D6.20: Report on Short Term Missions 2022 (Y5)

Work Package 6

Responsible Partner: UoS, UK (P23)

GENERAL INFORMATION

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REPORT ON SHORT TERM MISSIONS Y5

Introduction

Short Term Missions (STMs) are small co-funded travel grants which facilitate the exchange of scientific expertise, methodologies, equipment, and facilities to our consortium members. The aim of these missions is to harmonise the existing approaches and methodologies within the One Health EJP (OHEJP).

These STMs drive research forward in a collaborative and non-duplicative fashion to strengthen the scientific capacity within the OHEJP, and to also contribute to the future prevention, preparedness, detection, and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

The OHEJP is able to co-fund up to ten STMs each year (each funded 44% by the EU). The Education and Training activities team from Work Package 6 (WP6) are responsible for co-ordinating the call, selection of applicants for funding and reporting on the short term missions funded each year.

Short Term Missions 2022 call

The STM 2022 call was launched on 20th January 2021, and an additional call was launched on December 2021. For both calls, a promotional email marketing strategy was used to disseminate the call information to the Scientific Steering Board (SSB), Project Management Team (PMT), Progamme Managers Committee (PMC), Project Leaders, PhD teams, and Communication Contact Persons. The email was visually attractive and incorporated a branded launch infographic and branded text.

The email contained instructions on how to access the 'Call for Short Term Missions 2022' group on the private space of the OHEJP website, where they could download the guidelines, application form and templates. Readers were asked to forward the email to those in their institute who may find it useful.

The launch of the call was also disseminated on the Education and Training Open Calls page of the OHEJP website in the form of call flyers containing interactive links to the guidelines and application forms. This page was promoted in the Education and Training activities monthly bulletins, the OHEJP consortium newsletter, and social media channels, Twitter and Linkedin, to increase traffic to the website.

Selection of Short Term Missions

The WP6 Team co-ordinate the call and selection of the STMs following the validated procedure which involves the following steps:

Applicants must fill in the STM application form, and are strongly encouraged to compile the following documents with the main application: a detailed work plan, financial budget template, letters of recommendatio from the home and hosting institute, *Curriculum Vitae*, and

finally a list of publications (if applicable). After the deadlines closed on 14th June 2021 (2022 call) and 15th May 2022 (additional call), the WP6 Team performed an eligibility check with pre-determined eligibility criteria.

Eligibility criteria specified that only scientific staff from, and PhD students based at, the partner institutes of the OHEJP were eligible to apply. STM applications would only be considered if they relate to the key OHEJP priority areas: One Health Missions- Veterinary, Food, Medical and or Environmental research; skills development missions (e.g. genomics, bioinformatics, big data epidemiology); exchange with researchers, policy makers, and risk managers to complement WP5- Science to Policy translation; risk research; integration of microbiological, risk assessment and surveillance activities; and finally, harmonisation of diagnostics tests, platforms and research. There was no limit for the duration of the STM, as long as the funds requested adhere to the OHEJP budgetary rules, and STMs could take place between January 2022 and the end of the project.

Each application and the associated supporting documents were sent to three independent reviewers. The reviewers were nominated by the SSB.

The WP6 Team compiled and reviewed the scores, detecting and resolving any typographical or administrative errors before submitting to the PMT. All 6 applications submitted in the 2022 call and 4 of the 5 applications submitted in the additional 2022 call were eligible and of high quality. WP6 suggested a decision to PMT based on the actual comments of reviewers and list of missions recommended for funding. This decision to fund 10 of the 11 submitted missions was approved by the PMT.

The final decision was then communicated to the SSB and applicants, and published on the OHEP website on a dedicated webpage for Short Term Missions 2022- https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

Impact of COVID-19

The COVID-19 pandemic affected the success of these missions in 2020 and 2021 due to the disruption to travel plans and local restrictions. One of four STMs funded for 2021 took place early in 2022. A total of eleven STMs took place in 2022.

Short Term Missions

1. Short Term Mission 1

1.1 Short Term Mission 1: Report

Surveillance and source-attribution of AMR based on metagenomic analysis.

Name of applicant	Ana Cristina Ferreira
Institute of Affiliation/	Institute: National Institute for Agrarian and Veterinary Research (INIAV), Portugal.
Contact information	Address: Av. da República, Quinta do Marquês, Edifício Principal, Piso 1, 2780 -157 Oeiras, PORTUGAL
Host institute and names of scientists involved in STM	National Food Institute, Technical University of Denmark, Lyngby, Denmark (DTU) - Professor Tine Hald - Senior Scientist Ana Sofia Duarte
Dates of STM	31st January to 11th February 2022
Call Topic	Short term missions 2022, Skills development missions
Research Domain	AMR
Key aims of STM or Workshop	During the visit Ana Cristina (AC) improved their skills for analyzing metagenomics data for the purpose of surveillance and source attribution of antimicrobial resistance (AMR). AC engaged in the whole process from the arrival of the samples in the lab to the final epidemiological data analyses, although the main focus was on the bioinformatic analyses and epidemiologically modelling.
Impact and relevance of scientific mission	The collaboration and knowledge exchange between Portugal and Denmark in the development of new approaches to surveillance and source-attribution of AMR based on metagenomics was an upgrade in the education and training of the applicant and enriched the researcher's CV. INIAV is the national reference laboratory for antimicrobial resistance in animals and food of animal origin and is in need of scientist with the skills to analyze and interpret metagenomics data. In fact, as part of DISCOVER, several metagenomic sequence data have been produced, namely, for the analysis of AMR determinants in several matrices such as aquaculture

	sediments. The applicant was part of the implementation of these new approaches in INIAV.
Benefits to OHEJP	Use of data from omics technologies (WGS and metagenomics) for source-attribution and metagenomics based surveillance allow the characterisation of antimicrobial resistance determinants, making possible to identify the potential for their dissemination. This mission contributed to the development of skills in metagenomics, bioinformatics, and AMR source-attribution approaches, needed in the frame of OHEJP consortium and in future collaborations. The training improved the participation of INIAV in WP3 of DISCOVER project-Methods: Assessment/Improvement- Tasks T3.4 related with source attribution of AMR based on metagenomics. Sharing of scientific expertise and methodologies allowed the harmonisation of approaches within the OHEJP network. Furthermore, it strengthened the collaboration and scientific capacity of partners contributing to future preparedness in the surveillance, detection and response of the EU to foodborne, AMR and emerging threats.

Summary

The short-term scientific mission (STM) "Surveillance and source-attribution of AMR based on metagenomic analysis", performed by Ana Cristina Ferreira from the National Institute for Agrarian and Veterinary Research (INIAV, Portugal), consisted of a 12-days training session at the National Food Institute, Technical University of Denmark (DTU, Lyngby, Denmark), hosted by Professor Tine Hald and Doctor Ana Sofia Duarte, from the Division for Global Surveillance.

The focus of this STM was to apply novel approaches and models based on metagenomic data for surveillance and to infer source attribution of AMR determinants. INIAV is the National Reference Laboratory for AMR in animals and food of animal origin, and is in need of scientist with the skills to analyze and interpret metagenomics data. As part of OHEJP DiSCoVer project (Discovering the sources of *Salmonella*, *Campylobacter*, VTEC and antimicrobial resistance), several metagenomic sequence data have already been and will be produced, namely, for the analysis of AMR determinants in several matrices such as aquaculture sediments.

The collaboration and knowledge exchange between Portugal and Denmark was an upgrade in the education and training of the applicant. Moreover, the training will improve the participation of INIAV in JRP-DISCOVER-WP3 Task T3.4, related with source attribution of AMR based on metagenomics. Sharing of scientific expertise and methodologies will allow the harmonization of approaches within the OHEJP network.

Furthermore, it will strength the collaboration and scientific capacity of partners contributing to future preparedness in the surveillance, detection and response of the EU to foodborne, AMR and emerging threats.

Technical Report

The focus of this STM was to learn novel approaches and models based on metagenomics data for surveillance and to infer source attribution of AMR determinants. INIAV is the National Reference Laboratory for antimicrobial resistance in animals and food of animal

origin and is partner on the OH-EJP DiSCoVer project (Discovering the sources of *Salmonella*, *Campylobacter*, VTEC and antimicrobial resistance), namely in JRP-DISCOVER- WP3 (Methods: Assessment/Improvement), Task T3.4 - Assessing and developing approaches for source attribution of antimicrobial resistance based on metagenomics.

The STM started on 31st January 2022, and consisted of a 12-days (two weeks) training session at the National Food Institute, Technical University of Denmark (DTU, Lyngby, Denmark), hosted by Professor Tine Hald and Doctor Ana Sofia Duarte, from the Division for Global Surveillance. The work activity plan previously defined was fully complied. The DTU Global Surveillance Division team, which conducts ongoing studies on monitoring of antimicrobial resistance focused on *Salmonella*, *Campylobacter*, *Staphylococci*, *Enterococci,* and *Escherichia coli*, is the EU Reference Laboratory for AMR and FAO-RC for AMR. Ana had the opportunity to attend the Global Capacity Building group meeting (group leader: Marienne Sandberg) and the meeting of the Research Group on Genomic Epidemiology (group leader: Frank Moller), which included a presentation by Timmie Lagermann on Relational Databases - Advantages of using relational databases, usually implemented using SQL (Structured Query Language) compared to non-relational databases (like ResFinder).

During the first week, Ana Sofia Duarte and Fatemeh Ashari Ghomil (from the research group on Genomic Epidemiology) introduced Ana to the bioinformatic procedures used at DTU for metagenomic data analysis, and how to work in Computerome, the National Life Science Supercomputing Center of Denmark (DTU super computer for analyzing big data). Ana got an overview of the metagenomics data available from INIAV and included in the OH-EJP DiSCoVer project.

After getting access to Computerome and learning about the pipelines used for metagenomics data, namely MGMapper and Kraken, Ana went through a dataset of 37 metagenomes from sediments. They applied the ResFinder tool to identify AMR determinants and learned about approaches for quantifying AMR determinants in a sample as well as how to interpret sequence reads. Alongside, Ana had the opportunity to spend two mornings in the laboratory with Christina Aaby Svendsen, to learn about the processing of samples for metagenomics shotgun sequencing, including the procedures used for DNA extraction.

The second week was dedicated to the application of metagenomics in surveillance. The main point of this STM was to learn how to use the metagenomics data together with epidemiological data for surveillance to explain trends as well as for source attribution. The last four days of training were mainly dedicated to programming on RStudio. Ana Sofia gave the researcher an overview of methods that can be used to describe the distribution (in read counts or relative abundance) of taxa and/or genes in the samples. The researcher was familiarised with the interpretation of heatmaps and PcoA plots as well as histograms, boxplots, qqplots and forest plots.

A dataset of 37 metagenomes from aquaculture origin (from Portugal) was used for training purposes. According to the available data, it was decided to use the HieRanFor, a package for running a Hierarchical Random Forest (HRF) analysis in R. In summary, the script was former developed by Ana Sofia and was adapted to this dataset. The necessary R packages for analysis were loaded in RStudio and all metagenomic data were prepared

for analysis. The dataset with counts of resistance genes normalised as FPKM was used. Hierarchy levels were defined for the dataset, for the hierarchical random forest model. After running the HRF algorithm a brief inspection on the model outputs was performed.

List of dissemination and communication activities

Not applicable

Scientific outputs

INIAV is the National Reference Laboratory for antimicrobial resistance in animals and food of animal origin and is partner on the OH-EJP DiSCoVer project, namely in JRP-DISCOVER- WP3 Task T3.4. This STM improved the participation of INIAV and strengthened the collaboration and scientific capacity of Portuguese partners, contributing to future preparedness in the surveillance, detection and response of the EU to foodborne, AMR and emerging threat.

Testimonial

I had a very supportive and friendly 12-day training on "Surveillance and attribution of AMR sources based on metagenomic analysis". Professor Tine Hald, Doctor Ana Sofia Duarte and team, from the Global Surveillance Division at the National Food Institute, Technical University of Denmark (DTU, Lyngby, Denmark), have a great experience and skills on this subject, and it was very useful to become part of their group and work together with them for two weeks. This collaboration allowed me to increase my knowledge and to improve skills, also contributing for a better contribution of INIAV in OH-EJP DiSCoVer project - WP3 Task T3.4. Additionally, the information and tools acquired will be applied to the analysis of metagenomic data for source tracking in aquaculture environments and in future projects in this area, where we hope to continue the collaboration started in this mission.

1.2 Short Term Mission 1: Case study

SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large
- OHEJP European network
  Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Surveillance and source-attribution of AMR based on metagenomic analysis

Theme: One Health, AMR
Home Institute: National Institute for Agrarian and Veterinary Research (INIAV), Portugal
Mission Hosting Institute: Technical University of Denmark (DTU Food), Denmark
Duration of Mission: 2 weeks

"...it was very useful to become part of the DTU group and work together for two weeks. This collaboration allowed me to increase my knowledge and to improve skills. The information and tools acquired will be applied to the analysis of metagenomic data for source tracking in aquaculture environments and in future collaborative projects in this area...."

Ana Cristina Ferreira, INIAV, Portugal

The aim of this STM was to learn novel approaches and models based on metagenomic data for surveillance and to infer source attribution of AMR determinants.

INIAV is the National Reference Laboratory for AMR in animals and food of animal origin, and is in need of scientists with the skills to analyse and interpret metagenomics data.

The STM gave Ana the opportunity to improve her skills for analysing metagenomics data for the purpose of surveillance and source attribution of AMR. Ana was engaged in the whole process from when the samples arrived in the lab to the final epidemiological data analyses, although the main focus was on the bioinformatic analyses and epidemiologically modelling.

Moreover, this mission will contribute to the development of skills in metagenomics, bioinformatics, and AMR source-attribution approaches, needed in the frame of the OHEJP consortium and in future collaborations. Use of data from omics technologies (WGS and metagenomics) for source-attribution WGS and metagenomics based surveillance allows the characterisation of antimicrobial resistance determinants, making it possible to identify the potential for their dissemination.

The training will improve the participation of INIAV in the OHEJP DISCOVER project methods: assessment/improvement- related with source attribution of AMR based on metagenomics.

Sharing of scientific expertise and methodologies will allow the harmonisation of approaches within the OHEJP network. Furthermore, it will strengthen the collaboration and scientific capacity of partners contributing to future preparedness in the surveillance, detection and response of the EU to foodborne, AMR and emerging threats.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 1: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

2. Short Term Mission 2

2.1 Short Term Mission 2: Report

Zoonotic pathogen detection in rats.

Name of applicant	Marieke de Cock.
Institute of Affiliation/	Institute: National Institute of Public Health and the Environment (RIVM).
Contact information	Address: Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven The Netherlands.
Host institute and names of scientists involved in STM	Friedrich-Loeffler-Institut (FLI) Prof. Rainer G. Ulrich
Dates of STM	14th March to 15th April 2022.
Call Topic	One Health Missions- Veterinary, Food, Medical and or Environmental research and Harmonization of diagnostics tests, platforms and research.
Research Domain	Emerging threats.
Key aims of STM or Workshop	The collaboration between RIVM and FLI provides opportunity to compare and harmonise pathogen detection methods between the institutes; share reference material and possibly test new detection methods. The laboratory at FLI has ample experience with detecting specific rat-borne pathogens such as Seoul orthohantavirus, cowpox virus and rat hepatitis E virus, and it has facilities to perform those analyses, which is currently not available at the RIVM. With this visit, the PhD student learned more about these detection techniques, with the aim to also set up these detection methods at RIVM.
Impact and relevance of scientific mission	The RIVM (a public health institute) and the FLI (a veterinary health institute) both work on the topic of rat-borne zoonoses. Rats and rat-borne zoonoses are a wicked problem, therefore collaboration between the various fields of science involved is important to achieve the best results. In this short-term mission, scientists from RIVM and FLI collaborated and exchanged scientific expertise and research methods. Also, within these two OHEJP network institutes, equipment and facilities were shared with the aim of harmonising pathogen detection methods of potential emerging threats. The different expertise backgrounds complemented one another in order to contribute to

	a One Health vision and approach. The STM was focused on detection methods for specific zoonotic pathogens carried and transmitted by rats, to get more insight in the prevalence and transmission of such infective agents that are able to cross species barriers. This STM strengthened the current collaboration between RIVM and FLI, and aims to promote future collaboration opportunities between these institutes. This STM to FLI also broadened the PhD student's knowledge about rat-borne pathogens and improved their laboratory pathogen detection method skills. These skills can then be applied for pathogen detection at RIVM. Visiting another research institute also improved Marieke's scientific knowledge and network.
Benefits to OHEJP	This STM: - Enhanced collaboration between OHEJP network institutes - Broadened approach to One Health problems related to rodent-borne diseases - Extended pathogen detection and transfer of knowledge and expertise

Summary

During this the Short Term Mission (STM), Marieke de Cock, PhD student on the OHEJP PhD project DESIRE at the RIVM (in the Netherlands), went to the Friedrich-Loeffler Institut (FLI) in Germany to test rat and mouse samples (total n=670) on different zoonotic pathogens. The rat and mouse samples were collected during Marieke's fieldwork. The samples were tested for Cowpox virus, rat Hepatitis E virus (rat HEV), Lymphocytic choriomeningitis virus (LCMV) and *Streptobacillus moniliformis*. For the Cowpox virus testing two different tests were used: qPCR (on DNA extracted from nasal septum samples) and Immunofluorescence assays (IFA) using heart fluid on cowpox-infected cells. Both tests were performed in the Biological Safety Level 3 (BSL-3) laboratory. No Cowpox virus positive rats or mice were detected. For rat HEV, liver samples were tested with both conventional PCR and qPCR. In total, 15 rats were positive for rat HEV. These samples were further sequenced and analysed. Mouse liver samples were also tested for LCMV by conventional PCR and one positive house mouse was positive, which is an interesting result since LCMV has not been detected in wild house mice in the Netherlands for years. This sample will be further sequenced by WGS. The rat samples were tested for *S. moniliformis* by both qPCR and serology in collaboration with two German partner institutes. The serology validation tests were still ongoing at the end of the STM. Using serology on heart fluid, 33% of the samples were considered positive. However, using qPCR on DNA extracted from salivary glands, only seven positive samples were detected (<2%). All the pathogens that were tested will be included in the paper about zoonotic pathogen carriage in wild rats from urban areas and in additional pathogen-specific papers. Next to the valuable test results, this STM contributed to interesting discussions about rodent borne (zoonotic) pathogen research, increased contact between FLI and RIVM and led to new collaborations in sharing of data and writing of papers between FLI and RIVM.

Technical Report

For the STM, different pathogens were tested on rat and mouse samples (total n=670) collected by the PhD student during fieldwork. These samples were tested for cowpox virus, rat Hepatitis E virus (rat HEV), Lymphocytic choriomeningitis virus (LCMV) and *Streptobacillus moniliformis*. The tasks for each pathogen will be discussed separately.

Cowpox virus: For the cowpox virus testing, two different tests were used: qPCR (on nasal septum DNA) and Immunofluorescence assay (IFA) using heart fluid on cowpox-infected cells. All work for this pathogen was performed in the BSL3 laboratory of Donata Hoffmann at FLI together with her and her technicians. No cowpox positive samples were detected in both IFA and qPCR. Some additional historical rat samples were send by the PhD student's supervisor. Of those samples, some were positive in IFA, but no virus could be detected with qPCR.

Rat HEV: All samples were tested with both conventional PCR and qPCR to detect positive samples for rat HEV in liver. In total 15 rats were positive for rat HEV. These samples were further sequenced. In addition, new primers were designed, because the primers used at first were from a paper published in 2012, and a recent mutation in the virus meant that not all rat HEV strains would be recognised by these primers. However, there wasn't enough time during the STM to test all the samples with these adjusted primer sets; this work was continued after the mission by FLI. All laboratory work was performed in the BSL2 lab of Rainer Ulrich.

LCMV: Mouse liver samples were tested for LCMV by conventional PCR. One positive house mouse was detected, which a very interesting results because this virus has not been detected in wild house mice in the Netherlands for years. This sample will be further sequenced by WGS. All laboratory work was performed in the BSL2 lab of Rainer Ulrich.

Streptobacillus moniliformis: Rat samples were tested for *S. moniliformis* by both qPCR and serology in collaboration with two German partner researchers (Tobias Eisenberg and Katja Schmidt). The serology tests still have to be published, but the test itself gave promising results. Using serology on heart fluid, 33% of the samples were considered positive. However, using qPCR on salivary gland DNA only 7 positive samples (<2%) were detected. Normally DNA from the tongue tip DNA is used for the test, so the use of DNA from salivary glands during the STM could have led to lower prevalence estimates.

All the pathogens that were tested will be included in a paper about zoonotic pathogen carriage in wild rats from urban areas. Next to that, this STM contributed to interesting discussions about rodent borne (zoonotic) pathogen research and new collaborations (both papers and sample sharing) between FLI and RIVM.

Scientific outputs

The mission contributed to the detection of specific rodent-borne pathogens, which will be included in a paper about the effect of urban greening on rodent abundance and pathogen prevalence and diversity in wild rats from urban areas (expected date: end 2022). Also, the LCMV virus was detected in one house mouse, which is an interesting result that will be included in a separate paper about LCMV in collaboration with FLI.

List of dissemination and communication activities

Not applicable.

Testimonial

For my 5-week short-term mission (STM) I went to the Friedrich-Loeffler-Institut (FLI), which is idyllically located on an island in the North-Eastern part of Germany. The work of the FLI focusses on farm animal health and welfare, and on the protection of humans from zoonoses. I was working in the laboratory of Prof. Rainer Ulrich and Prof. Donata Hoffmann, who are specialized in rodent-borne virus research. I brought my own rat and mouse samples that I collected during previous fieldwork (total n=670) to test them for specific viruses and one bacterium. During my STM, we tested the samples for Cowpox virus, rat Hepatitis E virus (rat HEV), Lymphocytic choriomeningitis virus (LCMV) and *Streptobacillus moniliformis*. For some pathogens multiple tests were used (e.g. IFA, serology, conventional PCR and qPCR). We detected rat HEV in brown rats, LCMV in a house mouse and *Streptobacillus moniliformis* in brown rats. No Cowpox virus was detected. *Streptobacillus moniliformis* was tested in collaboration with two other German research institutes. Not only did this STM result in new data for my PhD project, it also strengthened the collaboration between FLI and RIVM, both in the form of sample/data sharing and publishing papers together. Besides all the hard work, I also had a lot of fun during my stay! The saying 'Germans don't make jokes' does not really hold for the people I worked with there, who were all very nice and enthusiastic people with whom I laughed a lot! I can recommend all my fellow PhDs to work at another research institute for a while, not only to broaden your horizon, but also to have an unforgettable experience and to meet other researchers!

2.2 Short Term Mission 2: Case study

SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large OHEJP European network
- Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Image: Flickr

Zoonotic pathogen detection in rats

Theme: One Health, Emerging Threats
Home Institute: RIVM, The Netherlands
Mission Hosting Institute: FLI, Germany
Duration of Mission: 1 month

The Short Term Mission opened up the opportunity to compare and harmonise pathogen detection methods between RIVM and FLI, share reference material, and test new detection methods.

The mission focused on detection methods for specific zoonotic pathogens carried and transmitted by rats, to gain more insight in the prevalence and transmission of such infective agents that are able to cross species barriers.

The laboratory at FLI has experience in detecting specific rat-borne pathogens such as Seoul orthohantavirus, cowpox virus and rat hepatitis E virus, and it has the facilities to perform those analyses, currently not available at the RIVM. Marieke was able to learn these detection techniques, with the aim to set up these methods at RIVM.

The detection of specific rodent-borne pathogens will be included in a paper, expected towards the end of 2022, about the effect of urban greening on rodent abundance, pathogen prevalence, and diversity in wild rats from urban areas. During the STM, the team detected lymphocytic choriomeningitis virus (LCMV) in one house mouse, which is an exciting result that will be included in a separate paper about LCMV in collaboration with FLI.

As well as producing valuable test results, the mission strengthen the current collaboration between FLI and RIVM, opened up interesting discussions about rodent-borne (zoonotic) pathogen research, and led to new, unforeseen opportunities for sharing of data and the writing of papers.

The outcomes of this STM are a step forward in the harmonisation of pathogen detection methods and responses to potential emerging threats, in line with the One Health approach and vision.

Not only did this STM result in new data for my PhD project, it also strengthened the collaboration between FLI and RIVM, both in the form of sample/data sharing and publishing papers together. I can recommend all my fellow PhDs to work at another research institute for a while, not only to broaden your horizon, but also to have an unforgettable experience and to meet other researchers!"

Marieke de Cock,
RIVM, The Netherlands

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 2: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2021.

3. Short Term Mission 3

3.2 Short Term Mission 3: Report

Validation and exchange of modelling tools to assess the risk of human salmonellosis based on environmental factors using multiple sources of data.

Name of applicant	Laura C. Gonzalez Villeta
Institute of Affiliation/	Institute: University of Surrey
Contact information	Address: School of Veterinary Medicine, VMS main building, Daphne Jackson Rd, Guildford GU2 7AL, United Kingdom.
Host institute and names of scientists involved in STM	RIVM (Dutch National Institute for Health and Environment). Eelco Franz (ADONIS project leader / head of department) Lapo Mughini Gras (ADONIS WP leader) Linda Chanamé Pinedo (ADONIS PhD student)
Dates of STM	20th April to 20th May 2022
Call Topic	One Health Missions, Risk assessment
Research Domain	Foodborne Zoonoses
Key aims of STM or Workshop	1. Creation of a University of Surrey-RIVM collaboration framework, and exchange of perspectives. 2. Expertise and methodology exchange. The combined viewpoint of researchers and public health officials allows to contextualise and compare the results from the UK and the Netherlands in light if the local epidemiology of salmonellosis and the characteristics of the surveillance systems generating the analysed data, as well as the applicability of the methods used. 3. Validation of the model developed for England and Wales with Dutch surveillance data, to strengthen the interpretability of the analyses. Applying the conditional prevalence methodology to a different setting allows to assess whether the relationship between salmonellosis and exposure is universal. This will be followed by a joint interpretation of the findings and a joint publication. The comparison of the methods used allows for more robust

	results and a deeper understanding of the epidemiology of salmonellosis.
Impact and relevance of scientific mission	1) An EnvDis PhD thesis chapter will be written, adressing the impact of the environment on human salmonellosis. This triangulation would render the findings of the thesis much stronger. 2) A joint publication of the findings when applying the conditional prevalence methodology in the Netherlands. This is expected to happen within the duration of the EnvDis PhD project. 3) A long and durable collaboration between the two institutions in the long term, possibly lead to a unified programme of research integrating the two approaches over diverse geographic and socio-economics settings.
Benefits to OHEJP	The mission has contributed to make the outcome of the EnvDis PhD more robust and open the possibility of being readily applicable to different public health settings, as well as benefit the deliverables associated to both the projects while fostering institutions' exchange and collaboration.

Summary

What is the link between weather and human salmonellosis? The seasonal patterns of salmonellosis cases observed in the northern hemisphere summer are often thought to be connected to certain human behaviours (e.g., increased barbecues or big outdoor gatherings). The weather itself may influence the incidence in other ways, for example high temperatures tend to enhance growth of *Salmonella* in food. The project EnvDis analyses in detail how the weather impacts on salmonellosis.

Together with my supervisory team, we developed a novel statistical modelling approach to reproduce the probability of detecting salmonellosis cases exclusively due to certain weather factors. The methodology was built on 30 years of daily epidemiological data from the UK Health Security Agency (UKHSA) and a spatio-temporal matching weather database from the MetOffice. The model first estimates the probability to observe salmonellosis cases conditioned to certain weather factors. Based on this information and local weather data, the model reproduces relatively well the empirical patterns from epidemiological surveillance data for England and Wales. It also suggests that the most relevant weather factors that influence the incidence of the disease are maximum air temperature, relative humidity, and day length

Now, the question is whether our results are specific to the England and Wales or if the model could be applied to other countries different from the one it was built for. We wanted to investigate if the assumption that the link between salmonellosis and weather is universal and independent on the country. Thus, the purpose of the mission was to apply the model to the Dutch situation (i.e., using weather and census data from The Netherlands) but using the probability of salmonellosis cases conditioned to weather factors for England and Wales. We then compared the model with salmonellosis disease records from The Netherlands. The preliminary results indicate that the model captures the magnitude and key seasonal patterns of the Dutch data. However, the model also results in some secondary peaks in the incidence

in early spring not observed in the real data, perhaps related to differences in notification and health seeking behaviour.

Technical Report

EnvDis aims to estimate the probability of salmonellosis cases conditional to certain environmental exposures. It assumes that this conditional probability does not depend on time. In addition, it aims to incorporate mechanistic processes, such as bacterial growth in the food source of human transmission. By applying the conditional probability approach to a different country —the Netherlands in this case—, we wanted to test the ability of our model to identify the relationship between salmonellosis and weather exposure, irrespective of the country of study. This finding will enhance the robustness of our findings and will help to identify the limitations of the methodology.

A pooled analysis of reported cases of human salmonellosis in the Netherlands was carried out to validate the methodology using the conditional probability estimated for England and Wales. The data available to our collaborators at the RIVM consisted of 15 years of national surveillance data on salmonellosis cases reported to the Dutch National Institute of Public Health (including both epidemiological metadata and laboratory typing data of the isolated strains) at a daily resolution. The salmonellosis data was stored under a secure institutional IT system that required working under the institution's network. The epidemiological data was available at patient postcode (PC6 and PC4) resolution. Some data cleaning included the removal of travel-related cases, empty or unknown postcode, serotypes of non-foodborne interest (i.e., *S. enterica* serovar Paratyphi), and revisits of the same patient under 3 months. The final total of cases was 17,243.

The weather variables of interest for the same period of time included mean relative humidity and maximum air temperature. These were obtained from the open-resource Royal Netherlands Meteorological Institute (KNMI) website (https://www.knmi.nl/klimaat). Weather information was available at the coordinate points of the meteorological measuring station (RD projection). Demographic data was required for getting the salmonellosis prevalence figures. The yearly resident numbers were obtained from the Central Bureau of Statistics (CBS) (https://www.cbs.nl/nl-nl/maatschappij/bevolking) with a grid resolution of 500 metres).

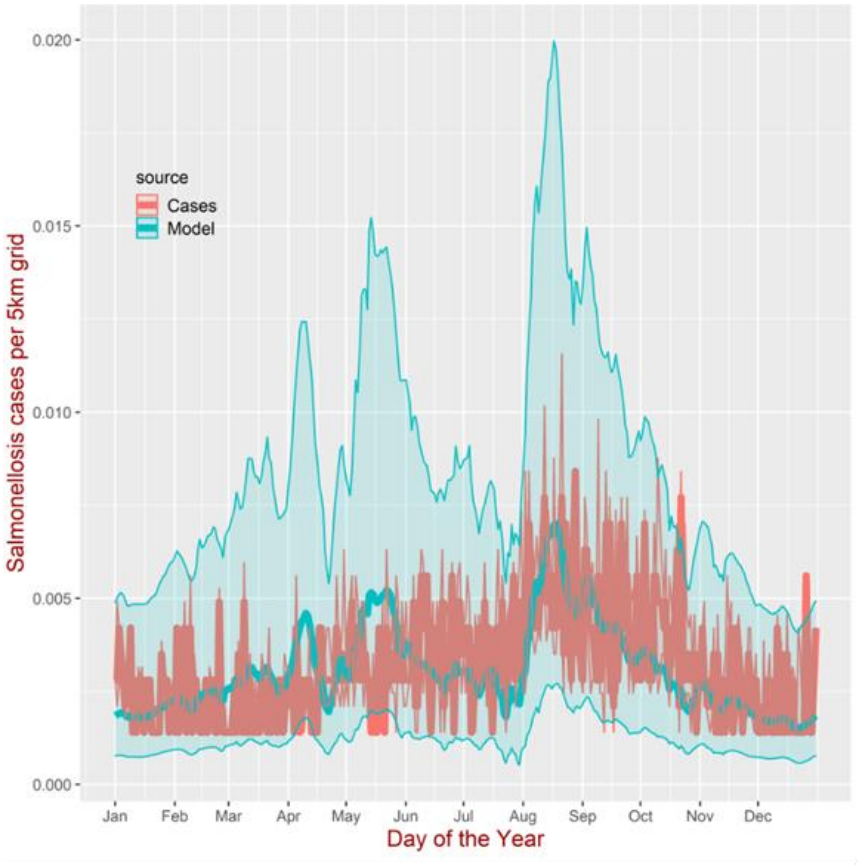
Guidance from our collaborators were fundamental to precisely adjust all the data sources to meet the same spatial unit: 10 of the 500m grid containing resident information were merged into the 5km final grid. The patient postcode was spatially assigned to the corresponding grid, and the weather values were assigned based on the nearest weather station in distance to the corresponding grid. Given the lack of residents' information in a yearly resolution prior to 2015, we focused our analysis from this year onwards.

Once the data was cleaned and ready to use, I started to review and adapt the R scripts wrote for the analysis in England and Wales:

1. *Remove reporting delays*: to estimate a probable day of infection from the date of the record creation at the RIVM. Local expertise was essential to adapt the code to the particularities of the country in terms of reporting delays. These delays included:

a. *Incubation period* for which a study from a NTS outbreak in Minnesota (Eikmeier, 2018) was used. We accounted for the two most common serotypes found in the NL: Enteritidis with (0.29%), and Typhimurium (0.27%), and applied an average of the rest of the serotypes available for the rest of the cases. The values were fitted to a gamma distribution.
b. *Time to healthcare*: the delay for an ill person to seek health care attention. A lag of 0-3 days was stablished for blood specimens, assuming a prompt urgency to seek medical attention. A lag of 7-11 days. was stablished for stool samples, where it is common to wait for self-resolution of a minimum of 7 days prior to seek medical attention.
c. *Time to hand out a sample*: time it takes for specimen to reach the diagnostic laboratory and analyse the sample: 0-2 days, adapted from the ECDC statistics.
d. *Time for the sample to get to the RIVM*: time for the specimen to reach the RIVM for typification: 1-2 days for blood samples and 1-3 for stools.

2. *Linkage of cases to a time lag*: As in England and Wales, we assumed that the impact of the weather is not instantaneous, instead there is a cumulative effect of weather over the bacterial population, and other potential factors, prior to the infection day. We assumed that this lag could be mimicked by averaging the relevant weather factors over the past 7 days. We have tried other values (14 and 2 days) but no significant difference was found.
3. Using the conditional probability and local weather and census data we can reconstruct the expected number of cases at each postcode at various times. The time-series then can be aggregated by months as shown in the figure below, comparing *cases simulated* by the model (blue) against the reported cases (red). The shaded areas represent the 25% and 75% quantiles. The variability is due to geographic and inter-annual variations (with different weather factors).



The predictions —based only on the simultaneous effect of maximum air temperature, relative humidity, and day length— show a mismatch for April and May that does not correspond to the reported cases. The main summer peak is however well detected.

The preliminary results show that the model captures the magnitude and key seasonal patterns of the Dutch data, however the model also results in some secondary peaks in the incidence in early spring not observed in the real data, perhaps due to bias in notification and health seeking behaviour.

Possible explanations for the discrepancy between predicted and empirical patterns are the limitation in the epidemiological data due to the few years analysed; and/or different population behaviours that have a significant impact on case reporting (e.g., different rates of seeking medical care). Further checks and analysis will help to elucidate this in the near future.

The work performed was delayed as per the work plan as follows:

Week 1: set accounts, access to the RIVM dataset, get and pre-process salmonellosis data. Find sources of demographic, country shapefile, and weather data. Introductory presentation of the methodology and feedback on ideas and next steps analysis.

Week 2: identify and translate the relevant demographic and meteorological dataset for the Netherlands and download the data.

Weeks 3 and 4: adjust all the data sources (cases, demographics, and weather) to the same geographic unit size. A grid of 5km was agreed and this was performed with assistance of the GIS experts of the RIVM.

During the visit there was not enough time to completely finalise the computer code built in the UK to the data of the Netherlands. This work continued in the institution of origin. However, it was enough to get the data required and perform the adjustments needed under close supervision of the host institute. At the same time, the mission successfully met the transversal activities, which included:

- Learning about the way of working in a Public Health institution. Moreover, Laura had the chance to attend and participate in scientific group meetings, get an insight of the current gastrointestinal disease work relevant to the Netherlands, present the work being performed to a RIVM-wide audience for feedback and comments, as well as see the daily modelling work performed behind the COVID-19 surveillance system.
- Collaboration and mutual knowledge exchange between the three PhD students involved (Laura, Linda and Annemieke), including modelling, epidemiology, and spatial analysis of foodborne diseases.

List of dissemination and communication activities

Not applicable.

Scientific outputs

1) A joint publication of the findings when applying the conditional prevalence methodology in the Netherlands. This is expected to happening within the duration of the EnvDis PhD project.
2) A long and durable collaboration between the two institutions in the long term, leading to a unified programme of research integrating the two approaches over diverse geographic and socio-economics settings.

Testimonial

I cannot thank the OHEJP consortium enough for allowing me to take part in this exchange. Although short, it has been a very positive experience that has not only complemented my PhD objectives well but has also increased my confidence in my project and enriched me with new contacts and experiences.

I felt very welcomed at the RIVM, my host institution. Everyone in the gastrointestinal disease group was very supportive and ready to give me a hand, have a brainstorming chat or give a piece of advice anytime! I could even meet in person Marieke, another OHEJP PhD student that I had only met in screen before (yes, she is taller than I imagined). From day 1 I felt that my mission was a success, as one of the most valuable objectives for me was to learn about the functions and daily work of a public health officer. It was inspiring to see such motivated and skilled scientists in action applying in practice what I am learning. It was a great inspiration and broadened my perspective on my next career steps.

The mission has not only helped me to accomplish the main objective of validating the model for my PhD, but has also helped me to enhance my networking and communication capacities, opening my mind to different ways of communicating and interpreting each other. By discussing the details of my project with other scientific peers and fellow PhD students, I got to know my project a bit better and improved my confidence to talk about my research in a friendly context outside of my university.

I also got to enjoy some free time cycling around the beautiful city of Utrecht and its surroundings, the stunning tulip fields in bloom with all the colours of the rainbow, the festive day at King's Day and its flea markets, the "borrelnootjes" or savoury snacks for drinks, and the boat parties along the canals. Overall, the whole experience was very enjoyable and worth remembering.

3.2 Short Term Mission 3: Case Study

Figure 3: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

4. Short Term Mission 4

4.1 Short Term Mission 4: Report

Application of spatial models to identify new environmental surveillance indicators on *Salmonella* and *Campylobacter* in pig and poultry.

Name of applicant	Antonio Rodríguez
Institute of Affiliation/	Institute: The National Institute for Agricultural and Food Research and Technology (INIA).
Contact information	Address: Ctra Algete a El Casar s/n, Spain
Host institute and names of scientists involved in STM	National Institute for Public Health and the Environment (RIVM). Arno Swart; Elisa Beninca.
Dates of STM	1st to 30th June 2022.
Call Topic	Skills development missions.
Research Domain	Foodborne Zoonosis.
Key aims of STM or Workshop	Machine learning applied to One Health surveillance data: Applications to Wild Boar Surveillance Plan of Andalucía (Spain); *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN) and the *Salmonella* and *Campylobacter* Spanish surveillance plan in poultry farms.
Impact and relevance of scientific mission	There is an urgent need to strengthen the surveillance programs of zoonotic diseases. The use of machine-learning algorithms for improving data visualisation, analysis and modelisation may provide useful pieces of information to this purpose. This STM was focused on the study and identification of risk factors of zoonotic pathogens (e.g. *Brucella*, *Salmonella* and *Campylobacter*) using surveillance data. We applied machine-learning algorithms (random forest, boosted regression trees) to identify the most important risk factors, to study their relationship with the zoonotic diseases, and to detect interactions between them. Results were spatially represented when possible to identify areas of high risk. During this STM we developed a procedure to fit machine learning based-models using surveillance data. The procedure developed during this STM will be applied to three datasets from three surveillance programs: • Wild Boar Surveillance Plan of Andalucía (Spain).

- *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN).
- *Salmonella* and *Campylobacter* Spanish surveillance plan in poultry farms.

Benefits to OHEJP

The procedure developed during this STM may allow to obtain information about the risk factors of zoonotic diseases useful to strengthen and improve the corresponding surveillance programs.
Not only academic articles will be published, but also the administrations in charge of these surveillance programs will have direct feedback from their data, since we will transfer our results directly to them by meetings and technical reports.
The first meeting will be with the Wild Boar Surveillance Plan of Andalucía (Spain) on the 15th of July 2022.

Summary

The aim of this mission was to develop a procedure to model surveillance data of zoonotic diseases with machine learning methods. We used the data from the Wild Boar Surveillance Plan of Andalucía (Spain) as first example. Concretely, we selected *Salmonella* serology of hunted wild boars as our first response variable. We sorted the data to organise them in an appropriate way for training machine learning algorithms. Additionally, we expanded the set of potential predictors adding climate and wild boar distribution information from published maps. We designed a modelling procedure valid for all the wild boar diseases in the dataset, based on the "tidymodels approach". This includes the processing of the dataset before the modelling (pre-processing step), the model application itself and its validation, and the study of the model output. The most important issue was the exploration of the best way to compensate the unbalance of the positives and negatives in the dataset, which can reduce either the sensitivity or the specificity of the model. We concluded that we could obtain acceptable results with the three methods we considered: to adjust of the positive-negative threshold by the ROC-curve, and down sampling and the up sampling of the dataset. We decided to use the latter because results seemed slightly better and is easy to implement. We prepared the script for providing a number of validation statistics based on the comparison between the predictions on the test data and the real positive and negative values. For studying the model output, we implemented the following methods: i) variable importance on the training data and on the test data; ii) partial dependence plots based on training and test data; iii) detection of interactions between predictors on the test data, and their partial dependence plots based on training and test data. The only work to do at this point is to optimise the dataset for the analysis. This procedure will be applied to the other wild boar diseases and to the databases facilitated by the other two surveillance programs: *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN) and *Salmonella* and *Campylobacter* Spanish surveillance plan in poultry farms.

Technical Report

The aim of this mission was to develop a procedure to model surveillance data of zoonotic diseases with machine learning methods. We used the data from the Wild Boar Surveillance Plan of Andalucía (Spain) as first example of data to work with. Concretely, we selected *Salmonella* serology of hunted wild boars as our first response variable. This decision was based

on two facts. First, this variable has a relatively balanced proportion between negatives and positives, and hence is potentially easier to model than a disease with an unbalanced ratio. Second, the database from the Wild Boar Surveillance Plan of Andalucía (Spain) comprises a wide variety of explanatory variables of different types (e.g. hunting ground management, wild and domestic species present in the area) and a large sampling size (> 2000 wild boars).

On the first week, we sorted the data to organize them in an appropriate way for training machine learning algorithms. Additionally, we collected climate and wild boar distribution information from published maps (https://confluence.ecmwf.int/; Bosch et al., 2017, 2012) to include them as potential drivers of the pathogens. This included the processing of raw climate variables (daily temperature and precipitation data) for obtaining a list of 19 bioclimatic variables (Fick and Hijmans, 2017).

On the remaining weeks, we designed a modelling procedure valid for all the wild boar diseases in the dataset, based on the "tidymodels approach". This includes the processing of the dataset before the modelling (pre-processing step), the model application itself and its validation, and the study of the model output. Concerning the pre-processing, we explored some methods of variable selection and the convenience of transforming categorical predictors to continuous dummy variables. Despite we discarded these methods; they could be employed in other datasets if necessary. We also decided to split the data in the training and the test datasets. The training data is the used to fit the model while the test data is used just to validate it. We used the data of the last hunting season (2020/2021) to validate the model, since the idea is to produce models of wild boar diseases able to predict where there is going to be a higher presence of a disease in the next hunting season, attending the data of the previous years.

Concerning to the model fitting, we focus on random forest models form the package "randomForest" (Breiman et al., 2012), although we explored the "xgboost" and "ranger" packages (Chen and Guestrin, 2016; Wright and Ziegler, 2017). Additionally, we explored the best way to compensate the unbalance of the positives and negatives in the dataset, which can reduce either the sensitivity or the specificity of the model. We concluded that we could obtain acceptable results with the three methods we considered: to adjust of the positive-negative threshold by the ROC-curve, and down sampling and the up sampling of the dataset. We decided to use the later because results seemed slightly better and is easy to implement. We prepared the script for providing a number of validation statistics based on the comparison between the predictions on the test data and the real positive and negative values: sensitivity, specificity, accuracy, kappa, auc of the roc curve and the true skill statistic.

For studying the model output, we implemented the following methods:

- Variable importance on the training data and on the test data.
- Partial dependence plots based on training and test data.
- Detection of interactions between predictors on the test data, and their Partial dependence plots based on training and test data.

The only work to do now is to optimise the dataset for the analysis, since some variables need for a revision of their writing and some spelling mistakes, and some translation from Spanish to English language to facilitate the publication of our results.

Thanks to the large dataset provided by the Wild Boar Surveillance Plan of Andalucía (Spain) and the work done in this short term mission, we have a data analysis tool that in a short period of time will allow to answer the following questions:

- Which are the most important risk factors (climate, wild boar population, wildlife and domestic animals, land covers, hunting ground management etc.) that shape wild boar diseases in Andalucía?
- How is the relationship of these factors with the probability of positive and negative to a disease of hunted wild boars?
- Which of these risk factors interact between each other concerning the risk of wild boar diseases?
- Taking the data from previous hunting seasons, can we fit a model able to identify the areas with higher and lower probabilities of positives to a disease, and represent the predictions in a map?

Additionally, this procedure has been automatised in an R script so can be easily applied to the data from the *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN) and the *Salmonella* and *Campylobacter* Spanish surveillance plan in poultry. Once again, the aim of this modelling will be to identify their main risk factors and study the sing and shape of their relationship with the presence of these pathogens.

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List of dissemination and communication activities

Name of the activity:	*Training to other team members of the lab. (Carlos Sacristán and Irene Sacristán) on the methods learnt and developed during the STM*

Date:	*12/07/2022-20/07/2022*		
Place:	*Animal Health Reshearch Centre (Madrid, Spain)*		
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories			
	Yes / No		*Yes / No*
Organisation of a Conference		***Participation to a Conference***	
Organisation of a Workshop		***Participation to a Workshop***	
Press release		***Participation to an Event other than a Conference or a Workshop***	
Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	
Training	***X***	***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categories			
	Number		*Number*
Scientific Community (Higher Education, Research)	***2***	***Media***	
Industry		***Investors***	
Civil Society		***Customers***	
General Public		***Other***	
Policy Makers			

Name of the activity:	*Meeting with the responsible people in charge of the Wildlife Epidemiological Surveillance Program of Andalusia (Spain). The aim is to give them feedback about the data for strengthen the surveillance program*		
Date:	*18/07/2022*		
Place:	*Water and Environment Agency from Andalusia (Spain) and Animal Health Research Centre (teleconference)*		
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories			
	Yes / No		*Yes / No*
Organisation of a Conference	***X***	***Participation to a Conference***	
Organisation of a Workshop		***Participation to a Workshop***	
Press release		***Participation to an Event other than a Conference or a Workshop***	

Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	
Training		***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categories			
	Number		*Number*
Scientific Community (Higher Education, Research)	***3***	***Media***	
Industry		***Investors***	
Civil Society	***2***	***Customers***	
General Public		***Other***	
Policy Makers			

Scientific outputs

We expect to send two publications in a highly ranked scientific journal in approximately six months, concretely corresponding to the datasets of the Wild Boar Surveillance Plan of Andalucía (Spain) and the *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN). We will send them to the journal Frontiers in Public Health, which is currently calling for manuscripts for a research topic: One Health Surveillance in Practice: Experiences of Integration among Human Health, Animal Health, Environmental Health, and Food Safety Sectors. This research topic has a deadline on the 31st of October 2022.

Additionally, we will share the information provided by our models with public administrations, which will be compiled in their technical reports, aimed for veterinarians, forest rangers, managers and other stakeholders.

Testimonial

I went to RIVM in Bilthoven (The Netherlands) granted by a OHEJP sort term mission, with the aim of analyse some large databases of zoonotic diseases. I had the chance to meet a new research centre and to collaborate with and take advice from experts on the field. I feel that we created a powerful synergy between guest and hosts, since each of us provided different backgrounds and knowledge. Consequently, we obtained results that we could not have obtained working independently. Moreover, not only I carried out an important advance in my research project, but also I acquired new skills that we will use in future projects. I would like to highlight that thanks to my short-term mission I pushed my data analysis skills to an upper level. Additionally, I would strongly recommend taking a short-term mission since it is a great chance to live in a different country for a short, but enough time to learn about its history,

culture and way of living. I truly believe this is a mandatory experience for a scientist, since it helps to have the necessary open mind to to ask, accept and reject hypothesis in an appropriate way.

4.2 Short Term Mission 4: Case study

SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large OHEJP European network Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Application of spatial models to identify new environmental surveillance indicators of Salmonella and Campylobacter in pig and poultry

I had the chance to meet a new research centre and to collaborate with and take advice from experts on the field. We obtained results that we could not have obtained working independently. I carried out an important advance in my research project and acquired new skills that we will use in future projects. I would like to highlight that thanks to my short-term mission I pushed my data analysis skills to an upper level."

Antonio Rodríguez
The National Institute for Agricultural and Food Research and Technology
Spain

Theme: One Health Missions - Skills development missions
Home Institute: The National Institute for Agricultural and Food Research and Technology (INIA), Spain
Mission Hosting Institute: National Institute for Public Health and the Environment (RIMV), The Netherlands
Duration of Mission: 1 month

The aim of this mission was to develop a procedure to model surveillance data of zoonotic diseases using machine learning methods. The model was tested on the Wild Boar Surveillance Plan of Andalucía (Spain) data, with the aim to determine the most important risk factors that shape wild boar diseases in Andalucía and study their interaction between each other.

During this mission, the model validation was performed in two main steps. First, a single response variable was selected for the study: the *Salmonella* serology of hunted wild boars. In the first week of the STM, the data was organised in an appropriate way for training machine learning algorithms. Additionally, climate and wild boar distribution information from published maps were collected, to include them as potential drivers of the pathogens. The results being conclusive, a modelling procedure valid for all the wild boar diseases in the dataset was designed. This includes the processing of the dataset before the modelling (pre-processing step), the model application itself and its validation, and the study of the model output. The best way to compensate the unbalance of the positives and negatives in the dataset was explored, to avoid reducing the sensitivity or specificity of the model. A script was prepared to provide several validation statistics based on the comparison between the predictions on the test data and the real positive and negative values. The variable importance was assessed on the training and test data, partial dependence plots based on these data were created and detection of interactions between predictors on the test data was conducted. The developed procedure successfully determined the most important risk factors that shape wild boar diseases in Andalucía and studied their interaction between each other. Using the data from previous hunting seasons, it also allowed to identify areas with higher and lower probabilities of boar disease and to represent the predictions in a map.

The STM has improved on the relationship between two existing One Health EJP partners and enhanced both current and future collaborations between the partner institutes. Additionally, this procedure has been automatised and can be easily applied to the data from the *Salmonella* and *Campylobacter* surveillance in the food chain by the Spanish Agency of Food Security and Nutrition (AESAN) and the *Salmonella* and *Campylobacter* Spanish surveillance plan in poultry farm.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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#OneHealthEJP #OneHealth #StrongerTogether

Figure 4: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

5. Short Term Mission 5

5.1 Short Term Mission 5: Report

Construction of dual labelled *E. coli* strains to study the effect of antibiotics and microbiota interventions on the horizontal transfer of ESBL genes in the *in-vitro* chicken caecal microbiota.

Name of applicant	Ingrid Cardenas Rey
Institute of Affiliation/	Institute: Wageningen Bioveterinary Research.
Contact information	Address: Houtribweg 39, 8221RA Lelystad, The Netherlands
Host institute and names of scientists involved in STM	University of Copenhagen Department of Veterinary and Animal Sciences One Health Antimicrobial Resistance Research Group (OHAR) Prof. Luca Guardabassi Dr. Mattia Pirolo
Dates of STM	2nd May to 8th July 2022.
Call Topic	Skills development missions.
Research Domain	Antimicrobial Resistance (AMR).
Key aims of STM or Workshop	1) To learn bacterial cloning methods (E.g. the Lambda Red recombineering technique) to produce dual labelled (fluorescent tagging of the chromosome and AMR-gene carrying plasmids) *E. coli* strains. 2) To enhance the research skills and enrich the knowledge of the PhD student. 3) To learn the basic concepts of fluorescence-activated cell sorting (FACS), a specialised type of flow cytometry.
Impact and relevance of scientific mission	The STM had a very positive impact on the PhD competences and skills development. Consequently, the new knowledge and skills acquired are an asset to the PhD project VIMOGUT since these will facilitate the advance and achievement of the project's goals. As for the knowledge and technical skills, the PhD student remarkably developed their bacterial cloning knowledge and skills. Additionally, they acquired knowledge of techniques like FACS, which can be applied to several

	AMR/microbiome research questions. Competencies and skills like interpersonal communication, teamwork, time management, and adaptability to work in new environments were exercised hugely.
Benefits to OHEJP	This short term mission will contribute to further achieving the objectives proposed in the OHEJP PhD project VIMOGUT, which holds high relevance as a priority research and integrative topic. VIMOGUT aims to study the chicken gut microbiome and find interventions that can help to reduce horizontal transmission of Extended Spectrum ß-Lactam (ESBL) genes. The VIMOGUT research aim falls into one of the OHEJP objectives to tackle AMR: "Strengthen infection prevention and control measures, including development and assessment of interventions that prevent the development and spread of AMR". Moreover, this STM was a valuable opportunity to collaborate and exchange knowledge with the One Health Antimicrobial Resistance (OHAR) research group at the University of Copenhagen on innovative alternatives to reduce AMR in a One Health context, which is also one of the highlighted goals of the OHEJP.

Summary

The goal of the Short Term Mission (STM) was to learn bacterial cloning methods (E.g. Lambda Red recombineering) using fluorescent reporter proteins like m-Cherry and GFP to produce dual labelled *E. coli* strains at the University of Copenhagen. Dual labelling (fluorescent tagging of the chromosome and AMR-gene carrying plasmids) of bacteria is a powerful tool to study plasmid-mediated antimicrobial resistance among complex *in- vitro* microbial communities simulated on *in-vitro* gut systems like the chicken caeca.

During her STM, Ingrid acquired experience in molecular cloning techniques and other skillsets which are key to the completion of her OHEJP research project VIMOGUT. VIMOGUT focuses on studying the chicken caecal microbiota and the effect of antibiotics and microbiota interventions on the horizontal transmission of Extended Spectrum ß Lactam (ESBL) genes.

The acquired knowledge and the dual labelled *E. coli* strains produced in this STM will be used in an *in-vitro* chicken caecal model i) to track the spread of ESBL genes among the caecal microbial community and ii) to study the effect of targeted microbiota interventions on the horizontal transmission of ESBL genes.

Finally, the STM opened up significant cooperation channels between the AMR group at Wageningen Bioveterinary Research and the One Health Antimicrobial Resistance (OHAR) research group at the University of Copenhagen to work towards innovative alternatives to reduce AMR in a One Health context, which is also one of the highlighted goals of the OHEJP. The output of the work performed during the STM and upcoming *in-vitro* experiments are expected to be published as a collaborative research article in a scientific journal by the end of 2023.

Technical Report

Antimicrobial resistance (AMR) continues to be a major global concern that threatens public and animal health and endangers the global economy. Despite the extensive efforts undertaken by multidisciplinary groups, resistance levels are still high in bacteria causing foodborne infections, as shown in the European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food for 2018/2019. Horizontal gene transfer (HGT) occurs via plasmids and it is one of the most important transmission mechanisms that contribute to the spread of AMR genes worldwide.

This STM enabled the PhD student to reach the objectives proposed in the OHEJP PhD project VIMOGUT, which holds high relevance as a priority research and integrative topic. VIMOGUT studies the chicken gut microbiota and microbiota interventions to reduce horizontal transmission of Extended Spectrum ß-Lactam (ESBL) genes. Thus, VIMOGUT's aim falls into one of the OHEJP objectives to tackle AMR: "Strengthen infection prevention and control measures, including development and assessment of interventions that prevent the development and spread of AMR".

During this STM the PhD student learned bacterial cloning methods (E.g. Lambda Red recombineering) using fluorescent reporter proteins to produce dual labelled *E. coli* strains at the University of Copenhagen. Fluorescently tagged chromosome and AMR-gene carrying plasmids of commensal *E. coli* strains will further be used to track the horizontal transmission of AMR genes among complex microbial communities like the chicken caeca.

The tasks performed during the STM are described below:
Five commensal ESBL *E. coli* strains that originated from broiler caeca and belonging to the collection of The Dutch National Reference Lab (WBVR) were used for the bacterial cloning experiments. All strains should meet the inclusion criteria of i) carrying an ESBL (bla_{CTXM-1} and bla_{SHV-12}) or AmpC β-lactamase (bla_{CMY-2}) genes, ii) the genes must be carried by highly prevalent plasmids in the broilers production like IncI1α plasmid and iii) susceptibility to three antimicrobials: chloramphenicol (CHL), tetracycline (TET) and kanamycin (KAN). Two fluorescent reporter proteins were used for chromosome and plasmid tagging, namely, mCherry and Green fluorescent protein (GFP).

Task 1. Dual labelled strain construction - Insertion of the m-Cherry fluorescent red marker into the bacterial chromosome

Chromosomal tagging of wild-type and control lab strains was attempted using the vector (plasmid) pGR636-CHL^R. This vector carried a gene cassette *CHL^RlacIq-pLpp-mCherry* encoding the mCherry protein, and a chloramphenicol resistant marker. The gene cassette was inserted into the bacterial chromosome via two different methods: i. Using the transposon Tn7 and ii. Using the Lambda Red recombineering system.

a. Transposon Tn7

Transposon Tn7 has a site-specific mode of insertion and a unique attachment at the attTn7 chromosomal site. This method was employed because it is known to have good efficiency; transposon Tn7 inserts at a very high frequency into a single conserved site that is present in most bacteria. The carried experimental workflow is shown in Figure 1.

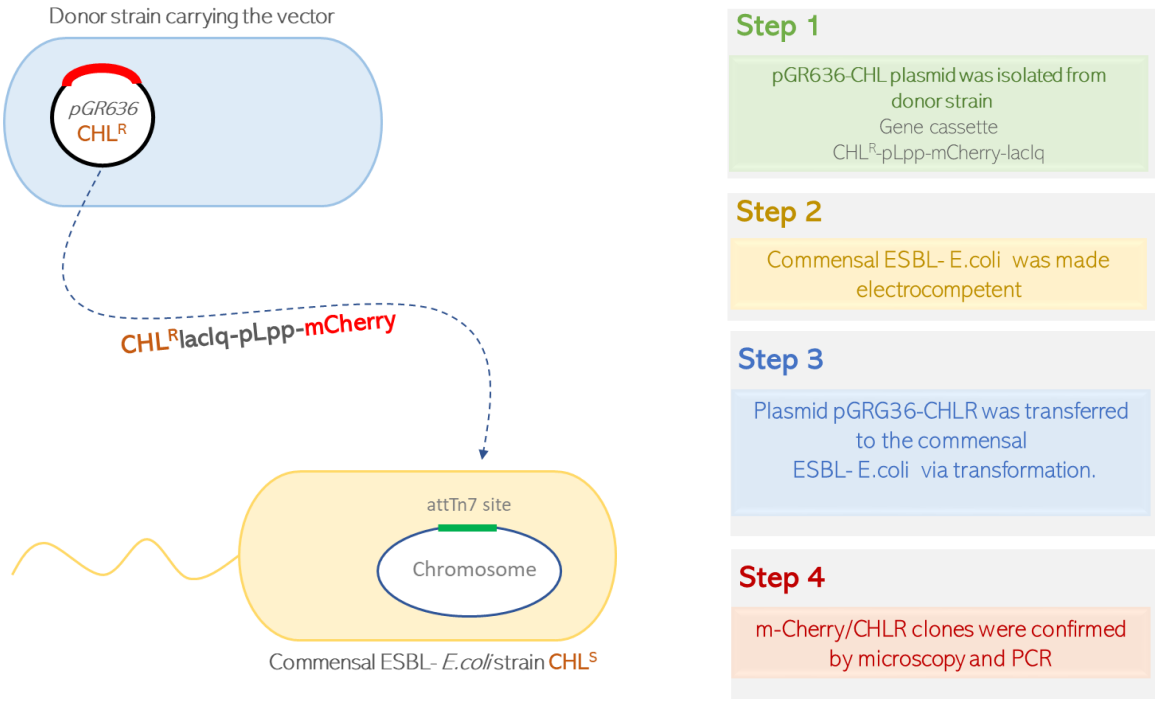

Figure 1. Experimental workflow for the insertion of the m-Cherry fluorescent red marker into the bacterial chromosome using the Tn7 transposon.

Results and conclusions: Despite transposon Tn7 inserting at a very high frequency into the attTn7 site which is present in most bacteria, none of the ESBL- *E. coli* strains acquired the m-Cherry fluorescent tag in their chromosome using this method. A possible explanation for these results is the presence of restriction-modification systems in the strains which actively degrade foreign DNA. Two solutions proposed for future experiments are the *in-vitro* methylation of the gene cassette before transforming it into the bacterial cell or genome analysis of the candidate strains to predict the presence of a restriction-modification system. To ensure that the technique was performed correctly, lab strains with mutations in the restriction system (DH5α) were also included in the experiments as controls. Control DH5α strains acquired the m-cherry tag successfully (see figure 2).

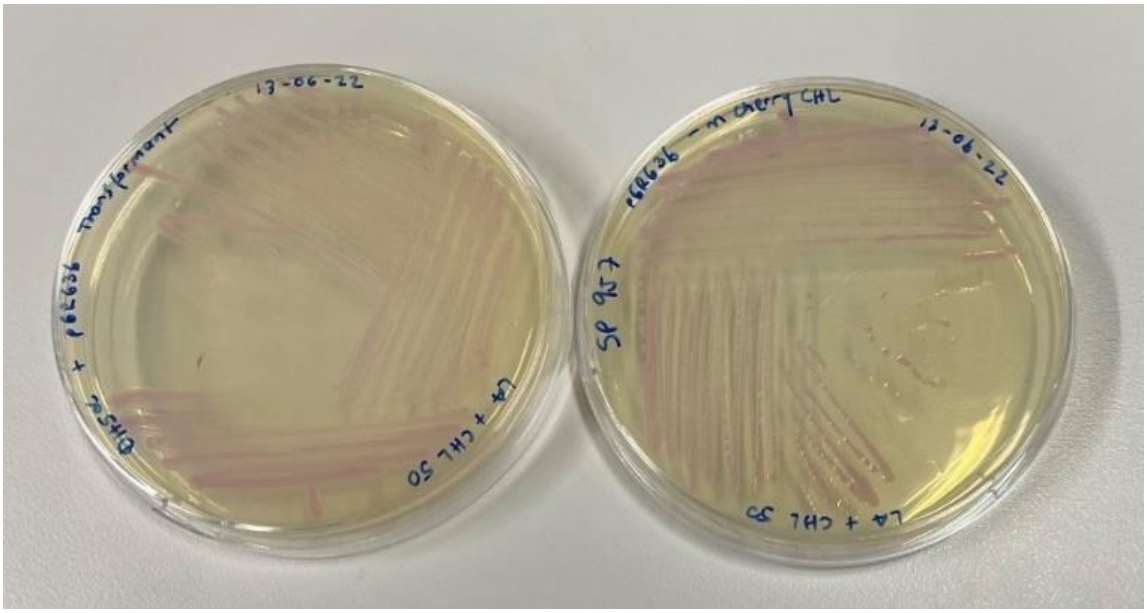

Figure 2. DH5α (left) and pGR636-CHLR donor (right) strains showing pink colonies which confirm the presence of m-cherry fluorescent protein.

b. Lambda Red recombineering system

The lambda Red system uses three proteins from the Lambda bacteriophage to facilitate homologous recombination between foreign DNA (in our case, the gene cassettes carrying the fluorescent proteins of our interest; m-Cherry and GFP) and the host bacterial genome (e.g. the chromosomal pseudogene *ybeM* and IncI1α plasmids of the ESBL *E.coli* strains). A summary of the steps followed during the experimental work is shown in Fig. 3 for m-Cherry chromosomal tagging and Fig. 4 for GFP plasmid tagging.

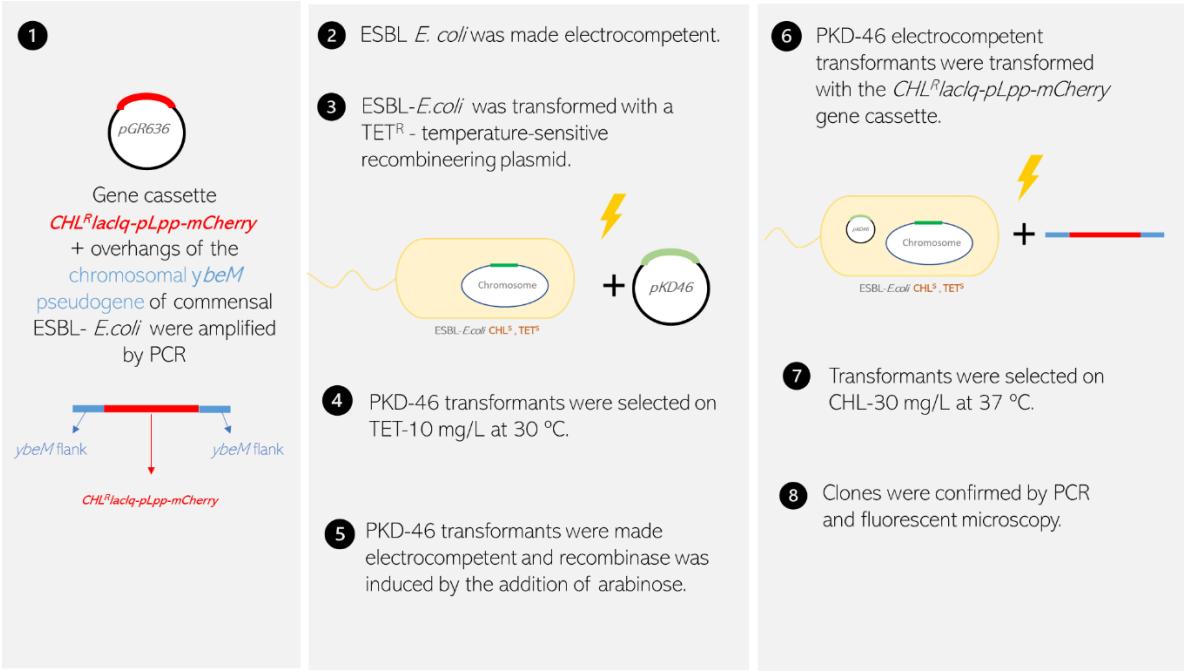

Figure 3. Experimental workflow carried out for the insertion of the *m-Cherry* fluorescent red marker into the bacterial chromosome using the Lambda Red recombination system.

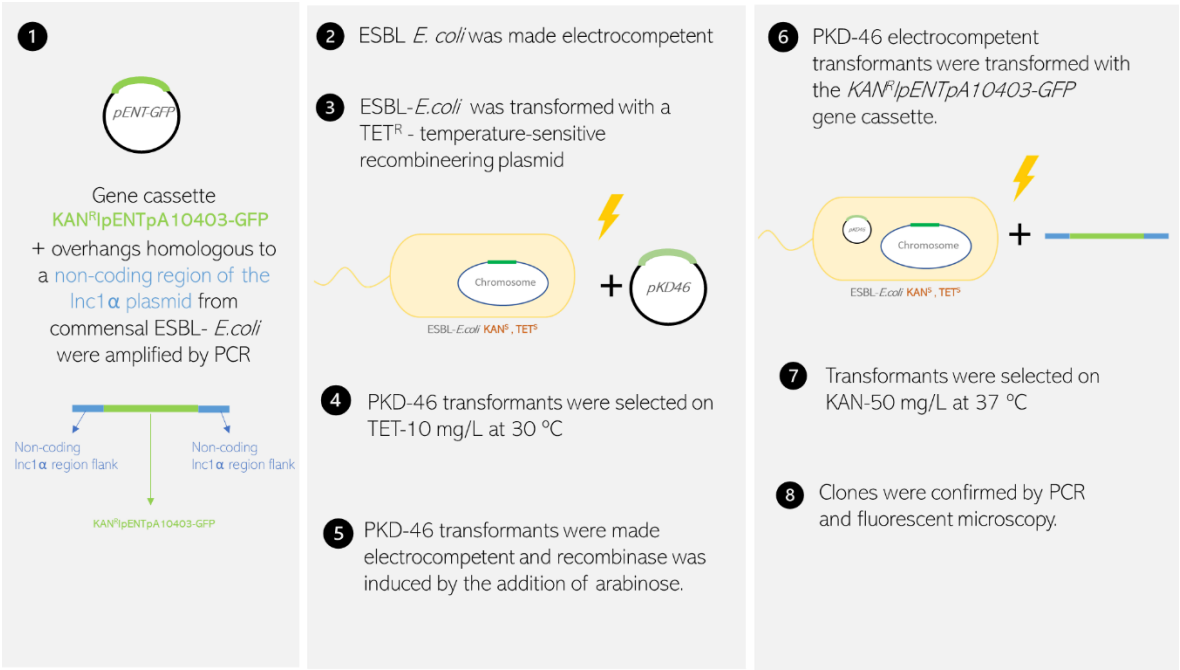

Figure 4. Experimental workflow carried out for the insertion of the *GFP* fluorescent green marker into the non-coding region of the IncI1α plasmids using the Lambda Red recombination system.

Results: Steps 1-4 were successfully achieved both for m-Cherry and GFP tagging; all ESBL-*E.coli* strains successfully acquired the recombinant plasmid PKD46 needed for the insertion of the gene cassette carrying the fluorescent protein. Despite multiple attempts, team discussions and optimisation of the cloning protocols, positive results were not obtained after the bacterial transformation (steps 6-8). Non-fluorescent clones were recovered. Possible reasons that explain these results are i) Lambda red recombineering is a powerful tool to modify bacterial genome but it has low efficiency, the recombination frequency of gene insertion is on average ~5 x 10^{-4} per viable cell; ii) Similarly as described above, the presence of strong restriction-modification systems in the ESBL-*E.coli* strains. Previous studies have reported that the presence of endogenous nucleases can potentially hinder recombination. With these concepts in mind, the PhD plans to repeat the experimental work at WBVR and apply additional techniques like DNA *in vitro* methylation, bacterial genome analysis to detect and remove endonucleases and using different Red protein expression plasmids like pSIM6.

After additional troubleshooting lab work, the PhD student expects to obtain fluorescent clones like the ones shown in Fig. 5 from dual labelled ESBL- *E.coli* control cells.

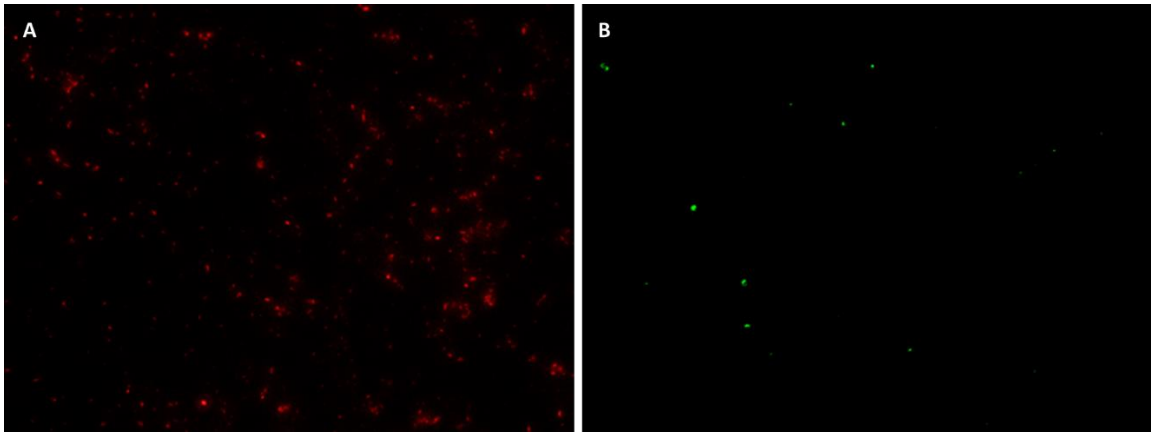

Figure 5. Control ESBL. *E. coli* fluorescent strains under an inverted widefield fluorescent microscope Axio observer 7 (ZEISS, Germany) using appropriated filters to visualise m-cherry and GFP expression. **A** m-Cherry labelled control ESBL- *E.coli* cells expressing red fluorescence. **B** GFP labelled control ESBL-*E.coli* cells expressing green fluorescence. Images were taken at Wageningen Bioveterinary Research after the completion of the STM to confirm the viability of the cells after transportation. Control cells were obtained as a courtesy of the OHAR group at the University of Copenhagen.

Task 2. Double labelled strain growth test and FACS analysis:

Due to technical issues, these two activities could not be fully carried out as proposed. However, the PhD student acquired strong theoretical knowledge of the techniques/analyses and had the opportunity to discuss and maintain contact with experts on the topic. These two analyses are crucial for the completion of future *in vitro* experiments, thus, the practical part will be carried out in the facilities of Wageningen Bioveterinary Research and Wageningen Univerisity in the upcoming months with the dual labelled strains produced and acquired during the STM.

Final conclusions:

- The main goal of the STM was successfully achieved. The PhD student learned the bacterial cloning techniques needed for the completion of future *in vitro* experiments and developed and strengthened her lab skills. Moreover, the PhD student gained extra confidence in designing experiments and working independently and cooperatively which is a key competence in the research field.

- Engineered ESBL-*E.coli* strains were successfully transported to WBVR to continue with the molecular work and further analysis will be performed on them in the upcoming weeks to pave the start of the *in vitro* experiments. Dual labelled strains are an asset for the group's research because they allow addressing several relevant research questions in the domain of AMR.

- Bacterial cloning is a challenging research area that requires time and repeatability of the experimental work. Technical issues and challenges were encountered during the

whole process which positively enhanced and enriched the skills and knowledge of the PhD student.

- The STM opened up significant cooperation channels between the AMR group at Wageningen Bioveterinary Research and the One Health Antimicrobial Resistance (OHAR) research group at the University of Copenhagen. The output of the work performed during the STM and upcoming *in vitro* experiments are expected to be published as a collaborative research article in a scientific journal.

List of dissemination and communication activities

Name of the activity:	Poster carousel, Microbial ecology group.		
Date:	19-24 June 2022		
Place:	Nunspet, The Netherlands		
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories			
	Yes / No		*Yes / No*
Organisation of a Conference		***Participation to a Conference***	
Organisation of a Workshop		***Participation to a Workshop***	Yes
Press release		***Participation to an Event other than a Conference or a Workshop***	
Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	Yes
Training		***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categories			
	Number		*Number*
Scientific Community (Higher Education, Research)	**50**	***Media***	
Industry		***Investors***	
Civil Society		***Customers***	

General Public		***Other***	
Policy Makers			

Name of the activity:	OHEJP side event "Lessons Learnt from a European Multidisciplinary Initiative" ONE2022 Conference		
Date:	22 June 2022		
Place:	Brussels, Belgium		
Specify the Dissemination and Communication activities linked to the One Health EJP project for each of the following categories			
	Yes / No		*Yes / No*
Organisation of a Conference		***Participation to a Conference***	Yes
Organisation of a Workshop		***Participation to a Workshop***	
Press release		***Participation to an Event other than a Conference or a Workshop***	
Non-scientific and non-peer-reviewed publication (popularised publication)		***Video/Film***	Yes
Exhibition		***Brokerage Event***	
Flyer		***Pitch Event***	
Training		***Trade Fair***	
Social Media		***Participation in activities organized jointly with other H2020 projects***	
Website		***Other***	
Communication Campaign (e.g. Radio, TV)			
Specify the estimated number of persons reached, in the context of this dissemination and communication activity), in each of the following categories			
	Number		*Number*
Scientific Community (Higher Education, Research)	***>150***	***Media***	
Industry		***Investors***	
Civil Society		***Customers***	
General Public		***Other***	
Policy Makers			

Scientific outputs

The acquired knowledge and dual-labelled *E. coli* strains constructed in this STM will be used in *in-vitro* experiments to study the horizontal transmission of ESBL genes in the chicken

caecal microbiota. The results of these experiments are expected to be published as a research article in a scientific journal or presented at scientific meetings or conferences. The manuscript is expected to be under peer review or published by the summer of 2023.

Testimonial

A short term mission should be a must-do for all young researchers! It was a priceless experience that enriched my knowledge and skillsets and expanded my network. Working in another lab with young researchers from around the world boosted my creativity and widened my perspective on science. I built up and exercised my lab skills, critical thinking and cooperation in a multidisciplinary group.

I am very grateful to the OHAR group at the University of Copenhagen. I enjoyed it to the fullest every single day! I was challenged daily with new knowledge and experimental work but also strongly supported by a wonderful work team!

5.2 Short Term Mission 5: Case study

SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large
- OHEJP European network
  Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Construction of dual labelled E. coli strains to study the effect of antibiotics and microbiota interventions on the horizontal transfer of ESBL genes in the in vitro chicken caecal microbiota

Theme: One Health Missions - Antimicrobial Resistance (AMR)
Home Institute: Wageningen Bioveterinary Research (WUR), The Netherlands
Mission Hosting Institute: University of Copenhagen, Denmark
Duration of Mission: 2 months

A short term mission should be a must-do for all young researchers! It was a priceless experience that enriched my knowledge and skillsets and expanded my network. I built up and exercised my lab skills, critical thinking and cooperation in a multidisciplinary group. I was challenged daily with new knowledge and experimental work but also strongly supported by a wonderful work team!"

Ingrid Cardenas Rey
Wageningen Bioveterinary Research, The Netherlands

The aim of this mission was for the PhD student to learn bacterial cloning methods using fluorescent reporter proteins, to produce dual labelled *E. coli* strains. Dual labelling (fluorescent tagging of the chromosome and AMR-gene carrying plasmids) of bacteria is a powerful tool to study plasmid -mediated antimicrobial resistance among complex *in vitro* microbial communities simulated on *in vitro* gut systems like the chicken caeca. This STM enabled the PhD student to reach the objectives proposed in the OHEJP PhD project VIMOGUT, which studies the chicken gut microbiota and microbiota interventions to reduce horizontal transmission of Extended Spectrum ß-Lactam (ESBL) genes.

During this mission, five commensal ESBL *E. coli* strains that originated from chicken broiler caeca and belonged to the collection of The Dutch National Reference Lab were used for bacterial cloning experiments. All strains carried an ESBL (blaCTXM-1 and blaSHV-12) or AmpC β-lactamase (blaCMY-2) gene on plasmids highly prevalent in the broilers production and were susceptible to three antimicrobials. Two fluorescent reporter proteins were used for chromosome and plasmid tagging, namely, mCherry and Green fluorescent protein (GFP). Bacterial cloning is a challenging research area that requires time and repeatability of the experimental work. Technical issues and challenges were encountered during this multi-step process. However, the PhD student learned the bacterial cloning techniques needed for the completion of future *in vitro* experiments and developed and strengthened her lab skills.

The STM opened significant cooperation channels between the AMR group at Wageningen Bioveterinary Research and the One Health Antimicrobial Resistance (OHAR) research group at the University of Copenhagen. The output of the work performed during the STM, and upcoming in vitro experiments are expected to be published as a collaborative research article in a scientific journal.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 5: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

6. Short Term Mission 6

6.1 Short Term Mission 6: Report

Microbiological and molecular techniques for *Brucella* identification.

Name of applicant	Albena Dimitrova Angelova Gergana Mateva Mihail Vladimirov Milanov
Institute of Affiliation/ Contact information	Institute: NDVRI. Address: Bul "Pencho Slaveykov 15", 1606, Sofia, Bulgaria.
Host institute and names of scientists involved in STM	ANSES, French Agency for Food, Environmental and Occupational Health & Safety Dr Claire Ponsart Dr Luca Freddi Dr Guillaume Girault
Dates of STM	10th to 16th October 2022.
Call Topic	Skills development missions.
Research Domain	Emerging Threats - Zoonotic Bacterial Diseases.
Key aims of STM or Workshop	1. Theoretical presentation: critical steps for *Brucella* culture. 2. *Brucella* culture (Demonstration and practice). 3. *Brucella* typing (Demonstration). 4. Theoretical presentation: principles and main molecular approaches, protocols. 5. Practical demonstration of initial steps :preparation of samples, inactivation and DNA extraction. 6. Practical demonstration of Real Time PCR : preparation of mix, distribution of mix and DNA into plates, PCR step. 7. Practical demonstration of Suis Ladder : Mix preparation and amplification steps, Gel preparation. 8. Practical demonstration of HRM PCR : preparation of mix, distribution of mix and DNA into plates, PCR step. 9. Practical demonstration of Suis Ladder : distribution of PCR reaction on gel, electrophoresis. 10. Molecular epidemiology : main applications, perspectives.

	11. Critical points of each method Reading / Interpretation of results. 12. Discussion - Evaluation/administrative issues.
Impact and relevance of scientific mission	The STM focused on a standardised approach to working with potentially infectious materials, including Biosafety/Biosecurity training consistent with CDC/USDA and OIE standards, training in microbiological techniques, and development of a consistent approach to phenotypic testing such as biovar determination using standard biochemical and microbiological tests.
Benefits to OHEJP	The short term mission (STM) was conducted under one of the One Health European Joint Programme (OHEJP) projects, IDEMBRU. The developed skills were applied to samples, obtained for the purpose of the project.

Summary

A primary concern is adhering to rigorous biosafety standards while handling lab-based, animal and environmental samples from brucellosis cases. Brucellosis is among the leading lab-acquired infections, so comprehensive biosafety training is paramount. The training was focused on a standardised approach to working with potentially infectious materials, including Biosafety/Biosecurity training consistent with CDC/USDA and OIE standards, training in microbiological techniques, and development of a consistent approach to phenotypic testing such as biovar determination using standard biochemical, microbiological tests and molecular diagnostics and typing. A one-week session was organised at ANSES during the last quarter of 2022. The training was done in France, because it is the only facility within our OHEJP IDEMBRU group where participants from all countries can be accommodated in BSL3 settings. The training demonstrated OIE standards, focusing on microbiological and molecular techniques for *Brucella* identification from strains. Standardised protocols and biosafety operating procedures were explained and demonstrated including culturing from different sample types, *Brucella* genus and species identification, and molecular typing (Bruce-ladder, MLVA-16, qPCR). Practical demonstrations were performed at each step and each participant had the opportunity to partake in the training session. After each practical demonstration there was enough time to answer questions regarding the workflow of each method. Also part of the STM were theoretical presentations that highlighted the importance of the methods and critical steps in performing them were given special attention.

Technical Report

Day 1 – On the first day we arrived by plane in Paris, France and proceeded to the hotel in Maisons-Alfort, in which we stayed for the remainder of the STM training.

Day 2 – In the morning on the second day, after a welcome, in which we met the team, responsible for the training and introduced ourselves, we overviewed the agenda for the training which would take place in the following days. This overview was done as a means of a round table, which allowed both parties to communicate better and clear out any questions that may have arisen beforehand.

After the round table we proceeded with the first task : Theoretical presentation: critical steps for *Brucella* culture. A throughout presentation on the methods of *Brucella* culturing (plating, media, strain handling, etc.) was carried out by Dr. Ponsart, Dr Freddi and Dr Girault. The most important parts were highlighted and given extra attention, so that we can better understand the importance of handling a Biosafety 3 pathogen, such as *Brucella*.

After finishing with the first task the second one was initiated: *Brucella* culture (Demonstration). A practical application of all safety measures mentioned in the first task (such as the necessary layers of clothing, face and head apparel (masks, hairnets) and how to handle ourselves in Biosafety level 3 laboratory were done before entering the laboratory. Dr Luca Freddi was the tutor for this task and before lunch he explained and demonstrated the microbiological aspects such as sample handling and plating of *Brucella spp*.

After lunch, for the duration of the afternoon we again returned to the laboratory and under the supervision of Dr Freddi and his assistant we took an active role in isolating *Brucella* bacteria from tissue samples and plating on the specific media. This practice was part of the second task: *Brucella* culture (Demonstration and practice) Both of our tutors were kind enough to help us with any difficulties that arose and answered any questions.

Day 3 – The third day begun with a rapid overview of day 2. There were discussions on specific points and questions and answers session was carried out. After that we focused on the third task: *Brucella* typing (Demonstration) which was done as a 2-part training (before and after lunch). We were introduced to the methods for *Brucella* typing as an after-isolation practice and the ways that we can implement it in our laboratory. After the training there was a discussion panel and after that the team evaluated any administrative issues that could occur in the process of implementation.

Day 4 – The fourth day begun with a rapid overview of the agenda. It was performed by holding a round table, in order to see if all topics up until this day were taken care of. The remaining topics on which the training would focus were discussed and any questions were answered. After a short break, the host team gave a presentation, titled Principles and main molecular approaches, protocols of testing (task 4: Theoretical presentation: principles and main molecular approaches, protocols). After that, Dr Girault and his team conducted a practical demonstration of the initial steps of molecular testing in his laboratory (task 5 : Practical demonstration of initial steps : preparation of samples, inactivation and DNA extraction). This involved preparation of samples, inactivation and DNA extraction. We were given the opportunity to repeat these steps and to familiarise ourselves with all the specifics when preparing samples for molecular testing. After lunch the second part of the training (task 6 : Practical demonstration of Real Time PCR: preparation of mix, distribution of mix and DNA into plates, PCR step) took place in the same laboratory. We were introduced to the apparatus and protocols, required to perform effective RT-PCR. Discussions of implementing said protocols in the laboratories in NDRVI were also brought out and commented on. The training for day 4 ended with the last part - Practical demonstration of Suis Ladder: mix preparation, amplification steps and gel preparation, which was part of task 7: Practical demonstration of Suis Ladder: Mix preparation and amplification steps, Gel preparation.

Day 5 – The fifth day began with a practical demonstration of HRM PCR. With careful supervision our team prepared the mix, distributed it together with the isolated DNA into plates

and performed the PCR. Again, this was per task 8: Practical demonstration of HRM PCR: preparation of mix, distribution of mix and DNA into plates, PCR step. After this task, before lunch we participated into the planned practical demonstration of Suis Ladder: distribution of PCR reaction on gel, electrophoresis (task 9) and immediately after that there was a presentation titled Molecular epidemiology: main applications, perspectives (task 10). At 14:00 we had a round table, in which we discussed the critical points of each method: things to look-out for, important steps, etc. We interpreted the results, obtained from the previous day with a following discussion and again critical control points. The day ended with yet another round table in which evaluation and administrative issues were considered (tasks 11 and 12).

Day 6 – The sixth day marked the end of the training. After a final round table with the team and planning for future collaborations/trainings we left the institution (ANSES)

Day 7 – The seventh day saw our departure (via airplane) to Bulgaria.

List of dissemination and communication activities

Not applicable.

Scientific outputs

Not applicable.

Testimonial

Having been recommended to finance the training of our team in ANSES (Paris, France) as a STM program (part of an OHEJP project) was a very easy and straightforward experience. Having heard nothing but positive opinions and review I was amazed at the very pleasant, down to earth communication with the OHEJP/STM team. The help with sorting out the required documents and quick response times were nothing but a plus to an already pleasant experience.

The work and acquired skills during the STM helped developed a strong foundation when working with microbiological and molecular methods when working with potentially infectious bacteria (such as *Brucella*). I discovered the STM through a recommendation from a colleague in ANSES. The people that we interacted and learned from (the EURL *Brucella* team) I have met previously and had already good relations with them. Nevertheless the experience and new knowledge that I have gained through the STM were very valuable and helped broaden our expertise regarding *Brucella* diagnostics. ANSES is an institution I was familiar with from my previous visits, but meeting new people and going into facilities, such as a state of the art BSL 3 laboratories helped our team understand better the specific requirements and caution that needs to be exercised when dealing with dangerous pathogens.

The experience gained from the STM first and foremost would help our institute to finish successfully our tasks in the IDEMBRU project, which we are part of. Also disseminating that knowledge to other colleagues would further help and enhance everyday tasks in our laboratories.

6.2 Short Term Mission 6: Case study

Microbiological and molecular techniques for Brucella identification

Theme:	One Health missions
Home Institute:	NDVRI, Bulgaria
Mission Hosting Institute:	ANSES, France
Duration of Mission:	1 week
STM Researchers:	Albena Dimitrova Angelova Gergana Mateva Mihail Vladimirov Milanov

The STM was an amazing experience and has provided newfound knowledge regarding the skill and requirements for microbiological manipulation and molecular diagnostics, especially when working with potentially infectious bacteria, such as Brucella. Disseminating this knowledge to other colleagues will further help and enhance everyday tasks in our laboratories"

Albena Dimitrova Angelova, NDVRI, Bulgaria

The aim of this mission was for the three researchers to learn standardised microbiological and molecular techniques, including DNA extraction and real-time PCR, for the identification of *Brucella spp*. As Brucellosis is the among the leading lab-acquired infections, learning effective biosafety/ biosecurity whilst working with the causative agent is paramount. This STM enabled the researchers to complete objectives in the OHEJP IDEMBRU project, which studies the zoonotic potential, virulence and persistence markers in isolated strains of *Brucella spp*.

During this mission, theoretical training was provided by the host institute on the critical steps for *Brucella* culture followed by principles of molecular approaches. Prior to the researchers undertaking any laboratory activities, practical demonstrations on bacterial isolation, culture and typing were provided, followed by demonstrations of molecular techniques including DNA extraction, real-time PCR protocols, gel electrophoresis and High-Resolution Melting PCR. Each day, round table discussions provided additional insight into each step of the training. A final presentation on molecular epidemiology afforded further knowledge on applications, perspectives and interpretation of data. The researchers strengthened existing and developed new laboratory skills that will assist their future research.

The STM provided valuable training, allowing for the isolation and identification of *Brucella spp.*; further experimentation will be performed on *Brucella spp.* strains, under rigorous biosafety standards, at the NDVRI laboratories in Bulgaria.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 6: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

7. Short Term Mission 7

7.1 Short Term Mission 7: Report

Tolerance of biofilm forming bacteria to disinfectants after repeated disinfectant exposure.

Name of applicant	Emma Brook
Institute of Affiliation/	Animal and Plant Health Agency
Contact information	Address: Animal and Plant Health Agency, Woodham Lane, New Haw, Addlestone, Surrey, KT15 3NB
Host institute and names of scientists involved in STM	Norwegian Veterinary Institute Ane Mohr Osland; Lene Karine Vestby
Dates of STM	25th April to 13th May 2022.
Call Topic	One Health Missions- Veterinary, Food, Medical and or Environmental research
Research Domain	Foodborne Zoonosis.
Key aims of STM or Workshop	To examine what happens to bacteria that survive disinfectant treatment, due to the protective measures of biofilms, by repeated exposure to disinfectants of bacteria in biofilm.
Impact and relevance of scientific mission	Methods for disinfectant efficacy testing against planktonic foodborne bacteria are well established, yet methods for efficacy of disinfectants for biofilms are still novel, particularly the effect of repeated exposure to disinfectants on the survivability of bacteria in biofilms. This STM will promote conformity of current test methods and models used in biofilm and disinfectant testing. Knowledge gained could be used in the application of disinfectants for the control of foodborne pathogens, and management of disease outbreaks.
Benefits to OHEJP	The STM has improved on the relationship between two existing EJP partners and enhanced both current and future collaborations between the partner labs. The STM has supported research in which both labs are currently working on together (OHEJP BIOPIGEE), through improving working arrangements and understanding of methods.

Summary

Methods for disinfectant efficacy testing against planktonic foodborne bacteria are well established, yet methods for efficacy of disinfectants against biofilms are still novel, particularly the effect of repeated exposure to disinfectants on the survivability of bacteria in biofilms. Bacteria within biofilms are less susceptible to biocide due to the protective matrix. This study aimed to establish the effect of repeated disinfectant exposure on the tolerance of biofilm forming bacteria.

Monoculture biofilms of *Staphylococcus aureus* (1323-323; isolated from milk from a goat with clinical mastitis) were grown on sterile glass slides, before being exposed to 0.5%, 1%, and 1.5% of Benzalkonium chloride (BAC) disinfectant. A control group was run alongside, exposing biofilm to sterile physiological water. Following neutralisation of the disinfectant, biofilm was removed via mechanical action and plated for enumeration. Persisting bacteria, from each condition, were isolated to prepare next-generation biofilms. These were then exposed to further disinfection using the same concentration to which they were previously exposed. This was repeated over nine consecutive experiments. Each experiment obtained a mean starting point of >7 Log10 bacteria/biofilm on the control coupons. The Log10 reduction in bacteria/biofilm was calculated for each disinfectant concentration, for each experiment. Paired t-tests showed statistically significant differences for 0.5% BAC in experiments three, four, five, six and eight ($p = <0.001$, $p = 0.042$, $p=0.006$, $p=2.608e-06$, and $p=0.020$, respectively), at 1% BAC in experiments six and seven ($p = <0.001$ and $p=0.037$, respectively), and 1.5% BAC in experiment six ($p=6.021e-05$). However, One-Way ANOVA's, followed by Dunnetts multiple comparison test, showed significance only in experiment six.

The results of this study have shown that increased tolerance to biocides by biofilm forming bacteria can occur. However, conflicting statistical results suggest the need for further investigation. Tolerance, indicated by decreased Log10 reduction in bacteria/biofilm, is not consistent between concentrations of BAC nor across consecutive experiments. This variation in results requires further investigation. Future studies should expand on the data collected in this study, to help develop knowledge on the role of biofilm producing bacteria in the dissemination of biocide resistance and factors affecting the variability in biofilm behaviour.

Technical Report

Introduction

The use of biocides as disinfectant is important in the prevention and control of the transmission of food-borne zoonosis. However, tolerance to biocides is increasingly observed. Bacteria which persist after ineffective biosecurity measures, such as poor cleaning and disinfection protocols, contribute to the dissemination of biocide resistance (Smith, Gemmell et al. 2008, Moretro, Schirmer et al. 2017, Nordholt, Kanaris et al. 2021, Fernandes, Gomes et al. 2022). Bacteria commonly persist after cleaning due to biofilms (Fernandes, Gomes et al. 2022). Biofilms form when bacteria encase themselves in a self-produced extracellular polysaccharide and protein matrix, and often adhere to surfaces. Bacteria within biofilms are less susceptible to biocide exposure than planktonic bacteria (Peng, Tsai et al. 2002) due to the protective matrix (Wales, Gosling et al. 2021). Difficulty in removing biofilms from various surfaces, which are hard to reach, is a major issue in the food industry (Vestby, Moretro et al.

2009, Giaouris, Heir et al. 2014). Whilst mechanical removal of the biofilm can improve the efficacy of disinfection protocols, some bacteria may persist and contribute to the spread of biocide resistance. Consequently, investigation into the prevalence and dissemination of biocide tolerance via biofilms is becoming increasingly prioritised. This study aimed to examine what happens to bacteria that survives disinfectant treatment, due to the protective measures of biofilms, after multiple repeated exposures to disinfection treatment.

Method

The method follows Vestby et al. (2015) with slight modifications.

Biofilm formation

The isolate (1323-323, isolated from milk from a goat with clinical mastitis) was prepared from a cryovial stock culture stored at -80^{0}C, and plated onto blood agar before incubation for 21 ± 3 hours at 37^{0}C ± 1^{0}C. One or more colonies were added to 5ml of sterile physiological saline (SPW) and adjusted to 0.5 McFarland using an optical densitometer. Then 500µl was added to 10ml TSB with 1% glucose and 1% NaCl, before addition of a sterile glass coupon. The suspension with coupon was then incubated at 37^{0}C for 24 ± 2 hours. A mean of 7 Log10 bacteria/biofilm was desired.

Preparation of disinfectant concentrations

A pilot study (data not shown) was performed to establish suitable disinfectant testing concentrations. The pilot examined a range of concentrations based on Vestby, Lonn-Stensrud et al (2010) and Ebrahimi, Hemati et al (2015).

Benzalkonium chloride 50% (BAC) (supplier - 63249 Aldrich, Merck KGaA, Darmstadt, Germany) was diluted in sterile distilled water (SDW) to final concentrations of 0.5%, 1% and 1.5%. Each concentration was performed in technical triplicate and biological duplicate (total of six replicates per concentration) in each experiment. The disinfectant concentrations were made fresh on the day.

Disinfection

Each coupon containing biofilm was separately rinsed of bacterial suspension and loosely adhered biofilm, by dipping three times in approximately 40ml SPW. The coupons were then moved to at least 10ml of disinfectant for a contact time of five minutes. Controls were performed using SPW. Then the coupons were moved to at least 15ml of Dey Engley neutralising buffer for approximately one minute, before the wash step was repeated. Lastly, the coupons were moved to a falcon tube containing 5ml SPW with 20-30 sterile glass beads. Cell scrapers were used to thoroughly remove biofilm from both sides of each glass slide. The coupon was discarded and the tubes vortexed for 40 seconds.

From each sample 200µl was added to the first column of a microtiter plate. Then 20µl of each sample was serial diluted using a multi-pipette in SPW. Following dilution blood agar plates were inoculated and incubated at 37^{0}C for 24 ± 2 hours.

Consecutive experiments

Following incubation, the plates were counted, and the Log10 bacteria/biofilm calculated. For each disinfectant concentration and control, the replicate with the median Log10 bacteria/biofilm was identified, for each biological replicate. The dilution plate used to calculate the cfu/biofilm for the chosen replicate was then used to prepare the test suspension for the next test, as per the steps previously described, and all disinfection steps were repeated.

Isolates were repeatedly exposed to the same concentration of disinfectant over a total of 9 experiments.

Validation checks

Validation controls were prepared by placing washed biofilm into 20ml SPW for 1 minute. The coupons were then moved to a falcon tube containing 5ml SPW with 20-30 sterile glass beads. A cell scraper was used to remove the biofilm, and the coupon discarded. The tube was vortexed for 40 seconds before the sample was diluted to 10-5 in SPW. The dilutions were plated on blood agar and incubated at 37^{0}C for 24 ± 2 hours.

Neutraliser toxicity validations (NTV) were performed, in duplicate, to ensure the neutraliser was non-lethal towards the organism. Washed biofilm were placed into 25ml Dey Engley neutraliser for 1 minute. The coupons were then moved to falcon tubes containing 5ml SPW with 20-30 sterile glass beads, and a cell scraper was used to remove the biofilm. The tubes, minus the coupon, were vortexed for 40 seconds before the dilution to 10-5 in SPW. Each dilution was plated onto blood agar and incubated at 37^{0}C for 24 ± 2 hours. The neutraliser was deemed not toxic if the counts were >0.5 of the validation control plate counts.

Disinfectant neutralisation validations (DNV) were performed, in duplicate, to ensure the neutraliser could inactivate the disinfectant. 1ml of the 6% disinfectant was added to 25ml neutraliser for 1 minute. After the contact time, the washed biofilm was placed into the neutraliser for a further 1 minute. The coupons were then moved to falcon tubes containing 5ml SPW with 20-30 sterile glass beads, and a cell scraper was used to remove the biofilm. The tubes, minus the coupon, were vortexed for 40 seconds before the dilution to 10-5 in SPW. Each dilution was plated onto blood agar and incubated at 37^{0}C for 24 ± 2 hours. The neutraliser was deemed effective at inactivating the disinfectant if the counts were >0.5 of the validation control plate counts

Statistical Analysis

Data were analysed using Microsoft Excel and RStudio. The mean and standard deviations (SDs) for all samples were calculated. Paired t-test's were run for each concentration. Data sets were assessed for homogeneity of variance with Levene's test and for normality using Shapiro-Wilk test. The three disinfectant concentrations were also evaluated by One-Way ANOVA, and Dunnett's multiple comparison post-hoc test. The control group was evaluated by Kruskal-Wallis rank sum test. Statistically significant differences were established for a probability of 5% ($p<0.05$).

Results

Validation checks

The plate counts for both the NTV and DNV were >0.5 of the control plate count. Confirming that the neutraliser was not toxic to the *S. aureus* and capable of neutralising the disinfectant.

Biofilm Growth

Each experiment obtained a mean >7 Log10 bacteria/biofilm on the control coupons.

Homogeneity of variance was confirmed using the Levene's test ($p=0.520$). The Shapiro-Wilk normality test showed the data to be not normally distributed ($p=7.652e-05$). Thus, the Kruskal-Wallis rank sum test was used to evaluate any significant change in biofilm growth across the nine experiments. No significant difference observed ($p=0.502$).

Log10 Reduction in Bacteria/Biofilm Following Repeat Disinfectant Exposure

The Log10 reduction in bacteria/biofilm observed in the treatment conditions was calculated from the difference between the mean Log10 bacteria/biofilm in the control condition and the Log10 bacteria/biofilm for each replicate, for each concentration. A decrease in the Log10 reduction in bacteria, following repeated exposure, was used as an indicator for increased tolerance.

Figure 1 illustrate the mean Log10 reduction in bacteria/biofilm observed when the biofilm was exposed to 0.5%, 1% and 1.5% BAC disinfectant concentrations, across nine consecutive experiments. The lowest mean Log10 reduction in bacteria/biofilm is observed most frequently at 0.5% concentration, in five out of nine experiments (56%). The highest mean Log10 reduction in bacteria/biofilm is observed at 1.5% concentration, in eight out nine experiments (89%).

Statistical Analysis

Paired T-test was performed to compare the Log10 reduction in bacteria/biofilm seen in the first experiment with each consecutive experiment, for each disinfectant concentration. At 0.5% BAC significance was observed between experiment one and experiments three, four, five, six and eight ($p = <0.001$, $p = 0.042$, $p=0.006$, $p=2.608e-06$, and $p=0.020$, respectively). At 1% BAC significance was observed between experiment one and experiments six and seven ($p = <0.001$ and $p=0.037$, respectively). At 1.5% BAC significance was observed between only experiment one and experiment six ($p=6.021e-05$). To consider the type 1 error rate observed via multiple testing, One-Way ANOVA's were also performed for each disinfectant concentration. The assumption of homogeneity of variance was confirmed via Levene's test (0.5% BAC $p=0.172$, 1% BAC $p=0.186$, 1.5% BAC $p=0.054$), whilst the Shapiro-Wilk normality test was used to confirm normal distribution of data (0.5% BAC $p=0.083$, 1% BAC $p=0.079$, 1.5% BAC $p=0.185$). The One-Way ANOVA's showed significance for all three concentrations (0.5% BAC $p = <0.001$, 1% BAC $p=0.001$, 1.5% BAC $p=0.008$). A Dunnett's multiple comparison post-hoc test was performed using the mean of the first experiment as a control, to compare with the means of the other experiments. Significance was observed between only experiment 1 and experiment 6, in all three concentrations (0.5% BAC $p=8.1e-06$, 1% BAC $p=6e-05$, 1.5% BAC $p=0.006$).

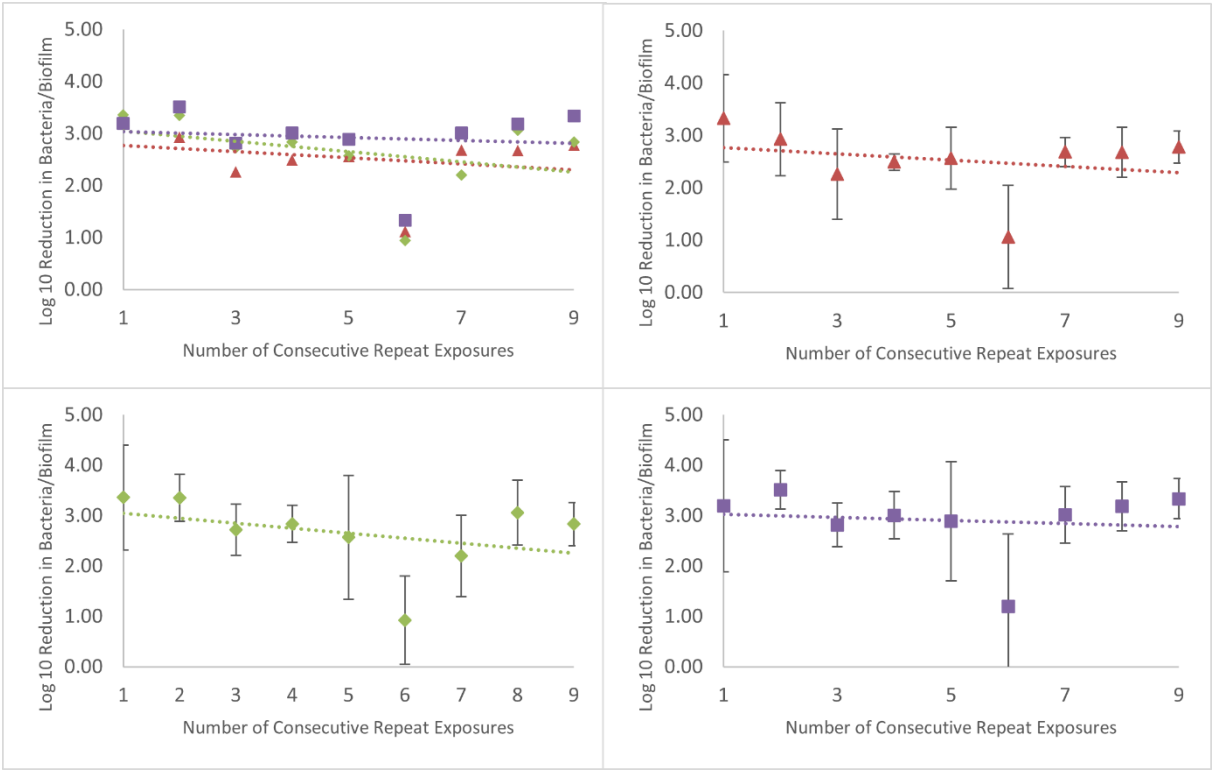

Figure 1 – Top left - Average Log10 reduction in bacteria/biofilm after exposure to 0.5% (red triangles), 1% (green diamonds) and 1.5% (purple squares) BAC disinfectant, with linear trendlines (corresponding colours), over nine consecutive experiments. Top right and bottom two - Average Log10 reduction in bacteria/biofilm after exposure to 0.5% (top – red triangle), 1% (middle – green diamond), 1.5% (bottom – purple square) concentration of BAC disinfectant, with error bars, over nine consecutive experiments.

Discussion

A consistent mean >7 Log10 bacteria/biofilm was successfully obtained across all nine consecutive experiments in the control condition, with no statistically significant variation observed. Thus, repeated isolation and exposure to test procedures did not influence the biofilm forming capabilities of the *S. aureus*.

A paired t-test was performed to compare the mean Log10 reduction in bacteria/biofilm in the first experiment with each consecutive repeated exposure. A significant difference was observed in all experiments except for experiments two, seven and nine at 0.5% BAC concentration. Whilst significance was observed in experiment six for both 1% and 1.5% BAC, as well as in experiment 7 for 1% BAC. Thus, according to the paired t-test, repeated exposure does increase the tolerance of *S. aureus* in biofilm to BAC disinfectant, to varying degrees at different concentrations.

However, the paired t-test does not consider type 1 errors observed due to multiple testing. Consequently, a One-Way ANOVA, followed by Dunnetts multiple comparisons test, was performed for each BAC concentration. For all three BAC concentrations significance was only observed between experiment one and six. Due to the extent to which the p-value for experiment six varies from the other eight p-values and taking into consideration the graphical presentation of the data across all nine experiments (Figure 1), it is assumed experiment six is an anomalous result. Therefore, the One-Way ANOVA's suggest that repeated exposure to BAC disinfectant does not increase the tolerance of S. aureus in biofilm. This supports evidence from Fernandes, Gomez et al. (2022) who showed biofilms regrown from persister cells, following

initial disinfection, produced antimicrobial susceptibility similar to those of the original biofilms, following a singular exposure to disinfectant.
The conflicting statistical results suggest the need for further investigation to establish a strong statistically supported conclusion on whether repeated exposure to biocide increases the tolerance of biofilm forming bacteria.

Further investigation should look at a wider range of disinfectant concentrations and repetitions to provide a more extensive dataset. A slight dose dependent effect was observed in the Log10 reduction in bacteria/biofilm between the three concentrations of BAC disinfectant in this study. This dose dependent effect was expected, however the variation between each concentration was not as pronounced as anticipated based on preliminary testing (data not shown). Inclusion of a wider range of concentrations would provide additional information on the influence of repeated exposure. For example, investigation of a concentration known to produce >4 Log10 reduction could provide knowledge on whether repeated exposure induces tolerance to the extent to which a disinfectant is no longer deemed efficient against bacteria in biofilm (<4 Log10).
Additionally, the biofilm-forming capability of the isolates following exposure and recovery was not established within each experiment. It would be of interest to observe whether any increase in tolerance is a result of increased biomass or biofilm production. Elekhnawy et al. (2021) found excessive exposure of *K. pneumoniae* to BAC disinfectant to lead to enhanced biofilm formation, and associated tolerance to BAC.

Furthermore, additional repeat exposures and an expanded panel of organisms, as well as disinfectants, would be beneficial to the understanding of the increasing emergence of biocide resistance. For example, peracetic acid has been shown to be more effective against biofilms than BAC (Barroso, Maia et al. 2019). Likewise, *Salmonella* has been shown to be more susceptible to QAC and chlorhexidine-based disinfectant, in comparison with *E. coli* and Gram-positive bacteria (Aarestrup and Hasman 2004).
Overall, the results of this study have shown that increased tolerance to biocides can occur in biofilm forming bacteria. However, further investigation is required to understand the variability in tolerance and mechanisms involved, and to help develop improved knowledge on the role of biofilm producing bacteria in the occurrence of biocide resistance, especially in food processing environments.

List of dissemination and communication activities

Not applicable.

Scientific outputs

The study will be presented as a short communication. The journal of choice and date of publish are yet to be decided.

Testimonial

I feel very privileged to have spent three weeks visiting the Norwegian Veterinary Institute. The people I have encountered during my visit have not only openly shared their knowledge and

skills, but also welcomed me with open arms. Everyone was friendly and willing to help, and from day one I felt as if I was part of the team.

Throughout the duration of the trip, both my personal and professional confidence grew. I improved on my communication skills and developed my knowledge surrounding disinfectants and biofilms. The information shared with me, by my colleagues at the Norwegian institute, has been invaluable to my learning and development. I have gained experience in a wider range of methods used in biofilm and disinfectant research, as well as with a species of bacteria which I do not normally work with.

The mission has allowed both parties to exchange a variety of skills and to allow harmonisation of approaches. The methods and techniques learnt will be applied to ongoing and future projects back at my home institute. Likewise, the relationship between the two institutes has only strengthened and enhanced collaboration on both current and future projects. I have thoroughly enjoyed my time at NVI and look forward to maintaining the relationship into the future.

7.2 Short Term Mission 7: Case study

Figure 7: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

8. Short Term Mission 8

8.1 Short Term Mission 8: Report

Metagenomics tools for the study of SARS-COV-2 in animals.

Name of applicant	Carlos Sacristán Yagüe.
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Host institute and names of scientists involved in STM	Departamento de Doenças Infeciosas / Núcleo de Bioinformática, National Health Institute Doutor Ricardo Jorge (INSA). Portugal. Dr. Vítor Borges and Dr. João Gomes.
Dates of STM	6th to 19th November 2022.
Call Topic	Skills development missions.
Research Domain	Emerging threats - Molecular diagnostics.
Key aims of STM or Workshop	The main goal of the present short-term mission was to provide the applicant with the opportunity to expand his training to a higher and more practical level within his field of research by learning the application of metagenomics and complete sequencing within a One Health context. These tools are essential for modern surveillance and diagnostics of infectious diseases, and will be especially useful for the applicant's career development. Additionally, the contact with the colleagues from Portugal was essential to build a professional network and exchange knowledge and experience between the Portuguese and Spanish scientific institutions.
Impact and relevance of scientific mission	One of the knowledge gaps in the SARS-CoV-2 pandemic is understanding the evolution of the infection in animals (pets, minks and wildlife) over time and the epidemiological role they play (e.g. potential reservoirs). The use of metagenomics in animal samples is key to determine whether a virus has mutated during a spill over event. This STM allowed the adaptation of modern metagenomics and bioinformatics tools to our research center. Such tools are valuable because the promote assess to key point, such as whether the SARS-CoV-2 infecting animals has changed as a result to an

	adaptation to a novel host species, but also to infer information regarding this virus' evolution in selected animal populations (e.g. mink farms). Our results will greatly contribute to the current understanding of the SARS-CoV-2 epidemic and its evolution in pets and other animals, one of the main goals of the COVRIN integrative project.
Benefits to OHEJP	The Short Term Mission 2022 "Metagenomic Tools for the Study of SARS-CoV-2 in Animals" will promote the adaptation of modern metagenomics and bioinformatics tools in our research roup (Epidemiology and Environmental Health, Centro de Investigación en Sanidad Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Consejo Superior de Investigaciones Científicas) during 2023. This mission facilitated the collaboration between INIA and INSA, through their common One Health goals, that are also shared by the OHEJP mission. This collaboration will be essential to promote a One Health approach using metagenomics in Spain, and ultimately, in the Iberian Peninsula.

Summary

The short term mission "Metagenomic tools for the study of SARS-CoV-2 in animals" was conducted at the Bioinformatics Unit, Department of Infectious Diseases of the National Health Institute Doutor Ricardo Jorge (INSA), in Lisbon, Portugal, under the supervision of Dr. Vítor Borges and Dr. João.Paulo Gomes. During two weeks, the researcher closely followed the group's daily activities, and performed specific training activities designed to maximize their learning experience.

One of the main activities was getting familiar with the INSaFLUTELE-VIR suite; this easy-to-use open web-based bioinformatics suite was designed for the genomic surveillance of human seasonal influenza and SARS-CoV-2, and adapted to monkey pox virus (now renamed Mpox). This platform was proven to be an excellent surveillance tool during the SARS-CoV-2 pandemic. Aside from the practical and conceptual learning about how to use the platform and interpret the results, the researcher had the opportunity to follow and understand the development of a new module created by the group, designed to simplify metagenomic analyses and to identify new viruses. Carlos also learned about the ReporTree tool, a surveillance-oriented tool to strengthen the linkage between pathogen genetic clusters and epidemiological data through a hands-on training with the complete genetic information of different *Listeria monocytogenes* strains surveyed in Portugal.

Another important part of Carlos' training in Lisbon was the "wet lab". It included the use of nanopore sequencing with MinION for the complete sequencing of SARS-CoV-2 (with a previous amplification step by multiplex PCR), the diagnostics of MPox virus in a clinical sample without previous enrichment, and sequencing of a poliovirus of a case that was being studied by the group. The researcher also had the opportunity of participating in two important scientific and cultural activities celebrated at INSA: the "Young Researcher Day" and the "INSA Day". This short term mission at INSA was key for Carlos' postdoctoral research, especially related to the project COVRIN – focused on SARS-CoV-2 in wild and companion animals, viral discovery and surveillance of emerging infectious agents within

a One Health approach, as well for the creation of a collaborative relationship their institution (Centro de Investigación en Sanidad Animal, Instituto Nacional de Investigación y Tecnología

Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas) and colleagues in Portugal.

Technical Report

The short term mission "Metagenomic Tools for the Study of SARS-CoV-2 in Animals" was conducted at the Bioinformatics Unit - Department of Infectious Diseases of the National Health Institute Doutor Ricardo Jorge (INSA), the National Health Laboratory of Portugal, in Lisbon, Portugal. The mission occurred from November 6th through November 19th, 2022, and was supervised by Dr. João Paulo Gomes and by Dr. Vítor Borges.

1. Getting familiar with INSA

During Carlos' first week at INSA, he got familiar with the National Health Institute Doutor Ricardo Jorge (INSA) environment, installations and personnel, and met the director - Dr. Fernando Almeida, and all the members of Vítor Borges' team at the Bioinformatics Unit – Department of Infectious Diseases. He also met two veterinarians working as virologists (Dr. Sílvia Santos Barros and Dr. Margarida Henriques) of the Instituto Nacional de Investigação Agrária e Veterinária (INIAV), whom were visiting the group at the same time. During this first week Carlos participated in INSA's Young Researcher Day, celebrated on November 8th, 2022. This important activity allowed him to get familiar with the different research lines carried out in the three INSA headquarters in Portugal (Lisbon, Águas de Moura and Porto), such as the genetic surveillance of Monkeypox cases in Portugal, the study of food-borne pathogens, the toxicity of nanomaterials, and an historical review of the Portuguese Science History.

2. Wet lab. First week: Preparation of samples and libraries for next generation sequencing.

The first week Carlos started the "wet lab work" with Dr. Joana Isidro. It included the preparation of samples and libraries for next generation sequencing, and following the next generation sequencing analyses performed at INSA with viruses. The first activity was learning about the SARS-CoV-2 routine genomic surveillance, following by viral metagenomics using the TELEVIR protocol.

2.1. SARS-CoV-2 complete sequencing using nanopore technology

The main modification was that, instead of sequencing the samples with Illumina technology to sequence a large number of samples at a time, only three clinical samples were sequenced with the portable device MinION Mk1B (Oxford Nanopore Technologie), based on nanopore technology. These samples were previously confirmed as SARS-CoV-2 positive by real time PCR. Before sequencing, reverse transcription of the RNA was performed using the LunaScript RT SuperMix Kit (Ref. 174E3010L), New England Biolabs, as follows:

- 2 µL of LunaScript RT SuperMix Kit + 8 µL template RNA, in a final reaction volume of 10 µL.
- Incubation (on a thermocycler): 25 ^{0}C for 2 min, 55 ^{0}C for 10 min, 95 ^{0}C for 1 min and 4 ^{0}C for ∞

Subsequently, the generated cDNAs were submitted to 2 amplification reactions (1 for each primer pool), using the ARTIC v4.1 primers scheme (V4 https://github.com/articnetwork/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V4/SARS-CoV-2.primer.bed; V4.1 (changes to the V4): https://github.com/artic-

network/articncov2019/tree/master/primer_schemes/nCoV-2019/V4.1), and the NEBNext Q5 Hot Start HiFi PCR Master Mix (Ref. 174M0543L), as follows;

- 12.5 µL NEBNExt Q5 HotStart HiFi Master Mix + 3.6 µL Primer Pool (A or B) (10µM) + 6.4 µL Nuclease-free water + 2,5 µL template cDNA, in a final reaction volume of 25 µL.
- Amplification conditions (on a thermocycler): 98 ^{0}C for 30 sec, 35x [98 ^{0}C for 15 sec, 63^{0}C for 5 min], and 4^{0}C for ∞

After the initial amplification step, both amplicon pools of each sample were combined prior to library prep. Subsequently, a clean-up of the amplicons was performed using magnetic beads with the Agencourt AMPure XP, Beckman Coulter, (Catalog #: A63880), using equal volume (1:1) of SPRI beads:sample.

Finally, the flow cell was cleaned with the Flow Cell Wash Kit (EXP-WSH004) – ONT and the three samples were sequenced with the MinIOn as described in the the ARTIC nCoV-2019 sequencing protocol v3 (LoCost) V.3 - https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bp2l6n26rgqe/v3.

2.2. Next Generation sequencing

The second training with Dr. Joana Isidro was focused on viral metagenomics. In this case, two Mpox virus-positive samples confirmed by real time PCR were selected. For this experiment, the MinION Mk1B device was also used. The main difference with the SARS-CoV-2 complete sequencing technique previously performed was that, in this case, the samples were not amplified prior to sequencing. Instead, a metagenomics protocol designed by the Televir project was used. The pretreatment and DNA extraction were performed following the new version (https://doi.org/10.1016/j.jcvp.2022.100120) of TELEVIR (https://onehealthejp.eu/jrptele-vir/) wet-lab protocol. Rapid Barcoding Kit (SQK-RBK004) was used to identifying the samples. Subsequently, the flow cell was prepared for the sequencing step. The first step was to clean the flow cells with the Flow Cell Wash Kit (EXP-WSH004) – ONT. After that, the Flow Cell Priming Kit (EXP-FLP002) – ONT was used for sequencing.

3. Learning how to use the INSaFLU-TELE-VIR suite and use it for the analysis of the complete sequencing and metagenomics results. Metagenomics analyses. First week.

An important part of Carlos training was learning how to use the INSaFLU-TELE-VIR suite (https://insaflu.insa.pt/). INSaFLU-TELE-VIR is a user-friendly open web-based bioinformatics suite designed for genome-based surveillance of influenza virus and SARS-CoV-2, and adapted to Mpox and other viruses. The suite promotes two different approaches:

- routine genomic surveillance (from reads to mutation detection, consensus generation, virus classification, alignments, "genotype-phenotype", screening, phylogenetics, integrative Nextstrain phylogeographical and temporal analysis, etc.).
- metagenomics virus detection (from reads to virus detection).

The INSaFLU-TELE-VIR platform promotes the analysis of primary sequencing data obtained with Illumina, Ion Torrent and Oxford Nanopore Technologies reads. The goal of this suite is to provide a user-oriented "start-to-end" bioinformatics framework able to
enhance a strengthened and timely detection, as well as monitoring of viral (emerging) threats.

In order to learn how to use the platform, Dr. Vítor Borger shared with Carlos some SARS-CoV-2, Mpox and Influenza virus FASTQ sequences, obtained with Illumina and Oxford Nanopore Technologies. He explained Carlos in detail how to load the data, the different analysis performed with the suite (e.g., quality of the reads, construction of consensus sequence genome, selection of reference genome for comparison, mutations list, alignments, Pango lineage classification, construction of phylogenetic trees, use of Nextstrain for phylogeographic and temporal analysis and metadata navigation), and answered all of Carlos' questions when he analysed these FASTQ files. Additionally, the researcher analysed the results of the SARS-CoV-2 and Mpox next generation sequencing performed during the wet lab practices. He was able to follow the next generation sequencing analyses performed at INSA, especially those related to viruses.

4. Wet lab. Second week: Preparation of samples and libraries for next generation sequencing.

During the second week, the main wet lab activity was the next generation sequencing of a poliovirus identified in a clinical case. The training was conducted by Dr. Rita Ferreira, using the Televir field protocol to prepare the samples (https://protocols.io/view/televir-field-protocol-phd-b68brhsn). In this case, following laboratory preparation, the samples were sequenced using Illumina. Dr. Rita Ferrerira also showed Carlos the routine preparation of the SARS-CoV-2 positive samples for complete sequencing. INSA receives these samples from all over Portugal. The samples are then randomly selected for sequencing using Illumina equipment. This learning experience was especially useful because Carlos was able to learn how to work with large numbers of samples.

5. Bioinformatics analyses. Interpretation of the obtained data. Second week.

5.1. Running of metagenomics analyses with the INSaFLU-TELE-VIR suite. Interpretation of the obtained data.

During Carlos' second week at INSA, he continued the genomic surveillance analyses, especially focusing on the results obtained from the next generation sequencing of SARS-CoV-2 and Mpox clinical samples performed during the first week. The interpretation of the obtained data was done under the supervision of Dr. Vítor Borges.

During this week, Carlos learned how to use the metagenomics virus module detection (from reads to virus detection) with Dr. João Dourado. This open module, integrated in the INSaFLU-TELE-VIR suite, was under construction during the STM. Its goal is to simplify metagenomics processing and is especially useful for researchers that are not used to bioinformatics. The module was released to the public at the end of 2022. Carlos had the opportunity to see how is it used and test it with some samples before its release.

5.2. Genomic surveillance of bacterial outbreaks

During the second week, Dr. Vítor Borges also showed Carlos how INSA does the genomic surveillance of bacterial outbreaks in Portugal. The center counts with a vast complete genome sequencing database of clinical isolates of *Listeria monocytogenes* from Portugal, including retrospective sequencing of over 1000 strains. The team is also adapting the system to *Salmonella sp*., *E. coli* and *Campylobacter jejuni*. In order to perform the genomic surveillance, the construction of phylogenetic trees and detection of clusters of the novel clinical isolates is performed with the ReportTree tool. This automated surveillance-oriented pipeline allows the detection of pathogenic genetic clusters and their characterisation in terms of geotemporal spread or linkage to clinical and demographic

data, and can be used for a wide variety of species, being especially useful for working under a One Health approach. In order to learn how to use the tool, some *L. monocytogenes* clinical isolates, were used for practice, working with core genome MLST data. The identification of the *L. monocytogenes* strains was performed with Chewie Nomenclature Server (chewie-NS, https://chewbbaca.online/), that classifies it according to a database of 1748 loci. ReportTree was then used to construct minimum spanning trees, thus being able to identify the bacterial isolate's cluster of origin. The ReportTree tool is also used with SARS-CoV-2.

6. INSA Day

On November 15th, 2022, the center celebrated the INSA Day, which celebrated the Institute's foundation, 123 years ago. It was a scientific and cultural event, with a master talk about the impact of climate change over Health, a message from Dr. Andrea Ammon (director of the ECDC), a round table about the future of health in Portugal that brought together experts in agriculture, animal sciences, medicine, environment, climate, and communication, a speech by Manuel Pizarro (Ministry of Health of Portugal) and INSA's directive council. Finally, the day ended with a show by Henk van Twillert & Vento do Norte Band.

7. Meeting with Dr. Rita de Sousa

Dr. Rita de Sousa is a senior researcher at INSA. She is responsible for the Rickettsial Unit at the Center for Vectors and Infectious Diseases and for the Diagnostic and Research of Viral Gastrointestinal Infections. Carlos and her met to discuss the hepatitis E virus study that Carlos is conducting with wildlife in Spain. Dr. de Sousa provided valuable insights for the project, and shared her experience working with that pathogen. In the future, a collaboration will be established to study this infectious agent.

8. Presentation about the INIA and Carlos' personal research line to the INSA colleagues

On November 16th, Carlos did a short talk about his research lines, developed in Spain and Brazil during the last years, focused on the surveillance of infectious agents in wildlife. He also presented the current research lines performed at the Epidemiology and Environmental Health Group (EYSA) of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria/Consejo Superior De Investigaciones Científicas, as the COVRIN project (WP3).

Overall, this period at INSA provided Carlos a solid theoretical and practical background in next generation sequencing and bioinformatics analyses, that will be key to strengthen the collaboration between INIA and INSA, and gave him the opportunity to meet top researchers working with infectious diseases under a One Health approach.

List of dissemination and communication activities

Not applicable.

Scientific outputs

The Short Term Mission 2022 "Metagenomic Tools for the Study of SARS-CoV-2 in Animals" provided me the training to diagnose and characterize future SARS-CoV-2 cases in animals using metagenomics, which is key to determine whether a virus has changed/mutated during a spill over event. At this moment, we have not diagnosed novel cases in animals, but currently have the means to face them, and to determine the source and timing of infection (shedding

light into the epidemiology of this novel virus) and the presence of viral mutations as an adaptation to novel hosts.

One of the outputs of the project will be the adaptation of modern metagenomics and bioinformatics tools in our research group during 2023. Another output of the project is the collaboration with Dr. Rita da Sousa in our research line on hepatitis E virus in wildlife.

Testimonial

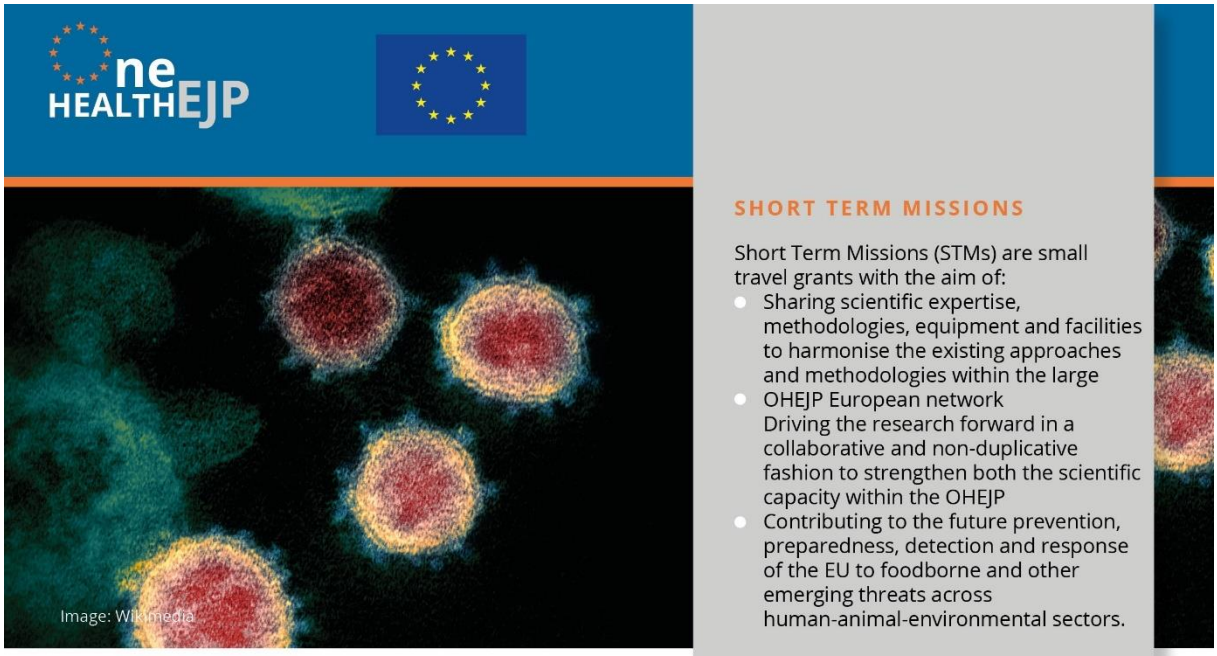
The two weeks of Short Term Mission titled "Metagenomic Tools for the Study of SARS-CoV-2 in Animals", conducted at the Bioinformatics Unit, Department of Infectious Diseases of the National Health Institute Doutor Ricardo Jorge (INSA), in Lisbon, Portugal, under the supervision of Dr. Vítor Borges and Dr. João Paulo Gomes, were a great personal and professional experience.

During this period, I closely followed the group's daily activities, and performed specific training activities designed to maximize my learning experience. It included learning to use the open web-based bioinformatics suite INSaFLU-TELE-VIR suite, designed by the group for genomic surveillance and viral metagenomics, and of the ReprotTree tool, created for the genomic surveillance of bacterial outbreaks and SARS-CoV-2. Additionally, I learned how to prepare samples and libraries for next generation sequencing, how to do nanopore based sequencing, and how to interpret next generation sequencing results.

I had the opportunity of getting acquainted with the National Health System of Portugal, and participated in several cultural and scientific activities carried out at INSA during my stay. I also gained valuable skills, that will allow me to use metagenomics in my future research, including the diagnosis and characterization of SARS-CoV-2 cases in wild and domestic animals using cutting-edge technology - one of the goals of the COVRIN Project.

This STM gave me the tools to adapt modern metagenomics and bioinformatics tools to our research center's studies'. I would like to thank the One Health EJP and the COVRIN integrative project for the opportunity and support, and specially to Dr. Vítor Borger, Dr. João Paulo Gomes, and their team at the Bioinformatics Unit - INSA for the training I received and their welcoming and friendly disposition.

8.2 Short Term Mission 8: Case study



SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large OHEJP European network
- Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Metagenomic Tools for the Study of SARS-CoV-2 in Animals

...a great personal and professional experience. I closely followed the group's daily activities, and performed specific training activities designed to maximize my learning experience. I had the opportunity of getting acquainted with the National Health System of Portugal, and participated in several cultural and scientific activities carried out at INSA during my stay. I gained valuable skills, that will allow me to use metagenomics in my future research.

Carlos Sacristán Yagüe
INIA, Spain

Theme:	One Health, Skills Development Missions
Home Institute:	INIA, Spain
Mission Hosting Institute:	INSA, Portugal
Duration of Mission:	2 weeks

The aim of this mission was to expand the Carlos' training to a higher and more practical level by learning the application of metagenomics and completing sequencing within a One Health context. These tools are essential for modern surveillance and diagnostics of infectious diseases and are key to shed light on the epidemiology of novel viruses.

During the mission, Carlos had the opportunity to learn how to use the INSaFLUTELE-VIR suite: an easy-to-use open web-based bioinformatics suite that was designed for the genomic surveillance of human seasonal influenza and SARS-CoV-2, and recently adapted to monkey pox virus (now renamed Mpox). This platform was proven to be an excellent surveillance tool during the SARS-CoV-2 pandemic and a new module has been designed by researchers in the mission hosting institute to simplify metagenomic analyses and identify new viruses. Carlos also learned about the ReporTree tool, a surveillance-oriented tool to strengthen the linkage between pathogen genetic clusters and epidemiological data and was trained on the use of nanopore sequencing with MinION. The MinION was used during the mission for the complete sequencing of SARS-CoV-2, the diagnostics of MPox virus and sequencing of a poliovirus. The "Young Researcher Day" and "INSA Day" took place during the mission, showcasing scientific and cultural activities. It allowed Carlos to learn about the different research lines carried out in the three INSA headquarters in Portugal and to expand his professional network.

The mission provided Carlos the training to diagnose and characterize future SARS-CoV-2 cases in animals using metagenomics, which is key to determine whether a virus has changed/mutated during a spill over event. One of the outputs of the project will be the adaptation of modern metagenomics and bioinformatics tools in the COVRIN project. The mission also strengthened further the collaboration between the institutes, with a new collaboration established to research on hepatitis E virus in wildlife.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 8: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

9. Short Term Mission 9

9.1 Short Term Mission 9: Report

Genotypic Characterisation of antimicrobial susceptibility and isolation of *Aeromonas* and *Vibrio* phages from water samples.

Name of applicants	Sandrine BARON Laetitia LE DEVENDEC
Institute of Affiliation/	Anses
Contact information	Ploufragan-Plouzané Niort Laboratory BP53 22440 PLOUFRAGAN France
Host institute and names of scientists involved in STM	Bundesinstitut für Risikobewertung (BfR, German Federal Institute for Risk Assessment) Dr. Jens A. Hammerl and Dr. Claudia Jäckel, head and deputy head of the Consultant Laboratory for Vibrio spp. in food (KL-Vibrio).
Dates of STM	18/11/2022 to 10/12/2022
Call Topic	One Health Missions- Veterinary, Food, Medical and or Environmental research - Skills development missions.
Research Domain	Antimicrobial resistance (AMR) and Emerging threats.
Key aims of STM	This STM aimed to: • Share technical expertise about (i) *Aeromonas* isolation from environmental samples (ii) phage isolation, • Being trained to refine bioinformatical analysis of French isolates data, • Initiate a close collaboration on aquaculture AMR issues between Anses and BfR, • Prepare new funding proposals as well as identify and contact other partners to be added to this new consortium.

Impact and relevance of scientific mission	Fostering One Health research to improve prevention, detection and control of AMR by: • Transferring techniques for the improvement of detection and typing techniques for phages, and their role in AMR transmissions. • Identifying possible uses of phages for the treatment of *Vibrio* and/or *Aeromonas* in aquaculture. • Bridging the gap of knowledge about AMR dynamics in *Vibrio/Aeromonas* and its spread.
Benefits to OHEJP	Collaboration and knowledge exchange on One Health subjects between institutes from the OHEJP consortium, further strengthening the network.

Summary

The objective of the mission was to share technical knowledge on the detection and characterisation of *Aeromonas* and *Vibrio* genus and to establish a collaboration between the unit Mycoplasmology-Bacteriology and Antimicrobial Resistance in Anses (UMBA, Anses - Ploufragan-Plouzané-Niort Laboratory) and the Consultant Laboratory for *Vibrio spp*. in food (BfR) on a shared interest: the study of the dissemination of antibiotic resistance in aquatic environments.

During this mission, two members of staff from UMBA were trained by colleagues in BfR on phage cultivation, phage enumeration and conservation methods. The two researchers from BfR learned how to isolate *Aeromonas* strains from water samples collected during the STM. Now the two teams have common protocols for the study of the *Aeromonas* and *Vibrio* genera. UMBA is involved in a research group, which aims to improve the methodology of antimicrobial susceptibility testing of aquatic bacteria. The BfR is now part of this group and participates to studies, co-coordinated by the UMBA, aiming to determine threshold values so called Epidemiological cut off value (Ecoff) used to study the antimicrobial susceptibility.

During the STM, the two teams were able to initiate the sharing of the Maldi-Tof databases for the improvement of the identification of species of the genera *Vibrio* and *Aeromonas*.
The sequencing of a hundred *Vibrio cholerae* strains, isolated along a salinity gradient in French estuary, will allow a better understanding of the role of Integrative Conjugative Element in the dissemination of AMR in the aquatic environment.

The possibility of a collaboration on the *Klebsiella* genus (exchange of strains, AMR/virulence determinants and mobile genetic elements) was highlighted in order to improve the knowledge on the genetic diversity and AMR of this genus.
Three video-conferences with French and Canadian researchers took place to widen the circle of collaborations of the two teams. At the end of this STM, two research projects were elaborated.
A research project associating two Anses teams, the BfR team and a Turkish team was submitted to the PNREST call managed by Anses.
Another has been proposed to internal BfR call and aims to investigate the genetic diversity of collection of *Aeromonas salmonicida* and *Vibrio spp*. using sequencing, to better understand the antimicrobial susceptibility of this species of critical importance in aquaculture.

Moreover a request for a thesis grant is underway to extend the collaboration on *Aeromonas* and *Vibrio* as an indicator of antimicrobial dissemination in aquatic environment"

This short-term mission strengthened the emerging collaboration between the two teams of the One Health EJP consortium. It has also led to a reflection on the use of phages to improve and reduce the use of antibiotics in aquaculture, farming sector where One Health approach is absolutely needed.

Technical Report

The duration of the mission was initially of four weeks, it was actually split into two periods (three weeks and one week). Thus, two persons from Anses/MBA unit had the opportunity to participate to this STM. Sandrine Baron, researcher, spent three weeks (from the 18th November to the 9th December) and Laëtitia Le Devendec, technician, spent one week (from the 3rd to the 9th December). The two first weeks were dedicated to the scientific exchanges between the scientists of the two teams, video conferences with French and Canadian partners, elaboration of proposals. The third week was dedicaded to the technical exchanges.

1. Phage detection

Phage is proposed as an alternative to antibiotic therapy. In European aquaculture, the therapeutic arsenal is very limited: only five antimicrobial agents are labelled and there is no commercial licensed vaccine against furunculosis, infection caused by *A. salmonicida*. The UMBA team conducted field studies which gave a large collection of *Aeromonas spp.* from aquatic environement and fish ponds and has the opportunity to collect water and fish samples at the request.

During the last week of the STM, Dr Hammerl and Dr Jäckel organised a practical training during which the French team practiced the handling for cultivation on specific media, counting and conservation of phages. The French team has now in hands detailed protocols from the BfR team, as well as phage and competent bacteria cultures, to be able to put into practice within Anses/UMBA.

The French team brought with them specific culture media for the detection of *Aeromonas sp.* from water and fish samples. Water samples have been collected nearby the BfR laboratory and the German team performed the entire handling : filtration, cultivation and presomptive identification. The BfR colleagues were also provided with the detailed protocol used by Anses/UMBA.

Before the STM, the protocol used in UMBA to perform ERIC-PCR has been transmitted to the BfR. This method is used for the rapid screening of *Vibrio* genomic diversity. During the STM, the transfer of the linked practical knowledge went on. Based of results obtained, it was decided to organise a ring trial with the two teams. In that interlaboratory assay, two methods of DNA extractions and two agars will be tested by both teams.

The protocols used for PCR identification and detection of virulence genes in *Vibrio* isolates were shared and control strains will be exchanged later (MTA have to be put in place between the two labs).

2. Investigation of AMR in Aeromonas

One aim of the STM was to investigate the AMR susceptibily of *Aeromonas* based on phenotypical results obtained in Anses. The susceptibility to colistin of *Aeromonas salmonicida* strains isolated from fish (n = 150) was tested in Anses/UMBA before the STM. Based on phenotypical results, it was not possible to make a subsampling of strains for sequencing. The decision to sequence the whole Anses/UMBA collection of strains was taken. Therefore, a project was submitted to an internal BfR call. In this project it is proposed to sequence the 150 *Aeromonas salmonicida* strains and an other set of strains of *Aeromonas spp.* collected from wastewater treatment plants. The aim is to gain data to inform or confirm the hypothesis of *Aeromonas* as a potential progenitor of *mcr* gene (resitance to colistin).

3. Preparation for future projects

The exchanges with colleagues from BfR, during the first two weeks allowed to better know the each other's fields of activity.Thus new topics of collaboration were identified.

Sandrine BARON was invited by Dr Martin Richter, Head of the Unit Diagnostics, Pathogen Characterisation and Parasites in Food, to present her research during the monthly meeting of the Unit. Exceptionnaly, Dr Eckart Straub (Jens-Andre Hammerl's predecessor), with whom S. Baron collaborated in 2020, attended the meeting.

Another meeting with Keike Schwartz (former PhD student of Dr E. Straub) made possible to progress on the valorisation of the work initiated in 2020 on *Aeromonas*.

- Phage and aquaculture

In Europe, aquaculture veterinarians have a limited therapeutic arsenal. Moreover, aquaculture, because of its direct link with the aquatic environment, is very much concerned with the dissemination of AMR. Thus, in this sector, efforts must be focused on (i) reducing/ improving the use of antitbiotics and (ii) developing alternative prophylactic treatments. In this context, phages seem to be a promising field, which nevertheless needs to be investigated for their actual role in the dissemination of AMR in aquatic environments. In the future, we will be looking for calls designed to support a common research project on phage and aquaculture in the european context.

- AMR and wild fauna

Initially, it was not intended to address the role of wildlife in the dissemination of antibiotic resistance during this STM, but it turned out that the subject was of interest for both teams. Moreover the BfR team had contacts and tools that they could share. Thus they started writing the draft of a research project, which aims to evaluate the genetic basis of the dissemination of antimicrobial resistance (AMR) in the environment with a particular focus on *E. coli* (Ec) in wild mammals (WM) as an invasive species and its possible role as an indicator or reservoir of AMR contamination.

- PhD grant

In 2021, both teams requested an internal grant in order to support a joint PhD project between the two teams, which was not accepted by Anses. This project has been improved during the STM and will be submitted again to the PhD grant call of Anses.

- Improvement of bacterial identification by Maldi-Tof.

The analysis of protein profiles via matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) mass spectrometry (MS) has emerged since last decade as a simple and reliable method to identify bacteria. Nevertheless, the reliability of the identification depends mainly on the quality of the database implemented by the supplier. Currently the databases mainly target the species of medical interest and therefore are not optimal for the identification to the species of genera such as *Aeromonas*, *Vibrio*, *Pseudomonas*.

During the STM, we agreed to pool the respective work of the two teams to improve the identification of the genera *Aeromonas* and *Vibrio* by Maldi-tof. Anses/UMBA, in collaboration with the Maldi-Tof platform in Anses-Nancy, is currently finalising a database dedicated to the identification of *Aeromonas*. Exchanges of strains with the BfR team were planned in order to test the robustness of the Anses base, last step before the publication by Anses of the results (of which the BfR will be co-author). Reciprocally, Anses/UMBA will transmit *Vibrio* strains to BfR for confirmation of identification with the upgraded BfR database.

In addition to the exchange of reference spectra, the two teams initiated discussions with the team of Professor Renaud Piarroux, who has developed an online application system based on Maldi-Tof spectra to identify yeasts and molds. The aim of this future collaboration is to broad his free online application to bacteria like *Aeromonas* and *Vibrio*.

List of dissemination and communication activities

Not applicable.

Scientific outputs

During the STM, a research project was drafted on the role of wildlife in the dissemination of AMR as a point of contact/exchange between wastewater and humans. The project has been submitted to the PRNEST call managed by Anses (5th January 2023).

The first draft of the publication "Determination of Epidemiological Cut Off value for *Vibrio cholerae*" was completed. The submission to special issue of Diseases of Aquatic Organism was scheduled for the end of March 2023.

A common interest on the improvement of Maldi-tof for the identification of aquatic bacteria was highlighted. A meeting with the team of Prof. R. Piarroud team was scheduled in March 2023.

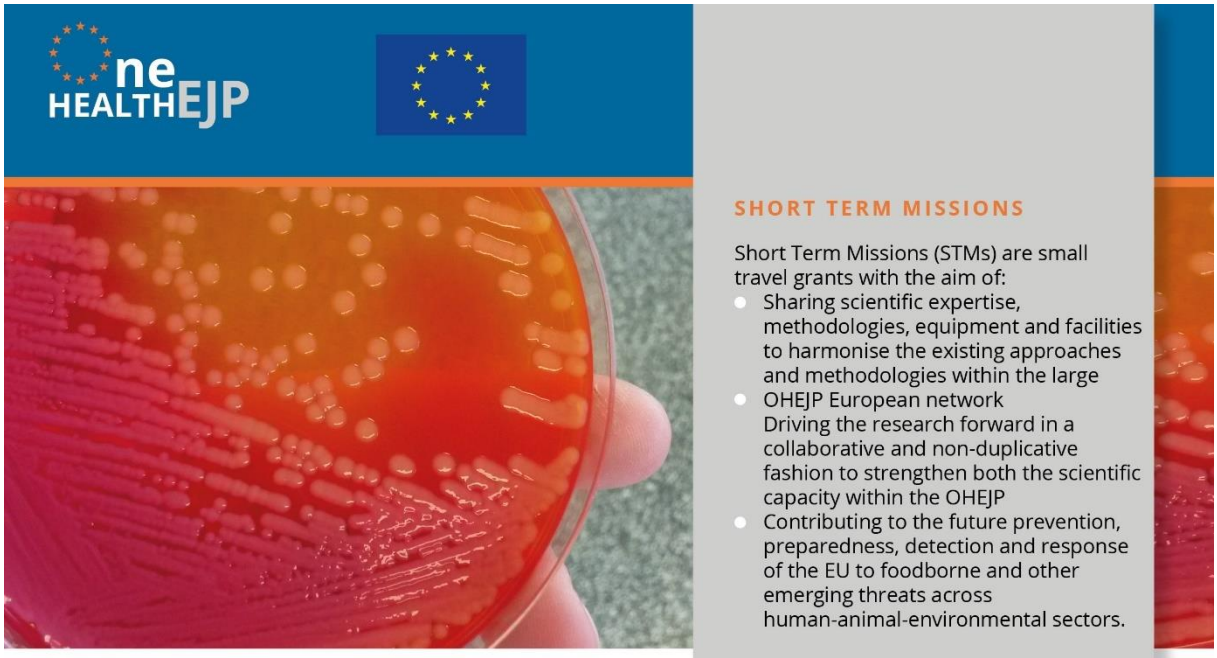
Strains of *Aeromonas*, *Klebsiella* and *Vibrio* were exchanged between the institutes, under MTA, to initiate new collaborative projects.

The first meetings concerning the Ecoff determination project for *V. alginolyticus* took place in January. The protocol of the study was drafted, for a start scheduled in March 2023.

Testimonial

This mission was a great opportunity to discuss the issue of the dissemination of antibiotic resistance in the aquatic environment in connection with aquaculture activities by sharing our experiences. Besides the improvement of the technical skills of both teams, the diversity of our fields of study (water, fishfarming vs food) opened up perspectives for new collaboration in a one health approach. This mission was an opportunity to meet colleagues with whom collaboration became obvious.

9.2 Short Term Mission 9: Case study



SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large OHEJP European network
- Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Genotypic characterisation of antimicrobial susceptibility and isolation of Aeromonas and Vibrio phages from water samples

Theme: One Health, Skills Development Missions
Home Institute: ANSES, France
Mission Hosting Institute: BfR, Germany
Duration of Mission: 4 weeks

"This mission was a great opportunity to discuss the dissemination of antibiotic resistance in the aquatic environment in connection with aquaculture activities. In addition to the improvement of the technical skills of both teams, the diversity of our fields of study (water, fish farming vs food) opened up perspectives for new collaborations, using a One Health approach."

Sandrine Baron and Laetitia Le Devendec ANSES, France

The objective of the mission was to share technical knowledge on the detection and characterisation of *Aeromonas* and *Vibrio* genus and to establish a collaboration between the institutes, to study of the dissemination of antibiotic resistance in aquatic environments.

During this mission, Sandrine and Laetitia were trained on phages' cultivation, enumeration and conservation methods. They now have in hands the detailed protocols, as well as phages and competent bacteria cultures, provided by the BfR team. They will be able to put the methods into practice in Anses. Sandrine and Laetitia travelled to BfR with culture media for the detection of *Aeromonas sp.*, from water and fish samples. Water samples have been collected nearby the BfR laboratory and the German team performed the entire handling: filtration, cultivation and presomptive identification. The partner teams established common protocols for the study of the *Aeromonas* and *Vibrio* genera and initiated the sharing of the Maldi-Tof databases, for the improvement of the species identification of bacteria from the *Aeromonas* and *Vibrio* genera. The two institutes are also now both involved in a research group, which aims to improve the methodology of antimicrobial susceptibility testing in aquatic bacteria and determine Epidemiological cut off values (Ecoff). Meetings and video conferences with French and Canadian researchers took place, to discuss research ideas. At the end of this STM, two research project proposals were elaborated.

This short-term mission strengthened the emerging collaboration between the two teams from the One Health EJP consortium. It has also led to a reflection on the use of phages to improve and reduce the use of antibiotics in aquaculture, a farming sector from which a One Health approach is absolutely needed.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

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Figure 9: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

10. Short Term Mission 10

10.1 Short Term Mission 10: Report

Stable Intra-specific genomic bacterial classification systems.

Name of applicant	Eve Zeyl Fiskebeck
Institute of Affiliation	Norwegian Veterinary Institute (NVI)
Contact information	Elizabeth Stephansens vei 1, 1433 Ås, Norway
Host institute and names of scientists involved in STM	Institut Pasteur Sylvain Brisse, Carla Parada Rodrigues, Melanie Hennart
Dates of STM	14 to 18th November 2022
Call Topic	One Health missions
Research Domain	Foodborne Zoonoses, AMR and/or Emerging threats
Key aims of STM or Workshop	Gaining knowledge about usage and design of a stable classification system and nomenclature approach for bacterial strains (cgLIN codes) and understanding advantages and limitations of its use in genomic epidemiology.
Impact and relevance of scientific mission	Previous isolate nomenclature/classification systems (e.g., serotyping, MLST typing) do not always satisfy two major criteria, such as long-term stability and congruence with phylogenetic relationships, which are essential for routine epidemiological surveillance (e.g., source attribution and outbreak investigations). During Eve's stay with S. Brisse team (Biodiversity & Epidemiology of Bacterial Pathogens) at the Institut Pasteur in the context of this OneHealth short term mission, Eve was able to understand the rational process that was used to develop the new cgLIN nomenclature system for *Klebsiella pneumoniae* species complex (KpSC) (Hennart et al. Mol Biol Evol 2022). Furthermore, Eve was able to apply this nomenclature system to a new dataset of *Kp*SC including ~300 isolates from a Norwegian collection. Eve also gained understanding of how to develop a similar stable nomenclature system for other species, and better comprehended the rationale and choices that have been made during development of the

	nomenclature system. Moreover, she has also gained better understanding on the limitations of this method. Eve is now able to not only encourage, but also to assist other scientists in applying cgLIN codes on their own datasets. A lecture presenting this method to scientists at the NVI will be given during the first quarter of 2023. This STM resulted in the establishment of an initial contact between the teams, a contact which is invaluable to the dissemination of the use of this nomenclature system to new users and to the development of this nomenclature system to other pathogens.
Benefits to OHEJP	This STM allowed knowledge transfer and strengthened the collaboration between OneHealth partners, and ideas of further developing the nomenclature system to other bacterial pathogens has been discussed. NVI is currently looking for funding that would allow to continue this collaboration. Having gained the experience with and the ability to train other scientists will strengthen activities in OneHealth context to address existing and emerging threats of foodborne and zoonotic agents as well as animal pathogens (e.g., Discover and BeOne).

Summary

The aims of this STM were to gain a deep understanding of the theoretical and developmental aspects of the newly developed bacterial stable classification and nomenclature system (cgLIN, Hennart *et al.* Mol Biol Evol 2022), as well as practical experience.

This nomenclature system has the advantage of providing a nomenclature that will be stable over time. The cgLIN (core genome Life Identification Number) classification code is based on core genome MLST (cgMLST) and on an adaptation of Life identification numbers (LIN, Vinatzer, B.A. et al. 2017). The cgLIN codes provides direct information about the placement of the genome within the evolutionary tree, and allows to directly evaluate the degree of relatedness when comparing isolates. Moreover, during development with the *Klebsiella pneumoniae* species complex (KpSC), it was shown that establishment of backward compatibility with previous nomenclature system (species, subspecies, sublineage, clonal group) is possible, which will enable building on previous knowledge of MLST identifiers and their epidemiology or pathogenesis, when employing this new nomenclature system.

cgLIN classification codes are attributed following sequence types determined based on the core genome sequence type (cgST, a unique combination of cgMLST alleles). The stability of the cgLIN nomenclature system is provided by coding of genomes, rather than groups. Careful identification of the major genetic discontinuities that occur at the different relatedness levels between genomes belonging to a species/species complex is used to determine the most appropriate allelic distance thresholds allowing to define clusters resulting from these genetic discontinuities. For *Kp*SC, 10 hierarchical levels of relatedness have been chosen to be represented by 10 numerical bins with numerical identifiers that then constitute the cgLIN code. The 4 first code bins represent the 4 deepest hierarchical levels of relatedness, while the 6 following bins have been chosen to delineate 6 epidemiologically relevant similarity

relatedness levels. Each of these similarity level corresponds to a specific number of pairwise differences between genomes.

During the STM, Eve applied this classification system on a *Kp*SC dataset that had not previously been analysed, and acquired knowledge that will allow promoting usage and adaptation of this nomenclature system for genomic epidemiology of zoonotic and infectious diseases. Knowledge transfer by introducing this method to scientists at the Norwegian Veterinary Institute (NVI) was planned for the first quarter of 2023. Moreover, this STM facilitated contact with OneHealth partners (S. Brisse lab, team: Biodiversity and Epidemiology of Bacterial Pathogens at Institut Pasteur and NVI) and they are currently looking for funding that would allow us to build on this newly acquired knowledge and collaborate on the development of cgLIN for other bacterial pathogens.

Technical Report

2 Theory: gaining in depth understanding of the classification system.

Initial discussions with the host provided an overview on how the nomenclature system was designed and identified some areas where the applicant had poorly understood the method following article reading.

The stable nomenclature system uses a classification code (cgLIN) that represents the placement of any new genome according to the most closely related genome previously classified. The code is attributed following cgST identification (unique combination of alleles determined by cgMLST3 scheme).
The classification code represents the hierarchical placement of the genome within the evolutionary tree. The successive code bins (CB) represent a series of hierarchical similarity clustering thresholds (see Fig. 5, Hennart et al. Mol Biol Evol 2022). In the case of *Kp*SC, the cgLIN code has been chosen to represent 10 hierarchical levels of relatedness. Hierarchical codes are attributed to new genomes according to the similarity levels to their most closely related genome previously classified (Fig S13 and S14, Hennart et al. Mol Biol Evol 2022). The first 4 CBs relate to which major phylogroup (species: CB1, subspecies: CB2), sublineage and clonal group (CB3, CB4) a genome belongs to. The second part of the code (6 following CBs) represent levels of similarities between genomes that were deemed epidemiologically relevant. Those 6 CB levels represent the hierarchical cluster membership of each genome. Each CB corresponds to hierarchical relatedness levels characterized by a specific number of cgMLST allelic mismatches (AM). In the case of *Kp*SC, CB1 to 4 corresponds to (610,585,191,43) AM, while CB5 to 10 corresponds to (10,7,4,2,1,0) AM, which coincides with (98.41, 98.89, 99.36, 99.68, 99.84 and 100%) similarity thresholds to previously classified genomes (calculated with scgMLST2 scheme for *Kp*SC5).

3 Practice: In-depth Understanding of the classification system.

Using a currently partially unpublished dataset *Kp*SC (submitted) from the KLEB-GAP project to perform the cgLIN nomenclature process, the HPC BIGSdb resources for *Klebsiella* was chosen to attribute cgLIN codes. Note that it is also possible to perform the classification via BIGSdb web-interface.
The main steps necessary to attribute cgLIN codes to new isolates are the following:

3.1 Sequencing data – quality control

3.1.1 Assembly quality control and species identification

The requirement of assembly quality for *Klebsiella* genomes to be accepted and analysed in BIGSdb include:

2.1.1 Contamination control: evaluated using ConFindr, KmerFinder, Kraken2 < 5%.

2.1.2 Species identification: performed with Kleborate and the lower acceptance threshold is defined as "acceptable identity".

2.1.3 Genome size: must be within the range [4.969.898 bp - 6.132.846 bp]

2.1.4 GC content: GC% must be within the range [56.35% -57.98%]

2.1.5 Contig number: must be composed of less than 500 contigs and contigs <200bp are not considered for further analyses.

Note that the data had already been checked by a colleague for quality (contamination detection with Kraken2, assemblies statistics with Quast and species identification performed with Kleborate. Only a short verification was ran manually using Quast and Kleborate. Conclusion: the data was deemed of sufficient quality.

3.1.2 Selecting isolates from this dataset that were not previously analysed in BIGSdb.

From an initial dataset composed of 500 KpSC isolates, only 297 isolates did not appear in the isolate database of BIGSdb (newly isolates/genomes). A private project was created for training purposes and these 297 genomes were uploaded, with a minimum set of required metadata (templates provided by BIGSdb).

3.2 MLST and cgMLST typing:

3.2.1 cgMLST typing was performed against the 629 loci scgMLSTv2 scheme (Bialek-Davenet et al. 2014), using the command line interface but this can be launched through BIGSdb web-interface for any user account. MLST typing is performed against the traditional scheme. The screening is done with blast using a blast word length of 30 for both schemes.

3.2.2 Search for new alleles not already present in the current MLST and cgMLST database (scan new). The search uses the following parameters: BLASTN identity of 90 and length coverage of 90 % for cgMLST and a blast word size of 30. At this stage, the new alleles are not included in the previous typing but they are reported. 2040 new cgMLST alleles were detected within the used dataset (mean 6.8 new alleles per isolate). No new MLST allele was detected.

3.2.3 Submission of new alleles for curating and cgMLST typing on updated cgMLST scheme. The curator evaluates if those new alleles are accepted. Upon acceptance of the newly discovered cgMLST alleles, the cgMLST typing is rerun using the updated typing scheme.

3.3 cgST are then attributed to all isolates based on the typed cgMLST alleles. cgST was not attributed for 22 isolates (due to > 30 missing loci8).

3.4 cgLIN codes are attributed to each isolate, by using the cgST of each isolate.

4 Development, under supervision, of a classification system for a new bacterial species.

Method understanding for development + discussion: day 3. 4, 5

Establishing a stable cgLIN nomenclature system relies on several essential elements:

- The genomes employed during the design of stable nomenclature system MUST be representative of the genetic diversity of the species/complex. This is likely true for all species from which a cgMLST scheme has previously been developed. However, for

species from which current sampling remains limited, attempting to develop such a nomenclature system might result in nomenclature instability. Depending on the amount of genetic diversity sampled, it might be possible to limit the development to the first 4 CBs levels. The code could then e.g., be extended at a later stage when a sufficient amount of genetic diversity has been sampled. However, success of such attempts might fail for species lacking clear genetic distances discontinuities between clonal groups.

- A thorough filtering of the dataset employed to develop this method must be employed. The cgMLST scheme that is used must be of high quality: cgMLST alleles are only valid if they contain full coding sequences (CDS: start-stop codon, no frameshift) and the loci must be present in most isolates. Because no cgST will be attributed if alleles at > 30 loci of the scheme failed to be detected during cgMLST typing, it is important to only employ high quality assemblies. Potential outliers, atypical isolates (identified by <96% ANI distance from reference strains, of unusual genome size or GC content are discarded), potential contaminated genomes and hybrid genomes (resulting from large recombination/horizontal transfer) must be identified and discarded during the first stages of the nomenclature design. Isolates with > 30 missing alleles at the cgMLST scheme will not be further used. Obtaining a such high dataset quality to create cgLIN nomenclature system relies on iterative filtering of the genomic dataset.
- Throughout evaluation of the stability and robustness of clusters formed at different similarity thresholds inform the choice of hierarchical similarity thresholds that are used to establish the different CBs component of the cgLIN code. The dissimilarity distances must be calculated from modified hamming distances (normalized) to minimize the effect of missing alleles on the classification. Minimum spanning tree (MStree) clustering is employed to evaluate at which distance/similarity thresholds clusters are stable, thus identifying the major genetic distances discontinuities along the hierarchical levels of the taxa population structure. Subsampling followed by MStree reconstructions allows evaluating cluster robustness to sampling bias.
- cgLIN encoding. When reliable clustering thresholds for clustering are chosen, the encoding is effectuated. Hennart et al. Mol Biol Evol 2022 determined that the optimal input order of genomes during encoding is achieved through traversal of MSTree, guiding input order with a Prim's algorithm.
- Establishing backward compatibility of lineages and clonal groups with the previous MLST classification (4 first CBs). Hennart etal. Mol Biol Evol 2022 developed and used an inheritance algorithm enabling lineages (SLs) and clonal groups (CGs) naming in the new nomenclature by following as closely as possible the previously established MLST-bases naming scheme. This, was done by prioritising transfer of naming suffixes (numbers) to the most abundant groups (frequency rules), attributing naming suffixes to orphan minor groups last. E.g., in case of sequence type (ST) group composed of 2 CGs, then the majority CG is named after the ST, while the minority CG is named last.
  The backward compatibility is not complete, which is largely attributable to the fact that some previously defined STs groups were either para- or polyphyletic, while the new nomenclature is established to reflect the evolutionary relationships where CBs represents monophyletic clusters. But because knowledge about biological proprieties attributed to previous ST is likely associated to the properties of the majority CGs; this will allow continuity of knowledge building. It may also contribute in identifying some

biological properties that might have appeared incongruent following the previous classification system in the case of previous para- or paraphyletic grouping.

5 Method development

It was envisaged to develop a cgLIN classification system for *Providencia/P. alcalfaciens* as this is a species that have been shown to occasionally produce disease outbreak both in humans and animals, and can be transmitted through contaminated food and from environmental sources under poor hygiene conditions. This bacterium, is an emerging threat, and no MLST nor cgMLST scheme is available to characterise and communicate about this genus. This species was considered a suitable candidate for the development of a cgLIN nomenclature system prior to this STM, because Eve had access to a large unpublished genomes dataset. However, after discussion with Melanie Hennart and Sylvain Brisse, it became clear that the genomic diversity of the genus is not yet represented well enough. Therefore, attempts at establishing a stable cgLIN nomenclature system might be impossible at the current stage. Whether developing a nomenclature system to the major phylogroups and lineages would be realisable is also uncertain.

Sylvain Brisse, offered the possibility to work together to develop a cgLIN system on *Klebsiella oxytoca*. We are currently at NVI searching for possibilities to finance such a cooperation.

5.1 Feedback, discussion and going further : Usability and feature in a One Health perspective

As mentioned by Hennart et al. (2022), this new nomenclature should facilitate unambiguous communication for population biology and epidemiological surveillance of bacterial pathogens.
However, it is expected that those advantages will not be restricted to communication clarification.
This nomenclature system provides the ability to rapidly evaluate the degree of relationship between isolates without further analyses, which can e.g., allow to exclude isolates as not belonging to a same contamination source/outbreak, and to timely select a subset of closely related isolates, for further in-depth analyses. E.g., should the need of further inspection, for outbreak detection should occur. It allows for rapidly finding the most closely related known isolate that can be used as reference, i.e., for SNPs analyses, or eventually an isolate that can serve as outgroup, choice that is often directed by other highly time-consuming analyses. In this regard, using this nomenclatures system will certainly facilitate our surveillance investigations.
Moreover, it can also help in identifying hybrid/recombinant genomes, which usually provide an analytical challenge when their nature is unknown.
Furthermore, this system may provide a solution, if extended, for improving automation of surveillance, e.g., for outbreak detection. It might be possible to use e.g., the 4 first CBs to automatically trigger a cascade of sub-analyses (e.g., cgMLST sub-schemes). It is possible to trigger a cascade of sub-analyses that will lead to ultimate resolution of analyses of genomes under-comparison. Such hierarchical analyses could facilitate reconstruction of Phylogenetic supertrees which could e.g., be used in research and surveillance of AMR/virulence.

List of dissemination and communication activities

Not applicable.

Scientific outputs

Knowledge transfer: presentation and discussion with scientists at NVI occured during the first semester of 2023.

Testimonial

The One Health Short Term mission (STM) program allows competency transfer between One Health collaborators. This program provided the opportunity to consolidate a collaboration between One Health partners (Institut Pasteur and the Norwegian Veterinary Institute). The STM allowed me to understand and practice applying the newly developped cgLIN (core genome Life Identification Number) bacterial nomenclature system (Hennart *et al.* Mol Biol Evol 2022). This nomenclature system is based on core genome MLST (cgMLST) analysis and on an the adaptation of Life identification numbers (LIN). While this system first has been established for *Klebsiella pneumoniae Species complex,* it certainly will be adapted to other bacterial pathogens. Moreover, I gained experience in applying this nomenclature system on a new unpublished *Klebsiella pneumoniae Species complex* dataset including ~300 isolates from a Norwegian collection. This training allowed acquiring practical competence in using the classification system in BIGSdb, and allowed reflecting on the rationale behind the choices of the methods that were used during the development of the cgLIN nomenclature system. I now have a better understanding on how the classification system can be used for genomic epidemiology of zoonotic and infectious diseases. I am now able to and will disseminate this knowledge further to other scientists within our institution. Moreover, I will promote usage and adaptation of this nomenclature system to other bacterial pathogens. This will certainly strengthen the activities in One Health context to address existing and emerging threats of foodborne and zoonotic agents as well as animal pathogens (e.g., Discover and BeOne).

10.2 Short Term Mission 10: Case study

Figure 10: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.

11. Short Term Mission 11

11.1 Short Term Mission 11: Report

Application of 'Single-Cell Genomics' for the study of the bacterial reservoirs of plazomicin resistance determinants.

Name of applicant	Bosco Matamoros Rodríguez.
Institute of Affiliation/	Universidad Complutense de Madrid
Contact information	Address: Laboratorio 202, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avenida Puerta de Hierro s/n, 28040, Madrid, Spain.
Host institute and names of scientists involved in STM	Karlsruhe Institute of Technology Anne-Kristin Kaster and Gunnar Sturm
Dates of STM	1st March to 31st May 2022
Call Topic	One Health Missions- Veterinary, Food, Medical and or Environmental research. Skill development missions (e.g. genomics, bioinformatics, big data epidemiology).
Research Domain	Antimicrobial Resistance.
Key aims of STM or Workshop	Analyse the bacterial reservoirs of plazomicin and next-generation aminoglycoside resistance genes using single-cell genomics.
Impact and relevance of scientific mission	The studied resistance genes are a threat to the application of old and novel aminoglycosides, therefore understanding their potential dissemination routes before they become widespread offers some room to establish preventive measures.
Benefits to OHEJP	Extending the ideas and concept of One Health to European institutes that are not part of the consortium as well as lying the foundation of future collaborations in the future.

Summary

The aim of this mission was the development of a single cell workflow for the detection of low abundance reservoirs of next-generation aminoglycoside resistance mechanisms. The main objective was to label single cells from complex environments with fluorescence markers to detect the genes of interest. This Short-Term Mission is framed within the OHEJP PhD METAPRO project and is expected to produce results that complement the ones already produced with the metagenomic analyses.

Three different labelled probes were designed for the detection of the aminoglycoside resistance gene *npmA*, a gene that confers high level of resistance to all known aminoglycosides, including plazomicin, apramycin and other next-generation aminoglycosides. To test the probes, the resistance gene was introduced in two plasmids with different copy numbers to use as positive controls, and the same plasmids without the resistance gene were used as negative controls. All these control plasmids were introduced in *E. coli* cells and a classical fluorescence *in situ* hybridisation (FISH) protocol was performed with the three probes independently to check their labelling efficiency. Two out of the three probes designed showed promising results to be used in environmental samples. Samples have been taken to evaluate the potential sorting of *npmA* positive cells and is planned to be performed in the upcoming months.

This Short-Term Mission has opened a new collaboration channel between two research groups with different scopes for the study of antimicrobial resistance with a One Health approach. The collaboration between the partner institutes is expected to last longer than the extend of the mission and we expect to produce interesting results than could potentially be published as a research article in a scientific journal.

Technical Report

Aminoglycoside importance has risen with the current situation of antimicrobial resistance, where first-line antibiotics became ineffective against many bacterial infections. The better understanding of aminoglycoside-associated toxicity and the relative ease to modify their chemical structure to produce novel compounds resilient against common resistance mechanisms, has made aminoglycosides a greatly attractive antimicrobial class. This fact is well highlighted with the approval of plazomicin in the US in 2018, a novel last resort aminoglycoside, based on a modification of the structure of sisomicin, to fight complicated urinary tract infections. In addition, the veterinary antibiotic apramycin has drawn a lot of attention lately to be used in human medicine due its unique structure and because of the low number and low prevalence of enzymes conferring resistance to it. To date, this compound has shown efficacy and safety in animal models and in a Phase I clinical trial in humans, which puts this antibiotic a step closer to the clinic. Furthermore, efforts to modify apramycin structure to produce next-generation aminoglycosides capable of overcoming the enzymatic modification mechanisms that already limit the application of this antimicrobial are being made in the form of the so called “apralogs”. Altogether, these and other current projects based on the modification of the current aminoglycosides offer a needed alternative in the fight against antimicrobial resistance.

The resistance gene *npmA* encodes a 16S rRNA methyltransferase that acts modifying the adenine located at position 1408 of the decoding site of the 30S subunit of the ribosome. This methylation impedes the binding and action not only of plazomicin, apramycin and most of the clinically important aminoglycosides, such as gentamicin, amikacin, but to all the potential next-generation aminoglycosides that uses these compounds as scaffold. Despite this broad range resistance, the number of reports of this methyltransferase is scarce. To date, only two articles show the presence of this 16 rRNA methyltransferase. It was discovered in 2007 in *Escherichia coli* and a few years ago it was proposed that *Clostridioides difficile* may be a potential reservoir of this enzyme. This latter suggestion is nowadays supported by the fact that the 49 isolates harbouring *npmA* present in the NDARO database belong to this bacterial species. However, the origin and main reservoir of this resistance gene is still unknown.

Over the last few years, metagenomic approaches have improved our understanding of the uncultured fraction of bacterial populations enabling the prediction of novel bacterial taxa and detection of antimicrobial resistance genes in non-pathogenic bacteria. Several metagenomic studies are based on the production of "metagenome-assembled genomes" (MAGs), a close representation of individual genomes existent in a metagenomic sample. However, the common association of antimicrobial resistance genes to mobile genetic elements, the difficulty of obtaining MAGs from low abundant species and the potential production of chimeric MAGs limit the application of metagenomics for the study of the link between resistance genes and bacterial species. Nevertheless, single-cell genomics have emerged as a potential technology to circumvent this problem. The physical isolation of individual cells through targeted cell sorting and the analysis of their genomes offers a solution for the study of antimicrobial resistance genes in their bacterial reservoirs.

The One Health EJP PhD project METAPRO studies the presence of resistance determinants to plazomicin and other next-generation aminoglycosides in different ecological niches, including human, animal, and environmental sources. Using a metagenomic approach the METAPRO project has identified that the resistance gene *npmA* might be part of the commensal gut microbiota of various animal species, including pigs, poultry and even humans. However, the relative abundance of the *npmA* gene in this environment is extremely low, bordering in most of the cases on the limit of detection. Consequently, commonly used metagenomic analyses do not offer an accurate identification of the bacterial reservoir that is maintaining this important resistance determinant nor enough genetic context for the evaluation of the potential dissemination routes that it may undergo in a close future. Therefore, they believed that the application of single-cell genomics was the best option to evaluate how and where this resistance gene is maintained to prevent its mobilisation to pathogenic bacteria. In this regard, application of fluorescence *in situ* hybridisation coupled with cell sorting is a great solution for the study of the bacterial reservoirs of *npmA* in the gut microbiota of pigs and poultry.

Task 1: Collection of faecal samples and conservation in protective media.

Based on preliminary results obtained in One Health EJP project METAPRO, it was concluded that the resistance gene *npmA* might be a common component of the gut microbiota from pigs and poultry. Therefore, new samples were collected from these two animal species to conduct single-cell genomics. A total of 15 new faecal samples were collected from broiler chickens, obtained a week before the slaughter of the animals. In addition, another 15 faecal samples were collected from pigs, 10 from post-weaning piglets at an age of 41 days and 5

from piglets 62 days old. Samples were collected in a latex glove and deposited in a plastic sterile container where an anaerobic atmosphere generation bag and an anaerobic indicator were introduced to maintain the conditions as similar as possible to the gastrointestinal tract. Plastic containers were kept at 4ºC until their arrival to the laboratory where they were processed. 2 grams of each faecal sample were homogenized 1:10 (w/v) with phosphate-buffered saline buffer (PBS) + glycerol to a final concentration of 30% glycerol and aliquoted in cryotubes conserved at -80ºC to preserve cell viability for downstream single-cell applications. In addition, the rest of the samples were directly frozen at -80ºC for genomic applications.

Task 2: DNA extraction and PCR check of *npmA* gene from faecal samples.

To check whether the newly collected faecal samples are suitable for single-cell genomics, their genomic DNA was extracted using DNeasy PowerSoil Pro kit from QIAGEN following the instructions provided by the manufacturer. Oligonucleotides targeting the *npmA* resistance gene were designed to amplify almost the full length of the gene, to be able to identify any potential variants of the gene that we might encounter, based on the data of our metagenomic analysis. PCR reactions were conducted to test for the presence of the gene in all the samples. All the samples collected from swine origin resulted positive to the gene in all the cases. However, some poultry samples seemed to be negative to the presence of *npmA*. It might be due to the abundance of the methyltransferase being below the limit of detection or because it is not present in all the animals.

Task 3: Design of fluorescence labelled probes for the detection of *npmA*.

Based on preliminary data, *npmA* abundance in the gut microbiome is very low. Therefore, designing several probes labelling the *npmA* gene might be needed to obtain enough fluorescence signal to be able to identify the resistance gene in such a complex matrix as a faecal sample. Probe design was conducted using of the NCBI Primer Blast tool to identify regions of 15 to 25 nucleotides of length in the gene that might be suitable for its identification. In addition, to better improve signal intensity, all the probes were designed to not only target the corresponding DNA sequence but also to hybridise to the potential messenger RNA that the *npmA*-harbouring bacteria might be producing. Hybridisation efficiency of all potential probes was analysed using mathFISH and only probes with hybridisation efficiencies of 1 or closer to 1 were used for further analysis. Probe specificity was evaluated using NCBI BLAST. Probes were selected to match less than 80% to bacteria that could be found on the gut microbiome. In the end, 3 probes were selected for further in vitro analysis.

Task 4: Fluoresence *in situ* hybridisation (FISH) against *E. coli* harbouring *npmA*.

To evaluate whether the probes designed *in silico* perform as expected and are suitable for the detection of *npmA* bacterial reservoirs, the resistance gene was inserted in a low (~5 copies) and a high copy plasmid (~20-40 copies). These plasmids were subsequently introduced in *E. coli* K-12 (as well as the empty plasmids to be used as a control) and were used to evaluate the affinity of hybridisation as well as the fluorescence signal derived of their use. A conventional FISH protocol described in the literature (Haroon et al., 2013) was applied with small variations of the final probe concentration to achieve the best results for each probe. Briefly, 1.5 ml of overnight culture was pelleted by centrifugation and washed with increasing ethanol concentrations (50%, 80% and 98%) for bacterial dehydration. Pelleted dehydrated cells were resuspended in 45 µl of hybridisation buffer (40 µl Tris-HCl 1M, 360 µl NaCl 5M, formamide 30%, 998 µl H2O, 2 µl SDS 10%) and probe to specific final concentration (2.5 - 8 ng/µl) was

added. Hybridisation was conducted for 3 hours at 46ºC. Afterwards, cells were washed with 500 µl of washing buffer (250 µl Tris-HCl 1M, 255 µl NaCl 5M, 125 µl EDTA 0.5M, 11870 µl H2O, 12.5 µl SDS 10%) twice and resuspended in 500 µl PBS. Correct fluorescent labelling of the *E. coli* cells containing npmA was validated via fluorescence microscopy. Probe 1 and probe 2 showed a labelling of more than an 80% of the cells in the positive controls while not labelling any cell in the negative controls (Figure 1). On the other hand, probe 3 did not efficiently bind to the positive target at the tested concentrations.

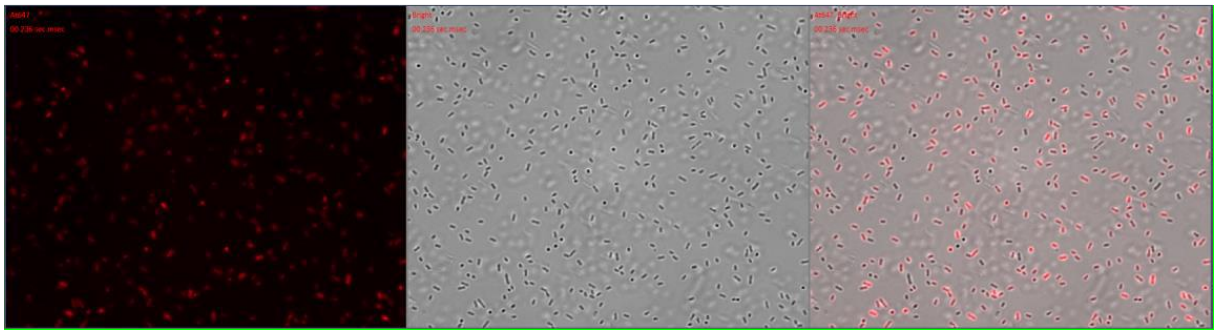


Figure 1. Microscopic image of fluorescence in situ hybridisation (FISH) of *E. coli* K-12 with a high copy plasmid containing npmA gene using probe 2 at a concentration of 15 ng/µl.

Probe 1 was further tested via flow cytometry showing that, consistently, a 50% of the cells labelled with the fluorophore can be distinctively separated from the negative control population. Ratios of 50:50 and 90:10 of negative control to positive control respectively were used to confirm these results (Figure 2).

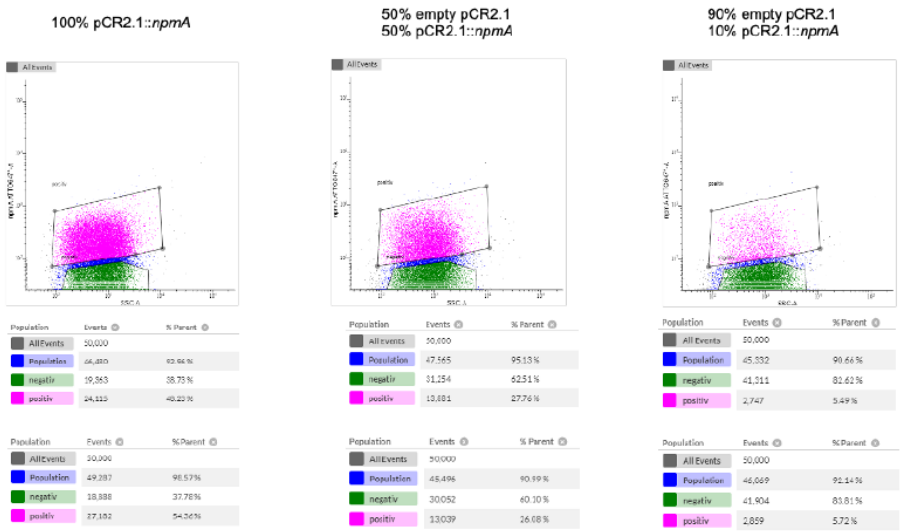


Figure 2. Flow cytometry results of different ratios of positive control and negative control of FISH experiment using probe 1 at a concentration of 8 ng/µl

Probe 2 was also tested using the same methodology and a similar result was obtained. Around an 80% of the cells stained with probe 2 were detected by fluorescence microscopy, and approximately a 50% of the labelled cells could be efficiently sorted from mock populations.

Task 5: Fluorescence *in situ* hybridisation (FISH) from faecal samples.
Task to be performed in June 2023.

Task 6: Fluorescent activated cell sorting (FACS) of faecal samples and single-cell genomics.
Task to be performed in June 2023.

List of dissemination and communication activities

Not applicable.

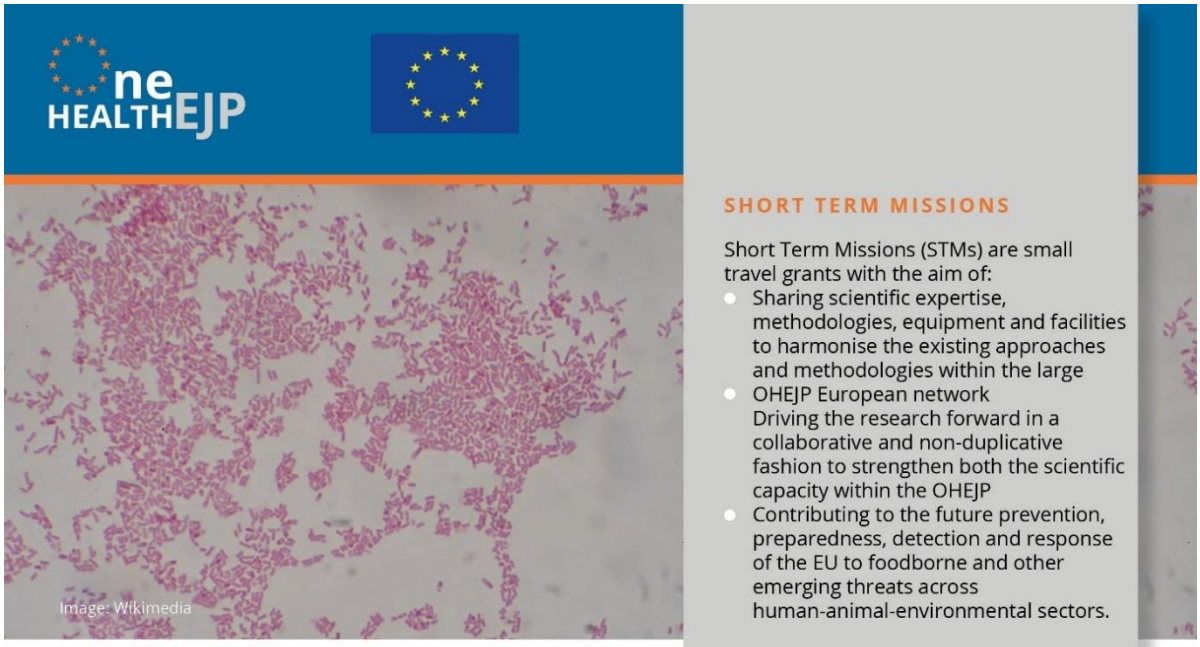
Scientific outputs

The intention is to combine the results obtained during this Short Term Mission with the rest of experiments and analyses performed in the METAPRO PhD project to write a scientific article, with all the findings regarding the prevalence and localisation of next-generation aminoglycoside resistance genes. The writing of this research article is expected to start at the end of 2023 if the experiments work as expected.

Testimonial

Thanks to the One Health EJP and their Short Term Missions I have had the chance to expand my skill sets and learn new methods that complement very well my research. Having the opportunity to know a new research centre and discuss my project with the very talented people that took me in has helped me expand my critical thinking and strengthen my professional confidence. Working in a big research group with people with very different points of view has made me love even more science and better understand the needs and the paths to take to become a better scientist. I believe we proposed a very ambitious project to do it in a very limited time, but I feel that everything has flowed very dynamically, and we have achieved more than we thought that we will. We built a long-lasting collaborative network that I trust can give promising results in a close future. On a personal note, I enjoyed my stay in Karlsruhe and I met incredible people that I hope stay close for many years. If everything has worked so well is because the human quality of the people at KIT that welcomed, helped me and made me one of them from day one.

11.2 Short Term Mission 11: Case study



SHORT TERM MISSIONS

Short Term Missions (STMs) are small travel grants with the aim of:

- Sharing scientific expertise, methodologies, equipment and facilities to harmonise the existing approaches and methodologies within the large OHEJP European network
- Driving the research forward in a collaborative and non-duplicative fashion to strengthen both the scientific capacity within the OHEJP
- Contributing to the future prevention, preparedness, detection and response of the EU to foodborne and other emerging threats across human-animal-environmental sectors.

Application of 'Single-Cell Genomics' for the study of the bacterial reservoirs of plazomicin resistance determinants

Theme: One Health Missions , Skills Development missions, Antimicrobial Resistance (AMR)
Home Institute: Universidad Complutense de Madrid, Spain
Mission Hosting Institute: Karlsruhe Institute of Technology, Germany
Duration of Mission: 3 months

Thanks to the One Health EJP I have had the chance to expand my skill sets and learn new methods. Having the opportunity to know a new research centre and discuss my project with very talented people has helped me expand my critical thinking and strengthen my professional confidence. We built a long-lasting collaborative network that I trust can give promising results in the close future.

Bosco Rodríguez Matamoros
Universidad Complutense de Madrid, Spain

The aim of this mission was for the PhD student to learn about the development of single cell workflows, for the detection of low abundance reservoirs of next-generation aminoglycoside (such as plazomicin) resistance mechanisms. The main objective was to label single cells from complex environments with fluorescence markers specific for the genes of interest. This STM enabled the PhD student to produce results that complemented the ones already produced within the OHEJP PhD METAPRO project using metagenomic analyses.

During this mission, three different labelled probes were designed for the detection of the aminoglycoside resistance gene *npmA*, a gene that confers high level of resistance to all known aminoglycosides, including plazomicin, apramycin and other next-generation aminoglycosides. To test the probes, the *npmA* resistance gene was introduced in two plasmids with different copy numbers to use as positive controls, and the same plasmids without the resistance gene were used as negative controls. All these control plasmids were introduced in *E. coli* cells and a classical fluorescence in situ hybridisation (FISH) protocol was performed with the three probes independently to check their labelling efficiency. Two of the designed probes showed promising results and have the potential to be used with environmental samples. Samples have been taken to evaluate the potential sorting of *npmA* positive cells and is planned to be performed in the upcoming months.

This STM has opened a new collaboration channel between two research groups with different scopes for the study of antimicrobial resistance with a One Health approach. The collaboration between the partner institutes is expected to last longer than the extend of the mission and we expect to produce interesting results than could potentially be published as a research article in a scientific journal.

One Health EJP has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773830.

@OneHealthEJP ONE Health EJP
#OneHealthEJP #OneHealth #StrongerTogether

Figure 11: This case study can be found on the OHEJP website: https://onehealthejp.eu/community/education-and-training/short-term-missions-2022.