenge in many cities of the industrialised world, not only The Netherlands. The relevance of the project and its results for regions outside The Netherlands could have been identified.

Reviewer 3: PMT member
Both reviewers scores are aligned.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer
Yes it was described.

Reviewer 2: External Scientific reviewer
One partner is mentioned (FLI). More collaborations could have been possible, especially with partners of different scientific fields.

Reviewer 3: PMT member
Both reviewers scores are aligned.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer
Maybe in the future.

Reviewer 2: External Scientific reviewer
In theory yes. However, difficult to assess since only results from one of four thesis chapters are presented.

Reviewer 3: PMT member
Both reviewers scores are aligned.

The full Final Thesis Report for the DESIRE PhD project can be found, here.

PhD9-FBZSH3/AMR2.1-UDOFRIC

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Taylor Hanford	PhD Student	Animal and Health Plant Agency	United Kingdom
John Rodgers	PhD Lead Supervisor	Animal and Health Plant Agency	United Kingdom
Isabelle Kempf	PhD Second Supervisor	Anses	France
Noel McCarthy	Other Supervisor	Trinity College Dublin	The Republic of Ireland
Muna Anjum	Other Supervisor	Animal and Health Plant Agency	United Kingdom
Katell Rivoal	Other Supervisor	Anses	France
Manal AbuQub	Other Supervisor	Animal and Health Plant Agency	United Kingdom

Summary of the work carried out in the PhD project

A limited summary is provided due to a confidentiality clause.

The UDOFRIC PhD project began in M27, in 2020 and is expected to be completed in M63, in 2024. This PhD project has utilised *Campylobacter* isolates from the APHA archives and associated information (phenotypic, genomic, epidemiological meta-data) from surveillance and research across the food chain to investigate temporal trends in the development and diversity of FQ resistance in UK broiler flocks. This project has conducted *in vitro* and *in vivo* competition trials between closely related FQ-resistant and FQ-susceptible isolates; to determine if FQ resistance leads to increased survival, growth, or colonisation. The findings from this project will provide information to policymakers to help make more informed decisions to curb the increasing levels of FQ resistance in *Campylobacter* in the chicken broiler industry.

Dissemination activities of this UDOFRIC PhD project were disseminated at a range of national (i.e., Warwick Medical School PGR Symposium, Warwick University, UK) and international (i.e., International Society for Animal Hygiene's annual 20th annual congress in Berlin) conferences.

Work carried out on the PhD, scientific results, and outcomes

Limited non-confidential summaries of each thesis chapter have been provided.

Chapter 1. Temporal trends and epidemiological analysis of fluoroquinolone resistance in broiler production

This section has collated UK national surveillance data from 1995 to 2020. Initial work included the filling of data gaps to align all datasets with the appropriate information, namely determining FQR profiles and of *Campylobacter* isolates and using whole genome sequences to determine MLST profiles. Using this information, FQR was traced across production variables, such as farming method used to rear the broiler chickens, the season when the chickens were sampled, the age of the chickens at the time of sampling and the abattoirs where the chickens were processed.

Chapter 2. Sequence analysis of whole genome sequences and trends in fluoroquinolone resistant sequences

Eight isolates were randomly selected from each month from the years 1995, 2008, and 2020 where possible and underwent short-read whole-genome sequencing, using the Illumina NextSeq platform at the APHA Central Sequencing Unit.

A genotypic profile for FQR in *Campylobacter* was then identified and validated against existing phenotypic information. A phylogenetic tree of sequences was then determined on both the large and small scale. Closely related FQR and FQS isolates were then selected for gene-by-gene analysis to spot any genetic variation that may lead to a fitness advantage in *Campylobacter*.

Chapter 3. In vitro fitness study: Comparative growth and survival kinetics of fluoroquinolone susceptible Campylobacter jejuni strains and their isogenic resistant mutants

This chapter aims to assess if the acquisition of FQR leads to increased fitness, measured by growth capabilities and survival in both faeces and food matrices models. This study measured the growth rate of FQS and FQR isolates. This section also assessed the survival capabilities of isolates on faeces and measured the ability of our *C. jejuni* strains to survive on a food matrices model in chilled conditions.

Chapter 4. In vivo study: Colonisation potential of fluoroquinolone susceptible Campylobacter jejuni strains and their isogenic resistant mutants

Using specific pathogen free chickens, this study compared the colonisation potential of FQR and FQS isolates used in Chapter 3. Colonisation was measured over a 35-day period with faecal samples taken at regular intervals throughout the trial before final caeca samples were taken.

PhD self-assessment

The initial PhD project hypothesis, as outlined in the proposal, was “FQ use in broilers plays a key role in the development of FQ resistance in *Campylobacter* and consequent risk of human disease with FQ strains”. The work carried out by the student in the last three years has addressed this question by tracing FQR *Campylobacter* in broiler chickens throughout production variables such as farming method used to rear the chickens sampled, the abattoir of processing, the age of the birds at the time of slaughter, the date at which the birds were

sampled and MLST groups. It has identified significant trends in the data to address areas for further research. Results from *in vitro* models has provided insight into the ability of FQR *Campylobacter* mutants to pass not only within a broiler flock but to humans through contaminated food produce. Finally, *in vivo* competition models, using specific pathogen free chickens (SPFCs) have been used to assess the colonisation capabilities of FQS *C. jejuni* against their FQR mutant counterparts in chickens.

Original objectives set out in the project proposal was to feed into topics such as:

1. Development and harmonisation of NGS-based methods for AMR determinants.
2. Source attribution and transmission routes.
3. Epidemiological studies.
4. Model systems to study host/microbe interactions.
5. Epidemiological studies in the dynamics of AMR in human and animal populations including horizontal gene transfer and selection of AMR.

This project has addressed each of these five areas of research. Future work by the student includes a publicly available thesis and scientific publications which will pass on the findings from this PhD project contributing to scientific knowledge in this area of research.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
UDOFRIC	D-PhD09-1.1	Completion of 9-month review	M33	M33	Confidential	
UDOFRIC	D-PhD09-1.2	Literature review of FQ in Campylobacter	M37	M38	Confidential	
UDOFRIC	D-PhD09-1.3	PhD Annual review	M38	M38		
UDOFRIC	D-PhD09-2.1	Description of the diversity of FQ resistance and acquisition of resistance variants over time.	M41	-	This report has been delayed due to difficulties in obtaining confidential data. Draft documents are currently being processed.	
UDOFRIC	D-PhD09-2.2	Report on the relationship between WGS and phenotype.	M42	-	Draft versions of this report are currently in process.	
UDOFRIC	D-PhD09-2.3	~~GWAS studies and identification of strains for fitness trials~~ Identification of strains for fitness trials	M45	-	No longer applicable previously replaced with "Identification of strains for fitness trials". This change in report topic has led to rescheduling of the deadline.	
UDOFRIC	D-PhD09-3.1	In vivo selection and characterization of isogenic resistant strains	M49	-	Report delayed due to difficulties regaining susceptible strain of wt-02 post passage. Further processing of the strain was needed ensure the validity of *in vivo* experiments.	
UDOFRIC	D-PhD09-3.2	In vitro fitness study: competition growth assays and growth kinetics	M51		Report delayed due to difficulties regaining susceptible strain of wt-02 post passage. Further processing of the strain was needed ensure the validity of *in vivo* experiments.	
UDOFRIC	D-PhD09-3.3	In vitro fitness study: comparison of survival on abiotic surfaces and on food matrices (e.g. chicken exudate model)	M53	-	*In vitro* trials reorganised with *in vivo* trials to fit scheduling with animal supplies and facilities. Final experimental work is needed to be completed at the students return to the APHA in M59. This delay was due to the student contracting COVID over the Christmas period, therefore preventing his return to France from the UK.	
UDOFRIC	D-PhD09-3.4	In vivo study: comparison of colonisation using chicken models	M57	-	All *in vivo* experiments are now complete with the writing up of the results currently underway.	

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD Reference	Milestone number	Milestone name	Mxx (M1 = Jan 2018, M60= Dec 2022)	Mxx (M1 = Jan 2018, M60= Dec 2022)	Yes/No	
PhD09-FBZSH3/AMR2.1-UDOFRIC	1	Completion of literature review	M37	M37	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	2	Completion of data collation and identification of data gaps	M33	M33	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	3	Completion of training in bacteriology and MIC	M36	M36	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	4	Completion of 9-month review	M33	M33	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	5	WGS and bioinformatics training	M36	M36	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	6	Annual review	M38	M38	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	7	Completion of phenotyping and WGS	M39	M43	**Yes**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	8	Descriptions of genomic diversity and temporal trends in resistance	M41	M65	**No**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	9	Relationship between WGS and phenotype	M42		**No**	
PhD09-FBZSH3/AMR2.1-UDOFRIC	10	Identification of strains for fitness study	M45	M45	Yes	
PhD09-FBZSH3/AMR2.1-UDOFRIC	11	Completion of 9-month review	M45	M45	Yes	
PhD09-FBZSH3/AMR2.1-UDOFRIC	M12	*In vitro* fitness models	M58	M58	Yes	
PhD09-FBZSH3/AMR2.1-UDOFRIC	M13	*In vivo* fitness models	M57	M58	Yes	

Publications and additional outputs

Publications

At the time this deliverable was submitted, the UDOFRIC PhD project has not published any scientific publications.

Additional outputs (i.e., poster/oral presentations)

The UDoFRiC PhD project disseminated these works through a range of oral and poster presentations at the following events:

- Oral presentation at International Society for Animal Hygiene's annual 20th annual congress in Berlin. 5-7th Oct 2022.
- Oral presentation – 180 second thesis at Journées Scientifiques et Doctorales de l'Anses (JSDA), Saint Malo, France. 18-19 Oct 2022.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University, UK. 22nd June 2022.
- Hanford, T., McCarthy, N., Kempf, I., Rivoal, K., Cawthraw, S., Anjum, M., Abu Oun, M., & Rogers, J. (2022). Identifying key characteristics in Fluoroquinolone Resistant Campylobacter throughout the production chain. Poster presentation at One Health EJP ASM, Orvieto, Italy. 11-13th April 2022. Abstract available here.
- 3-minute thesis presentation & roundtable discussion at One Health EJP ASM, Orvieto, Italy. 11-13th April 2022.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University. UK. 26th May 2021.
- Poster presentation & 3-minute thesis presentation at One Health EJP ASM, hybrid event. 9-10th June 2021.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University, UK. 30th Sept 2020.
- 3-minute thesis, One Health EJP ASM 2020, online. 27-29th May 2020.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
EURL Campylobacter Proficiency Test 2022	WGS and cluster analysis of Campylobacter	01/06/2022	EURL
OH EJP Annual Scientific Meeting	Scientific Conference	11/04/2022-13/04/2022	OH EJP
Warwick University Medical school Post-graduate symposium	Scientific Conference	22/06/2022	Warwick University
20th Congress of the International Society for Animal Hygiene	Scientific conference	5-7 October 2022	International society for animal hygine
ANSES's Scientific and Doctoral Days (JSDA)	Scientific conference	18-19 October 2022	French Agency for Food, Environmental and Occupational Health & Safety (ANSES)
OH EJP Annual Scientific Meeting	Virtual scientific conference	09/06/21 - 11/06/21	One Health European Joint Project
Warwick University Medical school Post-graduate symposium	Virtual scientific conference	26/05/21	Warwick University
EURL-AR online training course	Training course	26/04/21 - 29/04/21	EURL

One Health impact

Whilst this project is ongoing some results from this project have yet to be written up and have therefore been excluded from this report. Thesis due date March 2024. This project has been a collaboration between three institutions within the EJP namely the University of Warwick, the APHA and Anses. It has drawn upon and shared the experiences of all supervisors from their respective institutions to further understand how fluoroquinolone resistance effects the fitness of *Campylobacter* within the chicken broiler industry. It has allowed the sharing of knowledge between these institutions and led to integration and alignment in the identification of FQ resistance in *Campylobacter* and its associated risk factors. Each institution has played a key role in the project with the APHA providing historic UK surveillance data and *Campylobacter* isolates, Anses providing facilities, training, and equipment for *in vitro and in vivo* testing and the University of Warwick training the student in scientific methods and communications. Each of the collaborators of this project have built strong connections, with some going on to work together on future projects.

This project has analysed information from public health surveillance which has monitored zoonotic disease (*Campylobacter*) and through this analysis has identified factors associated with FQR *Campylobacter* and its potential impacts on animal health and its potential transmission to humans. Findings from this project has confirmed the use of genotyping in the detection of FQR *Campylobacter*. Also, through the identification of *Campylobacter* groups with a high association with FQ resistance within chicken broiler flocks, policy makers may be able to put in place diagnostic strategies to prevent the spread of this emerging threat.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

By completing my PhD with the OHEJP doctoral programme I have had access to three institutions at the top of their fields. The collaboration of my supervisors has given me training from a range of experience and expertise. I have had regular contact, training, and examination from the University of Warwick throughout my PhD. Feedback from examiners at the University of Warwick provided me important feedback on my project plan and had a significant impact on its outcome. The APHA has been my main institution throughout the course of my PhD, it has given me great lab experience and trained me from a novice in bioinformatics. I have given a number of presentations and talks in my time at the APHA where employees have given me feedback on my work to better myself. I spent one year of my PhD at Anses in France due to the OHEJP collaborations. This was an exciting time of my PhD, and I learned a lot from my time there. I conducted my first *in vivo* experiments and was taught to a high standard. I was also able to design my *in vitro* experiments under the guidance of my supervisors and other members of the team at Anses which was an important part of my scientific training.

The OHEJP has also held three international conferences throughout the course of my PhD, and I have been able to (virtually) attend them all. These conferences gave me experience in presentations and public speaking which I will carry through my scientific career.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

This project was co-funded by the Veterinary Medicines Directorate, UK and VetBioNet (Grant Agreement Number: 731014).

Evaluation of the Final Thesis Report

	PROJECT	
CRITERION	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	3
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	3	4
Were all the milestones and deliverables completed?	2	4
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	1	1
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	5	4
Did the PhD student actively engage in Education and Training activities?	4	3
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	1	1
Was the PhD managed and implemented in accordance with the DMP?	1	3
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	4	4
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	3	3
Is there any direct or indirect impact of the project for national or international stakeholders?	2	3
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	3	4
Does the project outcomes have policy implications?	2	2
TOTAL	35/65	39/65

AVERAGE: 37/65

NB. These scores and comments are based on a confidential version of the Final PhD Thesis Report, which isn't fully outlined in D6.18.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Suggestions for future work have been included. These go beyond the work itself, although they are generic.

Reviewer 3: PMT member

Yes, there are recommendations given which address limitations of the ongoing study and questions which cannot be dealt with.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

According to the Final report the PhD objectives have been addressed. Nevertheless the results showed in the 4 chapters do not fully cover the original objectives mentioned. I suggest to better report objectives.

Reviewer 2: External Scientific reviewer

Based on included work. A significant amount of the work done was not shown in the report due to confidentiality.

Reviewer 3: PMT member

There is a gap between the hypothesis ""FQ use in broilers plays a key role in the development of FQ resistance in Campylobacter and consequent risk of human disease with FQ strains" and the objectives listed. The objectives as listed (not suitable to answer the hypothesis) might be addressed once the work is completed. But objectives are too ambitious (e.g. source attribution) to address each topic properly with appropriate methods to provide really new evidence.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

The deliverable D-PhD09-3.3 is to be completed soon. Some milestones are missing.

Reviewer 2: External Scientific reviewer

Progress good, changes to milestones appropriately justified.

Reviewer 3: PMT member

No, unfortunately for 7 out of 10 deliverables no completion date is given.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

The section is empty I cannot have idea about the interactions.

Reviewer 2: External Scientific reviewer

None reported.

Reviewer 3: PMT member

No information given, no interaction mentioned.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

There is evidence of interaction among the institutions, the OJP network and the student.

Reviewer 2: External Scientific reviewer

The PhD candidate benefitted from having support and supervision from two OHEJP partners (APHA, ANSES), from being able to participate in the OHEJP annual meetings and conferences.

Reviewer 3: PMT member

The main benefit seems to relate to the cooperation between several institutions. Participation in additional elements, e.g., summer schools offered by OHEJP are not mentioned.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

The student was enrolled in several activities during the PhD. Most of them are dissemination activities and only one seems a structured training.

Reviewer 2: External Scientific reviewer

Good training at the participating institutes and some online courses.

Reviewer 3: PMT member

Yes, linked to the training programme of the enrolled universities and institutions.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

No records have been added. Probably publications will follow the ending of the PhD.

Reviewer 2: External Scientific reviewer

Not yet. The studentship is not finished yet, thesis submission is in 2024.

Reviewer 3: PMT member

No publications mentioned, also no submission of manuscripts for review; not very clear whether the PhD thesis needs to include several published papers.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

Section 14 miss the DMP.

Reviewer 2: External Scientific reviewer

Not specifically indicated.

Reviewer 3: PMT member

No reference to the DMP.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

There is evidence of scientific dissemination during the PhD period.

Reviewer 2: External Scientific reviewer

PhD student participated and presented at conferences, participated in competitions. Appropriate for UK PhD student.

Reviewer 3: PMT member

Yes, several presentations and posters.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Will be higher once confidential parts are released.

Reviewer 3: PMT member

Very relevant once available but not clearly spelled out in the respective section.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Potential for future, but not yet with data produced. Long term vision required.

Reviewer 3: PMT member

Yes, in my point of view, new methods and technical approaches become available; not clearly spelled out in the paragraph.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Good collaboration between UK-France partners, potential to expand.

Reviewer 3: PMT member

Yes, this worked and is described.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Not yet. Potential for future.

Reviewer 3: PMT member

Yes, once the results are available, but needs still to be described.

The full Final Thesis Report for the UDoFRiC PhD project can be found, here.

PhD10-FBZSH3/AMR2.1-WILBR

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Olivia Turner	PhD Student	Animal and Plant Health Agency	United Kingdom
Muna Anjum	PhD Lead Supervisor	Animal and Plant Health Agency	United Kingdom
William Gaze	PhD Second Supervisor	University of Exeter	United Kingdom

A non-confidential version of the PhD Final Thesis report wasn't submitted to WP6 to form part of D6.18.

Summary of the work carried out in the PhD project

The WILBR PhD project began in M26 in 2020 and is expected to finish in M67, in 2023. A five-month extension was provided due to the SARS-CoV-2 pandemic. The WILBR project had a core aim, which was despite wild birds not being intentionally exposed to antimicrobials. antimicrobial resistance (AMR) is widespread in bacteria in some wild bird populations. The WILBR project aims to explore the likelihood of wild birds as a vector of transmission of AMR to farms.

Dissemination activities of this WILBR PhD project were enshrined in two peer-reviewed publication, in a journal including the Journal of Antimicrobial Chemotherapy, and a number oral and poster presentations at the national (i.e., Warwick Medical School PGR Symposium) and the international level (i.e., One Health EJP Annual Scientific Meeting, Italy).

Work carried out on the PhD, scientific results, and outcomes.

A non-confidential version of the WILBR PhD Final Thesis Report was not submitted for part of the public D6.18.

PhD self-assessment

In the original proposal it states that the project aims to specifically examine the role wildlife play in the occurrence and transfer of AMR and its contribution to the global AMR problem. It states that some of the major shortcomings of studies focusing on AMR and wild birds, are that these are one-off studies, based on an opportunistic sampling-scheme from limited geographic areas and often limited number of samples. This PhD has addressed this aim and worked to fill these knowledge gaps by carrying out a longitudinal and a cross-sectional study that resulted in over 1000 isolates undergoing whole genome sequencing.

Pig and gull faecal samples were collected for the longitudinal study and 615 *E. coli* isolates underwent whole genome sequencing (pig = 342; gull = 273). The results of this study have shown that on this low antimicrobial usage pig farm there is clonal expansion of MDR bacteria, with bacterial clones present in both pig and gull isolates across multiple timepoints. This is indicative of repeated interspecies transmission events over time and suggests that wild birds are contributing to the persistence of MDR bacteria in the pig populations on farm. The results also indicate that this continues to happen even in the absence of the selective pressure of antimicrobials.

The cross-sectional study provides an in-depth look at the role of wild birds in the transmission of AMR bacteria on an outdoor pig farm with a more conventional approach to antimicrobial usage. This study resulted in 472 *E. coli* and non-*E. coli* isolates from pig, wild bird, and environmental swabs undergoing whole genome sequencing. The aim of this study was to investigate the role of the farm environment, as well as wild birds, as a reservoir of AMR bacteria. The study also considers the role of non-*E. coli* isolates in the transmission of AMR between pigs, wild birds, and the environment.

The original project proposal aimed to include *E. coli* isolates from wild birds in Sweden as a comparison of wider geographical areas. However, because of the SARS-CoV-2 pandemic the PhD student was unable to carry out the planned 6-month secondment to SVA laboratories to isolate bacteria collected from Swedish samples. There was also a change in the PhD supervisory team during the project as the supervisor based at SVA moved to a job role outside of the agency, so no longer had access to Swedish surveillance data. As a result of this samples were instead collected from farms from different geographical areas of the UK, and international isolates were included in some sections of WGS analysis from OHEJP partner institutes and online databases.

The student will continue to finish analysing the data produced by this project, keeping the original aims of the project in mind when writing their final PhD thesis.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
WILBR	1	Literature review of contribution of wild birds to AMR in the farm environment	M32	M32	*Confidential as will be included in the introduction of final PhD thesis*	
WILBR	2	Completion of 9 month review (Y1)	M33	M33		
WILBR	3	Completion of 9 month review (Y2)	M45	M45		
WILBR	4	Completion of 12 month review (Y2)	M48	M48		
WILBR	5	Completion of 9 month review (Y3)	M57	M57		
WILBR	6	Completion of 12 month review (Y3)	M60	M60		

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD Referenc	Milestone number	Milestone name	Mxx (M1 = Jan 2018, M60= Dec 2022)	Mxx (M1 = Jan 2018, M60= Dec 2022)	Yes/No	
WILBR	1	Completion of literature review	M32	M32	Yes	
WILBR	2	Recruitment of farm for longitudinal study	M33	M30	Yes	Due to severe delays to fieldwork caused by the COVID-19 pandemic, this had to be changed to a cross-sectional study
WILBR	3	Collection of microbiological samples for longitudinal study	M48	M45	Yes	
WILBR	4	Molecular analysis of bacterial isolates and antimicrobial resistance	M60	M65	No	
WILBR	5	Characterisation of mobile elements transmitting/proliferating AMR	M60	M66	No	
WILBR	6	Completion of 9 month review (Y1)	M33	M33	Yes	
WILBR	7	Completion of 9 month review (Y2)	M45	M45	Yes	
WILBR	8	Completion of 12 month review (Y2)	M48	M48	Yes	
WILBR	9	Completion of 9 month review (Y3)	M57	M57	Yes	
WILBR	10	Completion of 12 month review (Y3)	M60	M60	Yes	

Publications and additional outputs

Publications

The WILBR PhD project has enshrined these works through peer-reviewed publications, which are these are:

- Francesca Martelli, Manal AbuOun, Shaun Cawthraw, Nathaniel Storey, Olivia Turner, Matthew Ellington, Satheesh Nair, Anais Painset, Christopher Teale, Muna F Anjum. (2022). Detection of the transferable tigecycline resistance gene tet(X4) in *Escherichia coli* from pigs in the United Kingdom. doi. 10.1093/jac/dkab439
- Nathaniel Storey, Shaun Cawthraw, Olivia Turner, Margherita Rambaldi, Fabrizio Lemma, Robert Horton, Luke Randall, Nicholas A Duggett, Manal AbuOun, Francesca Martelli, Muna F Anjum. (2022). Use of genomics to explore AMR persistence in an outdoor pig farm with low antimicrobial usage. doi. 10.1099/mgen.0.000782

At the time of this deliverable, once publication was uploaded to Zenodo, becoming gold standard open access and can be found, here.

Additional outputs (i.e., poster/oral presentations)

The WILBR PhD project disseminated these works through oral and poster presentations at the following events:

- Oral presentation at International Society for Animal Hygiene's annual 20th annual congress in Berlin. 5-7th Oct 2022.
- Oral presentation – 180 second Thesis at Journées Scientifiques et Doctorales de l'Anses (JSDA), Saint Malo, France. 18-19 Oct 2022.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University, UK. 22nd June 2022.
- Hanford, T., McCarthy, N., Kempf, I., Rivoal, K., Cawthraw, S., Anjum, M., Abu Oun, M., & Rogers, J. (2022). Identifying key characteristics in Fluoroquinolone Resistant Campylobacter throughout the production chain. Poster presentation at One Health EJP ASM, Orvieto, Italy. 11-13th April 2022. Abstract available here.
- 3-minute thesis presentation & roundtable discussion at One Health EJP ASM, Orvieto, Italy. 11-13th April 2022.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University. UK. 26th May 2021.
- Poster presentation & 3-minute thesis presentation at One Health EJP ASM, hybrid event. 9-10th June 2021.
- Poster presentation at Warwick Medical School PGR Symposium, Warwick University, UK. 30th Sept 2020.
- 3-minute thesis, One Health EJP ASM 2020, online. 27-29th May 2020.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Scientific Writing Skills Workshop	Development of skills in scientific writing	04-05/02/2020	Animal and Plant Health Agency
OHEJP Summer School	Global One Health	17-28/08/2020	Wageningen University
EURL-AR Training Course	Working with bacterial sequence data in relation to the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria	26-29/04/2021	Technical University of Denmark
Engaging in Professional Development	Workshop	14/06/2022	University of Exeter
Research and innovation to reduce the burden of antibiotic resistance: strengthening the European action	Conference	07/06/2022	UE France 2022
GW4 AMR Alliance Early Career Pathways	Webinar	19/05/2022	GW4 AMR Alliance
GW4 AMR Alliance Lightning Talk and Networking Session	Online networking session	28/02/2022	GW4 AMR Alliance

One Health impact

The results of this project have indicated that wild birds can play a role in the transmission and persistence of AMR bacteria in the farm environment. The results suggest that there can be repeated horizontal transmission events between pigs and gulls, but also vertical transmission of AMR bacteria within epidemiological groups on a farm. This data could help inform national and European surveillance networks, in the value of including samples from wild bird species. Results of this study could be further disseminated by the supervisory team as they are involved in two JPIAMR surveillance networks (Towards Developing an International Environmental AMR Surveillance Strategy; Wildlife, Agricultural soils, Water environments and antimicrobial resistance - what is known, needed and feasible for global Environmental Surveillance). Expanding existing surveillance networks to include samples from wild birds could help uncover more of these transmission events and assist in mitigating against the worst of them to prevent entry into the food chain.

The results of this project also have implications for biosecurity. Stakeholders can make recommendations to farmers on how to improve their biosecurity measures on farm to reduce close contact between wild birds and food producing animals and therefore transmission events of AMR bacteria. Wild birds are known to be opportunistic feeders so an example of

this could be to use covered feeders instead of open troughs on outdoor farms so that it is more difficult for wild birds to access.

This PhD project has been co-funded by the VMD who are involved in the development of UK government policy as a devolved agency of the Department for Environment, Food and Rural Affairs. There have been multiple knowledge transfer events between the PHD student and the co-funding body over the last three years. VMD has received annual reports from the PhD student and been happy with the progress made during their studies, they have also approved all posters and presentations prior to them being taken to workshops and conferences. This was one of the first studies carried out at APHA investigating the role of wild birds in the transmission of AMR bacteria, but there are now further projects being funded within the agency to study this and it is being incorporated into national surveillance schemes.

The student has presented their work at multiple UK-based and international conferences where international stakeholders were in attendance, increasing the reach of the research and exposure to policymakers and stakeholders.

This project has reinforced links with OHEJP ARDIG project partner institutes as the student utilised samples that were originally collected as part of the APHA's work on this project. The student contacted partner institutes to ask if they would share WGS data of *E. coli* ST744 isolates they had collected as part of their involvement in the project. The student received 56 isolates from seven partner institutes which included isolates from humans, wild birds, poultry, cows, and pigs.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

Being a part of the OHEJP doctoral training programme has granted me the opportunity to receive unique training opportunities in the form of the OHEJP Summer School (2020). On this course I was able to work with scientists from countries, institutions, and scientific backgrounds across the world that I would otherwise not have had the opportunity to interact with, broadening my horizons and way of thinking.

I have also been able to attend multiple international conferences, both in person and online, to present my work. This has allowed me to improve my scientific communication skills resulting in me winning prizes for 'Best poster presentation' and 'Best oral presentation by an early career researcher' at conferences. This has also given me the opportunity to network with my fellow OHEJP PhD Students and other scientists working within One Health.

Through OHEJP I have also been able to easily access WGS data from OHEJP partner institutes that were involved with OHEJP ARDIG project. As a result of this I could increase the size of my dataset and try to gain further insights into the potential origins of MDR E. coli isolates from the APHA ARDIG farm.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The WILBR PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- Attended workshops and participated in online networking sessions run by the GW4 AMR Alliance. This is a One Health AMR research consortium at the Universities of Bath, Bristol, Cardiff, and Exeter which aims to tackle the global threat of antimicrobial resistance.
- This PhD overlaps with the OHEJP ARDIG project, where wild birds on farm have already been sampled.
- I have also received *E. coli* ST744 WGS data from OHEJP stakeholders involved in the OHEJP ARDIG project that has been included in my second results chapter. These stakeholders were: L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Universidad Complutense de Madrid (UCM), University of Surrey (UOS), National Veterinary Institute (NVI), Public Health England (PHE), German Federal Institute for Risk Assessment (BfR), Institut Pasteur (CNR).
- The PhD has been co-funded by the Veterinary Medicines Directorate (VMD) which is an executive agency of the Department of Environment, Food and Rural Affairs (Defra) within the UK Government. VMD contributes to DEFRA's objectives to protect public health and meet high standards of animal welfare. Their work also helps the Food Standards Agency to protect and improve the safety of food people eat. A report by the PhD student was also submitted to VMD detailing the progress of the project as well as receiving approval on all external presentations of the WILBR research.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	3
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	5	4
Were all the milestones and deliverables completed?	3	5
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	4	4
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	4
Did the PhD student actively engage in Education and Training activities?	5	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	2	2
Was the PhD managed and implemented in accordance with the DMP?	3	4
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	4	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	5	4
Is there any direct or indirect impact of the project for national or international stakeholders?	4	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	4	5
Does the project outcomes have policy implications?	3	4
TOTAL	50/65	53/65

AVERAGE: 51.5/65

NB. The PMT member who reviewed the SUSTAIN PhD project provided a global overview of the PhD Final Thesis Report, outlined below:

It is clearly stated in the report that the PhD is unfinished and only a draft version is presented, and some parts/chapters are still unfinished/incomplete. The information provided in the report, however, was enough for the external reviewers to be able to conduct the assessment. This internal reviewer agrees, the information needed to answer all the evaluation criteria questions is given in the report, in particular the results of the studies are clearly shown. The majority of the scores given by the external reviewers are above average (scores 4 and 5), 4

scores are average (score 3) and 2 scores are slightly (score 2), no scores 1 (not at all) are given. As a whole, the scores reflect fairly and adequately the good quality of the studies and the report. Most scores are accompanied/motivated by a comment. The comments of both external reviewers are to a large extent in agreement between them. However, for some questions the scores and comments differ, for example regarding the progress of the report reviewer 1 scored 3 because the PhD and some sections were unfinished while reviewer 2 scored 5 because all milestones and deliverables were completed. Scientific publications received score 2 by both reviewers and this internal reviewer agrees, this is because only 2 publications are shown and the PhD student is not first, second or last author in any of them. Dissemination and communication activities are very good, and the student has won presentation prizes, this is reflected in the high scores. One health impact received in general high scores, mostly 4 and 5, however, in this reviewer's view scientific impact is clear shown while policy impact could have been more strongly demonstrated. In my opinion, this deliverable can be finalised.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

There are thorough suggestions on what future work should be conducted.

Reviewer 2: External Scientific reviewer

Additional research is proposed in the form of tracking and sampling wild birds and further farms. However, with the data that was collected here and in previous research that was part of the literature review, I believe that the role of wild birds is quite evident. It would've been good if the student had shown a wider awareness and possibly proposed further research into biosecurity measures that can reduce the contact of wild birds with farm animals or other changes in legislation that aim to reduce the presence of wild birds on farms.

Reviewer 3: PMT member

See above.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Yes, there were some issues during the project (among others related to the SARS-CoV-2 situation) but how these were handled and overcome are satisfactory addressed and explained in the report.

Reviewer 2: External Scientific reviewer

The project proposal has not been seen so deviations beyond those that the PhD-student reported cannot be assessed. Deviations due to SARS-CoV-2 are apparent when sampling on farms and in other countries are not possible. Despite this difficulty, a longitudinal study on UK farms was carried out, which is analysed as planned, as far as I can assess.

Reviewer 3: PMT member

See above.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Overall yes se comment above, Although the PhD us unfinished, and Chapter 3 and 4 is also unfinished in the report only presenting some data.

Reviewer 2: External Scientific reviewer

As far as can be assessed, all milestones and deliverables have been completed, although not all are publicly available yet, until the PhD student has finished her thesis examination.

Reviewer 3: PMT member

See above.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Yes.

Reviewer 2: External Scientific reviewer

The student has interacted very much with the JRP ARDIG. Based on the report, no further interactions have been noticed.

Reviewer 3: PMT member

See above.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

Exchange and courses.

Reviewer 2: External Scientific reviewer

The student has engaged in several OH-EJP related training courses and network opportunities, including the OH-EJP summer school.

Reviewer 3: PMT member

See above.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

Yes.

Reviewer 2: External Scientific reviewer

The student has used opportunities for training very well, both locally form her university and within the OH-EJP network, but also abroad.

Reviewer 3: PMT member

See above.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

The student is part of two articles published in well-regarded peer-reviewed journals, although non as a main, second or last author. Although the PhD us unfinished and based on the material and results there are more than likely that additional publications will be available in the near future. Chapter 3 and 4 is also unfinished in the report only presenting some data.

Reviewer 2: External Scientific reviewer

So far, one peer-reviewed publication is listed on which the student is not first or second author, which is mostly described in Chapter 1 of the results section. Chapter 2 is a chapter that appears nearly ready for publication, but it is not mentioned if that work is currently under review. Chapters 3 and 4 mostly consist of figures. While these figures show that the bulk of the work has been done in terms of sample collection and analysis, the lack of description and discussion of the results is an apparent problem. Another paper is listed but the results from that study are not in the report and are therefore assumed not to be part of the thesis, although the student has undoubtedly contributed to this work.

Reviewer 3: PMT member

See above.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

See the comment above regarding scientific publications.

Reviewer 2: External Scientific reviewer

The DMP is not mentioned in the report, and this is difficult to assess. It appears that all relevant data, mostly WGS, was made publicly available upon publication. Furthermore, all reports were made available on Zenodo, so it appears that there is no issue here.

Reviewer 3: PMT member

See above.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The student has attended several conferences and webinar, both as part of OH-EJP activities and beyond. Furthermore, she has won two presentation prizes, showing she is very familiar and comfortable in communicating her research.

Reviewer 3: PMT member

See above.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

Very relevant especially in providing comparing data from the environmental and the specifically wildlife to that available from domesticated animals and humans which are lacking.

Reviewer 2: External Scientific reviewer

While the results of chapters 1 and 2 are very relevant, this is difficult to assess for chapters 3 and 4 as they are not yet in a finished state.

Reviewer 3: PMT member

See above.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

Giving data on the relatedness of AMR in pigs and gulls, which is need for agencies and a bases for establishing a surveillance and risk assessment of AMR in wildlife, it's also important data for the farmers in regard to influence of wildlife on the occurrence of microorganisms in their farms and the potential dissemination to and from farms to the environment and wildlife.

Reviewer 2: External Scientific reviewer

I believe that the results clearly show the need for further research, as the PhD student describes as well. However, it would've been nice to see if the future perspectives had had more depth to it.

Reviewer 3: PMT member

See above.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

It builds on previous projects (ARDIG) and the exchange of information and strains between partners is central for the project. It also has exchange with projects and networks financed through the JPIAMR.

Reviewer 2: External Scientific reviewer

The student has described the collaborations abroad within the OH-EJP network, mostly with the NVI, SVA and other institutes within the network. Furthermore, outside the network but within the UK, a collaboration with the University of Exeter was established in the form of a secondary supervisor from this institute.

Reviewer 3: PMT member

See above.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

No direct outcomes but it's likely that the results can have impact on future policies.

Reviewer 2: External Scientific reviewer

Currently, no policy implications are foreseen, but an opening to further research into the relationship of AMR on farms and in wildlife has been established nicely. Based on further research, it can be envisioned that biosecurity on farms may be further regulated in the future.

Reviewer 3: PMT member

See above.

The full Final Thesis Report for the WILBR PhD project can be found here.

PhD11-FBZ4/5- EnvDis

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Laura C. Gonzalez Villeta	PhD student	University of Surrey	United Kingdom
Giovanni Lo Iacono	PhD Lead Supervisor	University of Surrey	United Kingdom
Joaquin Prada	PhD Second Supervisor	University of Surrey	United Kingdom
Alasdair Cook	PhD Second Supervisor	University of Surrey	United Kingdom
Gordon Nichols	Other Supervisors	The UK Health Security Agency	United Kingdom
Theo Kanellos	Other Supervisors	Zoetis	United Kingdom

Summary of the work carried out in the PhD project

The EnvDis PhD project began in M25, in 2020 and is expected to finish in M65, in 2023. A five-month extension was provided due to the SARS-CoV-2 pandemic. EnvDis focused on the environmental component of the One Health approach. The seasonal pattern observed in the incidence of certain infectious diseases motivated to frame weather as an important factor driving cases and question its power to predict incidence. Using salmonellosis as an example, this PhD project explored the main weather factors driving disease in England and Wales and assessed the suitability of forecasting salmonellosis from weather data in a different spatial location as well as a different weather scenario.

The novelty of this study lies in the comprehensive exploration of the effect of 13 weather factors, at high spatio-temporal linkage, stratified in different combinations, which challenges other studies of single parameters in a silo. The good performance observed when reproducing the empirical data is a validation of the methodology. Without fitting the data, this approach enables drawing objective conclusions from complex weather interactions, facilitating the investigation of relevant factors influencing in human salmonellosis incidence, such as different weather factors alone and in combination, the effect of temperature in bacterial growth in food as well as characterisation of the universality of short-term weather and long-term climate to influence salmonellosis incidence. In more detail the to achieve these aims, the following objectives were outlined:

1. Review existing knowledge on non-typhoidal salmonellosis in humans, sources of infection, the effect of weather and climate modulating microbial growth and disease incidence, as well as common approaches used to model this relationship.
2. Investigate the effect of temperature on the growth of *Salmonella* in the main foodstuffs associated with salmonellosis and the relevance it has on the overall incidence in humans. A mechanistic approach was used for this.

3. Identify relevant weather factors and their interconnected influence on the number of salmonellosis cases reported in England and Wales. A statistical model was developed to assess the power of weather in driving salmonellosis cases.
4. Test the universality of the effect of weather on salmonellosis, regardless of geographical location. The statistical model was applied to the incidence data from a different country from the one in which it was originally developed.
5. Estimate the impact that climate change may have on the patterns of salmonellosis incidence in the near future (2043) in England and Wales.

The research presented contributes to existing knowledge on the effect of weather and climate as modulators of disease, which is widely accepted but under-investigated. The methodology developed identified maximum air temperature, relative humidity, precipitation, dewpoint temperature, daylength and global radiation as some of the most relevant weather factors driving salmonellosis, and more importantly, characterised the influence each combination exerts on the incidence of disease. While not delving into the underlying mechanisms behind the effect of weather, the knowledge generated from this thesis contributes to generate hypotheses regarding mechanisms of disease co-occurrence with weather and lays the groundwork for future predictive tools.

Dissemination activities of this EnvDis PhD project were presented through oral and poster presentations at the national (i.e., The Neglected Tropical Diseases (NTD) workshop, Brighton, UK) and the international levels (i.e., 6th World One Health Congress).

Work carried out on the PhD, scientific results, and outcomes

The performance of the model for different weather factors was assessed visually by comparing the empirical cases with the reconstruction of the modelled cases averaged for all the years studied. No quantitative operator was used given that the subtle and varied performance of the different weather combinations cannot be captured by a test. Furthermore, each factor has a different scale, is measured with different units, and have more constant values (e.g., humidity) than others (e.g., of radiation), penalising a natural variability by a coefficient that only looked at the similarity of the curves. The analogous visual agreement of the simulations with empirical data validated the suitability of our model, and pointed to maximum and mean air temperature, relative humidity, precipitation, and global radiation as important explanatory variables in England and Wales. Daylength proved to be a good proxy for global radiation where necessary. At the same time, the astronomical nature of the day length implies reduced variability and measurement errors. Other variable combinations and time lags were explored with less to no relevance.

Results from England and Wales

The model reproduced the main seasonal peak of salmonellosis incidence between August and September. Out of the various weather factors examined, the simulations utilising the combination of mean air temperature, relative humidity, and day length demonstrated a strong correlation with historical salmonellosis records, which are considered the gold standard. This finding highlights the suitability of these factors for effectively estimating the risk of salmonellosis in specific areas. Other weather factor combinations also showed good predictive power and are discusses as part of the supplementary material of the main thesis. Outdoors weather conditions exhibited a better agreement than the estimated indoor values.

The model was considered suitable for providing an estimate of the risk of salmonellosis for England and Wales from weather forecasts.

The effect of the different weather combinations on the risk of contracting salmonellosis is discussed in detail together with line plots in the thesis. A generalised higher risk for mean temperatures between 10 – 20ºC, followed by a decrease for greater temperatures. The risk was also higher for longer days, but not the longest (i.e., June and July). The combined values of day length and mean temperature that posed a higher risk were typical for March to October for the studied dates. Relative humidity did not seem to have a relevant influence by itself, given that all the profile values in the figure were overlapping without a different pattern for the different values.

Results from the application of the model in the Netherlands

The main summer seasonal trend is captured in the reconstruction of the time series. The model also projects a smooth decline of the peak until September resulting in greater number of salmonellosis cases as compared to the reported ones. However, the model also produces isolated spikes not observed in the empirical data, especially in June, and identifies an overall 25% of underreporting of cases over the entire series. This outcome suggests that the effect of weather on salmonellosis incidence is not limited to a specific geographical location but rather has a universal effect.

Results from the application of the model in a climate change scenario

The conditional incidence was applied to the near-future climate projections up until 2043. Land projections were downloaded at daily, 60km-grid resolution for the 15 members or possible scenarios produced by the global climate model HadGEM3-GC3.05 (Met Office Hadley Centre, 2018) for the RCP 8.5, available for bulk download here. Each projection provides an example of climate variability in a changing climate, which is consistent across many climate variables at different times and spatial locations. Annual population projections by regions were downloaded from NOMIS for England, and from StatsWales for Wales in a shapefile format.

Overall, i) the current summer seasonality is split in two in August and September, with an increasingly pronounced trough in between them. This phenomenon does not seem to be explained by temperature, since temperature is projected to be continuously higher from June to November. However, the average precipitation during August and September are lower than the historical records. On top of the double peak, ii) a smooth increase in modelled cases is detectable at the end of May for all years as well as iii) a crest at the end of April in 2040. Both events coincide with decreased average rain projections. By 2043 a flattening effect is observed, levelling out the historically marked peak of September with an iv) smooth increase in cases from January to April and v) from April to mid-June. These events coincide with an increase in predicted temperature and precipitation. The subsequent vi) decline in cases in the month of July match with decreased precipitations. It is pending to determine whether the model predicts an overall decrease in the total number of cases as compared to the current level of observed cases.

PhD self-assessment

The main objective of investigating the environment as a modulator of salmonellosis incidence in humans was broadly achieved. Different approaches (mechanistic, spatial, statistical), as well as several environmental drivers were explored (weather conditions, land use, animal presence, farming systems). Due to data availability, the focus was laid on the weather component, and in-depth analysis were performed (time lags, evaluation of weather factors in different combinations, climate change effect), resulting in the creation and validation of the conditional incidence model as the main output of the thesis.

The performance of the model with surveillance data from a different geography was planned to be done in New Zealand. However, given the uncertainty in international travel due to the SARS-CoV-2 pandemic, a short-term visit to the National Health Institute of the Netherlands was scheduled instead. The model built with UK data was applied to Dutch salmonellosis surveillance data, testing the universal effect of weather as shown with the model, as well as identifying improvements to the methodology (i.e., feeding the model with years with comparable incidence rates, normalisation discrepancies, *etc*.) and strengthening the results.

The initial project proposal mentioned the application of the model in *Leptospirosis*. However, efforts were focused in giving more solid results on the *Salmonella*-weather interface with a deeper exploration and improvement of the model, benefiting from the hight-resolution weather data. Nonetheless, the model was written in a generic fashion and the source code is publicly available so that it can be adapted to other infectious agents, where adequate resolution data is available. The results define the probable incidence for a certain day and location given the forecasted weather. Therefore, both direct and indirect effect of the weather factors with greater influence over human salmonellosis are presented graphically, which require a detailed and careful interpretation.

Finally, the educational side the project satisfactorily met my expectations. Through research development courses, attending scientific conferences, networking with experts, and discussing research gaps with peers and supervisors, I consider having substantially grown as a scientist, confident of my critical thinking and able to expose scientific results in an accurate manner. I have also gained valuable coding and disease modelling knowledge that will guide my professional career as a risk modeller in a public health agency.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name	Delivery date from AWPs (month)	Date delivered (month)	Comments
PhD11-FBZ4/5-EnvDis	D-PhD11-1.1	Mandatory Trainings	M30	M30	
	D-PhD11-1.2	Presentation of findings (e.g. conferences, internal school seminars) Y1	M36	M35	
	D-PhD11-1.3	Attending Relevant Training courses Y1	M36	M36	
	D-PhD11-1.4	End of Year Confirmation Report Y1	M39	M46	
	D-PhD11-2.1	Presentation of findings (e.g. conferences, internal school seminars) Y2	M46	M47	
	D-PhD11-2.2	End of Year Progress Review Y2	M48	M48	
	D-PhD11-3.1	Presentation of findings (e.g. conferences, internal school seminars) Y3	M60	M60	
	D-PhD11-3.2	End of Year Progress Review Y3	M60	M60	
	D-PhD11-3.3	Completion/Submission of thesis	M60	Due M66	*Accumulated delays derived from COVID*

Milestones

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD11-FBZ4/5-EnvDis	M-PhD11-1	Test and discuss findings of the model for Salmonellosis	M48	M35	Yes	Included in Deliverable 1.2
	M-PhD11-2	Validate the model with data from another European country	M55	M54	Yes	
	M-PhD11-3	Write up thesis	M60	Due M72	Ongoing	Public link to be shared after corrections on final thesis done

Publications and additional outputs

Publications

At the time this deliverable was submitted, the EnvDis PhD project has not published any scientific publications.

Additional outputs (i.e., poster/oral presentations)

The EnvDis PhD project has disseminated these works through oral and poster presentations at national and international events. These were:

- Enrich your research profile with an experience abroad. *Oral presentation.* OHEJP Final School, online. 5-7th December 2022.
- *Poster presentation* at Postgraduate Researcher Showcase – Building Connections, Doctoral College and Surrey Research Park, UK. 30th November 2022.
- *Oral presentation* on the latest findings on my project. The Neglected Tropical Diseases (NTD) workshop, Brighton, UK. 14th October 2022.
- *Poster presentation* at ONE conference organised by EFSA, Brussels, Belgium. 21-24th June 2022. Further details in report.
- *Oral presentation* at Journal Club to the Gastrointestinal Diseases team while visiting the RIVM, the Netherlands. 10th May 2022.
- *Poster presentation* at OHEJP Annual Scientific Meeting, Orvieto, Italy. 11-13th April 2022. Further details in report.
- *Poster presentation* at inaugural Open Research Lecture: challenges of Open research, University of Surrey, UK. 8th April 2022. Further details in report.
- Ongoing progress in the understanding of the environmental drivers of human salmonellosis using modelling. *Oral presentation.* Modelling in Animal Health conference (ModAH2), online. 16th September 2021. Further details in report.

- *Poster presentation* at OHEJP Annual Scientific Meeting, hybrid event. 9-11th June 2021. Further details in report.
- Project progress video presentation as part of the Veterinary Health Innovation Engine group (vHIVE). *Oral presentation.* 6th World One Health Congress, online. 30th October to 3rd November 2020. Further details in report.
- *Poster presentation* at 6th World One Health Congress, online. 30th October to 3rd November 2020. Further details in report.
- *Oral presentation* at University of Surrey School of Veterinary Medicine Research Celebration Event, online. 9th September 2020. Further details in report.
- 3-minute Thesis Competition, *Oral presentation* at University of Surrey, Doctoral College, 1st June 2020 and OHEJP Annual Scientific meeting, online, 27-29th May 2020. Further details in report.
- *Poster presentation* at OHEJP Annual Scientific meeting, online. 27-29th May 2020. Further details in report.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Demonstrating in Laboratories	Teaching	29/01/2020	University of Surrey – Degree for Higher Education
Assessment and Feedback	Teaching	03/02/2020	University of Surrey – Degree for Higher Education
Workshop on Git	IT	04/02/2020	University of Surrey
Introduction to Teaching in Higher Education	Teaching	07/02/2020	University of Surrey – Degree for Higher Education
Introduction to HPC	IT	24/02/2020	University of Surrey
Presentation skills	Communication	12/02/2020 19/02/2020	University of Surrey - ELSP
Intermediate R	IT Coding	20/05/2020 - 22/05/2020	Datacamp
Infectious disease modelling	Research Modelling	01/06/2020 - 12/06/2020	Coursera - Imperial College
Introduction to Infectious Disease Modelling and its Applications	Research Modelling	15/06/2020 - 26/06/2020	London School of Hygiene & Tropical Medicine
Statistics and R	IT	07/10/2020 – 17/10/2020	Coursera- HarvardX
French B2	Languages	07/11/2020 - 28/04/2021	University of Surrey – GGA
Managing your supervisor	Communication	15/12/2020	University of Surrey – Doctoral College RDP
Genomics & Bioinformatics workshop	Research Bioinformatics	14/12/2020- 24/12/2020	University of Surrey – Arnoud van Vliet
Research Data Management and Open Data	Research	19/01/2021	University of Surrey – Library Learning Services
Digital innovation for One Health practitioners	Research	15/02/2021- 19/02/2021	OHEJP CPD Workshop
Versatile Emerging Infectious disease Observatory (VEO)	Research	25/02/2021	OHEJP Cogwheel workshop
Building your professional network workshop	Communication	19/03/2021	University of Surrey – Doctoral College RDP
Performing Data analysis in R studio	IT Coding	26/03/2021	University of Surrey - Academic Skills and Development
Public engagement Workshop	Communication	01/04/2022	University of Surrey
The Conversation media training	Communication	20/04/2021	University of Surrey – Doctoral college
Storytelling for researchers	Communication	10/05/2021	University of Surrey - ESRC Impact Acceleration Account

Communication with impact	Communication	12/05/2021	University of Surrey - ESRC Impact Acceleration Account
German A1	Languages	05/10/2021-31/03/2022	University of Surrey – GGA
Introduction to Git	IT	27/05/2022	University of Surrey – Open Reproducibility Society
LaTeX for academic documents	IT	28/10/2022	University of Surrey – Open Reproducibility Society
Demonstrator of practical sessions	Teaching	03/2020-12/2022	University of Surrey
Research assistant collaboration	Research Lab work skills	10/2021 – 01/2022	University of Surrey

One Health impact

The model developed in this thesis describes the effect that weather has in salmonellosis incidence conditional to specific weather factors. It helps to formulate theories around the mechanism underlying the interface seasonality-disease. As future research, we recommend a complementary mechanistic approach where the effect of the different weather variables acts in the biologic processes of *Salmonella* are available. The conditional incidence could be applied in conjunction with in-depth experimental and social research to infer robust mechanistic theories.

EnvDis has contributed to the existing knowledge on *Salmonella*, providing great insight into the mechanism of seasonality underlying the final reporting of cases, such as population behaviour and environmental effects on host and pathogen. To date, this is the first study that analyses the interconnected effect of weather factors on the risk of salmonellosis in the context of climate change. This information could be used by health professionals and Public Health officers to be more alert to the potential increase in cases and outbreaks at unusual times of the year, allowing early detection and preventive measures to be taken. The model allows to estimate salmonellosis notifications based on weather forecast. If other diseases especially relevant to weather variation, this model could assist in better preparedness in the event of new or extreme weather events.

By utilising the provided open-source code, this methodology can be applied where long-term, high-resolution surveillance data is available, making it suitable for other countries and infectious agents. Incorporating animal disease data would be highly beneficial and serve as an ideal complement to surveillance data, considering the interdependencies between human and animal health. This integration would provide a more comprehensive understanding of the overall disease landscape and facilitate a holistic approach to health monitoring and control. Therefore, we encourage health institutions of other countries to actively participate in sharing surveillance data and applying the model to their area. Such collaboration and data sharing will enrich the model with broader information, thereby enhancing the robustness and reliability of the model's conclusions. A first application of the model was carried out successfully during the short-term mission in the Netherlands. The idea was well-received, demonstrating that one month is sufficient to evaluate the model's performance in a different location through

collaborative efforts. At the same time, we are aware of the interest that the climate change application is generating, and we are expecting high interest when the work is published.

Upon integrating robust datasets from diverse geographic locations into the model, it becomes feasible to incorporate automated applications, such as R Shiny, into regular national surveillance systems. This integration would enable swift risk identification during unexpected weather conditions. The implications for the One Health approach are self-explanatory, as the model has demonstrated that the environment plays a fundamental role in disease dynamics. By highlighting these connections, the model reinforces the importance of adopting a holistic and collaborative approach to disease prevention, surveillance, and control within a One Health framework.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

- Dissemination of achievements and results, which helped a lot to give relevance and visibility to the project by the excellent dissemination of the communications team.
- Continuous learning: many learning opportunities such as cogwheel workshops and scientific meetings.
- Contribution to the development of interpersonal skills, with special place laid presentations skills with the PhD students given the chance to present their research and participate to the 3-minute thesis competition.
- Networking: interaction and networking with other project, PhDs from other institutions, and international organisations. Thanks to the consortium, I met the collaborators of the Netherlands where I did a short-term mission and participated to the Doctoral Workshop, organised by “La Cité della Solidarité Internationale” and the collaboration of the United Nations in Geneva.
- Funding that allowed the attendance to conferences and relevant courses.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The EnvDis PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- 23 Things International, a global and collaborative online programme that provide researchers to online research tools for all disciplines, and profile-building opportunities.
- RX One Health course, a field-based experiential learning course focused on One Health core competencies organised by the University of California, Davis.
- Doctoral workshop, an event for doctoral students on the field of environmental health, organised by La Cité della Solidarité, Geneva health forum and UNITAR.
- Short-Term Mission at the RIVM within the gastrointestinal disease group, for a wonderful exchange of expertise, methods, and collaborative paper.
- Liaise with UKSHA Salmonella and gastroenteric experts for exchange of ideas.
- Attendance to the ONE EU, the conference organised by EFSA in 2022 in Brussels with a poster presentation.
- Attendance to the 6th World One Health Congress with a poster presentation.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	4	5
Were all the milestones and deliverables completed?	4	4
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	3	4
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	4
Did the PhD student actively engage in Education and Training activities?	4	4
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	1	1
Was the PhD managed and implemented in accordance with the DMP?	1	1
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	3	1
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	4	4
Is there any direct or indirect impact of the project for national or international stakeholders?	4	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	3	4
Does the project outcomes have policy implications?	3	4
TOTAL	42/65	45/65

AVERAGE: 43.5/65

NB. External Scientific Reviewers provided limited comments on the EnvDis PhD project.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

The core objective has been achieved. Other objectives have only been sketched but justified (e.g. moving the application of the model to The netherland insetead that in new Zealand due to travel disruption)

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Two of the objectives are only described to a limited extent; i.e. review of existing knowledge on non-typhoidal Salmonella, and effect of temperature on the growth of Salmonella in the main foodstuffs. Deviations are well justified.

Reviewer 2: External Scientific reviewer

There were changes in the project plan to accommodate for restrictions during the pandemic, and the PhD student overcame all challenges to meet the objectives.

Reviewer 3: PMT member

Seem to be accomplished. Only the thesis delivery was delayed. Considering the cause for this, the delay is acceptable.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Completion of thesis is ongoing and thus delayed due to SARS-CoV-2.

Reviewer 2: External Scientific reviewer

The only milestone and deliverable missing are the PhD thesis submission.

Reviewer 3: PMT member

Not many interactions dexcribed. This project seems to have developed without connceting to other OHEJP initiatives (JIPs and JRPs).

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

External interactions are described, not so much with JRPs or JIPs within the consortium.

Reviewer 2: External Scientific reviewer

Unclear what are the standards to compare. Three events listed.

Reviewer 3: PMT member

Yes.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

As described, not different from other PhD programmes. The PhD student attended conferences and courses that were related to OHEJP.

Reviewer 3: PMT member

Yes.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

As described, not different from other PhD programmes. The PhD student attended courses and workshops at a variety of institutions, and some were within the OHEJP programme.

Reviewer 3: PMT member

The relevant section was not filled. No publications listed.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

No scientific publications are listed, neither peer reviewed nor others.

Reviewer 2: External Scientific reviewer

No publication listed.

Reviewer 3: PMT member

See above.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

No information available.

Reviewer 2: External Scientific reviewer

No publication listed.

Reviewer 3: PMT member

The student participated in some conferences and workshops. Not clear whether the project's achievements were presented.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

None is listed in the report. However, in the deliverables some activities are listed. In my opinion these should also have been listed there.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Very relevant.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

The project could have a good impact if the model developed will be consolidated and validated also for other diseases.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

To a certain extent. The absence of connections with JRPs and JIPs is a limitation, though.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Might have some. If the model developed will be consolidated and validated also for other diseases.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

n/a

The full Final Thesis Report for the EnvDis PhD project can be found here.

PhD12-FBZSH9-AptaTrich

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Noah Emerson Brosseau	PhD Student	Universite de Paris Est a Creteil	France
Isabelle Vallee	PhD Lead Supervisor	Anses	France
Gregory Karadjian	PhD Second Supervisor	Anses	France

Summary of the work carried out in the PhD project

The AptaTrich PhD project began in M23, in 2019 and is expected to be completed in M67, 2023. A four-month extension was provided due to the SARS-CoV-2 pandemic. To facilitate the detection of *Trichinella* in humans and animals, an accurate, sensitive, portable, and practical tool is envisaged. In recent decades, aptamers have emerged as attractive alternatives to antibodies due to their superior chemical properties. In addition to their therapeutic activities, aptamers have displayed great diagnostic versatility, showcased by their integration into various biosensing platforms. In light of this, the excretory/secretory (ES) antigens of infectious and non-infectious *T. spiralis* muscle larvae (ML) were analysed by mass spectrometry in search of potential biomarkers of infection and possible aptamer targets. In parallel, a whole-larva Systematic Evolution of Ligands by Exponential Enrichment (SELEX) approach was devised to isolate aptamers specific for the muscle larvae of *T. spiralis*. Mass spectrometry identified a *T. spiralis* venom allergen-like protein (TsVAL) that was determined to be significantly over-expressed in non-infectious ML. Furthermore, this protein's identification was supported by *in vivo* studies, which demonstrated its strong immunogenicity in mice and potential to elicit a protective immune response. Following whole-larva SELEX, Next-generation sequencing (NGS) and bioinformatics analysis identified a panel of enriched aptamers. Muscle larvae binding was evaluated by fluorescence confocal microscopy, which identified a subset of candidates with superior binding to the target. Collectively, the results of this thesis provide a solid basis for future research towards the development of both aptamer-based therapeutics and diagnostics.

Dissemination activities of this AptaTrich PhD project were enshrined in a peer-reviewed publication in the Journal of Veterinary Microbiology, four oral and poster presentations at national (i.e., Non-coding Genome 11th Edition 2022, France) and international level (i.e., OHEJP ASM 2022, Orvieto, Italy).

Chapter 1. Identification and evaluation of a potential T. spiralis infection biomarker

Previous reports have demonstrated the importance of oxygen in modulating *T. spiralis* infectivity. While ML maintained in culture media under aerobic conditions appear to suffer from reduced infectivity, those cultured under anaerobiosis appear to retain it. Moreover, aerobiosis appears to stimulate increased larval motility, while larvae maintained in total anaerobiosis adopt an immobile coiled shape (Bolas-Fernandez, 2002; Bolas-Fernandez *et al*., 2009). These similar results have also been observed previously in our laboratory.

A significant difference in the quantity of protein was observed (***p*** = 0.02). A total of 5171 ± 833 µg and 2305 ± 1037 µg of ES protein was produced in the aerobic and anaerobic conditions, respectively, suggesting an increase in metabolic activity and up-regulation of gene expression and protein synthesis in aerobically maintained ML.

The ES proteins of *T. spiralis* ML maintained under aerobic and anaerobic culture conditions were migrated on 4 – 20% polyacrylamide gels and imaged with Coomassie blue staining. The ES proteins produced under the aerobic conditions yielded approximately 18 protein bands with molecular weights between 10 – 100 kDa, while the proteins produced under anaerobic conditions yielded approximately 13 protein bands. More specifically, the additional protein bands produced under the aerobic condition appear to be smaller molecular weight products ranging in size from 10 – 50 kDa, which correspond mostly to non-tyvelosylated proteins (Robinson & Connolly, 2005).

T. spiralis ML ES proteins were fractionated using reverse phase liquid chromatography (RPLC) and injected into the LC-MS system for analysis. While the resulting chromatogram profiles of ES proteins secreted under aerobic and anaerobic conditions shared similarities at lower retention times (from 5 – 20 min), some notable increases in intensity were observed in the aerobically-produced protein samples at retention times of 12 and 18 min. Moreover, the aerobic sample generated a pronounced spike in intensity between 20 and 30 min.

For top-down sequencing and protein identification, the MS/MS spectra were searched using the MASCOT search engine against the *T. spiralis* UniProt database (including 33,860 sequences). The effect of culture condition (aerobic vs. anaerobic) on protein secretion profiles were visualised using a principal component analysis (PCA), which displays the obvious clustering of aerobic and anaerobic proteins. This further supports the previous findings that there are major characteristic differences between the proteins of each group. Of the total protein hits, 22 and 14 were found to be significantly over-expressed in the aerobic and anaerobic conditions, respectively.

Of the 7 differentially expressed proteins identified, AE-1, identified as “Secreted venom-like allergen” was significantly over-expressed (11-fold) in the aerobic condition with a protein score of 135.66, indicating a high confidence match. In the anaerobic condition, a 12-fold increase in the expression of AN-3, identified as "WD-repeat containing protein", was also determined with a protein score of 13.15. However, due to the known immunogenicity of previously identified helminth venom-like allergens, the *Trichinella spiralis* secreted venom allergen-like protein (TsVAL) identified in this study was further scrutinised as a potential vaccine candidate.

Bioinformatics analyses revealed that the full-length TsVAL protein was composed of 380 amino acids, containing a signal peptide located at 1-18aa, and a CAP domain located at 141-301aa. AlphaFold successfully predicted a three-dimensional structure of TsVAL with regions of high and low prediction confidence. From this, an alpha helical structure spanning 16 residues from 31aa - 46aa was identified (EKVTDQEKIEQLLEKY). Furthermore, ABCPred and NetMHCIIpan-4.0 identified this motif as a probable B-cell and MHC class II binding epitope, respectively. Finally, absolute surface accessibility (ASA) analysis determined an estimated ASA of 1109 Å2 for the peptide sequence of interest.

T. spiralis muscle larvae (ML) maintained under aerobic and anaerobic culture conditions displayed different phenotypes. While the ML exposed to the aerobic environment were highly mobile, those in the anaerobic medium adopted a sedentary, tightly coiled position, a finding previously observed in our laboratory (unpublished) and elsewhere (Bolas-Fernandez, 2002; Bolas-Fernandez *et al*., 2009). Moreover, the aerobic condition appeared to stimulate a significant increase in ES protein production compared to the ML maintained under anaerobiosis. In addition to this apparent up-regulation of protein secretion, SDS-PAGE confirmed the presence of additional protein bands in the aerobic condition, suggesting that the presence of oxygen may have stimulated the expression of genes that were otherwise suppressed under anaerobiosis. Additional differences were also observed during mass spectrometry analysis. The proteins produced under each condition displayed subtle and major differences, as illustrated by their chromatogram profiles. Taken together, it appears as though the majority of proteins differentially expressed in the aerobic condition have higher retention times and are smaller than 50 kDa. Mass spectrometry also revealed the two protein clusters by a principal component analysis (PCA), further suggesting major characteristic differences in the proteins produced under aerobic and anaerobic conditions. From the MS/MS spectra, a number of proteins were found to be differentially expressed in each condition. Significantly, AE-1, identified as "secreted venom-like allergen" was over-expressed with a high protein score, implying that this protein may perform important tasks under aerobic environments. To develop a multiple antigenic peptide (MAP) vaccine, the TsVAL protein was scrutinised using various bioinformatic programs. While the three-dimensional structure of the protein appeared to contain a highly accessible epitope, some predicted domains of the protein were classified as having very low confidence scores. In light of this, it is highly likely that the exact positioning of the chosen epitope is not accurate or comparable with that of the protein in its native conformation. In addition to its predicted high accessible surface area (ASA), ABCPred and NetMHCIIpan-4.0 managed to also identify the 16 amino acid epitope as a potential immunogen.

Chapter 2. Evaluation of TsVAL by vaccination

During preliminary vaccination trials, mice were injected subcutaneously with either adjuvant (control group) or a mixture of adjuvant and *T. spiralis* venom allergen-like immunogenic peptide (TsVALp; treatment group). Blood samples were retrieved from each mouse at different time points (J0, J14, J28, and J63) to monitor the levels of TsVALp-specific IgG and IgG2a by ELISA. At J0, J14, and J28, mice belonging to the control and treatment groups displayed no detectable levels of TsVALp-specific IgG. However, two weeks after the second injection, mouse #3781 displayed a pronounced increase in TsVALp-specific IgG, with optical density (OD) levels increasing 45-fold from 0.045 on J14 to 2 on J28. Additionally, in both groups, multiple mice exhibited increases in OD values following the challenge with *T. spiralis*

muscle larvae on J28. This indicates that naturally induced IgG could indeed recognise the TsVAL peptide use in the vaccine, supporting the previous proteomic identification of a TsVAL.

In IgG2a ELISA experiments, the levels of VALp-specific IgG2a were monitored in adjuvant- and TsVALp-vaccinated mice. In adjuvant vaccinated mice, the levels of anti-TsVALp IgG2a remained unchanged throughout the duration of the experiment, even after challenging with *T. spiralis* muscle larvae. However, TsVALp-vaccinated mouse #3781 displayed a surge in anti-TsVALp IgG2a, with OD values increasing from 0.06 on J14 to 1.4 on J28 and 2.6 on J63. As with the adjuvant-vaccinated mice, 4 out of 5 TsVALp-vaccinated mice displayed no noticeable IgG2a response following the J28 challenge with *T. spiralis* muscle larvae.

To further investigate protective immunity induced by the TsVALp vaccine, *T. spiralis* ML were recovered after 5 weeks of infection by the previously described artificial digestion method and counted by microscopy. In the vaccinated and control groups, the average parasitic load was estimated at 24,960 and 27,550 ML, respectively. However, while 4 out of 5 TsVALp-vaccinated mice displayed no significant reduction in ML burden, mouse #3781 displayed a 83.7% reduction in parasitic load (4,500 ML) compared to the average of the adjuvant vaccinated group, suggesting a strong correlation between IgG2a response and parasite clearance.

The previously identified 16 amino acid peptides belonging to the *T. spiralis* venom allergen-like protein (TsVAL) was integrated into a multiple antigenic peptide (MAP) construct to yield a MAP TsVALp vaccine. Following subcutaneous injections of female OF1 mice with the formulated *T. spiralis* venom allergen-like protein multiple antigenic peptide formulation (TsVALp), a significant increase in anti-TsVALp IgG was observed in 1 out of 5 mice, suggesting that the vaccine may have been either incorrectly administered in the 4 unresponsive mice or in the 1 mouse that displayed a significant response. The more specific observation of a strong IgG2a response in mouse #3781 is indicative of a T helper type 1 (Th1) response, known for promoting cell-mediated activities. Because the vaccine was administered at the level of the abdomen, it was hypothesised that a faulty intraperitoneal injection may have led to the direct activation of the-cell mediated Th1 response in mouse #3781, indicated by high IgG2a titre. To test this hypothesis, a new set of intraperitoneal vaccinations was conducted, and IgG levels were monitored by ELISA. Despite the wide variance in IgG levels observed in the intraperitoneally vaccinated mice, the mean OD values on day 28 of the vaccination schedule were determined to be significantly different. This finding supports the hypothesis that the TsVALp vaccine may be more effective at stimulating immunity when administered intraperitoneally.

Throughout this study, the levels of anti-TsVALp IgG appeared to increase gradually following challenge with *T. spiralis* ML on day 28. This increase was observed in the mice of both the control group and TsVALp group. This result suggests that natural infection of T. spiralis in mice promoted the generation of IgG antibodies against an antigen containing the same peptide sequence presented in the TsVALp MAP construct. This ultimately supports the previous findings, which describe the identity of a TsVAL protein containing this peptide. However, the levels of IgG2a showed no reactivity to the TsVALp following ML challenge, implying that another IgG isotype was generated against this epitope.

Finally, protective immunity, assessed by the number of ML recovered after 5 weeks of infection, appeared to correlate strongly with the ELISA results. This finding indicates that the levels of IgG2a induced by the TsVALp MAP vaccine were partly responsible for the significant reduction in ML burden observed in mouse #3781. Furthermore, the relative low variance of ML burden observed in all other mice suggest that the ML challenge protocol was properly conducted.

Chapter 3. Selection of aptamer candidates

During the SELEX process, many steps of optimisation were performed to ensure the production of high-quality ssDNA to be used in future SELEX cycles. In addition, PCR amplification for the generation of dsDNA products was also optimised using a method of 'preparative PCR'. Of the three different methods of ssDNA generation that were tested, the method of lambda exonuclease digestion appeared to be optimal and allowed for the continuation of multiple cycles of SELEX. More specifically, the digestion of dsDNA PCR products by lambda exonuclease yielded the specific 80 nucleotide sense (+) strand of DNA that was required to perform further SELEX cycles.

This method of ssDNA generation allowed us to perform a total of 16 rounds of whole-larvae SELEX. This is advantageous since the selection of aptamers against complex targets often require several rounds of SELEX in order to enrich the ssDNA library. Throughout whole-larva SELEX, qPCR melting curve analysis was used to estimate sequence diversity and assess overall enrichment. Following 15 rounds of whole-larvae SELEX using the lambda exonuclease method, ssDNA retrieved from the ML was subjected to qPCR amplification and melting curve generation. Initially, from rounds 10 to 13 (R10-R13), a noticeable shift in amplification curve was observed, where the decrease in fluorescence becomes gradually less pronounced in R013. More specifically, though the products of R10 and R11 produced mostly identical amplification curves, a shift was observed from R12 to R13. Furthermore, the quantity of ssDNA belonging to R12 and R13 are similar with close Ct values, meaning that the shift in curve is likely influenced primarily by a change in diversity from high to low rather than from differences in sequence pool concentration. Surprisingly, the reduction in fluorescence in R14 and R15 was more pronounced than the products of R13, suggesting that in these rounds of SELEX, conditions did not favour the selection of sequences specific for the target.

These results are supported by the subsequently produced melting curves. From R10 to R13, the release of fluorescence decreases at lower temperatures (64°C) but increases at higher temperatures (76°C), suggesting a gradual increase in the proportion of more stable homoduplexes from R10 to R13. As with the amplification curves, the melting profiles of R10 and R11 are mostly identical, and the most pronounced shift in melting temperature is observed following R13 of SELEX, where a narrow melting peak is produced at 76°C. However, in the following rounds of selection (R14 and R15), a gradual decrease in fluorescence release at high temperatures is observed. These results mirror those of the amplification curves and suggest a possible over-selection in rounds 14 and 15, where conditions may not have favoured the selection of those sequences selected in R13 of SELEX.

The ssDNA recovered from various rounds of three independent whole-larvae SELEX experiments were subjected to High-Throughput Sequencing (HTS) and subsequent bioinformatic analysis with the PATTERNITY-seq® software for a more sensitive and quantitative evaluation of the whole-larvae SELEX process. More specifically, with NGS and PATTERNITY-seq® analysis, it was possible to identify and quantify specific sequence families in each round of SELEX. Sequence family F009, identified during SELEX02, appeared to have experienced the strongest selection, as its frequency increased from 0.0007% in R05 to 0.1066% in R08, corresponding to 7 and 897 reads, respectively. However, upon closer inspection, sequence family F029 appeared to experience the strongest event of selection, having displayed the highest frequency increase compared to all other sequence families. This occurred following R08 of SELEX02, where the family frequency increased from 0.0026% in R07 to 0.0446% in R08, corresponding to 22 and 375 reads, respectively.

In addition to using read frequency as a determinant of enrichment, a sequence alignment matrix was generated to compare the primary structures of the 200 most enriched sequence families. From this, 155 of the most enriched sequences families appeared to share a conserved 13-mer motif (5'-TTTCAATTTACCA-3') in their 40-mer variable regions. Furthermore, an 11-mer stem-loop motif belonging to the 13-mer sequence was found in 65 families and displayed an increase in frequency from 0.001% and 0.0006% in R05 to 0.11% and 0.08% in R08 of SELEX01 and SELEX02, respectively. In SELEX03, the same 11-mer stem-loop motif was found in 99 families and increased in frequency from 1.47% in R12 to 2.76% in R16.

To qualitatively evaluate aptamer binding to *T. spiralis* muscle larvae (ML), the panel of aptamer sequences obtained from HTS and PATTERNITY-seq bioinformatic analysis were subjected to a confocal microscopy fluorescence assay. Biotinylated aptamer sequences were conjugated to Streptavidin Phycoerythrin (SA-PE) and subsequently incubated with *T. spiralis* ML prior to confocal imaging. The first negative control, consisting of ML without aptamer or SA-PE, produced very mild fluorescence originating mostly from within the larva. Furthermore, the second negative control, composed of ML incubated with SA-PE, displayed almost identical fluorescence, meaning that SA-PE could not bind the ML. In contrast, all aptamer sequences could bind the ML surface, albeit with varying degrees of consistency. Equally interesting, some aptamers appeared to bind the ML surface less uniformly than the others, indicating the possible recognition of specific surface structures, resulting in the 'spotted' labelling displayed by aptamer F025. However, the labelling patterns were generally quite variable among all sequences, with some ML emitting no apparent fluorescence. This high variability ultimately made it impossible to conclude the binding of any specific structures.

Aptamer sequence F059 appeared to bind *T. spiralis* ML with the highest consistency at a rate of 88.6 ± 10%. To investigate its possible targets more accurately, three-dimensional confocal microscopy images were constructed. As observed previously, the negative control, having been treated with the SA-PE conjugate, displayed a fluorescence profile consistent with that emitted naturally. Conversely, in the presence of additional binding competitors, ML incubated with SA-PE-conjugated aptamer F059 displayed small 'specks' of fluorescence that appeared to be restricted to its surface.

To further evaluate specificity, aptamer F059, which displayed considerable binding to *T. spiralis* ML, was challenged with the ML of *T. britovi*, *T. nativa*, and *T. pseudospiralis* to determine species-specificity. In all cases, the aptamer appeared to bind the ML surface in a similar fashion. Binding patterns were more accurately described by generating three-dimensional images, which show the 'spotted' pattern adopted by the aptamer. Due to the variability in binding patterns, it was difficult to identify specific surface structures. However, these results do indicate that aptamer F059 is not species-specific.

In an effort to optimise whole-larva aptamer selection, a preparative PCR amplification protocol was developed. While the main purpose of this procedure was to determine the optimal number of amplification cycles required for the accurate amplification of desired 80bp dsDNA, it also served as an important 'checkpoint' to monitor SELEX. The apparition of DNA products larger than the intended target of 80bp was indicative of a failing SELEX experiment, while the more specific amplification of 80bp products was a good indicator of a successful SELEX experiment. Not surprisingly, the results of preparative PCR appeared to be heavily influenced by the chosen method of ssDNA generation. The PCR products of ssDNA recovered using the magnetic streptavidin bead method produced a wide range of increasingly high molecular weight DNA, while ssDNA recovered using the method of exonuclease digestion resulted in a higher proportion of desired 80bp dsDNA. This finding could be explained by the results of ssDNA generation, which depict exonuclease digestion as the most capable method of accurate ssDNA generation, successfully producing the desired 80-mer ssDNA. Conversely, the streptavidin bead method appeared to produce a mixture of dsDNA and ssDNA, which may have impacted the subsequent results of preparative PCR. Taken together, these findings imply that the whole-larva SELEX method requires a careful balance between the quality and quantity of dsDNA and ssDNA in order to selectively amplify adequate amounts of desired product for the following rounds of selection.

The exonuclease method of ssDNA generation was further implemented in a total of three whole-larva SELEX experiments (SELEX01, SELEX02, SELEX03). Sequence enrichment was monitored qualitatively using qPCR melting curve analysis to estimate sequence pool diversity. Whether caused by an insufficient degree of selection stringency, or by the high heterogeneity of potential targets on the larval surface, the melting curves only indicated enrichment after 12 rounds of SELEX. This sudden decrease in sequence diversity may have been induced by the reduction in target quantity from 1000 ML to 500 ML. Following R13 of SELEX03, a dramatic release of fluorescence at higher temperatures, suggestive of sequence enrichment, appears to correspond closely with the results of NGS, which show the greatest increase of high-frequency sequences following R13 of selection. Furthermore, the very slight reduction in melting curve peak that occurs from R14 to R15 of SELEX03 is also present in the results of NGS, where a reduction in the proportion of high-frequency sequences from R14 to R15 is apparent. Taken together, these findings further validate the use of this combined approach, using qPCR melting curve analysis as a complementary tool to gain information on major global increases in sequence pool enrichment, and NGS as a confirmatory tool to identify enriched sequences of interest more sensitively and specifically.

When comparing the results of the confocal microscopy aptamer binding assay with those of NGS and bioinformatics analysis, some interesting conclusions can be drawn. Firstly, the consensus sequence of SELEX03 family F000, which will be referred to as F000_53T, appeared to perform relatively poorly, binding at a rate of 37 ± 7.55% despite its high frequency

in the final rounds of selection. In contrast, aptamer F059, which bound at a rate of 89 ± 10%, was identified in R07 of SELEX01 and occupied only 0.005% of the total sequence pool. Another promising candidate, aptamer F029, which demonstrated a binding rate of 82 ± 10%, appeared to undergo the greatest increase in frequency compared to all other sequences, increasing from 0.0026% in R07 to 0.0446% in R08, equating to a 17-fold increase in frequency. Therefore, NGS successfully identified better performing aptamers during the earlier rounds of whole-larva SELEX. These findings suggest that the frequency of a sequence family in the final rounds of SELEX may not be an accurate predictor of its ability to bind the target of interest. Instead, a round-to-round enrichment ratio may be a more sensitive determinant of a sequence's performance. This evidence further supports NGS and bioinformatics analyses as highly sensitive tools capable of identifying potentially interesting aptamer sequences during the earlier stages of SELEX. This early detection of aptamers is highly valuable. By limiting the number of selection cycles, time is saved, costs are reduced, and the negative experimental effects of PCR bias are mitigated.

One of the most interesting pieces of information brought about by NGS and bioinformatics analysis was the identification of a highly conserved 13-mer sequence belonging to the 40-mer variable region of several sequence families in all three SELEX experiments. The nucleotides belonging to this sequence appeared to be pivotal in the formation of a stem-loop secondary structure found to be highly selected in all three SELEX experiments. While the presence of this structure in aptamer F059 may have contributed to its high binding rate, aptamers F105 and F107, which also shared this motif, did not display the high performance demonstrated by aptamer F059. Although aptamers F059, F105, and F107 all adopted identical stem-loop structures, the apparent variance in their binding performances indicate that the nucleotides belonging to other portions of the sequence are likely important contributors of target binding.

Following aptamer synthesis, the effects of truncation were simulated *in silico* by removing the 20-mer primer-binding regions flanking the 40-mer variable region. Interestingly, the removal of these segments appeared to have a significant influence on the secondary structures adopted by many of the aptamers. However, as evidenced by sequences F009, F739, F059, F086, F105, F107, and F127, structural motifs whose nucleotide compositions belonged exclusively to the variable region were not affected. Furthermore, of the four sequences that demonstrated binding rates greater than 80%, three (F059, F086, F739) appeared to have conserved specific structural motifs following truncation, implying that these structures could serve as important mediators of target binding. Exceptionally, aptamer F029 presented an impressive binding rate of 82 ± 10% even after having undergone dramatic post-truncation structural changes. However, further *in silico* investigation would reveal an alternative structure, one whose free energy value (ΔG) closely resembled that of the original predicted structure. Interestingly, this alterative structure appeared to have conserved the 8-mer stem-loop motif present in the 80-mer parent sequence. Because both of these predicted structures share near identical ΔG, values, this supplementary finding provides a possible explanation as to why aptamer F029, despite its dramatic post-truncation structural change, could consistently bind the target. Furthermore, aptamer F000_53C, an 80-mer sequence that displayed a binding rate of 77 ± 3.6%, appeared to experience a pronounced reduction in binding capabilities following truncation. The resulting 40-mer sequence, F000_33C, appeared to undergo significant structural changes and demonstrated a poor binding rate of only 25 ± 4.5%, suggesting that the secondary structure of aptamer F000_53C, specifically

the stem-loop motif of the 40-mer variable region, was likely important in target binding. Moreover, it is interesting to note that the 19-mer stem-loop motif present in aptamer F000_53C was also predicted in aptamer F739, whose binding rate was estimated at 80 ± 7%, indicating this structure as an important contributor of target recognition.

High-throughput sequencing (HTS) provided large quantities of sequence information at different time points of the whole-larva SELEX procedure. This helped to trace the molecular evolution of different sequence families and allowed for the construction of an empirical genealogical evolutionary (EGE) tree. With an improved understanding of the evolutionary dynamics of sequence family F000 during SELEX03, it was inferred that a single point mutation (53T>C) could improve the binding characteristics of consensus sequence F000, referred to as F000_53T. Interestingly, this point mutation, transforming aptamer F000_53T into F000_53C, resulted in the regeneration of the highly conserved 13-mer sequence (5'-TTTCAATTTACCA-3') that was identified in numerous sequence families from all three SELEX experiments. This single mutation subsequently resulted in a dramatic change in secondary structure. Moreover, the performance of aptamer F000_53T was significantly improved following mutation, with its binding rate increasing from 37 ± 7.5% to 77 ± 3.6%. Interestingly, the single nucleotide substitution resulted in the adoption of a 19-mer stem-loop motif in aptamer F000_53C. As mentioned previously, this motif was also found in aptamer F739, whose binding rate resembled that of aptamer F000_53C.

Confocal fluorescence microscopy was used to assess the selected aptamer's ability to bind the ML target. To do so, biotinylated aptamers were first incubated with fluorescent streptavidin-phycoerythrin (SA-PE) protein conjugate. While strictly qualitative, this method allowed us to estimate the degree of aptamer binding and facilitated the differentiation between strong and poor binding sequences. However, due to the nature of the assay, the binding affinities of each aptamer could not be determined. Furthermore, it should be noted that the large SA-PE complex, estimated at 296-kDa, could theoretically interfere with the ability of some aptamers to bind their target. Moreover, it is unclear whether aptamer-SA-PE conjugation interferes with the secondary structure of the aptamer, which could also impede target recognition. In light of this, it may be interesting to conduct further fluorescence assays using smaller fluorophores that are less likely to disrupt aptamer structure and target interaction. Because aptamer F059 appeared to bind *T. spiralis* ML consistently, it was further challenged with the ML of three additional *Trichinella* species (*T. nativa*, *T. britovi*, and *T. pseudospiralis*). Aptamer binding was more sensitively detected by generating three-dimensional images using the confocal microscope, which demonstrated that aptamer F059 lacked species specificity. Assuming that aptamer F059 is specific for a single surface structure, this result would imply that the target is shared among all species. Alternatively, aptamer F059 may bind to structures that are closely similar between each species.

The primary objective of this project was to develop an aptamer-based method for the accurate and sensitive detection of *Trichinella spp*. in both animals and humans. While this primary objective was not met, the results of the project lay a solid foundation for further work in the field and represents the first ever study focusing on aptamer selection against *Trichinella*. In addition, this work represents the first study of aptamer selection against an organism of this size. This work provides a novel method of aptamer selection against *Trichinella* whole larvae, which has been rigorously optimised and could theoretically be applied to other parasites. Using this novel whole-larva SELEX strategy, a myriad of potential aptamer sequences have been identified using next generation sequencing and a bioinformatic approach known as PATTERNITY.seq. In total, 18 aptamer sequences have been synthesised and evaluated using confocal microscopy. Though some sequences appear to bind better than others based on the results of confocal microscopy, further quantitative evaluation using an aptamer-based ELISA assay against the surface and/or excretory/secretory proteins of *Trichinella* could provide a more accurate measurement of aptamer affinity.

A major deviation from the primary objectives included a vaccination study using a peptide-based vaccine. This peptide was derived from a protein, the *Trichinella spiralis* venom allergen-like protein (TsVAL), which was initially identified during a biomarker discovery study. While this protein could still serve as an interesting biomarker of infection, given its success as a vaccine candidate against other nematode infections, it was further studied as a potential vaccine candidate. The purpose of this study was two-fold. For one, we sought to determine whether this protein could elicit a protective response in mice, which has been observed using proteins of the same family. Secondly, we wished to evaluate the potential of this protein as a diagnostic marker of infection. The results of this study illustrated a potential of this protein as a vaccine candidate, eliciting a dramatic reduction in muscle larvae in one mouse. Furthermore, ELISA results of mice demonstrated antibody specificity for the protein peptide throughout the course of infection, meaning that this protein could indeed serve as a biomarker of infection.

Unfortunately, the complexity of aptamer selection, specifically the selection against such a complex target as a whole larva, required much optimisation. This meant that the final objective, producing an aptamer-based tool of detection, could not be met within the allotted time of the project. We are, however, confident that the results of this project have brought us significantly closer to reaching this objective, as 18 aptamers sequences have been identified as potential candidates. With the optimised selection protocol produced throughout this study, and the identification of a potential biomarker of *Trichinella* infection, the TsVAL, it would be logical to perform further aptamer selection on this protein to yield aptamers specific for TsVAL to be used in a future diagnostic tool. Furthermore, because the aptamers generated throughout this study appear to bind the surface of the muscle larvae, and because some surface proteins appear to me secreted during infection, it is not inconceivable that such aptamers could equally bind some proteins that are secreted within the host throughout infection.

Progress of the project: milestones and deliverables

Deliverables

PhD Project Reference	Deliverable number *(Include original number, if different from the actual one)*	Deliverable name *(Include original name, if different from the actual one)*	Delivery date stated in Annual Work Plan 2021 *(month)*	Actual Delivery Date *(month)*	If not achieved: Forecast achievement date *(month)*	Comments *(Please mention: public or confidential, Zenodo reference, reason and justification of delay, other comments)*
AptaTrich	D-1	*T. spiralis* are fixed in ethanol	M24	M27	-	
AptaTrich	D-2	Aptamers set on whole larvae are selected	M29	M48	-	
AptaTrich	D-3	Aptamers set on stage specific proteins are selected	M36	-	M63	Will probably not be achieved
AptaTrich	D-4	*T. spiralis* super aptamers are identified	M42	-	M57	
AptaTrich	D-5	Article submitted to peer-review journal	M46	-	M59	
AptaTrich	D-6	Article on the use of the aptamers-based test submitted	M51	-	M61	Will probably not be achieved
AptaTrich	D-7	Thesis manuscript is written, and thesis defended	M57	-	M63	No-cost extension was accepted by OHEJP

** 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

PhD Project Reference	Milestone number	Milestone name	Delivery date stated in Annual Work Plan 2021 *(month)*	Achieved (Yes / No)	If not achieved: Forecast achievement date *(month)*	Comments
AptaTrich	M-6	The test is operational (reproducible, repeatable, sensitive and specific)	M51	No	M61	Will probably not be reached
AptaTrich	M-7	The test is able to detect the Three Trichinella species in pig serum	M52	No	M63	Will probably not be reached

Publications and additional outputs

Publications

The AptaTrich PhD project has enshrined these works through peer-reviewed publications, these are:

- Le Dortz LL, Rouxel C, Leroy Q, Brosseau N, Boulouis H-J, Haddad N, Lagrée A-C, Deshuillers PL. (2022). Optimised Lambda Exonuclease Digestion or Purification Using Streptavidin-Coated Beads: Which One Is Best for Successful DNA Aptamer Selection?, *Methods and Protocols*. 5(6):89. doi: 10.3390/mps506008
- Brosseau, N. E., Vallée, I., Mayer-Scholl, A., Ndao, M., & Karadjian, G. (2023). Aptamer-Based Technologies for Parasite Detection. *Sensors* (Basel, Switzerland), 23(2), 562. doi: 10.3390/s23020562

NB. At the time of this deliverable being written, only one publication has been uploaded to Zendo, which is gold standard open access, and can be found here.

Additional outputs (i.e., poster/oral presentations)

The AptaTrich PhD project disseminated these works through oral and poster presentations at the following events:

- Poster Presentation at Journee des Sciences de la Vie et de la Sante a Creteil, Salons de l'Aveyron, Paris, France. 19th Oct 2022.
- Oral Presentation at Journée de la recherche ENVA, Ecole Vétérinaire d'Alfort, Ile-de-France, France. 20th September 2022.
- Poster Presentation at Non-coding Genome 11th Edition 2022, Institut Curie, Paris, France. 11-18th May 2022.
- Oral Presentation – 3-minute thesis presentation at OHEJP ASM 2022, Orvieto, Italy. 11-13th April 2022.
- Oral Presentation at Journee des Sciences de la Vie et de la Sante a Creteil, Creteil, Ile-de-France, France. 16th February 2022.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Training in expérimentation animale Conception et Réalisation de procédures (Rongeurs-Lapin)	Animal experimentation	28/09/2020 - 12/10/2020	l'Ecole Nationale Vétérinaire d'Alfort (ENVA)

One Health impact

Luckily, though the final objective of the project has not been met, some very interesting results have been produced throughout this project. We are confident that the many results of this project will have a significant impact in the domains of foodborne zoonoses, specifically that of *Trichinella* infection. For one, the development of this novel whole-larva SELEX strategy, while specific for the selection of aptamers against the whole muscle larvae of *Trichinella spiralis*, also provides valuable methodology for the generation of aptamers against other targets. Because of this, laboratories focusing their research on other pathogenic organisms may also learn from the results of this thesis. Though much work is still required to reach a stage where an aptamer-based tool could be used to detect *Trichinella* for surveillance purposes, a solid foundation has been provided. From this work, valuable connections have been made between several research groups in Canada and some European countries. This has allowed us to share our knowledge on the subject of aptamer selection, helping to improve the SELEX methodology in these laboratories.

In totality, the results of this thesis provide extremely valuable information and a strong contribution to the scientific community. Under a One Health framework, which focuses efforts on improving the health of animals, the environment, and humans, this research provides potential vaccination, therapeutic, and detection tools that could be used in different aspects to improve the One Health. The discovery of a potential vaccine candidate, which still requires much further investigation, could prove to be of high value. Given that the transmission of *Trichinella* to humans is accomplished through the consumption of infected meat products, the elimination of *Trichinella* from animals destined for human consumption would ultimately eliminate the requirement to test animals for the parasite. Despite ongoing efforts, no such

vaccine exists, in animals or humans. Therefore, the identification of the TsVAL throughout this project could serve to improve current vaccination efforts to provide protective immunity in animals, effectively eliminating the parasite from the food chain. Until this time, the improvement of the current method of *Trichinella* detection in animals, which is based on the lengthy and costly protocol of artificial digestion, could serve to cut costs and allocate funds to other domains of research, such as vaccine study. Though this project focused primary on the development of an aptamer-based method of *Trichinella* detection, the development of an effective vaccine is especially attractive, as this has been used previously to protect farm animals against helminth infection.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

As a part of the One Health EJP, this PhD project allowed me to meet other OHEJP students and members working on different projects. Most notably, I was given the chance to attend three Annual Scientific Meetings throughout the course of this work. Additionally, the OHEJP program connected me with individuals also interested in parasite detection using aptamer-based strategies.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The AptaTrich PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- A mission to the Research institute of the McGill Universite Health Centre (RI-MUHC) in Montreal, Canada, was conducted throughout this thesis to acquire training in Mass spectrometry in the Dr. Momar Ndao laboratory. This has strengthened potential future collaboration between the researchers in France and Canada.
- During the thesis, a collaboration was made between members involved in the AptaTrich project and members of the OHEJP JRP PARADISE project, which focused, in one respect, on the selection of aptamers against *Cryptosporidium* and *Giardia*. in addition, communications were established with researchers in Canada, who successfully isolated *Cryptosporidium parvum*-specific aptamers capable of detecting the parasite using an electrochemical microfluidic aptasensor.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	5	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	4	5
Were all the milestones and deliverables completed?	3	5
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	-	4
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	5	5
Did the PhD student actively engage in Education and Training activities?	4	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	4	4
Was the PhD managed and implemented in accordance with the DMP?	-	4
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	4	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	4	5
Is there any direct or indirect impact of the project for national or international stakeholders?	4	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	5	5
Does the project outcomes have policy implications?	4	5
TOTAL	46/65	61/65

AVERAGE: 53.5/65

NB. Note that reviewer 1 did not provide scores for two questions, which may affect the overall score.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

The PhD student recommends future research from the conclusions of the project, which will lead to an in-deeper evaluation of the aptamers identified in terms of performance and target specificity. Both will be crucial for defining the extend of aptamers use.

Reviewer 2: External Scientific reviewer

A very good description of future perspectives is provided at the end of the manuscript which gives interesting ideas for the continuation of the different projects.

Reviewer 3: PMT member

Both Reviewers score as 5 with clear reasoning.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

The primary objective of the project was not met; however, this was clearly analysed and justified on the basis of the hard procedure for aptamer selection for such a complex target as a whole *Trichinella* larva.

Reviewer 2: External Scientific reviewer

Not all the objectives were met but the reasons were clearly indicated in the manuscript. In addition, some objectives could not be met due to the lockdown situation in France following the SARS CoV-2 pandemics.

Reviewer 3: PMT member

Both Reviewers gave a high score. The primary objective of the project was not met but the PhD candidate shows mature reflection on this.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

Not all the milestones and deliverables were completed.

Reviewer 2: External Scientific reviewer

Considering the lockdown situation and the challenging nature of the projects, the applicant did a fantastic job and achieved many of the milestones.

Reviewer 3: PMT member

Reviewer 1 gives average score; Reviewer 2 gives the highest score as the situation was challenging due to SARS-CoV-2 related restrictions. Only two out of seven deliverables had been submitted at the time of writing the report, neither of them was submitted by their original deadline. Both are available on Zenodo: brief documents. Deliverables in particular are important in EU-projects. When the expected specific result is not reached, a deliverable can nevertheless describe the work done. None of the two milestones were reached, and there is a comment that they will probably not be reached. Perhaps feedback for planning milestones and deliverables for future projects: for high-risk research where, final results are not known at proposal stage, naming the progress-measuring points so that progress and advancing science is considered achievement is often wise.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Not specified.

Reviewer 2: External Scientific reviewer

To the best of this reviewer's knowledge, no such interactions occurred but these were not necessarily planned.

Reviewer 3: PMT member

Score lacking from Reviewer 1. Important! This seems to affect the total score, perhaps unfairly? Even an option 'not at all' would have given one point, this lack of score gives zero points. This is more a matter of how OHEJP counts the points than something for the PhD candidate to do. The report mentions collaboration with PARADISE, which is a OHEJP JRP. This has likely not been clear to the Reviewers, as the expression 'JRP' is not used in the report.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

Yes, the PhD student declared a continuous exchange of information during his PhD with individuals also interested in parasite detection using aptamer-based strategies. He had the chance to meet other OHEJP students and members working on different projects, and to attend three Annual Scientific Meetings.

Reviewer 2: External Scientific reviewer

The PhD student gained highly valuable training and knowledge in multiple experimental techniques and was capable of carrying out multidisciplinary projects. In addition, the PhD student integrated very well in both international and national scientific environments. The PhD student could also carry out part of the work in the laboratory of a collaborator in Canada.

Reviewer 3: PMT member

Both Reviewers score as 5 with clear reasoning. Very nice to read that the OHEJP ASMs were valuable for the PhD candidate!

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

The PhD student actively engaged in education and training activities on animal experimentation, more precisely designing and carrying out experimental procedures in rodents and rabbits.

Reviewer 2: External Scientific reviewer

The PhD student successfully carried out all the education and training required by a French doctoral school (i.e., over 100h of training during the three-year long PhD).

Reviewer 3: PMT member

Both Reviewers gave a high score.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

Yes.

Reviewer 2: External Scientific reviewer

The PhD student published one review article as first author, a methods article as co-author, and a research article with the PhD student as first author will be submitted soon.

Reviewer 3: PMT member

Both Reviewers score as 4. The report mentions that OHEJP was not acknowledged in one of the publications. The one where OHEJP is acknowledged is available on Zenodo.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

Along the final report of the project there are evidence supporting the management and the implementation of the PhD in accordance with the Data Management Plan.

Reviewer 2: External Scientific reviewer

The PhD student and the Thesis advisors set up a data management plan and which was followed during the thesis. However, this reviewer does not have more details on how exactly this was done.

Reviewer 3: PMT member

Score lacking from Reviewer 1. This seems to affect the total score, perhaps unfairly? Even an option 'not at all' would have given one point, this lack of score gives zero points. This is more a matter of how OHEJP counts the points than something for the PhD candidate to do.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

Yes.

Reviewer 2: External Scientific reviewer

The PhD student presented results obtained during his thesis at various conferences both as oral and poster communications. The student participated in other conferences, seminars, and congresses.

Reviewer 3: PMT member

Both Reviewers gave a high score. The international dimension of the dissemination activities was relatively limited, likely largely due to the SARS-CoV-2 related restrictions.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

Even if the primary objective of the project will not be met, the results of the project constitute the basis for further work on both, foodborne zoonosis, and advanced technologies.

Reviewer 2: External Scientific reviewer

Developing diagnostic methods for human *Trichinellosis* is of high importance and clearly meets the objectives of One Health EJP.

Reviewer 3: PMT member

Both Reviewers gave a high score.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

Yes, this project could provide potential detection and vaccination tools that could eliminate the parasite from the food chain, therefore it could have an important impact for national and international stakeholders.

Reviewer 2: External Scientific reviewer

There is no direct implication however, the identification of solid detection methods for human *Trichinellosis* would certainly be of high interest to the pharmaceutical industry.

Reviewer 3: PMT member

Both Reviewers score as 4.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

Yes, in the final report is stated that valuable connections have been made between several research groups in Canada and some European countries.

Reviewer 2: External Scientific reviewer

This project allowed to start collaboration with partners in France and in Canada and will certainly permit to foster yet other collaborations.

Reviewer 3: PMT member

Both Reviewers score as 5 with clear reasoning.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

If the primary objective is reached, the project outcomes may have implications in policy.

Reviewer 2: External Scientific reviewer

The results stemming from this project certainly do have strong implications in future research directions that will need to be taken in order to develop diagnostic tools for monitoring human *Trichinellosis*.

Reviewer 3: PMT member

Both Reviewers gave a high score.

The full Final Thesis Report for the AptaTrich PhD project can be found, here.

PhD13-FBZ8/AMR2-VIMOGUT

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Ingrid Cardenas Rey	PhD candidate	Wageningen University and Research. Wageningen	The Netherlands
Mike Brouwer	PhD Lead Supervisor		
Arjan de Visser	PhD second supervisor		
Kees Veldman	Other supervisor	Wageningen Bioveterinary Research. Lelystad	The Netherlands

Summary of the work carried out in the PhD project

The VIMOGUT PhD project began in M20, in 2019 and is expected to be completed in M69, in 2023. The PhD project VIMOGUT brought together *in vivo* and *in vitro* studies to better understand the role of the chicken gut microbiota as an important ecological niche for the spread of AMR genes in the environment.

The PhD student has studied the successional dynamics of caecal microbiota of developing broilers in relation to colonisation by ESBL- producing *Escherichia coli* (ESBL-Ec) in a commercial broiler farm. Our results suggested the presence of ESBL-*E. coli* is associated with mild but consistent reductions in broilers' caecal microbiota richness and transient microbiota compositional differences. We further documented the increasing prevalence and clonal spread of ESBL-*E. coli* in a single broilers' flock during a single production round and pointed at the farm environment as a likely source for ESBLs. We also advocated for additional research to more precisely understand whether the presence of ESBL-*E. coli* modulates the competitive landscape of the broiler microbiota or vice-versa. *In vitro* microbiota studies involving experimental ESBL-*E. coli* introduction could provide valuable answers to these questions, which can then be further confirmed under controlled animal experiments.

Another goal of the VIMOGUT PhD project was to contribute to more sustainable research by developing a cost-efficient and animal-friendly tool. We have developed an *in vitro* chicken caecal model capable of reproducing the physiological conditions required to culture the main members of the chicken gut microbiota. This milestone paved the road for future investigation on the effect of microbiota-targeted intervention studies on the horizontal transmission of AMR genes. In addition, the *in vitro* gut model also expands the possibilities of addressing other microbiota-AMR related research questions. Our results also show that optimisation steps of such tools are a continuous process from which we learned that the choice of experimental settings impacts the *in vitro* cultured microbial community composition over time. Thus, pre-testing parameters and generating automated workflows are essential to reduce experimental bias, generate reliable data and draw meaningful conclusions.

The knowledge acquired during the VIMOGUT PhD project sheds light on the relationship between AMR bacteria and the broiler gut microbiota and highlights the need to consider the natural dynamics of host-microbiota development and the environment in the colonisation by resistant bacteria. Moreover, an *in vitro* model has been developed as a research tool to explore microbiota-based strategies to reduce the spread of AMR genes. This project supports the One Health approach to minimise the spread of AMR from animals to humans via the food chain in line with policies of the Dutch Ministry of Agriculture, Nature and Food Quality, as well as EFSA, ECDC, EMA, WOAH, WHO and FAO to tackle AMR.

Dissemination activities of this VIMOGUT PhD project were enshrined in a peer-reviewed publication in the Journal of Veterinary Microbiology, and four oral and poster presentations at international conferences (i.e., One Health EJP Final School 2022).

Work carried out on the PhD, scientific results, and outcomes

Chapter 1. Succession in the caecal microbiota of developing broilers colonised by extended-spectrum β-lactamase-producing *Escherichia coli*

In this chapter, we studied the successional dynamics of the caecal microbiota of developing broilers in a commercial flock during their production life cycle in relation to ESBL-*E. coli* (ESBL-Ec) colonisation. Broilers were categorised as ESBL-Ec colonised (ESBL-Ec+) or ESBL-Ec non-colonised (ESBL-Ec−) by selective bacterial culturing of caecal content. We compared the richness, evenness, and composition of the caecal microbiota of both broilers' groups using 16S rRNA gene sequencing. We also assessed the combined role of age and ESBL-Ec status on the caecal microbiota development. We observed an increasing linear trend in the proportions of ESBL-Ec throughout the broilers' production round. Over time, the caecal microbial richness was consistently higher in ESBL-Ec− broilers, but significant differences between groups were found exclusively on day three. Bray–Curtis distance-based RDA (BC-dbRDA) analyses showed no explanatory power of ESBL-Ec status, while age explained 14% of the compositional variation of the caecal microbiota.

Exploring the successional dynamics of the caecal microbiota of broilers can reveal windows of opportunity to implement intervention strategies that reduce the spread of resistant commensal and pathogenic bacteria. However, little is known about how changes in the developing caecal microbiota affect the prevalence of ESBL-Ec or how the presence of ESBL-Ec affects the microbiota. Here, we studied the caecal microbiota of developing commercial broilers in conventional farming conditions and classified them as ESBL-Ec+ and ESBL-Ec−. We found no clear divergence between these two groups over time, suggesting that the presence of ESBL-Ec does not have consistent effects on the caecal microbiota of developing broilers.

All broilers tested negative for ESBL-Ec on days zero and one. ESBL-Ec was detected only from day two onwards, and its prevalence increased rapidly over time, suggesting that the farm environment was the likely source for ESBL-Ec colonisation. The AMR phenotype, ESBL-gene, plasmid typing and MLST results were identical for all samples, indicating a clonal spread of ESBL-Ec throughout the flock. The clonal distribution of ESBL-Ec has been associated with the high shedding of ESBL-Ec after colonisation in previous studies.

In contrast to the ESBL-Ec prevalence trends, the relative abundance of *Escherichia* and *Shigella* decreased over time in both groups of broilers. This reflects the broilers' microbiota dynamic development over time; as broilers age, the microbial diversity increases, causing shifts in bacterial abundance. Despite the continuous dominance of members of the phylum Firmicutes, *Escherichia* and *Shigella* persisted throughout the broilers' production life cycle. The observed changes in the caecal microbiota composition over time resembled those documented in previous studies: the relative abundance of Proteobacteria decreased as broilers aged, while the relative abundance of Firmicutes and Bacteroidetes gradually increased.

Consistent with the literature, the observed caecal microbial richness increased linearly as broilers aged in both groups. Overall, microbial richness was not significantly different between groups. However, a higher microbial richness was consistently observed in ESBL-Ec− compared to ESBL-Ec+ broilers over time. In line with this finding, ESBL-colonised broilers showed a much less diverse microbial composition on day three, with only five genera representing 86% of the total community. These results suggest small but consistent alterations in the microbiota's competitive landscape, which could be further explored with controlled, culture-based laboratory experiments.

As shown in previous research, host ageing is one of the drivers of microbiota composition. In our study, age explained 14% of the microbial composition variability. The succession of the broilers' microbial communities was observed in three stages. The first stage (days 0 – 4) was dominated by Firmicutes and Proteobacteria, mainly by families Clostridiaceae, Enterococcaceae and Enterobacteriaceae. A decrease in Proteobacteria (< 10%) and a complete dominance (> 80%) by members of the Firmicutes (families Ruminococcaceae, Lachinospiraceae, and Lactobacillaceae) characterised the second stage (days 5 – 14). In the third stage (days 21 – 35), Proteobacteria continued steadily decreasing (< 5%), while Bacteroidetes emerged, accounting for 16.6% of the total community. Firmicutes were still extensively represented principally by families Ruminococcaceae and Lachinospiraceae and the appearance of members of the Clostridiales. Despite the differences in study designs, these observations support previous results, in which successional dynamics were also characterised in 3 stages and represented by similar phyla and families. On the other hand, the colonisation by ESBL-Ec did not explain any variability in the caecal microbiota composition in this study. Our results match previous observations, in which asymptomatic gut carriage of ESBL-Ec was not associated with differences in microbiota composition in humans.

To the best of our knowledge, this is the first study assessing the differences in the microbiota composition and diversity of ESBL-Ec+ and ESBL-Ec− broilers from a commercial farm throughout the production round. A previous experimental study examined the microbiota of broilers colonised with ESBL-Ec and treated with competitive exclusion products on days 5 and 21. However, the study aimed to evaluate the effect of compartmentalisation and interventions on the transmission and prevention of ESBL-Ec colonisation in the broiler microbiota composition more than the effect of ESBL-Ec on the broiler’s caecal microbiota. Longitudinal-experimental studies which manipulate ESBL-Ec prevalence could reveal the process of colonisation of ESBL-Ec in the caecal microbiota and shed light on the relationship between AMR bacteria, the caecal microbiota, and potential avenues for microbiota-based

control of AMR. Our study highlights the need to consider the natural dynamics of host-microbiota development and colonisation by resistant bacteria.

Chapter 2. Establishment of a semi-automated in vitro model to simulate the chicken caecal physiological conditions and maintain the main caecal microbiota communities

This chapter describes the results of the set-up and optimisation of an *in vitro* chicken caecal model. Several test runs were carried out to evaluate the performance of the semi-automated workflow. For each run there was a particular focus on pH control behaviour and culture agitation by the pulsing stirring response. Further testing was performed to learn about the best gassing strategy to maintain the optimal anaerobic conditions for the maintenance of the *in vitro* cultured chicken caecal microbiota.

Effect of nitrogen gassing strategy on the in vitro cultured chicken caecal microbiota

All simulated physiological caecal conditions (dissolved Oxygen, pulsing stirring and temperature) except pH remained stable over time. The pH fluctuations were associated with microbial activity during the continuous feeding phase.

Microbiota analysis

Bacterial abundance: Regardless of the gassing strategy and experiment time, Firmicutes was the most abundant phyla observed, making more than 90% of the *in vitro* bacterial community. Bacteroidota, proteobacteria, and Actinobacteria were observed in less proportion but consistently until the end of the experiment.

Differentially abundant taxa were observed only at the family and genus levels. The *In vitro* bacterial community from the sparger method showed a significant higher abundance of the genera *Butyricicoccus, Lachnospira, Clostridioides*, and *Phyllobacterium* (Fig. 7A; ANCOM-BC, $\boldsymbol{p} < 0.001$).

Alpha diversity: Microbial richness fluctuated daily in both bioreactors. 721 amplicon sequence variants (ASVs) were detected in the initial caecal inoculum. During the batch phase, a decrease in ASVs was identically observed in both bioreactors (overlay 559 ASVs; sparger 562 ASVs). Microbial richness dropped markedly after the beginning of the continuous feeding. Still, it steadily increased until the last experimental day with 101 and 91 ASVs for Overlay and sparger, respectively. No significant differences were found in microbial richness between sparger and overlay (Kolmogorov-Smirnov test, $\boldsymbol{p} = 0.833$). We suggest that the low detection of ASVs on day 3 was associated with the beginning of the continuous feeding phase. A hypothesis is that the continuous removal of waste media culture after each feeding might select for fast-growing bacteria compared to slow-growing bacteria. Another possible reason for this result could be sampling and detection bias which calls for optimisation of methods for culture collection, including sampling time.

Beta diversity: *In vitro* microbiota community composition changes were determined using Bray-Curtis principal coordinate (BC-PCoA) and Adonis Permutation analyses. Changes in microbiota composition were associated mainly with experiment time (Adonis, $\boldsymbol{p} < 0{,}001$) than with the gassing strategy. These results can also be correlated with the substantial decrease

in microbial richness on days 2 – 8, depicted by clustering samples from the same experimental days.

PhD self-assessment

The two main objectives of this PhD were: i) to study the dynamic processes of the developing caecal microbiota of commercial broiler chickens in relation to ESBL-*E. coli* colonisation, and ii) to develop an *in vitro* caecal model that allows studying the effect of antibiotics and microbiota-targeted interventions on the horizontal AMR gene transfer dynamics in the *in vitro* chicken caecal microbiota.

To facilitate the achievement of these objectives, the PhD was set up in two components: *in vivo* and *in vitro* studies. The *in vivo* studies were performed with samples collected from broiler chickens from commercial Dutch farms. This characteristic of the *in vivo* study was a plus since it allowed us to gain insights into the natural dynamic of ESBL colonisation occurring on a farm and its relationship with the changes in the microbial community composition of broiler chickens over time. This advantage of the study also implied taking risks, like the inability to sample due to in-farm infection disease outbreaks.

The SARS-CoV-2 pandemic from 2020 – 2021 and an extensive Avian Influence outbreak in 2022 impeded the farm visits and sample collection, considerably delaying the completion of the second study on the relationship between chicken gut microbiota development of slow-growing broilers and ESBL-*E. coli* colonisation. As a result, a deviation from the work plan was considered. Namely, samples were collected from research facilities instead of commercial broiler farms under the same conditions. The animals were not subjected to additional handling or stress, and as such, we believe the project's ethics did not change with the plan deviation.

Similarly, to the *in vivo*, the *in vitro* work was also considerably affected by the SARS-CoV-2 lockdowns during the stages of equipment set up and training. The set-up of an *in vitro* chicken caecal model was a high-risk and challenging process which required considerable programming work and optimisation. Although the *in vitro* model produced preliminary expected data, recurrent technical difficulties (software/hardware-related issues) hindered the completion of the experiments to study the horizontal gene transfer dynamics in the *in vitro* chicken caecal microbiota after colonisation with ESBL *E. coli* strains and microbiota-targeted interventions in the proposed time. This problem was tackled by acquiring new equipment with a dedicated anaerobic module expected to reach anaerobic conditions more reliably. The new equipment was installed in February 2023, and transfer of knowledge, test experiments and optimisation are currently being carried out. Delayed *in vitro* intervention experiments are expected to take place in the third quarter of 2023.

In anticipation of the start of the *in vitro* intervention experiments, the PhD student performed a short-term mission at the One Health Antimicrobial Resistance (OHAR) research group lead Prof. Luca Guardabassi at the University of Copenhagen. During this period, the PhD student learned molecular cloning techniques to label the chromosome and ESBL plasmids of *E. coli* strains with fluorescent reporter proteins. Such strains will be used to track the spread of ESBL plasmids in the *in vitro* chicken caecal microbiota.

Despite of the adverse situations due to SARS-CoV-2 pandemic, Avian Influenza outbreaks, and technical issues, the PhD project has produced valuable data and has contributed to understanding the chicken caecal microbiota development dynamics in relation to ESBL-*E. coli* colonisation during the broiler chicken lifespan. As well, as to set up a reproducible *in vitro* chicken model that will allow further experimental work and the answer to microbiota and AMR-related research questions.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments *(Please mention: public or confidential, Zenodo reference, reason and justification of delay, other comments)*
VIMOGUT	D1	Manuscript on preliminary findings for the relationship between chicken gut microbiota development and ESBL-*E. coli* colonisation.	36	45	
VIMOGUT	D2	Manuscript on the relationship between chicken gut microbiota development of slow-growing broilers and ESBL-*E. coli* colonisation.	56		Public Due to the COVID-19 pandemic in 2020 and an extensive non-seasonal Avian Influenza outbreak in the Netherlands, no farm sampling has been possible.
VIMOGUT	D3	Manuscript describing the results of testing *in vitro* ESBL *E. coli* intervention strategies.	60		Public
VIMOGUT	D4	Manuscript to describe the host range of ESBL plasmids from *E. coli* into the *in vitro* microbiota and strategies to lower plasmid spread.	68		Public

Milestones

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
VIMOGUT	M1	Molecular technique training and 16S barcode sequencing.	22	22	Yes	
VIMOGUT	M2	Attend a course on analysis for 16S barcode sequencing.	24	24	Yes	
VIMOGUT	M3	Visit APHA for training on *in vitro* chicken gut model.	24		Yes	The training was performed online.
VIMOGUT	M4	Perform 16S barcode sequencing on currently collected samples.	27	27	Yes	
VIMOGUT	M5	Perform 16S rRNA gene analysis on initial experiment	30	31	Yes	
VIMOGUT	M6	Perform initial test runs on *in vitro* gut model to determine CFU for reliable ESBL colonisation.	36	-	No	Due to technical difficulties in the set-up of the model, this will be carried out after the installation/optimisation of new equipment, expected in May-June 2023.
VIMOGUT	M7	Write a manuscript on the relationship between chicken gut microbiota development and ESBL-*E. coli* colonisation.	36	56	Yes	Published on M56
VIMOGUT	M8	Perform 16S rRNA gene sequencing and analysis of caecal samples from OHEJP VIMOGUT.	50	-	No	Farm visits were rescheduled due to COVID-19 and avian influenza.
VIMOGUT	M9	Manuscript on the relationship between chicken gut microbiota development of slow-growing broilers and ESBL-*E. coli* colonisation	56	-	No	Farm visits for sampling were rescheduled due to COVID-19 and avian influenza.

VIMOGUT	M10	Experiments in the *in vitro* model for ESBL *E. coli* colonisation intervention strategies.	60	-	No	Due to technical difficulties in the set-up of the model, this will be carried out after the installation/optimisation of new equipment, expected in May-June 2023.
VIMOGUT	M11	Experiment in the *in vitro* model on the horizontal transfer of ESBL plasmids within the caecal microbiota	60	-	No	Due to technical difficulties in the set-up of the model, this will be carried out after the installation/optimisation of new equipment, expected in May-June 2023.
VIMOGUT	M12	Experiments in the *in vitro* model to evaluate the effect of microbial interventions on the spread of ESBL plasmids	64	-	No	Due to technical difficulties in the set-up of the model, this will be carried out after the installation/optimisation of new equipment, expected in May-June 2023.
VIMOGUT	M13	Write a manuscript describing the results of the experiments on the effects of antibiotics and intervention strategies on the transmission of ESBL plasmids in the *in vitro* caecal microbial community.	68	-	No	Expected after the installation of the new system and collection of data from the *in vitro* experiments.

Publications and additional outputs

Publications

The VIMOGUT PhD project has enshrined these works through a peer-reviewed publication, which is Gold Open Access, this is:

- Ingrid Cárdenas-Rey, Teresita d. J. Bello Gonzalez, Jeanet van der Goot, Daniela Ceccarelli, Gerwin Bouwhuis, Danielle Schillemans, Stephanie D. Jurburg, Kees T. Veldman, J. Arjan G. M. de Visser and Michael S. M. Brouwer. (2022). Succession in the caecal microbiota of developing broilers colonised by extended-spectrum β-lactamase-producing *Escherichia coli.* Animal Microbiome. 4 (51). doi: 10.1186/s42523-022-00199-4.

The above publication has been uploaded to Zendo, which is gold standard open access, and can be found here.

Additional outputs (i.e., poster/oral presentations)

The VIMOGUT PhD student went on a short-term Mission funded by the One Health EJP entitled: "Construction of double-labelled *E. coli* strains to study the effect of antibiotics and interventions on horizontal ESBL genes transfer in the chicken's caecal microbiome" at the One Health Antimicrobial Resistant group at the University of Copenhagen.

Information on the short term mission undertaken by the VIMOGUT PhD student, can be found here and here.

Furthermore, the VIMOGUT PhD project disseminated these works through oral and poster presentations at the following events:

- Cardenas-Rey, I. (2022). Dual labelling of bacteria with a dual purpose; insights of a nine week journey on experimental research and teamwork. *Oral presentation* on 1st day of One Health EJP Final School 2022, online. 5-7th December 2022.
- Cardenas-Rey, I., Gonzalez, T. J. B., Veldman, K., de Visser, A., & Brouwer, M. (2022). Effect of nitrogen gassing strategy on the *in vitro* cultured chicken caecal microbiota. *Poster presentation* at One Health EJP ASM 2022, Orvieto, Italy. 11-13th April 2022. More information can be found here.
- Cardenas-Rey, I., Gonzalez, T. J. B., Veldman, K., de Visser, A., & Brouwer, M. (2021). A semi-automated in vitro model to study AMR transfer dynamics in broiler chicken caecal microbial communities. *Poster presentation*. One Health EJP ASM 2021, Copenhagen, Denmark. 9-11th June 2021. More information can be found here.
- Cardenas-Rey, I., Gonzalez, T. J. B., Veldman, K., Ceccarelli, D., Jurburg, S., van der Goot, J., Bouwhuis, G., de Visser, A., & Brouwer, M. (2021). Caecal microbiota composition of broiler chickens colonised and non-colonised with ESBL *Escherichia coli*. *Poster presentation*. One Health EJP ASM 2021, Copenhagen, Denmark. 9-11th June 2021. More information can be found here.

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Scientific integrity	Skills and competences	03/03/23	Wageningen Graduate School
Supervising BSc & Master students	Skills and competences	09-10/02/23	Wageningen Graduate School
Workshop on human microbiome & e-DNA research for public health & the environment	Microbiome in health and disease and as tool to monitor diversity	26-27/09/22	National Institute for Public Health and the Environment (RIVM)
Microbial Ecology course	Microbial Ecology	19-24/06/22	PE&RC graduate school
Short Term Mission at the University of Copenhagen	Bacterial cloning - AMR	30/04/22 - 09/07/22	OH-EJP
Poster and pitching	Skills, competences and career development	10/03/22, 31/03/22, 07/04/22	Wageningen Graduate School
Workshop on plasmids as vehicles of antimicrobial resistance spread	AMR	21-25/03/22	International Centre for Theoretical Physics
Reviewing a scientific manuscript	Skills, competences and career development	17/02/22	Wageningen Graduate School
R Markdown	Data analysis	05-06/07/21	VLAG – WSG
Multivariate analysis course	Statistical analysis	23-29/06/21	PE&RC – Wageningen Graduate School
Mindful productivity for PhD Candidates	Personal development	28/05/21	PE&RC - Wageningen Graduate School
Effective and efficient verbal communication in academia and beyond	Communication	26/05/21	PE&RC - Wageningen Graduate School
Applications of omics technologies in poultry health and productivity: where are we now?	Omics	22/04/21	*IHSIG*
Global Tricycle Surveillance ESBL *E. coli*	AMR	03/03/21	WHO
7th OHEJP cogwheel workshop	Science	25/02/21	OH-EJP
Imposter syndrome	Personal development	24/02/21	CACTUS
Scicom IG meeting	Science Communication	16/02/21	WUR
Metagenomics webinar (DADA2)	Data analysis	21/01/21	Loop Genomics
Peer review discussion meeting	Communication	12/01/21	GSS-WUR
Cogwheel workshop	Science	25/11/20	OH-EJP/Horizon 2020
Writing course	Scientific writing	09-10/09/20	Leipzig University
Basic cultivation course	Bioengineering	12-15/10/20	Applikon
Advanced community ecological data analysis using vegan	Statistics/bioinformatics	09/07/20	University of Regina, Canada (online)
Introduction to multivariate data analysis using vegan	Statistics/bioinformatics	07/07/20	University of Regina, Canada (online)

One Health impact

To effectively combat the health threat of AMR for humans and livestock animals, we need to better understand how AMR genes arise and spread within and between host species and the general environment. VIMOGUT contributed to this aim in several complementary ways.

First, we have studied the prevalence dynamics of resistant bacteria like ESBL-*E. coli* in natural conditions. We have shown how the clonal spread of AMR genes occurred in a broiler population. Moreover, and confirming those findings, our ongoing longitudinal study on ESBL/*ampC* plasmid epidemiology suggests that despite routine cleaning, the same ESBL-*E. coli* clones are maintained in the environment and detected in different production rounds. These results emphasise the need for further development and improvement of farm biosecurity and management via recommendations or policies by the national authorities.

Second, we have considered an important workhorse in the battle against AMR: the gut microbiota. Alternative interventions besides antibiotic usage reduction need to be explored, like understanding and modulating the gut microbiota. Modifying such complex microbial communities can help tackle the spread of AMR bacteria and improve animal and human health. We have shown that changes in microbiota are observed during the natural colonisation of resistant bacteria and therefore offer an opportunity for modulation and natural exclusion of pathogens and resistant bacteria. This could be considered a One Health approach where the animal, the human, and the environment's health would benefit.

Third, we have developed an *in vitro* caecal model that will allow us to determine the fate of plasmid-encoded AMR genes and further study microbiota-targeted interventions that might help to reduce the spread of these genes in complex microbial communities like the chicken gut. Additionally, the *in vitro* chicken caecal model is a step ahead in doing sustainable research. Despite its limitations, this cost-efficient tool allows ample research with controlled parameters and contributes to using fewer resources, including the unnecessary use of animals in the first stages of research or hypothesis testing.

Ultimately, VIMOGUT outcomes will support the development of new intervention strategies to hinder the spread of AMR in animals and subsequently in humans and environment. This would result in the production of safer food with a lower risk of transfer of AMR genes from broiler chickens to humans via the food chain or alternative routes. This One Health approach is supported by all stakeholders mentioned, including EFSA, ECDC, WHO, FAO, and the Ministry for Agriculture, Nature and Food Quality in the Netherlands.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium.

Being a OH-EJP PhD student opened up different channels and opportunities, from gaining and exercising skills and competences to enriching the student's scientific knowledge.

Early in 2020, VIMOGUT collaborated with researchers from the Helmholtz Centre for Environmental Research – UFZ in Leipzig, Germany. This collaboration strengthened our knowledge on microbiome data analysis and generated fruitful ideas about studying the chicken caecal microbiota development on relation to colonisation with resistant. This work is currently published as a research article in the scientific journal Animal microbiome.

Moreover, valuable knowledge was exchanged with the Animal Plant Health Agency (APHA) in UK to establish an *in vitro* chicken gut model capable of reproducing the main physiological conditions of the caeca and maintain the main microbial members that populate this organ.

Other fruitful collaborations were set up with the One Health Antimicrobial Resistant (OHAR) group of the University of Copenhagen via a short-term mission (STM) in 2022. During the STM, the PhD student learned bacterial cloning techniques and developed and strengthened her lab skills to produce dual fluorescently labelled bacterial strains. The dual labelling of bacteria was essential to carry out the *in vitro* experiments that aim to study the effect of antibiotics and microbiota interventions in the horizontal spread of Extended Spectrum ß-Lactam (ESBL) genes. The output of this collaborative work is expected to be published as a research article in a scientific journal.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The VIMOGUT-AMR PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

1. Data collected during OHEJP JRP ARDIG is being further analysed during the VIMOGUT project.
2. During OHEJP ARDIG, samples from broiler farms were collected and further analysed during the PhD project VIMOGUT.
3. The *in vitro* chicken caecal model that was developed for VIMOGUT is currently also employed for the JPI-AMR STRESST project. Expertise that was gained through VIMOGUT is further utilised for the development of an *in vitro* waste water model to study the transfer of AMR genes.
4. Co-funding for the VIMOGUT project was received from the Dutch Ministry of Agriculture, Nature and Food Quality. Results of the VIMOGUT project have been shared with the ministry throughout the project's lifetime.
5. Link with national projects:
    a. National project for AMR monitoring in livestock in the Netherlands.
    b. National project on ESBL-prevalence in veal calves in the Netherlands.
    c. National project on epidemiology of ESBL-carrying plasmids using long-read sequencing in the Netherlands.
    d. National AMR monitoring in livestock project code: WOT-01-002-038

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	4	5
Were all the milestones and deliverables completed?	4	4
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	3	5
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	5	5
Did the PhD student actively engage in Education and Training activities?	5	4
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	4	3
Was the PhD managed and implemented in accordance with the DMP?	1	4
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	3	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	4	5
Is there any direct or indirect impact of the project for national or international stakeholders?	3	5
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	3	4
Does the project outcomes have policy implications?	2	3
TOTAL	45/65	57/65

AVERAGE: 51/65

NB. Reviewer 2 provided only scores, with no comments or feedback.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

The main research lines seem to have the implementation of the DASbox system and the use of fluorescent labelled plasmids. However, these will be difficult to finish by the end of the year.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Generally, agree with the score and the comments provided by reviewer 1, but the student did list more than just the DASbox. No comments were provided by reviewer 2, so it is difficult to evaluate the score.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

The research plan was clearly hindered by the SARS-CoV-2 and Avian flu pandemics. However, the shift towards *in vitro* work was well justified and well executed.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the scores and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

See above comments.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Difficult to evaluate the reviewers scores as no comments were provided on the milestones and deliverables.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Heavily lab-focused work, not many interactions possible with other projects and initiatives.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

The student participated in multiple conferences with his peers, and did a lab visit to the University of Copenhagen.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

Vast number of trainings followed (see training and education section for the VIMOGUT PhD project).

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

One peer-reviewed publication so far, but I would judge at least two more can be conceptualized from this work.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

No mentioning of a DMP in the document.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Seems strange that reviewer 2 has given a score of 4 for the DMP plan, when it is not mentioned? No score provided by reviewer 1.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

The student mainly participated in the activities of the OHEJP consortium, little outreach beyond this. However, the main result of the study remains to be published so probably more outreach foreseen in the near future.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1, although additional outreach might not be associated with publications? No comments were provided by reviewer 2, so it is difficult to evaluate the score.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

Additional insight in population dynamics of ESBL *E. coli*, and the development of an *in vitro* model system to study future interventions are both of great value in understanding and interrupting the flow of AMR.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

No direct impact, but this was only not the main goal of this research which aimed more at understanding the prevalence dynamics of resistant bacteria like ESBL - *E. coli* in chicken microbiota. The observation that the presence of ESBL - *E. coli* is associated with mild but consistent reductions in broilers' caecal microbiota richness and transient microbiota compositional differences is clearly interesting and will form the basis for further study.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, the studies may have impact in the future. No comments were provided by reviewer 2, so it is difficult to evaluate the score.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

Mainly interactions with local stakeholders, not necessarily international. However, the *in vitro* model built can (and might) be of high interest for OHEJP partners in the future.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score. It should be noted that *in vitro* chicken gut models have been published by other groups.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

The project was not designed, nor executed to lead to policy measures. So, this can't be blamed on the PhD student.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

Agree with the score and the comments provided by reviewer 1. However, no comments were provided by reviewer 2, so it is difficult to evaluate the score.

The full Final Thesis Report for the VIMOGUT PhD project can be found, here.

PhD14-FBZ4-ToxSauQMRA

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Filip Dámek	PhD Student	The National veterinary school of Alfort	France
Radu Blaga	PhD Lead Supervisor	The National veterinary school of Alfort	France
Delphine Le Roux	PhD Second Supervisor	The National veterinary school of Alfort	France
Marieke Opsteegh	Other Supervisor	The National Institute for Public Health and the Environment	The Netherlands
Arno Swart	Other Supervisor	The National Institute for Public Health and the Environment	The Netherlands

Summary of the work carried out in the PhD project

The ToxSauQMRA PhD project began in M23, in 2019, and finished in M62, in 2023. The project received a three-month extension due to the SARS-CoV-2 pandemic. The ToxSauQMRA project explored various aspects of addressing issues in pork production and reducing the spread of toxoplasmosis through pork and pork products. We successfully composed a comprehensive literature review of the most recent prevalence of *T. gondii* in selected European animal populations to develop a Bayesian statistical model that estimates age-specific seroprevalence for individual animal species, considering the effects of region, outdoor access, and sample matrix. The model and related resources were made publicly available to aid and standardise the conduct and reporting of future prevalence studies.

Furthermore, we investigated the potential effects of *T. gondii* genotype and stage on clinical response and tissue distribution in pigs, providing valuable insights into the epidemiology of *T. gondii* in pigs. The highest prevalence of the parasite was observed in shoulder muscles, with the highest parasite load observed in the uterus. Strain- and stage-related patterns were noted, and the infection with the type III isolate and tissue cysts resulted in significantly more positive tissues and higher parasite burden in tissue cyst-infected tissues. We have expanded on the previous findings by investigating the distribution of *T. gondii* in pig tissues after experimental infection, highlighting the importance of considering infection routes and genotype in the development of preventive strategies against *T. gondii.*

In the next step, we assessed the inactivation potential of salt, nitrites and nitrates on *T. gondii* in dry sausages and processed pork. We observed inactivation of the parasite in all tested recipes with salt concentrations above 2% after less than a day. In addition, we completed an assessment of the French and European country-specific meat products including their

composition and processing steps during production that might influence viability of potential *T. gondii* tissue cysts within.

Finally, we adjusted and updated the necessary databases for the development of novel heating and salting models applicable to meat and meat products. Our goal was to incorporate these models into the final quantitative microbial risk assessment (QMRA) model, which aims to estimate the relative contribution of different sources of *T. gondii* human infection. This novel QMRA model will provide an effective tool for decision-makers in the development of preventive strategies against the parasite.

Dissemination activities of this ToxSauQMRA PhD project were enshrined in a number of peer-reviewed publication, in journals such as Pathogens, and a range of oral and poster presentations at the national (i.e., Société Française de Parasitologie, Société Française de Mycologie Médicale, Lyon) and international levels (i.e., 15th International Congress of Parasitology).

Work carried out on the PhD, scientific results and outcomes

The PhD thesis is currently under embargo and no non-confidential summaries of these works were provided and more information on the PhD project can be found here.

PhD self-assessment

We have diligently adhered to the original project proposal and have successfully executed a majority of the promised actions. However, there were certain parts of the project that required a prompt adaptation due to various reasons.

As part of the original project, dry sausages and dry hams were to be produced. The dry hams were meant to be manufactured by INRAe Corte (Corsica), utilising two traditional salting techniques (a long one: 2.5 days/kg and a short one: 1 day/kg). Unfortunately, due to a local strike in the Marseille harbour, the hams were delayed by 10 days. This caused the sanitary quality of the meat to be questionable in terms of manufacturing. As a result, only the long salting technique was employed, and 300g of product was taken at D30 and D90 due to the SARS-CoV-2 pandemic.

Furthermore, we had to face complications in the supply chain and shortages of chemicals required for qPCR due to the SARS-CoV-2 pandemic, which caused delays in the project timeline. However, despite the challenges, all predetermined milestones were successfully achieved, and the outcomes were valorised in the form of presentations at national and international congresses, as well as publications in peer-reviewed journals.

The QMRA model for French meat products will be an integral part of the final QMRA model, which will be the final outcome of the linked OHEJP TOXOSOURCES. Thanks to collaborations in these interconnected projects, we can ensure that the final deliverable will be of the highest quality, meeting all expectations.

Progress of the project: milestones and deliverables

Deliverables

These weren't supplied by the PhD student or supervision team.

Milestones

These weren't supplied by the PhD student or supervision team.

Publications and additional outputs

Publications

The ToxSauQMRA PhD project has enshrined these works through three peer-reviewed publications, these are:

- Kauter J, Damek F, Schares G, Blaga R, Schott F, Deplazes P, Sidler X, Basso W. (2023). Detection of Toxoplasma gondii-specific antibodies in pigs using an oral fluid-based commercial ELISA: Advantages and limitations. *International Journal for Parasitology*. doi: 10.1016/j.ijpara.2022.11.003
- Dámek F, Swart A, Waap H, Jokelainen P,Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García G, Betson M, Davidson R.K, Györke A, Antolová D, Hurníková Z, Wisselink H. J, Sroka J, van der Giessen J. W. B, Blaga R, Opsteegh M. (2022). Systematic Review and Modelling of Age-Dependent Prevalence of *Toxoplasma gondii* in Livestock, Wildlife and Felids in Europe. *Pathogens*. doi: 10.3390/pathogens12010097
- Dámek F, Fremaux B, Aubert D, Thoumire S, Delsart M, Martin J. L, Vuillermet S, Opsteegh M, Jokelainen P, Le Roux D, Boireau P, Villena I, Blaga R. (2022). Inactivation of *Toxoplasma gondii* in dry sausage and processed pork, and quantification of the pathogen in pig tissues prior to production. Food and Waterborne Parasitology. doi: 10.1016/j.fawpar.2023.e00194

Each of these publications have been uploaded to Zendo, which is gold standard open access, and can be found here, here and here, respectively.

Additional outputs (i.e., poster/oral presentations)

The ToxSauQMRA PhD project further disseminated these works through oral and poster presentations at the following events:

- Modelling of age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felids." *Oral presentation.* La Journée Sciences de la Vie, Université Paris Est-Créteil (UPEC), Paris-Bercy, France. 19th October 2022.
- Systematic review and modelling of age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felids in Europe. *Oral presentation.* 6th international meeting on apicomplexan parasites in farm animals, Bern, Switzerland. 5-7th October 2022.
- Tropism and persistence of *Toxoplasma gondii*: from pork carcass to dry sausage & Detailed anatomical distribution of *Toxoplasma gondii* in tissues of experimentally

infected pigs & Comparison of *Toxoplasma gondii* distribution in tissues of experimentally infected pigs. *3 poster presentations.* 6th international meeting on apicomplexan parasites in farm animals, Bern, Switzerland. 5-7th October 2022.

- Systematic review and modelling of *Toxoplasma gondii* prevalence in animals of Europe. *Oral presentation.* La Journée de la Recherche de l'Ecole Vétérinaire d'Alfort (ENVA), Maisons-Alfort, France. 20th September 2022.
- Systematic review and modelling of age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felids in Europe & Tropism and persistence of *Toxoplasma gondii*: from pork carcass to dry sausage & Detailed anatomical distribution of *Toxoplasma gondii* in tissues of experimentally infected pigs. *3 oral presentations.* 15th International Congress of Parasitology (ICOPA2022), Copenhagen, Denmark. 21-26th August.
- Modelling of the age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felines. *Poster presentation & 3-minute thesis competition.* OHEJP Annual Scientific Meeting, Orvieto, Italy. 11-13th April 2022.
- Veterinary and public health research in France in relation to *Toxoplasma gondii*. *Oral presentation.* The Parasitological Seminar, Brno, the Czech Republic. 17 March 2022.
- Tropism and persistence of *Toxoplasma gondii*: from pork carcass to dry sausage. *Poster presentation.* La Journée Sciences de la Vie, Université Paris Est-Créteil (UPEC), Créteil, France. 16th February 2022.
- *Toxoplasma gondii* seroprevalence in European wildlife: a systematic review & Tropism of *Toxoplasma gondii* in the tissues of experimentally infected pigs. *2 poster presentations.* 13th European Multicolloquium of Parasitology (EMOP2021), Belgrade, Serbia. 12-16th October 2021.
- *Toxoplasma gondii* prevalence in animals in Europe: a systematic review & Tropism and persistence of *Toxoplasma gondii*: from pork carcass to sausage. *2 poster presentations.* 28th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP2021), Dublin, Ireland. 19-22 July 2021.
- *Toxoplasma gondii* prevalence in animals in Europe: a systematic review & Tropism and persistence of *Toxoplasma gondii:* from pork carcass to sausage. *2 poster presentations & 3 minute thesis competition.* OHEJP Annual Scientific meeting, Copenhagen, Denmark as hybrid event. 9-11th June 2021.
- *Toxoplasma gondii* seroprevalence in European wildlife: a systematic review. *Oral presentation* given on Filip' behalf by Dr Pascal Boireau. Société Française de Parasitologie, Société Française de Mycologie Médicale, Lyon 27-28th May 2021.
- Tropism and persistence of *Toxoplasma gondii*: from pork carcass to sausage and dry ham, a quantitative risk assessment. *Poster presentation & 3 minute thesis competition.* OHEJP Annual Scientific meeting, online. 27-29th May 2020.

One Health impact

The developed literature data extraction template will greatly simplify the reviewing process for future prevalence and epidemiology studies. In concordance, the data reporting template will guide authors in providing all the necessary data from their original research, ensuring there are no data gaps. Thanks to a standardized template format, these data can be easily utilized for future data extractions.

We have compiled a comprehensive database of *T. gondii* prevalence in European animal species, which includes corresponding ages and the European region of their origin. Additionally, we have collated an extensive database of both generic and country-specific meat product recipes, documenting the additives, ingredients, and processing steps used. These datasets will prove valuable for future risk assessment studies and models.

To ensure the highest possible quality of the collated data, experts from leading scientific institutions in Europe have actively contributed to this effort. The outcome was submitted for approval to representatives of private and public authorities in the respective countries.

These outputs have been instrumental in developing a of large-scale Quantitative Microbial Risk Assessment (QMRA) model for human *T. gondii* infection sources. By filling crucial data gaps, this model can aid in the development of preventive strategies that go beyond limiting the impact of *T. gondii* on public health. These strategies and models will play a crucial role in decision-making at both national and European levels, as well as within private meat production companies.

The literature reviews, data collection, and database development required significant involvement from experts across various fields such as public health, animal health, epidemiology, ecology, and others, all representing participating European partners. This collaborative effort resulted in the establishment of a robust network capable of achieving our ambitious goals. Regular meetings within this diverse consortium facilitated the exchange of exceptional expertise and provided an excellent platform for future collaborations.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

The One Health concept plays a crucial role in bridging individual fields of research, opening up new avenues to address existing research gaps and promoting creativity and innovative ideas that push the boundaries of scientific horizons. It was a privilege to share my research findings with like-minded professionals from other OHEJP projects, and the opportunity to engage in collaborative partnerships and network with peers was invaluable. The OHEJP meetings provided a unique platform for me to gain insights into diverse scientific perspectives that enriched my understanding of complex research topics beyond my own area of expertise.

In addition, the OHEJP consortium is a diverse and dynamic community of motivated and passionate scientists who are dedicated to advancing knowledge in their respective fields. The strength of this community lies in its collective ability to help troubleshoot individual setbacks and support each other in overcoming challenges. The power of this collaborative approach cannot be overstated, and I am grateful for the opportunity to be part of such a dynamic and supportive community that is working towards a common goal of improving public health through multidisciplinary research.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The ToxSauQMRA PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- The ToxSauQMRA PhD student has been involved in the OHEJP JRP TOXOSOURCES, as part of the Anses team, participating to the kick-off meeting (3-4.02, Copenhagen, Denmark) and since then participating to all the videoconferences of the various WPs, in which Anses is involved. He took a very active role in the activities of WP2 (human/animal prevalence review, questionnaires, meat product processing, QMRA model updating, *etc.*), helping the coordinators of this WP during his STSMs in RIVM. At the same time he participated in all the activities of WP3 (*T. gondii* detection in RTE salads).
- Similarly, as part of the Anses team, the ToxSauQMRA PhD student was involved in the national research project n° 0917003490 financed by the French Ministry of Agriculture through the France Agri Mer agency with the title: Study of the tropism and persistence of *Toxoplasma gondii*: from pork carcass to sausage, gathering the above-mentioned partners (Ifip, URCA, Inrae Corte), representing at the same time the fundament/basis of his PhD programme.

Evaluation of the Final Thesis Report

Not applicable as the PhD student submitted their thesis manuscript.

PhD15-FBZ5-TRACE

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Renate Hakze – van der Honing	PhD Student	National Institute for Public Health and the Environment	France
Eelco Franz	PhD Lead Supervisor	National Institute for Public Health and the Environment	France
Wim H.M. van der Poel	PhD Second Supervisor	Wagenigen Bioveterinary Research	The Netherlands
Hans Zaaijer	Other Supervisor	Sanquin	The Netherlands

Summary of the work carried out in the PhD project

The TRACE PhD project began in M25, in 2020 and is expected to be completed in M69, in 2023. A three-month extension was provided due to the SARS-CoV-2 pandemic. TRACE developed a protocol for optimal sample (pre)processing (enrichment) to do whole genome sequencing of Hepatitis E virus (WP1). We developed a new HEV-probe enrichment set based on probe capture enrichment to sequence all the different HEV strains. With this new probe set we were able to generate whole-genome sequences from samples of Ct values up to 30 from different origins. Due to several disappointments during the development of the whole genome procedure, we are still working on the last work packages. When we have generated enough HEV sequences of pigs, wild boar, and humans, we can start the phylodynamic work (WP2) and the quasispecies analysis (WP3).

We first started setting up a method for HEV analyses for timed phylogeny of known sequences from the NCBI. This should enable us to later apply this method for the sequences we generated ourselves. As the majority of the HEV sequences from NL only cover a small (493 nt) region of the viral genome, we have tested to what extent this fragment could be suitable in performing accurate diagnostics or inferring any viral population-scale processes. To do this, we started with a set of viral whole genome sequences that we had sequentially trimmed to various fragment lengths (down to 148 nt) and used each of the fragments to infer the richness and diversity of the viral sequence types, typing accuracy, and phylodynamic. By comparing the results obtained with each of the fragments we could draw conclusions on the utility of using particular genomic loci in molecular diagnostics, typing, phylogenetics and phylodynamic of HEV.

Dissemination activities of this TRACE PhD project were enshrined in four oral and poster presentations at the international level (i.e., One Health EJP, ASM 2022, Italy).

Work carried out on the PhD, scientific results, and outcomes

Chapter 1. Whole genome sequencing (WP1)

Using the HEV-specific capture enrichment method we were able to generate whole-genome sequences of different genotypes (Gt3, Gt4 and rat-HEV) up to a Ct value of 30, in faeces and tissue samples. Further optimization is needed for Ct values higher than 30. However, in several cases it was still not possible to generate whole genome sequences from HEV positive samples with a <30 Ct value after several attempts.

During our research to solve this problem and testing all the different steps in the whole procedure for several samples, we found a HEV RNA loss after the benzonase treatment. This indicates an RNA degradation during this step, meaning that the virus in the sample is no longer intact. However, in a probe based sequencing method the benzonase treatment cannot be omitted because of the interference of host and bacterial DNA/RNA in the sample. In a whole genome sequencing method based on HEV specific primer amplification a benzonase treatment not really important. In such case the specific HEV primers will only bind to the target RNA and there will be less interference with other DNA/RNA. Newly generated full genome sequences will be submitted to NCBI as the scientific paper will be published in an open access journal.

With the development of the new HEV capture enrichment method, we will now be able to generate full genome sequences that can be used to perform phylogenetic studies comparing HEV strains of different origins and from different hosts.

Chapter 2. HEV phylodynamic (WP2)

The final alignment of the whole genome sequences consisted of 1073 sequences and 6889 nucleotides.

From the HEVNet database we retrieved 1013 HEV sequences. Of these, only two covered the whole genome (-7:7225 and 21:7230 of reference strain MN614141), 163 sequences covered a fragment of ORF1 (with variable lengths from 242 to 371 nt, corresponding to positions 77:448 of the reference strain MN614141), and the majority of the sequences covered a fragment of ORF2 (with variable lengths from 148 to 1390 nt, corresponding to positions 5732:7121 of the reference strain MN614141). The alignment of these sequences has been trimmed to correspond to the 493nt and 148nt of ORF2 fragments.

Chapter 3. Identification of HEV virulence genes and HEV quasispecies (WP3)

The generated sequences derived from patients with acute hepatitis, and asymptomatic RNA positivity among blood donors and veterinary data generated in work package 1 will be analysed for genomic regions associated with HEV virulence and infectivity. This will be performed by linking the available sequences with the clinical data. In the generated whole genome sequences, we will look in de quasispecies of the virus, to identify non dominant sequences which can play an role in the changing virulence of the virus. When a chance of the equilibrium of the virus consensus will occur, the consensus sequence will change. These changes can play a role in the virulence of the virus and therefore need to be investigated. However due to the previously described delay in WP 1 we were not able to finish WP3. This

part of the work will not be completed by 30 June and therefore cannot be included in the final report.

Chapter 4. WP4 Data analysis and data evaluation

For the analysis and data evaluation in work packages 4 we need the results of the previous work packages. Unfortunately, we weren't able to complete these before the 30th of June and therefore will not be included in the final report.

PhD self-assessment

In WP1 we optimised and developed a sample (pre) processing (enrichment) method and a probe-based enrichment procedure for HEV that enables us to generate whole genome sequences of HEV from different origins and genotypes up to a Ct of 30.

In the project we did not yet succeed to perform HEV phylogenetic analyses using the full genome sequences we generated ourselves. Therefore, we set up a method for HEV timed phylogeny of known sequences from the NCBI. This should enable us to later to apply this method to sequences we generated ourselves.

Additionally, we analysed sequences of different HEV fragment lengths. Primarily the 493 nt fragment, which is sequenced most frequently in literature and often used for genotyping and subtyping. To aim was to assess the usability of these 493 fragments for performing accurate diagnostics or inferring any viral population-scale processes. To do this, we started with a set of viral whole genome sequences that we have sequentially trimmed to various fragment lengths (down to 148 nt) and used each of the fragments to infer the richness and diversity of the viral sequence types, typing accuracy, and phylodynamic. By comparing the results obtained with each of the fragments we could draw conclusions on the utility of using particular genomic loci in molecular diagnostics, typing, phylogenetics and phylodynamic of HEV.

The work of WP3 and WP4 will not be finished before the 30 of June. However, we will still want to carry out this analysis and we will continue this research. We will try to perform this before the 1 of September, the end date of the project.

Progress of the project: milestones and deliverables

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories*
PhD 15	D-PhD15-1	Sample processing protocol for HEV RNA positive target samples of different origin and associated deep sequencing procedure for HEV from such samples	Mxx (M1 = Jan 2018, M60= Dec 2022) 48	Mxx (M1 = Jan 2018, M60= Dec 2022)	*Please mention: public or confidential, the Zenodo reference, reason and justification of delay (for instance COVID), other comments* At the start of this project 2020, we had a delay due to the COVID-19 pandemic. Due to the producer/supplier of the enrichment product made a change in his product. And a first attempt to develop a new method was unsuccessful. We were not able to make the delivery date. We will try to complete this before 31 of August.	*If applicable* 2*
PhD 15	D-PhD15-2	Report/publication on HEV dynamics including information about the geographical origin of predominant virulent strains and identification of genetic traits changed over time.	48		D-PhD15-2 is depending on D-PhD15-1 We plan to submit the publication before 30 june.	5
PhD 15	D-PhD15-3	Identification of virulence genes and elucidation of the relationship between quasispecies and changing virulence of circulating HEV strains	64		D-PhD15-3 is depending on D-PhD15-1. We will try finish it at the end of 2023	5
PhD 15	D-PhD15-4	Report explaining HEV strain shifts and HEV disease outcomes related to HEV circulation and HEV variability. Evaluation of results for future anticipation and intervention as possible.	64		D-PhD15-4 is depending on D-PhD15-1. We will try finish it at the end of 2023	5
PhD 15	D-PhD15-5	Evaluation of results for future anticipation and intervention as possible.	64		D-PhD15-5 is depending on D-PhD15-1. We will try finish it at the end of 2023	5

** 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

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD 15	1	Detailed outline phD plan	M24	M24	Yes	

Publications and additional outputs

Publications

The TRACE PhD project has enshrined these works through peer-reviewed publications, these are:

- Renate W. Hakze-van der Honing, Frank Harders, & Wim H.M. van der Poel. (2022). Development of a whole genome sequencing method for Hepatitis E Virus. doi.10.5281.

The above publication has been uploaded to Zendo, which is gold standard open access, and can be found here.

Additional outputs (i.e., poster/oral presentations)

The TRACE PhD project disseminated these works through oral and poster presentations at the following events:

- Oral presentation at ISFEV in Santiago de Compostella, Spain. 16-20th May 2022.
- Hakze van der Honing, R., Harders, F., Franz, E. & Van Der Poel, W. (2022). Development of a whole-genome sequencing method for Hepatitis E virus. Poster presentation at One Health EJP, ASM 2022, Orvieto, Italy. 11-13th April 2022. More information can be found here.
- 3-minute thesis presentation & roundtable discussion at One Health EJP ASM 2022, Orvieto, Italy. 11-13th April 2022.
- Poster presentation & 3-minute thesis presentation at One Health EJP ASM 2021, hybrid event. 9-10th June 2021.

Transferrable Skills and Training

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Name of training	Key topic(s)	Date of training	Name of organisers
On the job training for whole genome sequencing	Sample preparation, whole genome sequencing	2020	WBVR
Immunology	Immunology	06-10/01/20	Utrecht Univercity
OH-EJP Continung professional development module 2021	Digital innovations for One Health Practitioners	15-19/02/21	German Federal Institute for Risk Assessment
Biopigee OH-EJP Workshop	HEV	14 /12/21	OH-FJP
On the job training to preform HEV annalysis with various programs as Jalview, Beaty and Beast, Tracer, tree viewer. Rstudio	Richneness and the divirsety of sequences, typing accuracy and phylodynamics.		RIVM
Internal audit course	Course to get a graduated auditor	24-25/10/22	WBVR

One Health impact

Development and harmonisation of deep sequencing-based methods for detection and tracing of foodborne zoonotic agents and emerging threats are important in the One Health approach. Data from this project will lead to improved surveillance and more harmonised data analyses on the foodborne zoonosis HEV. This will contribute to broader and flexible actions to detect actual hazards, main reservoirs, trends, and routes of transmission as well as common approach and timely analysis and data sharing which will be needed more and more with ongoing globalisation. Information sharing and harmonisation can be performed international and with national authorities.

HEVnet hosted with the RIVM is a collaborative inter-disciplinary network for sequence data repository for enhanced HEV molecular typing, virus characterisation and epidemiological investigations. HEVnet was set up in April 2017 with support of the ECDC and almost 15 institutes in 9 European countries working on HEV in public and veterinary health, environment as well as food and blood safety have already contributed more than 1600 HEV sequences to this database, with data on the origin (acute patients, RNA-positive asymptomatic blood donors, swine meat and swine faces), time and country of sampling. Most of these sequences where patrial sequences, with our developed whole genome method other groups can generate more whole genome sequences, which can be used for the HEVnet members.

In the One Health EJP there was a cooperation with the BIOPIGEE project. The infectivity assay developed in this project can be used in case the whole genome sequence method still lacks sensitivity. We can consider HEV culture to increase the amount of HEV virus, till the amount it is enough to sequence.

In the One Health EJP project MATRIX the HEV samples were used as a data base example. While, in the One Health EJP project SIMEX, HEV has been used as an example for an outbreak agent.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

In the OHEJP program the collaboration and integration with other HEV researchers in the Netherlands and international has been improved. This was achieved by working towards a common goal and interest.

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The TRACE PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- BIOPIGEE: In the BIOPIGEE project partial sequencing was performed in several EU counties during the same lime line. These sequences were compared with each other in a phylogenetic analysis and connections were made and differences in the sequences were discussed. In the BIOPIGEE project an HEV infectivity assay has also been developed. The infectivity assay can be used in case the whole genome sequence method still lacks sensitivity. We can consider HEV culture to increase the amount of HEV virus, till the amount it is enough to sequence.
- MATRIX: In the MATRIX project HEV was used as a data base example.
- SIMEX: In the SIMEX project HEV has been used as an example for an outbreak agent.
- ECDC: HEVnet was set up in April 2017 with support of the ECDC. It is a collaborative inter-disciplinary network for sequence data repository for enhanced HEV molecular typing, virus characterisation and epidemiological investigations. ECDC uses HEV sequences submitted in in HEVnet database. When the best predicted region has been identified for HEV typing, characterisation and epidemiology. Members of the HEVnet group and ECDC can be advised.
- EFSA: When we are able to identify the virulence genes of HEV in the consensus sequence or the quisispecies sequences, it can be used to estimate the virulence of the circulating stains in food products. This can be used by EFSA as an important tool for to predict the safety of food products which contain HEV.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	5
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	3	5
Were all the milestones and deliverables completed?	2	4
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	5	5
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	2	5
Did the PhD student actively engage in Education and Training activities?	2	5
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	1	4
Was the PhD managed and implemented in accordance with the DMP?	4	5
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	3	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	5	5
Is there any direct or indirect impact of the project for national or international stakeholders?	4	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	2	4
Does the project outcomes have policy implications?	1	4
TOTAL	38/65	60/65

AVERAGE: 49/65

NB. These scores and comments are based on a confidential version of the Final PhD Thesis Report, which isn't fully outlined in D6.18.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

Yes, clear ideas for future research, e.g., partial sequences for phylodynamic, understanding viral transmission, *etc*.

Reviewer 2: External Scientific reviewer

The student clearly explains the main conclusions of the thesis. Results are promising and will be the starting point of future work.

Reviewer 3: PMT member

Indeed the project results may well be the base for future research, setting a good ground of protocols and approaches to elaborate on the phylodynamics of HEV.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

Not all objectives were met, yet this was justified by issues with setting up WGS of HEV.

Reviewer 2: External Scientific reviewer

Yes, objectives are met although some analyses are still ongoing. The justifications are plausible and well explained, here par 7 and elsewhere. Deviations from the original are not present and I think results will be obtained soon because the method developed by the student is robust.

Reviewer 3: PMT member

Setting up WGS for organisms as HEV may indeed be tricky. Although not all the objectives were met, those that were compensate Setting up WGS for organisms as HEV may indeed be tricky. Although not all the objectives were met, those that were compensate.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

The milestone is achieved, while some deliverables are not available yet. Nevertheless, results to be included into the deliverables are almost ready. Based on this report and as stated by the student, deliverables will completed be soon.

Reviewer 3: PMT member

While the milestone has been achieved, most of the deliverables' completion is foreseen. While the justification seems reasonable the strict dependence of all the deliverables from D-PhD15-1 seems to be a weakness in the original design of the project.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Yes, the PhD student participated in many projects, both internal and external.

Reviewer 2: External Scientific reviewer

Yes, with two projects of the EJP, both were focused on HEV. Data and results were shared among projects.

Reviewer 3: PMT member

Nicely completed.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

It seems like the PhD student understood the question as added value for the stakeholders, not added value for the PhD student themselves.

Reviewer 2: External Scientific reviewer

In paragraphs 10 – 12 interactions, with international networks is clearly reported, including European authorities and HEV-Net, the network of HEV experts. These interactions were possible thanks to the OHEJP doctoral programme.

Reviewer 3: PMT member

Agree with the reviewer 1 comment on the possible misunderstanding of the added value's target. It seems that the student did not capture the sense of the question that was related with the awareness of the value acquired by the PhD student.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

Some training was followed, yet it seems like some training must have been forgotten to be listed.

Reviewer 2: External Scientific reviewer

Yes, the student attended different courses on different topics.

Reviewer 3: PMT member

Fulfilled. Although I would have expected mor training events for a PhD student as these are an important base for the education.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

No not yet.

Reviewer 2: External Scientific reviewer

Not yet, the paper is in preparation, but the summary of method is available on Zenodo.

Reviewer 3: PMT member

Apparently, there are 3 abstracts uploaded on the ZENODO platform that did not end into full papers yet. Two of them date to 2020 and 2021, I would have expected that these 2 papers were already out.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Yes.

Reviewer 3: PMT member

n/a

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

Yes, two conferences and a pitch.

Reviewer 2: External Scientific reviewer

Yes, by presenting two posters during the OHEJP meetings.

Reviewer 3: PMT member

Not much visibility was given to the work done in my opinion.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Very relevant, since it represents the OH approach, the sequencing data from human and animal strains are needed to manage foodborne outbreaks/cases caused by the virus.

Reviewer 3: PMT member

Very relevant.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Probably in the future.

Reviewer 3: PMT member

The project's results can indeed have an impact on stakeholders at both national and international level. They might impact much more if released with open access publications.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

Not clear from the report.

Reviewer 2: External Scientific reviewer

Yes, information, results and method were shared among several JRP projects.

Reviewer 3: PMT member

Agree with reviewer 1. this does not emerge from the report although part of the data produced were used in other projects.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

Not immediately. In the near future results collected by this and other studies will probably have policy implications.

Reviewer 3: PMT member

The project's outcome could have policy implication in perspective but as they are presented are too preliminary to formulate now the extent of such an impact.

The full Final Thesis Report for the TRACE PhD project can be found here.

PhD16-FBZ2/AMR6.1-Codes4strains

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Mélanie HENNART	PhD Student	Sorbonne University and Pasteur Institute Paris	France
Sylvain BRISSE	PhD Lead Supervisor	Pasteur Institute Paris	France
Alexis CRISCUOLO	PhD Second Supervisor	Pasteur Institute Paris	France

Summary of the work carried out in the PhD project

The Codes4strains PhD project began in M22, in 2019, and finished in M59, in 2022. Codes4strains focused on the tracking of bacterial pathogens through sources, geography and time using stable phylogenetically informative genome codes. Infectious diseases are a global public health concern, particularly due to antimicrobial-resistance in some pathogenic bacteria. *Klebsiella pneumoniae* is one of the most worrying multi-resistant bacteria. *Corynebacterium diphtheriae*, which causes diphtheria, remains largely susceptible to first-line antibiotics, including penicillin, and can be controlled through vaccination, but re-emerges when vaccination coverage is insufficient. Among the effective infection control measures, the accurate detection and identification of these pathogens, as well as their epidemiological monitoring, play a key role. In the recent years, the implementation of whole-genome sequencing (WGS) has revolutionised bacterial genotyping, by providing discrimination at the strain level. Genomic sequencing also enables the detection of variants and their important characteristics, such as virulence or antimicrobial resistance.

The research work of this thesis is structured around two main axes, which are:

1. The first axis provides bioinformatic analyses of the population structure of antimicrobial resistance in *C. diphtheriae*. Based on a large clinical isolates collection from metropolitan France and its overseas territories, a genome-wide association study (GWAS) was performed to determine the genetic basis behind resistance phenotypes, as well as their associations with diphtheria toxin production and other strain characteristics. A new penicillin resistance gene was discovered on a mobile element in *C. diphtheriae*. A genotyping bioinformatics tool, DIPHTOSCAN, was developed, to harmonise and facilitate the detection and genotyping of the main virulence factors and resistance genes of *C. diphtheriae*. We also developed strain nomenclatures from assembled genomes. DIPHTOSCAN further enables the prediction of biovars and of the toxicity of strains. The public availability of the tool, can be found here, and its ease of use will advance the genomic epidemiology of the agent of diphtheria, the clinical management of patients and knowledge on the links between animal and human diphtheria cases (particularly those caused by *C. ulcerans*). This

thesis also advanced knowledge on the genotype-clinical phenotype links in *C. diphtheriae*.

2. The second axis relates to infra-species genomic taxonomy. A new approach of genome-based classification and nomenclature of strains was developed using *K. pneumoniae* as a model. This work describes the design and implementation of a barcoding system that uses Life Identification Number (LIN) codes based on the core-genome MLST (cgMLST) typing approach. This innovative taxonomic strategy provides precise and stable strain nomenclatures and was applied to the 'one health' model *Klebsiella pneumoniae* species complex, a ubiquitous group of pathogens. The proposed unified taxonomy of *K. pneumoniae* strains will facilitate advances on the biology of its sub-lineages across niches, time, and space, and will endow surveillance networks from different activity sectors, including food microbiology and veterinary and human medicine, with the capacity to efficiently monitor and control the emergence of sub-lineages of high public health relevance.

Based on the contributions and concepts developed in the two above axes, several case studies were carried out: identification and characterisation of a new species (*C. rouxii*), which was previously misidentified as *C. diphtheriae*; genomic epidemiology studies of diphtheria in different world regions from animal and human clinical sources; showing that *C. rouxii* probably represents a novel zoonotic pathogen. These applications of genomic taxonomy in combination with antimicrobial resistance gene detection illustrate the potential of the methods and tools developed during this thesis to support genomic research and surveillance of pathogenic bacteria in a one health perspective.

The work of this thesis has shed light on important questions in the biology of *C. diphtheriae* and *K. pneumoniae* populations and has developed concepts and tools that meet the modern needs of medical microbiology and public health actors. The speed and reduced costs of sequencing offer the possibility for microbiology laboratories to have access to this new approach in routine. Their exploitation and interpretation with standardised and automated approaches, such as the ones developed in this thesis, will facilitate analysis of genomic sequence data, thus contributing, hopefully, to a better control of infectious diseases.

Dissemination activities of this Codes4strains PhD project were enshrined in a number of peer-reviewed publication, in journals such as Molecular Biology and Evolution, and a range of oral and poster presentations at the international level (i.e., One Health EJP Annual Scientific Meeting, Orvieto, Italy).

Work carried out on the PhD, scientific results and outcomes

Whole genome sequencing allows the tracking of pathogenic strains and informs infection control, diagnostics, and sometimes treatment strategies. Universal strain nomenclatures are necessary to track strains at a global level, and as they spread between the environment, food, animals, and humans. The core genome Multilocus Sequence Typing (cgMLST) approach is an accurate, reproducible, and portable strain genotyping method that underlies widely used strain nomenclatures, in which groups are generally determined by single-linkage clustering. However, cgMLST groups are unstable due to the possibility of group fusion upon subsequent sampling. Recently, a new coding approach named LIN (Life Identification

Number) was introduced by Marakeby *et al*. It provides a numerical code for each genome based on its similarity (estimated using the Average Nucleotide Identity, ANI) to the closest genome already encoded. As LIN codes are attributed to each genome rather than to groups, they are stable. A common feature of both approaches is that single linkage groups and LINcodes can be defined using several similarity cut-offs, in which case they inherently convey phylogenetic proximity information. cgMLST additionally provides, through the number of allelic mismatches among cgMLST profiles, intuitive human-understandable metrics of differences among strains involved in epidemiological events (e.g., '5 cgMLST allele differences'). In this PhD project, a novel genome-based genotyping approach has been developed, taking the best of the two above classification approaches, i.e., combining the advantages of cgMLST (discrimination, standardisation) with those of the LIN code approach (complete stability). The strain classification utility of cgMLST-based LIN code (cgLINcodes) systems, where the pairwise distance is based on the number of allelic mismatches, rather than ANI, has been developed and explored. The cgLINcodes approach was compared with other existing classification approaches: the SNP address and multi-level single-linkage classifications (Multilevel Single Linkage, MLSL). A novel way to define the population structure of bacterial species, called MSTclust (D-PhD16-4.3) has been developed; and the inheritance algorithm has been optimised to provide backwards compatibility of MLSL groups with previous nomenclature identifiers from 7-gene MLST (D-PhD16-4.4); the optimal input order for cgLIN encoding has been defined; the ANI metric has been compared with the cgMLST metric (D-PhD16-4.5); a method to identify recombination in cgMLST data (D-PhD16-4.6) has been devised; the MLSL identifiers have been incorporated into the BIGSdb platform to make them publicly available (D-PhD16-4.7); and a manuscript on the cgLIN codes concept and its implementation in Kp has been published, with comparison with multi-level single-linkage classifications (D-PhD16-4.2).

The cgLIN codes were implemented into the Institut Pasteur *K. pneumoniae* MLST and whole genome MLST database. In BIGSdb version 1.35.0, the new functionalities are: the definition of cgLIN code schemes, the assignment of cgLIN codes, and the nomenclature of cgLIN code prefixes.

The cgMLST profile of all isolates with less than 30 missing cgMLST-629 alleles were assigned to a core genome sequence type (cgST), and these were assigned a cgLIN code. For SL and CG levels, a nomenclature of cgLIN code prefixes was defined. All cgMLST profiles, cgLIN codes and their corresponding classification identifiers were made available for public use. All functionalities were implemented and are available from the website sequence query page.

The original code for assigning cgLIN codes was written in python and is available here. However, the second version was implemented in perl language in order to be incorporated into BIGSdb. In addition, we have compared the results of two algorithms.

Before the encoding of cgLIN code an order is defined by the Prim's algorithm (see details in D-PhD16-3.2).

We compared the orders generated by Prim's algorithm, either from BIGSdb or from the cgLIN script shared above. The results show that the two orders are different. However, whatever the order, the interpretation of the cgLIN codes is identical. Following this observation, we

decided to follow the order initially calculated (in the shared script), which is consistent with all our previous results generated and our previous reports.

We have highlighted that the accuracy of the calculation of the distances between the cgMLST profiles and the accuracy of the cgLIN code thresholds have an impact on the results. Therefore, the chosen solution is to calculate all the distances between the profiles and the thresholds of the cgLIN codes on the same machine and with the same programming language.

Integration into Pathogenwatch

Pathogenwatch provides species and taxonomy prediction for over 60,000 variants of bacteria, viruses, and fungi, and can be found here. The species *K. pneumoniae* is of particular interest. This platform presents many features such as it provides MLST and cgMLST profiles with available schemes, it predicts resistance genes, virulence loci, capsule and O-antigen biosynthesis loci, and it provides replicon typing.

We have recently collaborated with the platform's team to add our MLSL nomenclature, cgLIN code and cgLIN code prefixes. This integration is in progress.

Collaboration to test our approaches

Visitors and students will come to our lab to be trained on the different nomenclature approaches. We also contacted external collaborators to test our approaches on their datasets.

The PhD thesis and associated publications can be found here.

PhD self-assessment

The research project has been largely and successfully completed, as we have addressed most of the objectives stated in the initial work plan. We developed an innovative genomic nomenclature and typing approach called cgLIN codes, which combines the advantages of the cgMLST approach (reproducibility, standardisation) with the LIN (Life identification numbers) code approach (complete stability). LIN codes are attributed to each genome, and the code conveys a notion of phylogenetic proximity with other strains of the same species, based on the number of different alleles between the cgMLST profiles of the genomes.

A slight deviation from the initial plan is that we did not include the SNP address approach in the PhD work, even though it was originally planned. We did conduct a comparison between the cgMLST and SNP address approaches, and the results showed that the methods were not easily comparable. Besides, the SNP address approach has not been largely implemented (to our knowledge, only PHE/UKSHA uses it, on a restricted number of pathogens). Finally, our partner in charge of SNP address developments changed job and moved from PHE/UKSHA to the Netherlands, meaning he had no access anymore to the comparative databases. For these reasons, it was decided not to pursue the SNP address comparative evaluation.

To compensate, we used a similar approach to the SNP address, a multi-level grouping system based on cgMLST profiles (instead of SNPs in the SNP address approach), which allowed us to create a multilevel taxonomic barcode system. We also made sure that this new nomenclature approach would be linked to existing nomenclature systems, such as the 7-gene MLST, to make it backwards-compatible with this widely used language, and to facilitate adoption of the genomic nomenclature.

It should be noted that, even if this was not planed initially within this PhD work, our LIN code method was implemented and made available on the BIGSdb platform as well as in PathogenWATCH, two prominent genomic epidemiology and strain taxonomy global platforms (https://bigsdb.pasteur.fr/klebsiella/cgmlst-lincodes/ ; https://cgps.gitbook.io/pathogenwatch/technical-descriptions/typing-methods/klebsiella-lin-codes), allowing the scientific community to access and use it for other bacteria. These two achievements are very significant additional deliverables.

Finally, although our initial work plan aimed to apply this novel approach to *Escherichia coli*, we were unable to do so as our PHE partner moved away from *E. coli* surveillance. However, we largely compensated this loss by studying and applying our approaches to another pathogen, *Corynebacterium diphtheriae*, on which significant work was done during this PhD (see abstract and below).

Overall, this PhD project resulted in 10 scientific publications and the development of two important bioinformatics tools (DIPHTOSCAN and LIN codes) that are already being used by the scientific community.

Deliverables

PhD reference	PhD Project deliverable number	Deliverable name *(Original name, if different from the actual one)*	Delivery date from AWPs (month)	Date delivered (month)	Comments	Integrative categories
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-2.1	Pilot Genome set	M24	M24		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-3.1	cgMLST schemes for *Kp* and *Ec*	M36	M36		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-3.2	LINcodes algorithm defined and implemented on full dataset	M30	M30		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-3.3	SNapperDB *Ec* implemented for full datase	M33	M33		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-3.4	SNapperDB *Kp* implemented for full dataset	M36		Not performed; We did not create SNapperDB for the pathogen *Kp*.	None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.1	Simulated dataset analysed	M42		Not performed; 7,000 public genomes were sufficient to constitute an adequate real genomic dataset.	None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.2	Publication on LINcode approach and comparison with cgMLST and SNP address approaches	M48	M48		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.3	A novel tool to define the population structure of species, called MSTclust		M40		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.4	Optimisation of our inheritance algorithm to provide backwards compatibility of MLSL groups with 7-gene MLST		M42		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.5	Comparison of the ANI metric with the cgMLST metric		M42		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.6	Designed a method to identify recombination in cgMLST		M40		None

PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-4.7	Integration of MLSL identifiers into the BIGSdb platform to make them publicly available		M42		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-5.1	Method implemented in partners' labs.	M54	M56		None
PhD16-FBZ2/AMR6.1-Codes4strains	D-PhD16-5.2	PhD viva	M57	M59	16th November 2022	None

Milestones

PhD reference	Milestone number	Milestone name	Delivery date from AWPs	Actual delivery date	Achieved	Comments
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-2.1	Pilot genome dataset defined	M24	M24	Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-3.1	cgMLST schemes defined for *Kp* and *Ec*			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-3.2	LINcodes algorithm defined			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-3.3	SNapperDB databases set-up for both pathogens			No	Was completed for *Ec*; but not for *Kp*
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.1	Simulations completed			No	Not performed; 7,000 public genomes were sufficient to constitute an adequate real genomic dataset.
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.2	Publication submitted			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.3	A novel tool to define the population structure of species, called MSTclust			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.4	Optimisation of our inheritance algorithm to provide backwards compatibility of MLSL groups with 7-gene MLST			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.5	Comparison of the ANI metric with the cgMLST metric			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.6	Designed a method to identify recombination in cgMLST			Yes	

PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-4.7	Integration of MLSL identifiers into the BIGSdb platform to make them publicly available			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-5.1	LINcodes method disseminated			Yes	
PhD16-FBZ2/AMR6.1 - Codes4strains	M-PhD16-5.2	Viva presented			Yes	

Publications and additional outputs

Publications

The Codes4strains PhD project has enshrined these works through a number of peer-reviewed publications, these are:

- Hennart M, Crestani C, Bridel S, Armatys N, Brémont S, Carmi Leroy A, Landier A, Passet V, Fonteneau L, Vaux S, Toubiana J, Badell E, Brisse S. (2023). A Global *Corynebacterium diphtheriae* genomic framework sheds light on current diphtheria re-emergence. *Microbiology Spectrum*, 11(3), doi: 10.1128/spectrum.00006-23.
- Museux K, Arcari G, Rodrigo G, Hennart M, Badell E, Toubiana J, Brisse S. (2023). *Corynebacterium* of the *diphtheriae* complex in companion animals: clinical and microbiological characterisation of 64 cases from France. *Microbiology Spectrum*, 11(3). doi: 10.1128/spectrum.00006-23.
- Tessier E, Hennart M, Badell E, Passet V, Toubiana J, Biron A, Gourinat AC, Merlet A, Colot J, Brisse S. (2022). Genomic epidemiology of *Corynebacterium diphtheriae* in New Caledonia. *Microbiology Spectrum*, 11(3). doi: 10.1128/spectrum.04616-22.
- Arcari G, Hennart M, Badell E, Brisse S. (2023). Multidrug-resistant toxigenic *Corynebacterium diphtheriae* sub-lineage 453 with two novel resistance genomic islands. *Microbial Genomics*, 9 (1). doi. 10.1099/mgen.0.000923.
- Hennart M, Guglielmini J, Bridel S, Maiden MCJ, Jolley KA, Criscuolo A, Brisse S. (2022). A dual barcoding approach to bacterial strain nomenclature: Genomic taxonomy of *Klebsiella pneumoniae* strains. *Molecular Biology and Evolution*, 39(7). doi: 10.1093/molbev/msac135.
- Guglielmini J, Hennart M, Badell E, Toubiana J, Criscuolo A, Brisse S. (2021). Genomic epidemiology and strain taxonomy of *Corynebacterium diphtheriae*. *Journal of Clinical Microbiology*, 59(12). doi. 10.1128/jcm.01581-21.
- Badell E, Alharazi A, Criscuolo A, Almoayed KAA, Lefrancq N, Bouchez V, Guglielmini J, Hennart M, Carmi-Leroy A, Zidane N, Pascal-Perrigault M, Lebreton M, Martini H, Salje H, Toubiana J, Dureab F, Dhabaan G, Brisse S; NCPHL diphtheria outbreak working group. (2021). Ongoing diphtheria outbreak in Yemen: a cross-sectional and genomic epidemiology study. *The Lancet Microbe*, 2(8). doi: 10.1016/S2666-5247(21)00094-X.
- Hennart M, Panunzi LG, Rodrigues C, Gaday Q, Baines SL, Barros-Pinkelnig M, Carmi-Leroy A, Dazas M, Wehenkel AM, Didelot X, Toubiana J, Badell E, Brisse S.

(2020). Population genomics and antimicrobial resistance in *Corynebacterium diphtheriae*. *Genome Medicine*, 12(107). doi: 10.1186/s13073-020-00805-7.

- Huynh BT, Passet V, Rakotondrasoa A, Diallo T, Kerleguer A, Hennart M, Lauzanne A, Herindrainy P, Seck A, Bercion R, Borand L, Pardos de la Gandara M, Delarocque-Astagneau E, Guillemot D, Vray M, Garin B, Collard JM, Rodrigues C, Brisse S. (2020). *Klebsiella pneumoniae* carriage in low-income countries: antimicrobial resistance, genomic diversity and risk factors. *Gut Microbes*, 11(5). doi: 10.1080/19490976.2020.1748257.
- Badell E, Hennart M, Rodrigues C, Passet V, Dazas M, Panunzi L, Bouchez V, Carmi-Leroy A, Toubiana J, Brisse S. (2020). *Corynebacterium rouxii* sp. nov., a novel member of the diphtheriae species complex. *Research in Microbiology*, 171(4). doi: 10.1016/j.resmic.2020.02.003.

Each of these publications have been uploaded to Zendo, which is gold standard open access, and can be found, here, here, here, here, here, here, here, here, here, and here, respectively.

Additional outputs (i.e., posters/oral presentations)

The Codes4strains PhD project disseminated these works through a range of oral and poster presentations, these were:

- "Journées Boris Ephrussi" 2022, May 05th-06th 2022, Paris, France.
- OHEJP ASM 2022, Poster, 3-minutes thesis and roundtable April 11th -13th 2022, Orvieto, Italy /online.
- "Journées Boris Ephrussi" 2021, Poster: A new approach for naming bacterial strains, combining cgMLST and LIN codes, codes, May 27th -28th 2021, online conference.
- OHEJP Annual Scientific Meeting 2021, poster presentation and 3-minutes thesis, 09th -11th June 2021, hybrid event, Copenhagen, Denmark and online.
- One Health EJP Annual Scientific Meeting 2020, Poster: A new approach for typing bacterial strains, based on the joint use of cgMLST and LIN codes, and its application to *Klebsiella pneumoniae* species and 3-minutes thesis, 27th - 29th May 2020, online.

Tools developed were uploaded and stored on Gitlab to ensure the code is open access and sustainable, these can be found here:

- https://gitlab.pasteur.fr/BEBP/diphtoscan
- https://gitlab.pasteur.fr/BEBP/inheritance-algorithm
- https://gitlab.pasteur.fr/BEBP/LINcoding

The *K. pneumoniae* isolates database and the sequence database with LIN codes:

- https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates
- https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=query&scheme_id=18&submit=1

Training Event	Topic
Communicate effectively in writing	Objectives - Applying the principles of effective communication to concrete cases adapted to the needs of doctoral students - Learning how to write a document, in particular an article, adapted to its recipients in order to be read and understood Practicing using the methodology specific to each document and professional writing style Contents - Characteristics of writing in communication and marketing (objectives, targets, messages) - How to select relevant information and give it meaning - How to structure your writing (adapted plans and specific methodologies) - Writing to be read (principles of readability and popularisation of information) Improving the presentation of your documents (rules of form)
Speed up the writing of your thesiS	Objectives - Understanding the different approaches to the thesis - Managing the organisation of the thesis - Structuring and writing the document clearly Contents - Organisation of research work (project management approach) - General approach Searching for the main theme
Establishing the basis for a personalised professional project	• General aspects of the job market, organisations, functions and positions • Panorama of the results of surveys on the professional future of Sorbonne PhDs • Notion of Personalized Professional Project • Notion of competences and reflexive posture
Open access: general aspects	• Development of arguments and editorial solutions for Open access • Presentation of institutional and disciplinary open archives • Learning to use the portal HAL Sorbonne University
Conduct your interviews and meetings efficiently	• Preparing and structuring interviews and meetings Anticipate and anticipate objections or questions • Learn how to manage the types of interlocutors • Improve verbal and gestural expression techniques • Build your argumentation and develop your strength of conviction and persuasion

	• Use the educational tools in the right situation • Practice dialogue, participation and facilitation skills Learn how to regulate and manage voltages face-to-face or in a group setting
Open science, by and for the benefit of researchers	• Definition of open science • Institutional context • The need for openness in science
Ethics of scientific research	3h Workshop, based on the Dilemma Game of the Erasmus University of Rotterdam. • A maximum of 5 groups of 5 people are trained at each workshop. Each group must answer a dozen questions on scientific integrity, chosen according to the origin of the participants. When the group does not agree on a response, it must discuss it to seek consensus. • Pooling of group results, using the same consensus-finding and discussion process where this is not possible.
Discover the main principles of management	• History of management • Management styles and team types • Management and teamwork • The skills of the manager in situation
Seminar BIBLIO@PHD	• The program is focused on points of view from diff-erent actors of scientific publishing and varies depending on the years. • The programs and slides of prior seminars are available online: https://paris-sorbonne.libguides.com/bibliodoctorat
Discover the principles of effective written and oral communication.	• Decrypt the act of communication • Principles of effective written communication • Principles of effective oral communication
Research ethics and scientific integrity	• Open to all students and the whole scientific community of Sorbonne University. • Each conference focuses on a specific theme and is given by an internationally recognized speaker. Speakers are selected from the recognized resources (US Office of Research integrity, French OFIS, EUA-CDE, LERU). The lectures are captured in video for live broadcast on the other campuses of Sorbonne

One Health impact

The project has defined, implemented and evaluated:

(i) a novel bioinformatics strategy to classify and name strains within pathogenic bacteria, from the level of deep subspecific lineages down to shallower levels of diversity that differentiate epidemiological related strains from non-related ones. The general applicability of the LIN codes approach means that in the future the classification and nomenclature of strains of other pathogens could benefit from the PhD project outcomes. We have discussed the approach with one member of the ECDC, Erik Alm (Principal Expert Applied Molecular Epidemiology at European Centre for Disease Prevention and Control), who expressed interest in a dissemination towards ECDC's molecular surveillance networks and EU national focal points. By facilitating the intercommunicability on bacterial strains across sectors and countries in the future, the project is highly relevant to multiple topics and objectives of the One Health perspective: antibiotic resistance clonal dissemination, emerging pathogens, host-jumps/species barrier crossing, cross-sector transmission, public health, and basic microbiology integration. This project outcome has far-reaching impacts on possibilities to integrate efforts of agencies (e.g., at the international levels, ECDC, EFSA, PulseNet international) to detect, monitor, understand and control the spread of pathogens.

(ii) Our tool to scan the genomes of diphtheria clinical isolates, including those from reservoir animals (pet cats and dogs mainly) for virulence, resistance and other characteristics will facilitate their molecular epidemiology and the understanding of transmission from animals to humans, or between humans in local, national or global scales. This tool is already in use by the ECDC Microbiology and Molecular Surveillance team (Daniel Palm and Andreas Hoefer, pers. Comm.).

(iii) We have also described a novel zoonotic pathogen of the *Corynebacterium diphtheriae* complex (*C. rouxii*). Isolates of this species are *tox*-negative and the distinction of this species from *C. diphtheriae* will improve risk assessment and diagnostic of diphtheria.

Through this project, the PI Sylvain Brisse was invited at One Health international conferences, including a keynote at the One Health EJP ASM in 2022, and a talk at the World One Health Congress in Singapore (November 2023). He was also invited to talk at the prestigious international KlebClub webinar series (May 10th, 2022) to talk about *Klebsiella* strain taxonomy. He thus interacted with other colleagues in the field.

We have also started collaborating on the diphtheria EU 2022 emergence with ECDC and other national reference laboratories and public health agencies on diphtheria; and S Brisse gave a talk on genomics of resistance in diphtheria at the Wellcome Trust conference Antimicrobial Resistance, Genomes, Big Data and Emerging Technologies (Nov. 2020).

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

The annual conferences organised by OHEJP allowed me to get in touch with other researchers, including other PhD students. They also allowed me to share the results of the project with the One Health community, which gave an additional dimension to my thesis, i.e. to give a greater visibility to the project.

The OHEJP doctoral program allowed me to network with other One Health partners, such as those involved in the MedVetKlebs project.

Specific outcomes to highlight in dissemination and communications

During the project, we first developed a taxonomy method for bacterial strains. The cgLIN tool is in open access and has been implemented in the BIGSdb (PubMLST and Pasteur) and pathogen watch platforms, the two leading bacterial genomic epidemiology platforms. This novel approach was praised by some at the specialised congress IMMEM in Bath, was the object of a communiqué by the Pasteur press office (https://www.pasteur.fr/en/research-journal/news/improving-bacterial-strain-classification-more-effective-surveillance) and is being implemented for other pathogens by international colleagues. We also developed a bioinformatics pipeline, DIPHTOSCAN, which enables the harmonisation, and facilitates, genomic epidemiology of the agents of diphtheria. This tool is available publicly and is described in a bioRxiv preprint, here. It is being used by the ECDC Microbiology and Molecular Surveillance team to investigate the European diphtheria re-emergence in 2022.

Our tools have been distributed publicly and some of them are in use. For example, a comment on a publication on multidrug resistant carbapenem-resistant *Klebsiella pneumoniae* ST23, related to an ECDC risk assessment (https://www.ecdc.europa.eu/en/publications-data/risk-assessment-emergence-hypervirulent-klebsiella-pneumoniae-eu-eea), pointed out the

utility of our LIN code classification system, and underlined that its use would have avoided confusion raised by these publications, which was due to ST23 being made of two phylogenetically very distinct sub lineages of *K. pneumoniae* (https://doi.org/10.1093/jac/dkad028). The diphtOscan tool was presented to, and is being used by the ECDC Microbiology and Molecular Surveillance team and other public health microbiology teams in national reference laboratories, to investigate the European diphtheria re-emergence in 2022 (https://www.ecdc.europa.eu/en/publications-data/increase-reported-diphtheria-cases-among-migrants-europe-due-corynebacterium).

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The Codes4strains PhD project has interacted with key OHEJP stakeholders and national relevant projects. These are outlined here:

1. The novel LIN codes method developed during the course of the PhD project will naturally disseminate via the existing networks of collaboration in which the main investigators are involved: *K. pneumoniae* networks include MedVetKlebs (just finished JRP), KlebNET (JPIAMR support), SpARK (JPIAMR support), kleb-GAP, Nor-Kleb-Net (see MedVetKlebs final report), and KlebNET-GSP (funded by BMGF); and *Corynebacterium diphtheriae* surveillance networks at national and international levels include the French NRC @Pasteur, the ECDC, and international contacts.
2. The novel LIN codes nomenclature system will be used for wider communication and backwards-compatibility in the broad genomic epidemiology arena. Stakeholders such as EFSA, ECDC, national public health agencies including USA CDC, or WHO might end-up hearing from, evaluating, and adopting this system, but this will also percolate naturally via the adoption of the approach by the epidemiological surveillance community for *Klebsiella* or other pathogens.
3. Future interactions with application developers and bacterial nomenclature platform administrators will be established to compare this approach with comparable approaches in the field, such as HierCC (Zhou *et al*., 2021; EnteroBase, Warwick, UK) or PopPunk (Lees et al., 2019; https://poppunk.net/); or commercial software such as SeqSphere+ (Ridom& cgmlst.org). Following our publication on the cgLIN codes concept and its implementation in *K. pneumoniae*, Martin M. C. Maiden and Keith A. Jolley were interested in applying the method on other pathogens of interest to the One Health perspective (e.g., Campylobacter) or Clinical/Antimicrobial resistance (e.g., N. gonorrhoeae).
4. The tool is now available on PubMLST platform at Oxford and can be easily used by other partners.

Evaluation of the Final Thesis Report

Not applicable as the PhD student submitted their thesis manuscript, which can be found here.

PhD17-WP7-SUSTAIN

Final Thesis Report

PhD Supervision Structure

Name of the PhD student and supervision team, with full affiliations

PhD Project PhD and supervision Team	Position	Affiliation	Country
Sarah Humboldt-Dachroeden	PhD Student	Roskilde University	Denmark
Olivier Rubin	PhD Lead Supervisor	Roskilde University	Denmark
Ann Lindberg	PhD Second Supervisor	Swedish National Veterinary Institute	Sweeden

Summary of the work carried out in the PhD project

The SUSTAIN PhD project began in M20, in 2019 and is expected to be complected by M66, in 2023. SUSTAIN focused on addressing disparities between institutions working on One Health topics involving quantitative studies and a survey. This dissertation reveals drivers and constraints of the implementation of the One Health approach by investigating international non-governmental organisations, European Union (EU) agencies and some EU countries, and non-EU countries including Norway, Switzerland, and the United Kingdom. Additionally, two country cases, Sweden, and Italy, provide practical examples by demonstrating agenda setting as well as knowledge translation processes, and the work carried out in government agencies and networks.

The dissertation has different methodological approaches, these are: 1) a bibliometric analysis highlights challenges of cross-sector collaboration among scientists through publishing patterns and co-citation networks; 2) further, a literature review indicates challenges for the environment sector and governance of the One Health approach; 3) expert interviews were conducted to analyse One Health-related coordination, collaboration, and communication activities via the Swedish and Italian cases; and 4) a survey study investigated the role of governance, agenda setting, and policymaking for the One Health approach.

Through the analyses of the individual papers and their synthesis, the dissertation arrives at three overarching conclusions. First, there are institutional barriers for One Health implementation. The main barriers that the dissertation investigated are silo working, agendas, and government agency set-ups. Government agencies can be fragmented and, to approach this, governments must determine clear criteria when establishing ministries and government agencies. To implement cross-sector One Health activities, the analysis points towards establishing institutional One Health strategies and incorporating specific problem definitions when designing One Health projects to tackle coordination issues. Second, there are knowledge translation challenges among scientists and between scientists and policymakers. These challenges must be addressed by leaders, problem brokers, and policy entrepreneurs. Scientific knowledge must be translated across sectors and to policymakers to create heterophil networks, use the knowledge that exists within networks, and to provide opportunities for actors from environment, social, and political science sectors to contribute to

the knowledge pool. Third, there is a lack of understanding of the One Health approach. Efforts must be made to comprehend the approach and what it means generally, for institutes and for specific projects. Continuous capacity building for scientists must be performed to strengthen the use and operationalisation of the One Health approach.

For the OHEJP, the PhD project reveals opportunities to support One Health-related policymaking through considering knowledge translation and societal challenges. It proposes ways to maintain cooperative structures (e.g., with policy entrepreneurs and problem brokers, honing leadership skills and strengthening heterophil networks). It presents drivers and constraints of One Health institutionalisation. The PhD project's impact for the OHEJP stakeholders include practical examples about possible applications of social and environmental scientists and a call to engage eastern European countries in One Health-related activities. Lastly, the impact for national ministries and institutes are lessons that can be learnt from the cases to strengthen future One Health approaches and activities. The studies highlight the need to establish scope, strategies, and harmonise data for international surveillance activities to prevent duplication and strengthen efficacy.

The objective of the PhD project was to investigate the drivers and constraints of One Health institutionalisation in Europe. The aim was to enhance the understanding of the challenges in relation to One Health institutionalisation and how to address them. The dissertation is located within the social sciences and is article-based, meaning that per Roskilde University's regulations at least three articles have to be produced. Of the three articles, at least two must be single authored (without co-authors). Six publications were produced in total, five scientific articles of which two are co-authored and three single-authored, and one co-authored chapter of an edited book.

Dissemination activities of this SUSTAIN PhD project were enshrined in a number of peer-reviewed publication, in journals such as the Journal of Public Health Policy, and a range of oral and poster presentations at the national (i.e., Grasp Festival 2021, Denmark) and international levels (i.e., One Health Sweden meeting 2022).

Work carried out on the PhD, scientific results, and outcomes

Chapter 1. Institutional silos, sectors, and disciplines

In the scientific context, the One Health approach is mostly known and used among veterinary scientists, and something of a monopole has been established (Paper I). On the other hand, the environmental sciences are not as engaged, both due to a lack of initiation and invitation. This two-sided challenge represents on the one hand scientists at the public health, veterinary, and food agencies, who find it difficult to engage with scientists from environment institutes due to lack of knowledge on who to involve, and not perceiving a need to connect. On the other hand, scientists at the environment institutes do not find thematic overlaps and, if common themes do exist, they lack information on who to contact (Papers I–III). However, environmental aspects and conditions have been described in the literature as important for tackling many health-related issues (Jones *et al*., 2008; Redford *et al*., 2021; Zinsstag *et al*., 2018). Environmental conditions can help to predict stress as well as risks, and the environment affects humans and animals through a variety of pathways. They can be directly related to beneficial or adverse effects for humans, animals, the ecosystem, and its

biodiversity (Paper III). This also includes plant health, which has received little attention in One Health projects and activities thus far (Andrivon *et al*., 2021).

The public health disciplines are frequently represented and engaged within One Health activities (Papers I–VI). This includes public health professionals but does not always expand to medical professionals working in hospitals (Paper VI). Relations among veterinary and public health institutes exist, and they are often good (Papers II & VI). Agriculture, food, and climate are often categorised under the public health, veterinary, and environment sectors.

In addition to the public health, veterinary, and environment sectors, there is another sector that directly and indirectly affects all other sectors and their disciplines: the social sciences. Within the One Health approach, a stronger social science presence could possibly provide tools and techniques to assess and appreciate contextual factors that aid in the creation of projects and activities (Papers I & V). The sector is crucial, as it can contribute to an understanding of societal contexts, economic, and behavioural aspects, and it can be used to understand policymaking processes, institutions, and networks (Papers I–V).

Including different perspectives and tools of the social sciences can benefit One Health activities and shine some light into the black box of One Health policymaking (Degeling *et al*., 2015; Lapinski *et al*., 2015; Michalon, 2020). For One Health policymaking, the social sciences can assist in mediating and brokering between scientists and politicians by providing insight into policy processes and political institutions (Papers IV–VI). Further, the social science sector can reveal contextual as well as behavioural aspects underlying to One Health issues. Contributing to clarifying contextual aspects is to define the One Health approach in the beginning – as a fundament – of projects or activities. Because how the approach is defined depends on the context of where the One Health approach is implemented (Papers II & V). Clarifying and defining the approach can prevent the word ‘One Health’ from merely becoming a trend or ‘buzzword’4F (Paper V). It can facilitate the implementation and institutionalisation of the One Health approach by establishing the frame, scope, context, and capabilities.

Governments and government agencies

On the local and national levels, the sectors on the human–animal–environment interface are usually represented by government agencies, such as public health, veterinary, food, and environment agencies; by universities; and by other research institutes. The government agencies are mandated by legislation which defines their agendas and priorities. Within a government agency, there is often a fruitful environment that allows for the exchange of information and performance of interdisciplinary activities (Paper II). Engaging in activities across agencies can, however, be a challenge. The clash of interests due to different agendas and priorities, data security issues, competition, or lack of resources can impede cross-sector collaboration and communication (Papers II–VI). This is why the sectors are often referred to as silos. The coordination of activities, collaboration, and communication are easier within a silo, as there are clear common objectives and themes. Bridging to another silo entails more complex coordination, as potentially distinct interests and objectives must be addressed. Obviously, connecting three or even more sectors presents an even more complex web of agendas, interests, and priorities, all of which must be accommodated. Nevertheless, it is important to have those sectors with their respective focus and perspective. They provide specific knowledge that can be and is used for political decision making (van Thiel *et al*., 2012).

However, a mindful division of the topics within the sectors together with a cautious establishment of additional sectors is crucial (Paper VI). Being mindful and cautious helps to identify connections and complementary knowledge that can be important for decision- and policymaking (van Thiel *et al*., 2012). The case study provides two examples of how two topics are categorised differently among government agencies: In Italy, the National Institute of Health deals with food safety, but the veterinary institutes also work with certain food safety issues. In Sweden, in contrast, food safety is handled by the National Food Agency, a stand-alone agency. Further, water-related issues are categorised within the Institute for Environmental Protection and Research in Italy, whereas matters related to drinking-water are dealt with by the National Food Agency in Sweden, the Environmental Protection Agency taking care of other water-related topics (Paper VI).

Different countries obviously have different arrangements of government structures and divisions of services under the ministries. Some European countries (e.g., Germany and Belgium) have federal systems where the central government does not hold all the power, instead sharing it with the regional authorities (Papers IV & V). Along with increased bureaucracy, this can lead to disconnected governing approaches of national, regional, and local authorities (Paper VI). The different government systems – as well as under which ministry the public services are mandated – can have implications for the resources available, the power or influence of the institute, and how they operate and respond (Azfar *et al*., 2004). For example, the research in Paper V points towards some well-working arrangements of government agencies to tackle AMR (e.g., DANMAP in Denmark). Paper IV however, presents remaining challenges that the fight against AMR still faces. The challenges might be due to the lack of perceived urgency, judging AMR as a creeping crisis (Engström, 2021; Munkholm *et al*., 2021). Additionally, Paper IV describes how intricate institutional contexts cause issues, where multiple ministries and government agencies work on different aspects of AMR on the national level. The institutions do not always align their work (e.g., through similar analytical approaches and data harmonisation to make data comparable), which adds to the information complexity that compromises cross-sector collaboration and communication. To prevent information complexity, national governments must consider interconnections when creating government agencies. For this, governments must establish criteria that account for the needs of those agencies to allow for transparency and manageability (Paper VI; van Thiel *et al*., 2012).

Geographical silos

In addition to the challenges that can arise with establishing agencies and the distribution of topics, there are geographic distinctions that can impact agency productivity. For example, the geographic proximity of agencies within a country can facilitate collaboration (Paper II). Across countries, it can be more difficult to maintain such collaboration, as effort and funding is required (Papers V & VI). For instance, there are few co-citations and One Health-related publications of authors from eastern European countries, indicating little interaction (Paper I). Western (and especially Nordic) countries seem more inclined to implement the One Health approach than eastern European countries (Paper V). There can be many reasons for this, and the process can change as the One Health approach gains popularity and more international research funds support projects with a One Health perspective (Paper I). The scarcity of eastern European countries within interdisciplinary research projects and the lack of data from those countries for international surveillance activities is a challenge for the One

Health approach, as it leads to a fragmented picture, making data comparisons, disease prevention, and tracing measures more difficult (Boqvist *et al*., 2018). The eastern European countries and the countries working together with them would benefit from collaborations in terms of cross-fertilisation, knowledge creation, and sharing. To establish a comprehensive One Health approach, these disparities and differences should be evaluated and approached within institutes as well as by decision- and policymakers (Papers IV–VI). Policy entrepreneurs and problem brokers are actors who can be employed to tackle this disconnect, as they can use their skills to create networks and establish connections (Paper VI). Education promoting the One Health approach throughout the working lives of scientists can contribute to the dismantling of geographical silos by emphasising the cross-sector and cross-country connectedness of One Health-related topics (Paper II).

Bridging silos during outbreaks

Working together across disciplines and sectors can be particularly effective during 'war times'; that is, periods when there is a disease outbreak (Paper II). During such war times, which can involve food-borne disease outbreaks like Salmonellosis or Campylobacteriosis (Paper II) or infectious diseases like SARS-CoV-2 (Papers II & VI), funds are more rapidly allocated to tackle outbreaks, and cross-sector coordination is facilitated. During one food-borne disease outbreak in Sweden, however, the National Food Agency and Public Health Agency clashed over different agendas and priorities: the National Food Agency with interest for the food industry and economy; the Public Health Agency with interest for public health (Papers II & VI). Such conflicting agendas and priorities must be considered when establishing One Health surveillance and response activities. At the same time, different orientations can also be complementary and result in strong, knowledge-sharing networks (Papers II & V).

Additionally, in Sweden and Italy, the tackling of disease outbreaks revealed different perspectives between the medical, veterinary, and public health sectors; where the medical perspective usually focuses on the individual, the veterinary and public health perspectives can include both individual and population-wide perspectives. These differing perspectives can become a point of contention regarding the respective importance among practitioners and scientists (Paper VI). During the SARS-CoV-2 pandemic, Swedish and Italian scientists highlighted the value of both perspectives. In these two countries, the veterinary agencies have demonstrated their significant contributions to tackling the pandemic. Public health laboratories and hospitals usually have less capacity, processing smaller numbers of samples. Conversely, veterinary laboratories are used to test multitudes of samples, applying a herd or population approach (Papers II & VI). In addition to the laboratory capacity, combining the expertise of public health and veterinary scientists on SARS viruses, which is a subject studied in both fields, has contributed to improving practices regarding diagnosis, therapies, and the treatment of SARS-CoV-2-related maladies (Paper VI; Fenollar *et al*., 2021). The environment institutes also contributed to investigating the spread of SARS-CoV-2; in Italy, for example, studies were conducted to determine the presence of the virus in sewage (Paper III; La Rosa *et al*., 2021). Hence, all sectors related to the human–animal–environment interface were utilised in some manner to contain the spread of SARS-CoV-2 or to inform about it. A One Health approach was institutionalised as the countries invested effort in developing cross-sector activities and processes – whether intentionally or unintentionally One Health. The urgency of the outbreak facilitated the allocation of funds that were helpful to link sectors, work together, and share knowledge. The Italian case, however, displayed some persisting

difficulties across sectors (Paper VI). The veterinary agencies responsible for analysing the swabs for SARS-CoV-2 experienced challenges when working with medical doctors in hospitals. The main claim that was voiced concerned the lack of knowledge of medical doctors about the work and expertise of veterinarians. Unfortunately, within the PhD project, medical doctors working in hospitals were not interviewed and could therefore not provide their perspective, which would have been worthwhile. The reason for the difficulties among veterinarians and medical doctors might be rooted in different values and hierarchical thinking, which should be addressed to enable a levelled and fruitful environment for future collaboration (Huth *et al*., 2019). This can be approached via training and the education of scientists, explaining the meaning and actors involved in tackling multifaceted health issues (Papers II & VI).

The Swedish and Italian cases provide examples of the lessons learned in terms of how veterinary, public health, food (and sometimes environment) institutes communicate, collaborate, and coordinate One Health-related activities (Papers II & VI). Concretely, lessons can be learned about cross-sector meetings concerning outbreaks, One Health networks, and collaborative approaches to tackle disease outbreaks (e.g., Salmonellosis, Campylobacteriosis, and SARS-CoV-2; Papers II & VI).

Chapter 2. Learning new languages

Language and the translation of knowledge plays a tremendous role for the One Health approach, and it can address different actors and sectors (Papers II, IV, V, & VI). In this context, knowledge translation can contribute to the learning of new languages, 'languages' in the sense of different terminologies and the specialist jargon with which research, operations, methods, analytical approaches, and other specific issues are communicated. This provides opportunity for different actors to get to know and contribute to the One Health approach. Some actors were identified within the papers and will be presented in three different dimensions that depict knowledge translation among scientists, between scientists and policymakers, and between scientists and the public. Each of these dimensions provides potential ways to overcome translation issues, which can have practical implications for implementing the One Health approach.

Knowledge translation among scientists

The first-dimension addresses knowledge translation among scientists within different disciplines and sectors. Communication, data sharing, and understanding one another are key aspects of knowledge translation. The public health, veterinary, food, and environment sectors all have their own terminologies, methods, and analytical approaches, and while they overlap, they do not always align (Papers II, IV, & V; Mateus *et al*., 2022). Different technologies and analytical methods can produce results that are difficult and sometimes impossible to compare (Paper II). This provides challenges, both on national and international levels, when working together on cross-sector health issues, research projects, and when carrying out disease surveillance activities. Such difficulties can be in the form of fundamental communication-related issues when scientists talk with one another about the same issue from their respective sector-related perspectives, the difficulties being due to different terminologies. Methods and analytical approaches used in different sectors also reflect their own language, because using different methods and analytical approaches for the same issue (e.g., Salmonellosis

surveillance) can lead to different interpretations and difficulties when comparing findings. Hence, harmonising data can at least to some extent facilitate the comparison of data, which is crucial when investigating health threats that are able to cross borders (Papers II–IV). It can also enhance the efficient use of data, as similar samples or data are often gathered across the different sectors. Streamlining and communicating data can prevent the unnecessary duplication of efforts (Paper VI). Establishing cross-sector networks supported by strong and open-minded leadership can save resources by facilitating the exchange and brokering of knowledge across sectors (Papers IV & VI). Tools such as glossaries should be shared within networks, and they can help to clarify the terms and terminologies used in other sectors (Papers II & V).

A One Health strategy on an institutional level that problematises and defines each specific One Health topic will further help to clarify roles, responsibilities, and the expectations held to one another (Papers I, V, & VI). This can especially have positive implications for the environment sector as their role and potential contributions become clear, which could motivate future collaborations (Papers II, V, & VI). Further, capacity building in terms of scientist training and education can facilitate the learning of new languages and jargon, by promoting the understanding of One Health surveillance techniques and more generally by specifying the One Health approach in practice. Capacity building constitutes an important aspect of the One Health approach, as reflected in the high number of One Health networks reporting on it (Khan *et al*., 2018). Capacity building refers to individual development in terms of equipping scientists with skills and access to information, knowledge, and training. Ideally, this will lead to personal development and enable performance as well as adaption to new knowledge and contexts (Boyko *et al*., 2012). Paper V identifies the influencing factor 'lack of context' that typically hampers the knowledge translation process. Engagement with social, political, and economic scientists will prove fruitful, as their contributions can address this issue by using tools and expertise to unravel contextual and cultural aspects. While natural scientists might be somewhat accustomed to the One Health approach, this is a novel notion for many social scientists (Papers I & VI). Building bridges across the sectors and tackling homophily will facilitate knowledge sharing and learning. This can contribute to an enhanced understanding of the world as an interconnected web while acknowledging local, societal, and political realities (Papers I & VI; Craddock & Hinchliffe, 2015; Lapinski *et al*., 2015).

Knowledge translation between scientists and policymakers

The second-dimension addresses issues of knowledge translation among scientists and policymakers (e.g., bureaucrats, politicians). 'Lack of common language among scientists and policymakers' is an influencing factor interfering with the knowledge translation process (Paper V). Scientists who want to share their knowledge (scientific findings) are often faced with difficulties in their interactions with policymakers (Mateus *et al*., 2022). Or even one step before this: When seeking to catch their attention (Paper V). Policymakers often lack a background in the natural sciences and, in the same way, scientists do not necessarily have any training in the dissemination of science to non-scientists. The inability to break down complex scientific information and to create a 'compelling narrative'7F can lead to misunderstandings and render it impossible to convey the importance of a specific issue (Paper V). The fact that One Health issues often span several disciplines and sectors contributes to the difficulty of expressing an issue coherently and making it readily understandable. Education and training in research communication can better equip scientists

with the tools necessary to disseminate their research findings. Confident and eloquent expressions when sharing scientific findings within policy communities can help scientists to advocate and lobby for their topic and increase the likelihood of catching political attention (Papers IV & V). By doing so, they can become policy entrepreneurs, using their scientific expertise and storytelling competencies to translate knowledge (Paper II; Stone, 2019). Problem brokers can also be research communicators and help to frame scientific findings comprehensibly, as they are able to understand and speak the scientific and political languages (Rushmer *et al*., 2019).

Considering the One Health approach generally, many problems are already clearly outlined, and solutions have been proposed, such as conceptual frameworks, guides, or references to support One Health research, policies and implementation (e.g., Coker *et al*., 2011; FAO *et al*., 2008; Lebov *et al*., 2017; Rüegg *et al*., 2018; Papers I, II, IV, & V). However, political awareness is scarce and policy communities often struggle to generate interest (Connolly, 2017; Papers II & V). This indicates areas where policy entrepreneurs and problem brokers can be employed to facilitate the contact between scientific and bureaucratic knowledge and to communicate successfully with politicians (Papers II & IV). Here, policy entrepreneurs who employ the networker strategy and know how to exploit networks can use their connections to inform policymakers of problems (Stone, 2019). The problems that require attention fuel the problem stream; and when the policy and politics windows also align, it can stimulate innovation in terms of methodologies, technologies, and analytical approaches (Kingdon, 2014; Rogers, 2003). Capturing political attention can have implications for scientists working with the One Health approach, potentially resulting in policy change and increased funding for One Health-related research. Increased awareness of the One Health approach and the issues it addresses can entail a more widespread use of the One Health term in strategic reports (Paper I); for example, the European Commission adopted the 'European One Health Action Plan against Antimicrobial Resistance' (European Commission, 2017). Mentioning One Health in the title and body of the document sends a clear signal to the EU member states, demonstrating the EU's initiative and encouraging the use of the One Health approach.

Paper V has presented processes that can lead to policy change, including the roles and abilities of different actors. International actors such as those coming from EU agencies (e.g., ECDC, EFSA and the European Medicines Agency) were perceived as important to pushing One Health policies forward, and they can also act as policy entrepreneurs (Papers IV & V). Interestingly, based on the lack of co-citation networks of authors, there does not seem to be much scientific One Health-related exchange among these agencies (Paper I). The EFSA and ECDC have a memorandum of understanding to cooperate on common issues, explicitly mentioning the One Health approach (EFSA & ECDC, 2021). The bibliometric analysis indicates how greater effort should be invested in using each other's knowledge via co-authoring collaborations or by referencing one another (Paper I). Such increased mutual cross-fertilisation can influence policies relating to One Health issues, as they in fact advise and hence indirectly influence decisions made by the European Commission (Chatzopoulou, 2018; Wood, 2018). Establishing transdisciplinary One Health Research and Innovation governance, as suggested by Bronzwaer *et al*. (2022), could possibly aid the translation of knowledge between the agencies and set a research agenda that facilitates cross-sector collaboration. The Quadripartite were also perceived as important actors, especially due to the potentials lying in the organisations' combined forces and because of their prestige (Paper V). Building on this, the Quadripartite should identify policy entrepreneurs and problem brokers

within their organisations who utilise their storytelling ability to translate knowledge, creating powerful and compelling narratives to inform policymakers (Stone, 2019). Above all, national agencies were recognised to be the most important actors to push One Health policies forward, which is plausible as the government agencies (evidence producers) are indirectly involved in government policymaking (evidence users; Papers IV & V). Figure 12 summarises the potential actions that can be taken to foster knowledge translation among scientists and policymakers, as well as the implications this might have for the One Health approach.

Knowledge translation between scientists and the public

Lastly, the third dimension examines knowledge translation between scientists and the public. The public impacts and is affected by their environment, their own health, as well as the health of animals and ecosystems. The characteristics of different communities – of how society is built and governed – are crucial for preventing, mitigating, and combating health threats. This has been emphasised in the definition of the One Health approach by the One Health High-Level Expert Panel (WHO, 2022a). However, there is often a disconnect between scientists and those communities and societies. The determinants of health address some of these connections, focusing especially on the people, societies, as well as the communities and the environment in which they live (Barton & Grant, 2006). The Determinants of Health Model lacks a specific ecosystem perspective, including animals. Yet the model provides a valuable knowledge base for the One Health approach in relation to the interconnectedness of individuals, communities, and their socioeconomic, cultural, and environmental conditions (Barton & Grant, 2006).

To disseminate knowledge, researchers mostly publish their findings in scientific journals that rarely reach the public. However, the communication to the public can be important to move from theory to practise (Senabre Hidalgo *et al*., 2021). It can activate the politics stream, in which the public mood is a crucial element for setting agendas (Kingdon, 2014). Disseminating scientific findings to the public can make them aware of an issue, stimulate discussion, and can pressure policymakers (Paper II). Similar to communication issues among scientists and policymakers, breaking down complex One Health topics remains an obstacle in communications with the public (Mateus *et al*., 2022). Training in research communication or employing research communicators and problem brokers can facilitate the translation of knowledge and popularise science. Scientists can engage with the public via their research projects, engaging, welcoming, and encouraging the public to take part if the project allows. Public participation, such as citizen science or the co-creation of science, can intrigue the public, make them more aware of the One Health approach, and reveal the societal and contextual determinants of health (Paper IV; Senabre Hidalgo *et al*., 2021). Including the public in One Health-related research can lead to more local One Health activities and projects (Paper VI). If public participation is not possible, scientists can engage with the public in other ways, such as research fairs and festivals, social media, TV news, radio, and newspaper articles (Ross-Hellauer *et al*., 2020). A broad audience can be reached in this manner, enhancing knowledge about specific One Health topics and the whole approach in general (Paper IV). An enhanced understanding of the One Health approach among the general public can trigger discussions and initiate action on the political level (Papers II & VI; Haxton *et al*., 2015).

The One Health approach can also be brought to the public by implementing it in school education. This can give children the chance to become familiar with the approach already from a young age, which can enhance their knowledge of One Health, possibly even shaping their future education decisions (Paper II). A study of sustainability education showed that children who learn about topics relating to sustainability in school have an impact on their parents and their consumer habits (Walker, 2017). Knowledge translation between scientists and the public can have positive implications in terms of generally raising knowledge about specific One Health topics relevant for a specific area or context and by shaping public opinion and attracting political attention to those issues.

Chapter 3. Managing One Health

Individuals and groups of people who inspire and lead are important drivers for the One Health approach. They must have the ability to steer through complex national and international systems, being conscious of needs, interests, priorities of states, governments, institutes, and organisations (Stephen & Stemshorn, 2016). This chapter scrutinises the opportunities available to leadership and leaders to drive the One Health approach forward. It examines the role and importance of relationships, including the nature of close relationships, their opportunities, and pitfalls for the One Health approach, and the potential to form relationships with new, previously unknown connections.

The role of leadership

In the context of this dissertation, leaders are individuals who lead scientific projects and networks. They can be policy entrepreneurs and problem brokers. However, policy entrepreneurs, problem brokers, and leaders are not interchangeable terms. Problem brokers have limited functions with respect to agenda setting. They usually operate in the preceding steps, framing a problem and making an issue understandable (Knaggård, 2015). Similarly, policy entrepreneurs work towards framing a problem and go further to suggest policy changes. Some of the skills of leaders go even further than that, as they possess more resources to make policy innovations (Capano & Galanti, 2021).

To establish and maintain collaborations within networks and scientific projects, there is a need for leaders with decisionmaking skills who guide, enable innovations, and are trustworthy (Papers IV–VI; Rogers, 2003; Stokols *et al*., 2008). A leader must be able to problematise and define specific One Health issues to establish clear goals and objectives (Paper V). In the following, problematising specific One Health issues and the opportunities they entail will be described using the AMR example. Paper III outlined how Denmark demonstrated collaborative processes that problematise AMR. The DANMAP report includes information, data, and knowledge from different sectors and has explicitly mentioned how implementing cross-sector monitoring and surveillance and engaging different sectors is based on motivated leadership (Papers III & V). However, while One Health approaches such as the Danish approach to AMR surveillance provide a good example, they cannot always be easily translated to other contexts, such as to countries or regions like the EU (Papers IV & V). Projects, programmes, activities, interventions, and measures that work on a local level might not be translatable to other local or global contexts – and vice versa. Activities must be adapted to specific circumstances, preferably already in the planning stages (Papers IV–VI). For example, implementing AMR stewardship programmes must consider the many actors who

are affected and involved who have different aims and priorities. Additionally, microbes can easily cross state borders, meaning that AMR should not only be treated within countries but also – and ideally – in partnerships, collaborations, and networks with other countries (Paper IV). Apart from national actors, there are also multiple EU agencies and NGOs working with different aspects of AMR. Hence, leaders should facilitate among sectors and create inclusive networks to connect and create One Health activities that include all relevant actors (Paper V). The knowledge produced within such networks must be exploited to foster cooperation and communication (Papers IV & V). The aims and priorities of different actors must be accounted for and addressed to avoid miscommunication and shortcomings regarding mitigation and preventing the spread of diseases (Papers II & IV). Some international approaches, like the Joint Programming Initiative on Antimicrobial Resistance, which is a global funding coordinator, have successfully provided resources to collaborate on tackling AMR (Papers IV & V). Interdisciplinary projects like this facilitate cross-sector collaboration. Such projects can foster an understanding (and appreciation) of the counterpart's work (Papers II & VI).

However, combating AMR on an international level remains a challenge (Paper IV). This might be due to challenges in associating knowledge, which means conveying scientific information to the policymakers they find plausible and relatable (Papers IV & V). To tackle such challenges, engaging experts from the social, political, and economic sciences can aid in associating knowledge by using their methodological and analytical tools to assess circumstances and contexts. They can provide knowledge on contexts and policy processes from the local to the global levels, which can complement knowledge from the medical and natural sciences (Paper IV). Hence, engaging social, political, and economic experts can potentially induce innovation, as it allows for tailored, sustainable approaches (Papers IV–VI). Here, leaders must connect experts from the social, political, and natural sciences within networks to enable knowledge sharing. Based on this knowledge, leaders can then problematise issues and broker between sectors to establish relationships among the scientific and political sectors and to convey the scientific information comprehensibly to policymakers (Papers V & VI; Rogers, 2003; Tasselli *et al*., 2015). This is the setting for problem brokers and policy entrepreneurs, who can use their skills to promote, convince, and even persuade policy- and decisionmakers (Papers II, IV, & VI). The focus should also be on using already existing stewardship programmes and frameworks for implementing as well as evaluating AMR (and other One Health) activities and learning from studies that describe specific country cases (e.g., Jani *et al*., 2021; Lebov *et al*., 2017; Paternoster *et al*., 2017; Rüegg *et al*., 2018) in terms of their experiences with implementing the One Health approach (Papers I, II, & IV).

Close and distant relationships

Relationships, personal relationships in particular, are essential for the One Health approach, as they facilitate communication and make collaboration easier (Papers V & VI). Relationships promote the implementation and effectiveness of activities, as shown by the collaborations among Swedish agencies on One Health-related topics as well as the collaboration of Danish institutes and industry on AMR (Papers II & III). Here, trust is necessary to carry out One Health activities and seems to ensure reliability subjectively (Papers IV & V). However, relationships are often confined to the borders of ones one epistemic culture and work environment. There are usually strong ties among actors, as they invest time and emotion in

establishing the relationship (Rogers, 2003). This can lead to homophily and entail the exclusion of relevant actors and perspectives (Papers I & VI). Homophily describes the phenomenon whereby similar individuals (e.g., in terms of age, gender, work topic, education, values, believes, etc.) group together (McPherson *et al*., 2001). To combat homophily, establishing a One Health strategy within institutes that define the scope of activities and projects can help to determine relevant perspectives and actors who must come together (Paper VI). Leaders should clarify objectives as well as scope, ensuring the mapping of stakeholders in the design stage of the One Health activity (Paper VI; Bordier *et al*., 2021; Mazet *et al*., 2014). In so doing, leaders can facilitate cross-sector connections and promote heterophily. This would encourage actors to engage with individuals with whom they have no prior relations (creating weak ties), which can facilitate new connections, information sharing, and innovation (Paper VI; Rogers, 2003). Papers II and VI highlighted how seeking weak ties requires the availability and knowledge of where to find contact information. Institutions (e.g., government agencies, research institutes) should provide easy access to contact information. This will help to address collaboration shortcomings and avoid the exclusion of relevant actors, such as the oft-neglected actors from the environment sector (Papers I–III). It will also facilitate the inclusion of actors from the social and political sectors (Papers I, IV, V, & VI). Another possibility that can strengthen the collaboration of stakeholders from different sectors is to establish environments that facilitate knowledge sharing, such as interdisciplinary conferences, meetings, workshops, or summer schools (Papers I, II, & VI). Policy entrepreneurs and the problem brokers who are in such network environments can use or expand the networks to go from knowledge sharing to translation. The intermediaries can be used as brokers to connect sectors and actors. Additionally, there is already existing literature, guidance, and advice on establishing comprehensive One Health projects and activities as well as cross-sector and interdisciplinary collaborations, which can be used and adapted (Papers I & IV).

PhD self-assessment

Methods

Initially, an observation study was planned for 2020 that was supposed to be conducted at OHEJP stakeholder meetings. This methodological approach was abandoned due to SARS-CoV-2 lockdowns that hampered access to meetings.

The interview study was kept as part of the research design. Instead of doing them face-to-face, they were conducted both, online and face-to-face. This was due to travel restrictions. Conducting the interviews online did not compromise the content or the extent of discussions. The pandemic even opened up possibilities of adding questions about SARS-CoV-2 in relation to the One Health approach.

Fieldwork

Two research stays were planned: a three-month stay in Sweden at the National Veterinary Institute and a three-month stay in Italy at the Public Health Institute. The Swedish research stay was reduced to a one-week stay in autumn 2019, planned as an introductory meeting. While only a small fraction of the originally intended time, it nonetheless provided some useful contacts and insights into the institute structures. However, the full three-month research stay in Sweden would likely have provided opportunities that might have led to different and

possibly deeper impressions, collaborations, and a better understanding of the government agency itself. In Italy, a one-month research stay was accomplished at the Istituto Superiore di Sanità in autumn 2020. This period was productive in terms of conducting interviews and networking. However, strict SARS-CoV-2 regulations rendered it impossible to participate in ongoing meetings or other activities that might have provided in-depth insight into the institute processes and structures. One more month of fieldwork was conducted in Italy in autumn 2021, at which time I visited experts at regional veterinary institutes. While this deviated from the originally planned three-month research stay, the changes provided plenty of opportunity to network and conduct fieldwork. All in all, more interviews (both in Sweden and Italy) were conducted than initially planned, which demonstrates the flexibility of conducting online interviews.

Progress of the project: milestones and deliverables

The SUSTAIN PhD project did not use the standard method of milestones and deliverables to ensure the successful completion of the research project.

Publications and additional outputs

Publications

The SUSTAIN PhD project has enshrined these works through peer-reviewed publications, these are:

- Humboldt-Dachroeden, S. (2023). Translating One Health knowledge across different institutional and political contexts in Europe. *One Health Outlook.* 5, 1. doi: 10.1186/s42522-022-00074-x
- Humboldt-Dachroeden, S. (2022). A governance and coordination perspective – Sweden's and Italy's approaches to implementing One Health. *SSM – Qualitative Research in Health.* 2, 100198. doi: 10.1016/j.ssmqr.2022.100198
- Munkholm, L., Rubin, O., Bækkeskov, E., & Humboldt-Dachroeden, S. (2021). Attention to the Tripartite's one health measures in national action plans on antimicrobial resistance. *Journal of public health policy.* 42(2), 236–248. doi: 10.1057/s41271-021-00277-y
- Humboldt-Dachroeden, S. (2021). 12-Month PhD Report SUSTAIN. doi: 10.5281/zenodo.6364821
- Humboldt-Dachroeden, S. (2021). One Health practices across key agencies in Sweden – Uncovering barriers to cooperation, communication, and coordination. *Scandinavian Journal of Public Health,* pp 1-7. doi: 10.1177/14034948211024483
- Humboldt-Dachroeden, S., Mantovani, A. (2021). Assessing Environmental Factors within the One Health Approach. *Medicina.* 57(3), 240. doi: 10.3390/medicina57030240
- Humboldt-Dachroeden, S., Olivier, R., Frid-Nielsen, SS. (2020). The state of One Health research across disciplines and sectors – a bibliometric analysis. *One Health,* 10, 100146. doi: 10.1016/j.onehlt.2020.100146

Each of these publications have been uploaded to Zendo, which is gold standard open access, and can be found here, here, here, here, here, and here, respectively.

Additional outputs (i.e., poster/oral presentations)

The SUSTAIN PhD project disseminated these works through oral and poster presentations at the following events:

- "One Health knowledge translation among European scientists and policy actors" *Poster presentation* at ONE – Health, Environment, Society Conference 2022, Brussels, Belgium. 21-24th June 2022. DOI: https://doi.org/10.5281/zenodo.7611420
- "Knowledge translation challenges of the One Health approach in Europe" *Poster presentation* at One Health EJP Annual Scientific Meeting 2022, Orvieto, Italy. 11-13th April 2022. DOI: https://doi.org/10.5281/zenodo.7611595
- *Oral presentation* at the One Health Sweden meeting 2022, Uppsala, Sweden. 30th March 2022.
- *Oral presentation* at the Grasp Festival 2021, Panel 1: Leadership, collaborative governance and public-private partnerships, Roskilde, Denmark. 18th November 2021
- *Oral presentation* at the One Health EJP Annual Scientific Meeting 2020, Copenhagen, Denmark. 10th June 2021.
- Humboldt-Dachroeden, S., Rubin, O., & Frid-Nielsen, S. S. (2020). "The state of One Health research across disciplines and sectors –a bibliometric analysis" *Poster presentation* at One Health EJP Annual Scientific Meeting 2020, online. 27-29th May 2020. DOI: https://zenodo.org/record/7611129

Name of Training Event	Topic	Dates (DD/MM/YY)	Organising Institute
Workshop 1 & 2 Write an article for publication	Journal and editors; Structures of articles; how to write a good article	26.10.2021	Roskilde University
Quantitative research methods workshop	Sectional simple and multivariate regressions	16.09.2021	Roskilde University
Workshop on Research Communication	Use the journalistic toolbox; Find the good story in your research; Write to get read: Dissemination strategies	09.09.2021	Roskilde University
One Health EJP Summer School 2021 Global One Health - Environmental Issues in One Health (2 weeks)	From risk assessment to surveillance	06.08.2021	OHEJP
One Health European Joint Programme Cogwheel Workshop	Finding synergies and opportunities for knowledge transfer and/or collaboration.	25.11.2020	OHEJP
Research data management and the FAIR principles in Open Science	Storage of data; Metadata; data management plan; FAIR principles	05.11.2020	Roskilde University
One Health EJP Summer School 2020 Global One Health (2 weeks)	From Research to Practice	28.08.2020	OHEJP
Qualitative Research Interviews	Qualitative Research Interviews	12.06.2020	Roskilde University
Qualitative Research Methods	Interviews, observation, document analysis;	29.05.2020	Roskilde University

	surveys, cases, focus groups		
Research Design Course	Research design development, research questions	13.05.2020	Roskilde University
NVivo Beginners & Advanced	NVivo theory and practical training	7. & 10.01.2020	Roskilde University
Systematic literature search	Systematic literature search and analysis approaches	09.11.2019	Roskilde University
Research Ethics and Integrity	Ethics and Integrity	04.11.2019	Roskilde University

One Health impact

The PhD project has a unique angle on One Health. It investigates the implementation and institutionalisation of the One Health approach within public health, veterinary, food, and environment institutes. The results of this research can be used by regional or national authorities and stakeholders of the OHEJP to evaluate and consider their coordination and collaboration efforts. It produces useful knowledge and examples for institutes on challenges for collaboration and how to overcome those. It provides practical guidance on who to engage with and how, e.g., via establishing institutional One Health strategies. For international stakeholders, it can provide useful information about improvements of existing /planned surveillance programmes. This includes discussions about existing surveillance activities and specific aspects that must be improved, especially for antimicrobial resistance surveillance. Further contributions are discussions about strengthening cross-sector collaboration in terms of communication and harmonising data to prevent duplication as well as strengthen efficacy of disease surveillance programmes and collaborations. The impact for the stakeholders of the OHEJP also include practical examples about possible applications of social and environmental scientists and a call to engage eastern European countries in One Health-related activities.

On a political level, it will provide insight into sharing and translating knowledge between scientists and politicians. The PhD project reveals opportunities to support One Health-related policymaking through considering knowledge translation and societal challenges. It proposes ways to maintain cooperative structures (e.g., with policy entrepreneurs and problem brokers, honing leadership skills and strengthening heterophil networks).

The OEHJP has supported PhD project not only with facilitating contacts, but especially with its exceptional network. This has led to partner with the Swedish National Veterinary Institute and the Italian National Institute of Health. The collaborations included research and fieldwork stays at the institutes, where I conducted formal and informal interviews with experts and got to know the structures and procedures of the institutes. Additionally, it resulted in a co-authored scientific article (with Alberto Mantovani) in relation to environmental considerations within the One Health approach via the two cases of antimicrobial resistance and mycotoxins. Through the research stay at the Swedish National Veterinary Institute, I was introduced to

the NOVA project. I engaged in the project through supporting the development of the interview guide, interviewing Swedish and Norwegian experts and analysing the qualitative data. This has provided invaluable experiences that went beyond the topic of the PhD project and helped to better understand the One Health approach and all its components and actors.

Added value and benefits during the PhD resulting from being part of the OHEJP doctoral training programme and consortium

Being part of a large, EU-funded Horizon 2020 project provided invaluable access to stakeholders from different countries, sectors, and disciplines. Further, the OHEJP provided contacts for the interview as well as survey study and shared the survey invitations through their internal email lists. The Horizon 2020 project offered many opportunities for courses and networking, but also entailed an extensive amount of reporting. While extensive and time-consuming, these reporting procedures were a valuable contribution for the PhD project, as they taught among other things techniques to disseminate research findings. This was done continuously through conferences and engagement via social networking sites (e.g., LinkedIn, Twitter).

Further, the Annual Scientific Meetings provided opportunity to disseminate findings and learn about another ongoing research. Especially the in-person conferences led to fruitful discussions and provided opportunity to engage in conference organisation, where I facilitated a One Health quiz (OHEJP Annual Scientific Meeting, 2021).

Interactions with JRPs/JIPs or with external (global, EU, national or regional) relevant projects or initiatives and/or interactions with OHEJP stakeholders

The SUSTAIN PhD project interacted with key OHEJP stakeholders and national relevant projects. These were:

- Invited speaker at 11th One Health Sweden Scientific Meeting 2022: Pandemics and preparedness the 29-30 March. Presentation titled: ‘Importance of connecting early and senior project collaborators under the One Health umbrella’.
- JRP NOVA ‘Surveillance barriers and opportunities as perceived by veterinary practitioners’: I was involved in conducting an exploratory study on barriers and opportunities in food-borne disease surveillance from a One Health approach within four EU countries. I carried out online semi-structured interviews of veterinary practitioners (from Sweden and Norway) and analysed the interviews. This was led by Maria-Eleni Filippitzi.

CRITERION	PROJECT	
	Reviewer 1 (External Scientific Reviewer)	Reviewer 2 (External Scientific Reviewer)
Did the PhD student recommend future research from the conclusions of the project?	4	4
Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?	3	4
Were all the milestones and deliverables completed?	4	1
Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?	4	3
Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?	4	3
Did the PhD student actively engage in Education and Training activities?	4	4
Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?	4	5
Was the PhD managed and implemented in accordance with the DMP?	4	5
Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?	5	5
How relevant are the finalised project outputs to the One Health EJP aims and objectives?	5	4
Is there any direct or indirect impact of the project for national or international stakeholders?	5	4
Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?	4	4
Does the project outcomes have policy implications?	5	4
TOTAL	55/65	50/65

AVERAGE: 52.5/65

NB. The PMT member who reviewed the SUSTAIN PhD project provided a global overview of the PhD Final Thesis Report, outlined below:

The scores given by the reviewers are high, except for one score about the progress of the project (reviewer 2 scored 1), which is properly explained by the PhD student/supervisors. One of the main comments is from reviewer 1 and is about the single authorship of the majority of the papers. A clear response is provided by the PhD student/supervisors: it is a requirement of the Roskilde Univ. for social sciences. The remaining scores and comments are adequate

and fair and the changes in the report properly address the comments. In my opinion, this deliverable can be finalised.

Did the PhD student recommend future research from the conclusions of the project?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Were the PhD project objectives met as detailed in the original proposal or was a good justification provided for any deviation from the original proposal?

Reviewer 1: External Scientific reviewer

I think it is difficult to know if conducting online interviews compromised the content. This is not discussed as such, just concluded it did not. I think what surprises me is that the majority of papers have only got one author and that is the PhD-student. How did she get input to the papers? Especially in this field of One Health transdisciplinary is needed and much talked about from the authors side and then it is very surprising to find sole authorship. I think especially this huge topic of One Health would have benefited of (lots!) co-authors from different fields which could add different perspectives. I think one could also have discussed more about the methodologies that were used in the papers, for instance the "translational" process of the interviews- did this truly capture the essence?

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Were all the milestones and deliverables completed?

Reviewer 1: External Scientific reviewer

The SARS-CoV-2 pandemic obviously impacted the progress.

Reviewer 2: External Scientific reviewer

This section doesn't seem to have been filled in, I only see the instructions and example of what to do.

Reviewer 3: PMT member

See above.

Did the PhD student interact with JRPs/JIPs or external (global, EU, national or regional) relevant projects or initiatives?

Reviewer 1: External Scientific reviewer

Difficult to judge actually by reading this.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Did the PhD student highlight any added value or benefits during the PhD (e.g., Training, national & multi-national networking)?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Did the PhD student actively engage in Education and Training activities?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Did the PhD student publish their scientific outcomes in relevant peer-reviewed journals?

Reviewer 1: External Scientific reviewer

I think the sole authorship on majority of the papers & thus lack of co-authors from the respective areas making up OH is a lack work publications.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Was the PhD managed and implemented in accordance with the DMP?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Did the PhD student actively engage in disseminating the scientific outputs of the project (e.g., conferences, OHEJP blogpost, interviews etc.)?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

How relevant are the finalised project outputs to the One Health EJP aims and objectives?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Is there any direct or indirect impact of the project for national or international stakeholders?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Has this project built or strengthened collaborations with other partners of the One Health EJP and beyond? Was it described in the final report?

Reviewer 1: External Scientific reviewer

n/a

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

Do the project outcomes have policy implications?

Reviewer 1: External Scientific reviewer

I miss experiences/results from the NOVA projects that could have been drawn into the discussion section and perhaps also into the papers? She states that she visited the institute in charge of NOVA and that she participated in the interviews, but nothing about the results as far as I can see. I think also that SARS-CoV-2 is not a good example of a zoonotic disease, since it is not a classical zoonotic disease since primarily transmitted between humans and

not so easy from humans to animals (when look into details of trans pattern and the overall numbers!). I also think separated collection and analyses of data might not be the problem with OH, but the actual sharing of the information and co-work around this part. I think the appreciation of what lies in the WHOs definition of health is far broader; it is everything that impacts a human's life and defines if good health - and here is also access to nature, animals etc. included.

Reviewer 2: External Scientific reviewer

n/a

Reviewer 3: PMT member

See above.

The full Final Thesis Report for the SUSTAIN PhD project can be found here.