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Improving the risk assessment of antimicrobial resistance (AMR) along the food/feed chain and from environmental reservoirs using qMRA and probabilistic modelling

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Abstract

Efficient risk assessment of antimicrobial resistance (AMR) in environmental reservoirs, particularly agroecosystems, is critical for predicting threats to animal and human health due to infections unresponsive to antibiotic therapy. However, approaches currently employed for the risk assessment of AMR along the human food chain rarely rely on antibiotic resistance gene (ARG) environmental pathways connected to food production and related quantitative data. The present project aimed at improving the risk assessment related to the spread of AMR along the food/feed chain based on ARG quantification in agroecosystems and interconnected environments. The fellow received training and worked in close cooperation with the team on two ongoing research projects which involved: (i) the monitoring of ARGs in field soils and surface waters to identify and characterise food/feed chain-associated environmental reservoirs of AMR relevant at the national level; (ii) the evaluation of ARG dynamics in relation to agricultural practice within an international project assessing biodiversity as an ecological barrier for the spread of clinically relevant ARGs in the environment. ARG quantification was performed using single/multiplex real-time polymerase chain reaction (PCR) with tailor-made primers/probe sets according to in-house optimised and validated conditions. The assessment was completed by a comprehensive revision of available literature data for risk-ranking of ARGs along with a literature review exploring AMR quantitative knowledge gaps and the role of certain AMR determinants encoded on free extracellular DNA (exDNA) in their environmental spread.

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1. Introduction

1.1. General background on the risk assessment of AMR in agroecosystems

Agroecosystems are considered one of the reservoirs of antimicrobial resistance (AMR) due to risk to human health posed by agrochemicals as a factor contributing to the expression of antibiotic resistance genes (ARGs) in resident bacteria and to ARGs pre-existing in the animal gut (Ramakrishnan et al., 2019). Practice such as irrigation with reclaimed wastewaters and application of manure contribute to the contamination of agricultural soil and crops (Cerqueira et al., 2019a,b, 2020; Sorinolu et al., 2021), while surface runoff and water drainage systems are the primary pathways of ARG diffusion across ecosystem boundaries, in particular to adjacent waterbodies (Iwu et al., 2020). Although the highest concentrations of ARGs upon irrigation with water contaminated by antibiotics have been detected in soil and its surface (Pan and Chu, 2018), current wastewater treatment technologies only partially remove antibiotic residues, ARGs and ARB (Anthony et al., 2020).

Despite a large body of scientific literature addressing AMR, substantial knowledge gaps regarding the complexity of its evolution and spread decrease the accuracy of risk assessment for human health (Niegowska et al., 2021). Monitoring and surveillance of ARGs are the primary tools providing key information for accurate risk assessment of AMR dissemination, however available approaches addressing AMR spread in environmental settings are scarce (Claycamp and Hooberman, 2004; Ashbolt et al., 2013; Baker et al., 2016; Ben et al., 2019). In particular, dose–response models including the quantification of infection risks due to ARGs along the food/feed chain together with long-term holistic studies evaluating the accumulation and dissipation of ARGs related in agroecosystems are missing (FAO/OIE/WHO, 2020; Sorinolu et al., 2021). The development of high-quality risk assessment models requires quantitative data for relevant ARGs, including information on abundance and potential to confer resistance to various antibiotics belonging to distinct classes in pathogenic bacteria (Larsson et al., 2018). Additionally, few quantitative data are reported on the frequency of ARG transfer across ecosystem boundaries and the uptake rates of free extracellular DNA (exDNA) by potentially pathogenic bacteria.

1.2. Hosting site risk assessment projects addressing AMR

Investigation of AMR has been included under the One Health approach from an integrated perspective in which environmental, animal and human compartments are interconnected (WHO, 2017). The role of the environment, including agroecosystems, in halting or promoting the spread of AMR through pathogenic and non-pathogenic microorganisms is under investigation, as foreseen in the FAO action plan on AMR 2021–2025 (FAO, 2021). In this context, Austrian Agency for Health and Food Safety (AGES) is part of the ANTIVERSA project European Consortium which aims at studying biodiversity as an ecological barrier for the spread of clinically relevant antibiotic resistance in diverse terrestrial and aquatic ecosystems, focusing on the impact of ARGs and antibiotic resistant bacteria (ARB) on AMR diffusion in wastewater treatment plant (WWTP) effluents and soil fertilised with manure. The outcomes will help in developing strategies in support of water and soil quality.

Under assignment from the Austrian Ministry of Health and the Ministry of Agriculture, AGES is a leading part of the MARGINS project with the main objective to close quantitative knowledge gaps on ARG prevalence as naturally occurring background loads as well as on their modification and resilience to external inputs in the tested environmental reservoirs. To this end, a range of ARG targets are quantified in various agricultural environments allowing a future selection of few ARGs to be used as locally-specific AMR markers in a realistic scenario where routine monitoring of numerous resistance genes in parallel is not feasible for economic reasons. Additionally, the project foresees the development of optimised operating procedures for sample collection, analysis and data evaluation.

AGES is also partner in the One Health European Joint Programme (OHEJP) and is currently analysing the role of exDNA on the dissemination of AMR in agroecosystems in the framework of the FED-AMR project developed at the Division of Data, Statistics and Risk Assessment.

2. Description of work programme

The fellow was hosted by the Division of Data, Statistics and Risk Assessment at AGES. The hosting division has a long-standing experience in the risk assessment of toxic substances in food and related environments, and is a national unit of reference for the development of assessment and monitoring strategies for AMR.

2.1. Aims

The present work programme aimed primarily at improving the risk assessment of AMR of environmental origins and to evaluate the impact of clinically relevant antibiotic resistance genes identified in environmental compartments and along the food/feed chain on human and animal health. Narrowing known knowledge and data gaps which are still hampering a holistic AMR risk analysis as outlined in the One Health concept was one primary target of this fellowship. Providing a sound training for and presenting the tools already available for AMR risk assessment to the fellow, who should be capable of disseminating the obtained expertise at the end of the fellowship, was another objective.

2.2. Activities/Methods

2.2.1. Comprehensive revision of available data

To meet the objectives of the work programme, the fellow assessed and identified exposure pathways of AMR and ARGs in agroecosystems and connected environments, including the identification of potential pathways of ARG dissemination from the environment to human and animal pathogens, mainly along the food chain, as well as collection of data on the current knowledge status relating to environmental AMR. The identification of knowledge gaps of major relevance in the risk assessment of AMR in the environmental context, with focus on AMR spread through exDNA and quantitative information on ARGs related to the food chain and agroecosystems was used in a predictive modelling approach and used to prepare a draft of a literature review, currently under refinement for a publication in a peer-review journal.

To provide an evidence-based section of ARG targets the quantitative monitoring of AMR dissemination in environmental compartments, research trends of recent years were evaluated focusing on agricultural soil, wastewater, manure and food/feed ecosystems. The literature review was performed taking into account experimental approaches based on quantitative PCR (qPCR) and using appropriate keywords. Resulting ARG ranking was subsequently used to verify the relevance of gene targets selected for the practical part of the work programme.

2.2.2. Practical work – quantification of ARGs in environmental samples

During the practical part integrative of the work programme, the fellow completed laboratory tasks which included optimisation and validation of detection protocols, in particular: (i) validation of TaqMan qPCRs for the quantification of ARGs with improved positive controls used for generating standard curves for absolute quantification; (ii) validation of ARGs in a duplex approach that allows to assess two targets simultaneously with a significant reduction of sample volume, reagents and time necessary for the assay; (iii) determination of an optimal sample dilution to avoid PCR inhibition for the qPCR assays based on 16S gene target; and (iv) quantification of core and accessory ARGs in environmental samples (agricultural soil, forest soil, surface water) by qPCR with a total of 26 gene targets assessed (Table 1) and over 10,000 assays performed.

A subsequent analysis of the obtained data executed by the fellow indicated trends in ARG prevalence in agricultural soils with reference to fertilisation practice and their background concentrations in naturally occurring ecosystems. The movement of ARGs between different environmental compartments was assessed along with the impact of environmental conditions. Also, in a field plot experiment where the field was separated into three single plots of the same size, the variability of qPCR results between single plots and a composite soil sample representative of the whole field was determined in a methodological approach.

Table 1: Target genes quantified in soil and/or surface water samples

Target genes	Resistance mechanism	Inactivated class of antibiotics
Core targets		
<i>aph(3')-IIa</i>	Antibiotic inactivation	Aminoglycosides
<i>aph(3')-IIIa</i>	Antibiotic inactivation	Aminoglycosides
<i>bla_{TEM-1}</i>	Antibiotic inactivation	β-lactams
<i>ermB</i>	Target alteration	Macrolides
<i>sul1</i>	Target replacement	Sulfonamides
<i>tet(W)</i>	Ribosomal protection	Tetracyclines
<i>vanA</i>	Target alteration	Glycopeptides
<i>16S</i>	–	–
Accessory I ARGs		
<i>cmxA</i>	Efflux	Amphenicols
<i>ermF</i>	Target alteration	Macrolides
<i>mcr-1</i>	Target alteration	Peptide antibiotics (colistin)
<i>qacEΔ1</i>	Efflux	Quaternary ammonium salts
<i>qnrS(rt11A)</i>	Target protection	Fluoroquinolones (ciprofloxacin)
<i>tet(A)</i>	Efflux	Tetracyclines
Accessory II ARGs		
<i>bla_{OXA-10}</i>	Antibiotic inactivation	β-lactams
<i>dfrA1</i>	Target replacement	Diaminopyrimidine antibiotics (trimethoprim)
<i>sat-4</i>	Antibiotic inactivation	Streptothricin
<i>tet(M)</i>	Target alteration	Tetracyclines
<i>tet(O)</i>	Target alteration	Tetracyclines
<i>bla_{CTX-M-1-15}</i>	Antibiotic inactivation	Cephalosporin
<i>bla_{GES-1}</i>	Antibiotic inactivation	β-lactams
<i>bla_{KPC}</i>	Antibiotic inactivation	Carbapenems
<i>bla_{NDM-1}</i>	Antibiotic inactivation	Carbapenems, penicillins
<i>bla_{VEB-1}</i>	Antibiotic inactivation	β-lactams
<i>mecA</i>	Antibiotic target replacement	Carbapenem, cephalosporin, penam, cephamycin, monobactam
<i>aph(6)-Id (strB)</i>	Antibiotic inactivation	Aminoglycosides

ARGs: antibiotic resistance genes.

2.2.3. Risk assessment training

In addition to training modules foreseen by the EU-FORA Fellowship, the fellow followed face-to-face workshops with the hosting site experts on general issues related to risk assessment activities including risk assessment in food/feed with focus on novel foods and chemical substances including pesticide residues, heavy metals and veterinary medicinal products, classification and management of monitoring and risk assessment data in the national system and their communication to EFSA, introduction to the use of geographic information systems (GIS) for data projection, evaluation of risks and dynamic trends.

The fellow participated in the regular weekly meetings on the current scientific work carried out by the department group and in the OHEJP Working Group meetings and conferences on ongoing international efforts to harmonise AMR risk assessment according to the One Health concept. Supporting activities provided by the hosting site during the EU-FORA fellowship are summarised in Table 2.

Table 2: Training and supporting activities provided by the hosting site

Type of activity	Title/subject	Date/frequency
Trainings and workshops	Characterisation of exposure pathways of antimicrobial ARGs in naturally occurring ecosystems (agricultural soils, surface, ground and wastewater and along the food/feed chain)	8 February 2021
	Modelling and simulation of the spreading of infectious diseases and antimicrobial resistance genes using probabilistic modelling	9 February 2021
	Quantitative microbiological risk assessment (qMRA) for evaluation of AMR dissemination in natural environments (soil, water; food/feed chain)	10 February 2021
	Monitoring of AMR in the environment: case studies based on MARGINS and ANTIVERSA projects	15 and 16 February 2021
	Processing of laboratory and monitoring data for internal reporting and generation standardised reports for stakeholders (Data management)	11 and 19 March 2021
	Internal webinar on <i>C. difficile</i> - the environmental perspective	12 March 2021
	Compilation of risk maps using Geographical Information System	8 April 2021
	Introduction to risk assessments along the food/feed chain	19 and 27 April 2021
	Meetings	Group/Department meetings
OHEJP FED-AMR Working Group meetings		Monthly
Other activities	Introduction to the Reference Laboratories of Molecular Biology and Method Development	4 May 2021
	Introduction to the Reference Laboratory of Microbiology	5 May 2021

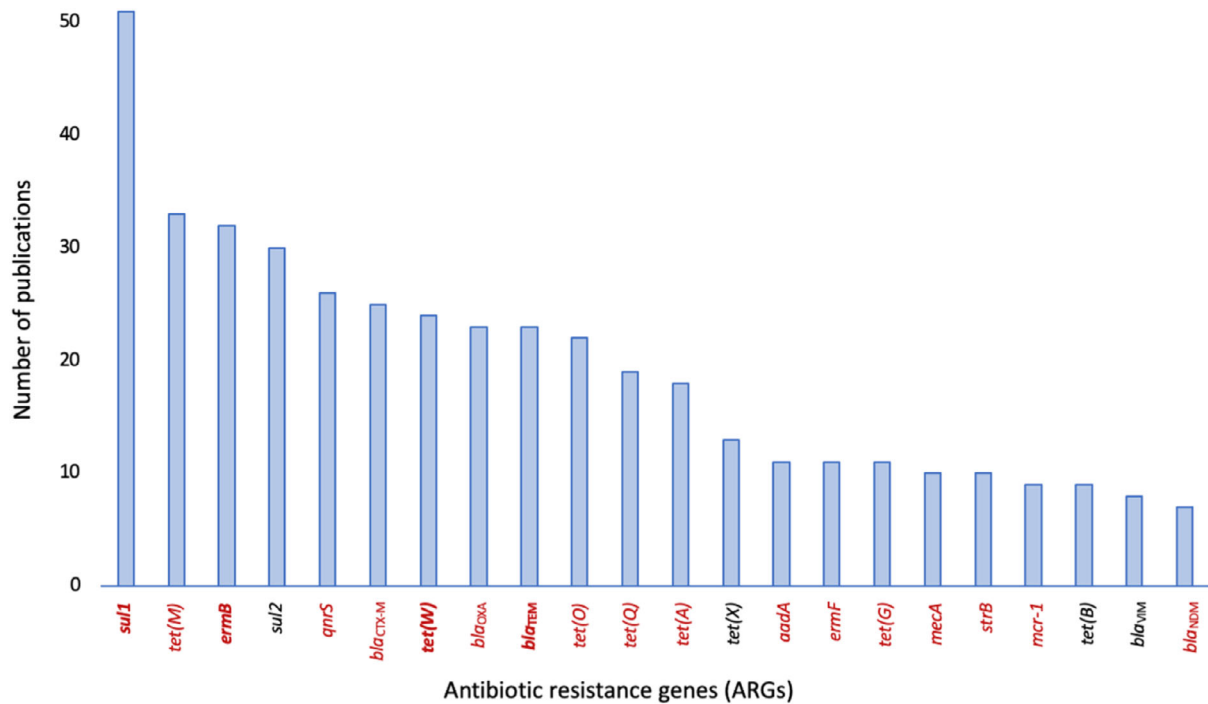
OHEJP: One Health European Joint Programme.

3. Results

The investigation of ARGs in natural ecosystems and those impacted by human activities is crucial due to uneven availability of data across environments and sources. In particular, occurrence and movement of ARGs from non-point sources to soil, plants and plant/animal food products, are less characterised (FAO, 2019). Although the mechanisms of AMR spread are widely described, the transfer of ARGs encoded on exDNA remains poorly evaluated and has been identified as the main knowledge gap in AMR assessment. The analysis of available literature data during the fellowship revealed that, besides background knowledge on exDNA behaviour in soil, little is known about the abundance of exDNA encoding ARGs in farmlands, its uptake by resident bacteria and persistence under various environmental conditions; even less is known when considering the food/feed chain and very few studies investigating exDNA-related ARGs in crops have been published so far.

One step ahead has been performed by assessing the transfer of ARGs via exDNA during cultivation of vegetables. A probabilistic modelling performed based on experimental data showed that the presence of exDNA in a cultivation system constitutes a key factor impacting the transformation rates of competent soil bacteria. Detailed description of the probabilistic model and outcomes will be published in a peer-review journal (in preparation).

The analysis of ARGs most frequently quantified using qPCR technique in farmlands, agricultural wastewater, manure and food/feed over the last years provided a ranking of gene targets relevant due to their frequency in the studies matrices, impact on human health (i.e. presence in pathogenic bacteria), determination of resistance covering various antibiotic classes occurring through different mechanisms. The ranking showed that 18 out of 22 most studied ARGs have been included in the qPCR analysis within the MARGINS projects, reflecting a widely recognised relevance of these targets for AMR assessment (Figure 1). Further analysis of ARG quantitative data generated during the practical part of the fellowship will show potential associations with agricultural practice, climate and site conditions as well as the extent to which AMR determinants spread across environments.



Red: accessory ARGs quantified within the MARGINS project. Black: ARGs non included in the MARGINS project.

Figure 1: The most frequently quantified ARGs in agroecosystems over 2017–2021 based on literature review Red bold: core ARGs quantified within the MARGINS project

4. Conclusions

Overall, the fellow acquired skills in defining the spreading pathways of AMR, ARGs and ARB along the environment–animals–humans axis. Increasing the conceptual knowledge underlying AMR development upon exposure to antibiotics with reference to natural conditions, being a primary objective of the work programme, introduced the fellow to further steps during the fellowship, such as definition of existing knowledge gaps in the current state-of-the-art, which particularly refer to very limited availability of quantitative data for specific ARGs present in environmental compartments.

The work programme allowed the fellow to gain practical knowledge in validation, optimisation and execution of analytical protocols for the quantification of clinically relevant ARGs in farmlands and connected environments. Through this activity, the fellow participated in the first environmental ARG quantification project at national level required for narrowing quantitative data gaps in the assessment of ARG dissemination pathways from the source to distanced environments. Also, by collecting data on the advancement of quantitative microbiological risk assessment (qMRA) models for AMR, the fellow acquired practical and theoretical basis for evaluating their suitability and applicability in environmental compartments as well as their overall potential for improvement of AMR risk assessment strategies.

5. Disclaimer

Detailed outcomes of the ARG quantification, data collection on the role of exDNA on AMR dissemination, ranking of ARGs for monitoring purposes and probabilistic modelling approach are intended to be published in peer-review journals (currently in preparation). In order to avoid copyright claims, they were not included in the present report.

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Abbreviations

AGES	Austrian Agency for Health and Food Safety
AMR	antimicrobial Resistance
ARB	antibiotic-resistant bacteria
ARG	antibiotic resistance genes
exDNA	free extracellular DNA
GIS	geographic information system
OHEJP	<i>One Health European Joint Programme</i>
PCR	polymerase chain reaction
qMRA	quantitative microbiological risk assessment
qPCR	quantitative polymerase chain reaction
WWTP	wastewater treatment plant