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Risk assessment of antimicrobial resistance along the food chain through culture-independent methodologies

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Abstract

Antimicrobial resistance (AMR) represents a major challenge for Public Health and the scientific community, and requires immediate and drastic solutions. Acquired resistance to certain antimicrobials is already widespread to such an extent that their efficacy in the treatment of certain life-threatening infections is already compromised. To date, the emergence and spread of AMR has been attributed to the use, misuse or indiscriminate use of antibiotics as therapeutic drugs in human, animal and plant health, or as growth promoters in veterinary husbandry. In addition, there is growing concern over the possibility of AMR transmission via the food chain. Food processing environments could act as potential hotspots for AMR acquisition and spread. Indeed, biocide use and exposure to food-related stresses and food processing technologies could presumably act as selection pressures for increased microbial resistance against clinically relevant antibiotics. Global AMR surveillance is critical for providing the necessary information to form global strategies and to monitor the effectiveness of public health interventions as well as to detect new trends and emerging threats. Surveillance of AMR is currently based on the isolation of indicator microorganisms and the phenotypic characterisation of the strains isolated. However, this approach provides very limited information on the mechanisms driving AMR or on the presence and spread of AMR genes. Whole genome sequencing (WGS) of bacterial pathogens is a powerful tool that can be used for epidemiological surveillance, outbreak detection and infection control. In addition, whole metagenome sequencing (WMS) allows for the culture-independent analysis of complex microbial communities, providing useful information on the occurrence of AMR genes. Both approaches can be used to provide the information necessary for the implementation of quantitative risk assessment of AMR transmission routes along the food chain.

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Keywords: Antimicrobial resistance, risk assessment, surveillance, food-borne pathogens, whole genome sequencing, metagenomics

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1. Introduction

1.1. Antimicrobial resistance

Antimicrobials have been used for several decades to fight infections in humans, animals and plants caused by pathogenic microorganisms. In the last two decades, the emergence and transmission of multidrug-resistant pathogenic bacteria has created an urgent need to understand the underlying mechanisms involved. This is necessary to combat this global phenomenon, which is one of the main public health challenges of the 21st century (Smith et al., 2016; WHO, 2015). The ability of bacteria to resist the action of one or several antimicrobial agents through bacterial gene mutations or by acquiring exogenous resistance genes carried on mobile elements is defined as antimicrobial resistance (AMR) (ECDC, 2017). Acquired resistance to some antimicrobials is already so widespread as to compromise their value for the treatment of life-threatening infections (EFSA BIOHAZ Panel, 2008). Harmonised outcome indicators have been jointly established by the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) to assist Member States in assessing the progress made in reducing antimicrobial use and AMR both in humans and food-producing animals (ECDC, EFSA BIOHAZ Panel and CVMP, 2017). The possibility of AMR transmission via the food chain is currently under investigation (Verraes et al., 2013; Bengtsson-Palme, 2017), but the relative contribution of the food chain to the global burden of infections caused by antimicrobial-resistant microorganisms remains unknown. Controlling the emergence and spread of resistant bacteria and resistance genes in primary food production and food processing must therefore be a priority to reduce the occurrence of untreatable infections.

Risk analysis schemes provide a framework for the evaluation and communication of risks related to foods. For a risk assessment to be successful, adequate surveillance systems should be implemented. Surveillance of AMR is currently based on the isolation of indicator microorganisms and their phenotypic characterisation, but this culture-dependent approach does not provide complete information on the mechanisms driving AMR or on the presence or spread of AMR genes throughout the food chain. Metagenomics is a powerful tool that allows culture-independent analysis of complex microbial communities and thus has potential applications in AMR surveillance (Bonham et al., 2017; Flórez et al., 2017; Walsh et al., 2017). It can provide access to all the genetic resources in a given environmental niche, which is essential for obtaining the genomes of fastidious or non-culturable microorganisms. Metagenomics could therefore facilitate the tracking of AMR genes and mobile genetic elements, providing the essential information for quantitative risk assessments that will allow for the identification of hotspots and routes of transmission of AMR in the food chain.

2. Description of work programme

2.1. Aims

The aim of the work programme was to familiarise the Fellows with the plethora of risk assessment methods and to train them in the execution of qualitative and quantitative risk assessments in relation to AMR. The specific objectives were: (i) to perform a complete qualitative risk assessment to characterise the role of the food supply chain in the spread of AMR; (ii) to develop and validate a toolbox for the execution of next-generation-sequencing laboratory experiments and bioinformatics analyses for use in AMR surveillance programmes. The application of these methods in global surveys may provide insights into the mechanisms of selection and spread of antibiotic resistance in food-related settings and help identify hotspots of AMR spread in the food chain. Thus, knowledge-based interventions could be developed to reduce the spread and dispersal of multidrug-resistant bacteria throughout the food chain.

2.2. Activities/Methods

The objectives of the work programme were as follows:

Objective 1: The EU-FORA Fellows were trained in risk assessment methodologies routinely used by the supervisors and collaborators at the host institution. The Fellows received training in risk prioritisation, identification of risk factors through regression analyses and in-depth training in specific risk assessment software tools. The Fellows used this knowledge to compile a report calculating the percentage of transmissions that could be considered food-borne for *Cronobacter*, histamine and marine biotoxins. This opinion report along with additional data was used by Professor Jesús A. Santos,

who is a member of the Scientific Committee of the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN), as part of a risk assessment study carried out by this Institution.

Objective 2: The Fellows were trained on the execution of a qualitative risk assessment of the role of the food chain in the spread of AMR. A systematic approach mimicking that followed by EFSA panels in their Scientific Opinions was used for hazard identification and characterisation. A review article presenting the findings of this exercise was published in the international open-access peer-reviewed journal, *Genes* (impact factor 3.6), in the special issue *Genetics and Genomics of Foodborne Pathogens*, with the title 'The present and future of whole genome sequencing (WGS) and whole metagenome sequencing (WMS) for surveillance of antimicrobial resistant microorganisms and antimicrobial resistance genes across the food chain'. At the time of writing, a second review article, 'Food processing activities as a risk factor for AMR spread through the food chain', is in preparation for submission to the international peer-reviewed journal, *Current Opinion in Food Science*. In addition, the Fellows developed two survey questionnaires, one addressed to food companies in Spain concerning the use of biocides and the cleaning and disinfection protocols used in the food industry, and the other directed to the general public across the EU and concerning consumer awareness and risk perception of antimicrobial resistance.

Over a thousand answers were collected from the risk perception questionnaire on antimicrobial resistance, thanks to help from EFSA, which distributed the questionnaire link to all EFSA focal points and thence to all Article 36 organisations. The questionnaire was also distributed via web-based social network platforms such as Facebook and Twitter, and through emails, food- and health-related professional groups and associations as well as in a printed format. In brief, initial results indicate that almost 70% of the participants considered they were well- or very well-informed about antibiotics, which could be explained by the facts that 72% had received an education or specialisation related to the health or food safety sector and 67% had a profession related to those sectors. More than 90% of the participants had obtained antibiotics by medical prescription and adhered to the prescription guidelines. Almost all participants (98.4%) were aware of bacteria being resistant to antibiotics, and the majority believed that the most likely cause is the inappropriate use of antibiotics at farms and in household and clinical settings. About a quarter of the participants believed that antibiotics are used in the EU to stimulate the growth of farm animals, and another quarter did not know the answer to this question. Almost half of the participants either thought that antibiotics used on farm animals are different from those used on people or did not know the answer to this question. Approximately 80% of the participants had received information about antibiotic-resistant bacteria in humans compared with 56% of the participants who had received information about antibiotic-resistant bacteria in farm animals and 45% who had received information about antibiotic-resistant bacteria in foods. Participants felt more confident receiving information about antibiotic resistance in farm animals from scientists and national and European Food Safety Agencies and Institutions than national governments, consumer organisations, farmers, food manufacturers and family and friends. Almost 20% of the participants had changed their eating habits in the past 12 months because of antibiotic-resistant bacteria in farm animals, and 34%, when buying a meat product, considered whether the animal had been bred without the use of antibiotics, although 40% did not know where to check that information. Interestingly, 80% of the participants would be willing to pay more for a meat product that had been obtained from an animal produced without antibiotics, and more than half felt strongly or very strongly threatened by antibiotic-resistant bacteria. According to the participants, the highest priorities for reducing resistance to antibiotics should be the implementation of legislation further restricting the use of antibiotics, informing consumers of the risks of picking up resistant bacteria, helping farmers to shift to modes of production that require less or no antibiotic use, and more investment in research to replace ineffective antibiotics. Information from this survey will provide an overview of consumers' current awareness and perception of antimicrobial resistance. This survey could serve as a baseline for developing new strategies or adjusting existing ones to increase public awareness. To support future risk communication strategies at the European level, analysis of the results of the survey is in progress to identify how different sociodemographic factors influence consumers' perceptions.

The second survey questionnaire, relating to the use of biocides and the cleaning and disinfection protocols used in the food industry, is ongoing at the time of writing. This questionnaire is divided into three sections. Section one includes business details and questions relating to quality management, section two mostly focuses on manufacturing procedures, and section three on cleaning and disinfection protocols and procedures.

Objective 3: The Fellows contributed to the development and validation of harmonised methods and protocols suitable for monitoring AMR genes through next-generation sequencing. At the time of

writing, standard operating procedures (SOPs) are being built for the execution of culture-independent analyses of samples in routine AMR surveillance of foods and food processing samples. The aim is to build a toolbox of methods and protocols that can potentially be used for the surveillance of AMR. For this, the Fellows attended a training course organised by the Institute of Food Science and Technology of the University of León focused on acquiring knowledge in the use of genomics and metagenomics tools. To achieve this, the Fellows participated in taking food and environmental samples (swabs) from a Spanish meat industry site on several occasions and extracting genomic DNA from over 150 samples using established protocols for metagenomic analysis. In addition, the Fellows were involved in the extraction of bacterial fosmid DNA from a metagenomic library previously built from dairy and environmental samples. The fosmid DNA was then purified, subjected to enzymatic restriction analysis and sequenced to identify genes associated with phenotypically increased resistance to antibiotics including ciprofloxacin, gentamycin, tetracycline, cefotaxime and ampicillin. Laboratory research was performed within the framework of the research project 'Identification of routes and mechanisms of antibiotic resistance spread throughout the food chain through culture-dependent and culture-independent methodologies' funded by the Spanish Ministry of Economy and headed by the mentor.

Objective 4: The Fellows participated in dissemination and outreach activities. Two reports were published in international peer-reviewed high-impact-factor journals. Both Fellows presented their work and research interests at seminars in the Institute of Food Science and Technology (ICTAL), which belongs to the University of León. Results were disseminated by the Press Office of the University of León on several occasions, e.g. meeting with the Dean of Research of the University of León, meeting with the EFSA scientific coordinator of EU-FORA, etc. In addition, two poster presentations by the Fellows were accepted for the 2018 EFSA conference 'Science, Food, Society – contextualising risk assessment' to be held in Parma, Italy, 18–21 September 2018. The title of the first poster was 'A European survey questionnaire on consumers' awareness and risk perception of antimicrobial resistance', focused on the overall results of the survey questionnaire on consumers' awareness and risk perception of antimicrobial resistance at the EU level. The title of the second poster was 'Consumer awareness in Greece of issues concerning antibiotic use and the risk of antimicrobial resistance in bacteria', which focused on the results of the same survey, particularly in Greece, which was the country contributing the highest number of answers to the survey questionnaire.

The Fellows also participated in a three-day training course (20 h duration, 1 ECTS) entitled 'Practical workshop on genomics and metagenomics applications in the fields of food microbiology and veterinary microbiology' organised by the Institute of Food Science and Technology of the University of León. The objectives of this workshop were to acquire knowledge of the main applications of genomics and metagenomics techniques in food science as well as to become familiar with the main bioinformatics tools available for genomics and metagenomics studies. In brief, the Fellows independently learned to solve complex problems in the fields of genomics and metagenomics (hands-on exercises in sequence quality control, assembly and genome annotation, mobile genetic elements, antimicrobial resistance genes, virulence genes and others), designed experiments, understood the limitations of the experimental approach and were trained in the review of scientific information relating to genomics and metagenomics. The Fellows also attended a conference entitled 'University-enterprise conference: knowledge transfer in the agrifood sector', organised by the Asociación de la Industria Alimentaria de Castilla y León (Vitartis) and academics from the University of León.

In addition, Erasmus agreements for exchange of students and staff were signed between the Department of Food Hygiene and Technology of the University of León, Spain (hosting site) and the Department of Food Technology, Alexander Technological Educational Institute of Thessaloniki (ATEI-The), Greece (the Fellow's home organisation). One undergraduate student from the Department of Food Technology of ATEI-The is already undertaking her Erasmus placement in the Department of Food Hygiene and Technology of the University of León. Erasmus agreements were also signed between the home institutions of the two Fellows hosted in the University of León: The Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Romania; and the Department of Food Technology of ATEI-The, Greece. An additional Erasmus agreement was signed between the Department of Food Technology of ATEI-The, Greece and the Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia (the home organisation of another participant in the EU-FORA Fellowship programme).

Two research grant proposals were also prepared jointly between the Laboratory of Food Microbiology, Department of Food Technology, ATEI-The, Greece (the Fellow's home organisation) and the Laboratory of Food Microbiology, Department of Food Hygiene and Technology and Institute of Food Science and Technology, University of León, Spain (hosting site) and submitted to national and European funding bodies. In addition, a postdoctoral fellowship proposal (Juan de la Cierva –

Formación Fellowship) was submitted to the Ministry of Economy of Spain by the Fellow from Dunarea de Jos University of Galati, Romania, in collaboration with the University of León in the area of Food Science and Technology.

Finally, the Fellows took Spanish lessons to get acquainted with both the language and cultural heritage of Spain, and also participated in outdoor activities organised by the University of León (talks/seminars, skiing lessons, mountain walks, etc.).

3. Conclusions

Both Fellows have so far successfully fulfilled the objectives and tasks of the work programme proposal. Activities performed allowed the Fellows to communicate and disseminate the results. In this context, the Fellows undertook to prepare manuscripts for publication in peer-reviewed journals; one has been published in the journal *Genes*, which has an impact factor of 3.6, and another is currently being written. In addition, two survey questionnaires have been developed, one focused on addressing the use of biocides and the cleaning and disinfection protocols used in Spanish food industries, and another focused on current awareness and consumer perceptions of antimicrobial resistance. Both survey questionnaires can provide a basis for future risk management and communication strategies at the European level. Two poster presentations were submitted by the Fellows and accepted for the EFSA 2018 conference 'Science, Food, Society – contextualising risk assessment' to be held in Parma, Italy 18–21 September 2018. Both posters were linked to the results provided by the questionnaire of the survey on consumer awareness and risk perception regarding antimicrobial resistance. Moreover, Fellows were familiarised with a three-day training course entitled 'Practical workshop on genomics and metagenomics applications in the fields of food microbiology and veterinary microbiology' organised by the Institute of Food Science and Technology of the University of León.

Certain objectives were slightly amended during the placement and/or new objectives were introduced that still served the same purpose: training the Fellows in risk assessment methodology. Monthly laboratory meetings were arranged with the supervisors to monitor the status of the work objectives. The Fellows adjusted and integrated very easily at the University of León and worked in harmony with the rest of the Department staff (Figure 1) .

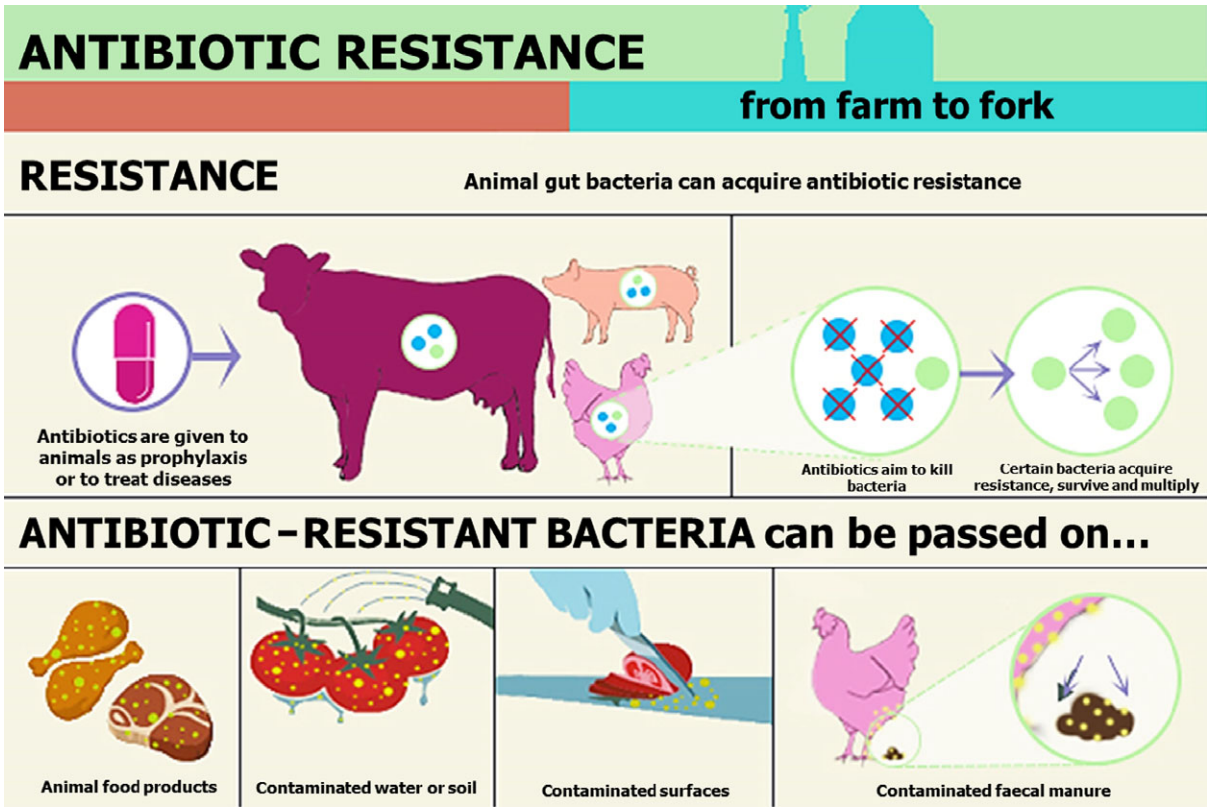

Figure 1: Schematic representation of antibiotic resistance spread through the food chain

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Abbreviations

AECOSAN	Spanish Agency for Consumer Affairs, Food Safety and Nutrition
AMR	antimicrobial Resistance
ATEI-The	Alexander Technological Educational Institute of Thessaloniki
ECDC	European Centre for Disease Prevention and Control
ECTS	European Credit Transfer and Accumulation System
EMA	European Medicines Agency
EU-FORA	European Food Risk Assessment Fellowship Programme
ICTAL	Institute of Food Science and Technology
SOPs	standard operative procedures
WGS	whole genome sequencing
WHO	World Health Organization
WMS	whole metagenome sequencing