EU-FORA SERIES 3

APPROVED: 7 September 2020

doi: 10.2903/j.efsa.2020.e181107

Identification of risk factors and hotspots of antibiotic resistance along the food chain using next-generation sequencing

Department of Food Hygiene and Technology, Institute of Food Science and Technology, Universidad de León, León, Spain,
I. Bergšpica, G. Kaprou, E. A. Alexa, M. Prieto-Maradona and A. Alvarez-Ordóñez

Abstract

Bacterial antimicrobial resistance (AMR) is considered to be very alarming following an upward trend and thus posing a primary threat to public health. AMR has tremendous adverse effects on humans, farm animals, healthcare, the environment, agriculture and, thus, on national economies. Several tools have been proposed and adopted by numerous countries after comprehending the need for antimicrobial stewardship and for a rational use of antibiotics. These tools include diagnostics for infections or AMR detection, for measuring and monitoring antibiotic consumption (e.g. surveillance tools) and for guiding medical doctors and veterinarians in selecting suitable antibiotics. In addition, it has been known that the food chain represents a leading vector for the transmission of pathogens to humans via various routes (direct or indirect). Considerable efforts have been made and are still in progress both at international and national levels in order to control and mitigate the spread of pathogens and thus ensure food safety. During the last decades, a new concern has risen regarding the food chain playing a potential major role in the transmission of resistant bacteria as well as resistance genes from the animal kingdom to humans. Several recent studies highlight the role of food processing environments as potential AMR hotspots contributing to this spread phenomenon. Next-generation sequencing (NGS) technologies are becoming broadly used in the AMR field, since they allow the surveillance of resistant microorganisms, AMR determinants and mobile genetic elements. Moreover, NGS is capable of providing information on the mechanisms driving and spreading AMR throughout the food chain. In the current work programme, the aim was to acquire knowledge and skills to track AMR genes and mobile genetic elements in the food chain through NGS methodologies in order to implement a quantitative risk assessment and identify hotspots and routes of transmission of AMR along the food chain.

Keywords: antimicrobial resistance, next-generation sequencing, food chain, risk assessment

Correspondence: eu-fora@efsa.europa.eu

Acknowledgements: This report is funded by EFSA as part of the EU-FORA programme.

Suggested citation: Department of Food Hygiene and Technology, Institute of Food Science and Technology, Universidad de León, León, Spain, Bergšpica I, Kaprou G, Alexa EA, Prieto-Maradona M and Alvarez-Ordóñez A, 2020. Identification of risk factors and hotspots of antibiotic resistance along the food chain using next-generation sequencing. EFSA Journal 2020;18(S1):e181107, 8 pp. https://doi.org/10.2903/j.efsa.2020.e181107

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

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1. Introduction

According to the World Health Organization (WHO), antimicrobial agents are indispensable drugs for the protection of human and animal health. Yet, the persisting emergence and spread of pathogenic microorganisms that are resistant to antimicrobial agents constitute a growing global concern (WHO, xxxx). Antimicrobial resistance (AMR) is one of the major health issues of the 21st century. A major factor leading to the spread of AMR is the use of antimicrobial drugs in veterinary medicine and human medicine, where misuse and overuse of antibiotics have been detected. However, another hotspot for AMR can be the food production environment.

Nevertheless, the relative drivers and the potential sources of AMR bacteria in the food chain are not well established yet. Overprescription of antimicrobial agents by clinicians is considered one the main sources of selection for AMR, and some antimicrobials are used in larger quantities in food production. These resistant bacteria can afterwards reach humans via the environment, food or other means (Hudson et al., 2017). According to the Center for Disease Control and Prevention (CDC), antibiotic-resistant bacteria can spread through the food chain in various ways. For instance, in the process of animal slaughtering and meat processing, contamination of meat (or other animal products) as well as processing equipment can occur by resistant bacteria. In addition, animal waste can be a source of resistant bacteria which can contaminate the surrounding environment. Furthermore, vegetables and fruits can get contaminated while being in contact with water, soil or fertilisers containing animal waste contaminated with resistant bacteria (Centers for Disease Control and Prevention, online). In other words, food and food processing environments (FPEs) could be reservoirs and vehicles of transmission of AMR to humans causing a major public health impact.

In order to bring antibiotic-resistant bacteria under control several actions could be implemented, such as in the fields of i) education and training, ii) surveillance, monitoring and record-keeping, iii) legislation and regulations, or iv) optimisation and reduction of irrational antibiotic use (Caniça et al., 2019).

Among the surveillance and monitoring tools, omics technologies (e.g. genomics, metagenomics and transcriptomics), serve the purpose of monitoring and controlling AMR in various One Health settings, mainly in respect to the selection and distribution of AMR in food-related settings. In addition, omics could facilitate the unravelling of the associated AMR mechanisms (Caniça et al., 2019).

Major improvements have been made in sequencing technologies during the last decade. These technologies are referred to as next-generation (or high-throughput) sequencing (NGS). NGS has considerably revolutionised the analysis not only of bacterial genomes but also of complex bacterial communities (i.e. intestinal or environmental microbiota). Regarding the AMR field, NGS facilitates both the identification of already known resistance genes and it also enables the prediction of novel ones. NGS has advanced the capacity of tracking bacterial clones (e.g. multidrug-resistant clones), and the identification of new antibiotic resistance genes (ARGs) as well as their genetic carriers (i.e. plasmids). Therefore, NGS paves the way for new perspectives in risk assessment and AMR surveillance while simultaneously it enables the comprehension of the AMR dynamics between pathogens and commensals stemming from various sources (e.g. environment, animals and humans) (Ruppé et al., 2019). The main advantages offered by NGS are: i) it facilitates clinical decision-making by providing various levels of information to guide the treatment with the appropriate antimicrobial; ii) it improves outbreak investigation and guides the interventions to control them; iii) it allows a retroactive analysis once new information appears (storing NGS data for future analysis); iv) it has the potential to link various fields such as clinics, food, environment and animals; v) it provides mechanistic information on the resistance. Regarding these latter advantages, unlike phenotypic tests – providing information only related to resistance/susceptibility to antimicrobials – NGS can shed light on the molecular basis for the resistance. Moreover, NGS has the potential to characterise novel resistance mechanisms once they arise (via the sequencing of isolates proved to be phenotypically resistant). This is an outstanding added value compared to conventional nucleic-acid based techniques (e.g. polymerase chain reaction, PCR) (Berendonk et al., 2017).

2. Description of work programme

2.1. Aims

The aim of the work programme was to acquaint the fellows in the execution of assessments to identify risk factors present in the food chain that could allow occurrence and spread of antibiotic-resistant microorganisms, AMR genes and mobile genetic elements in FPEs. The main focus was to

investigate the potential of NGS methodologies as a tool for the identification and characterisation of resistant bacteria and their AMR gene repertoire. The outcomes of the work programme may assist the design of knowledge-based interventions facilitating the reduction of the dispersal of multidrug-resistant microorganisms in the food industry.

2.2. Activities/methods

The objectives of the work programme are listed below:

Objective 1: Training of the fellows on the risk assessment methodologies routinely used by the mentor and other collaborators at the host institution.

The research fellows gained in-depth training in specific tools commonly employed by laboratory members of the supervisor and collaborators. The fellows were familiarised with the use of several software tools such as: Monte Carlo simulation distribution software (@Risk, Crystal Ball), software programs for quantitative microbiology and risk assessment models (Combase, FSSP) and last but not least risk ranking tools (RiskRanger).

Moreover, they received training in the use of the SPSS software program for processing the acquired data from the performed experiments in order to identify any correlations among the various parameters being analysed. In addition, several simulations were ran regarding various case studies employing the @Risk software allowing the fellows to obtain a more clear view and acquire hands-on experience on Monte Carlo simulations and the importance of distribution functions in the risk assessment process.

Objective 2: Execution of a qualitative risk assessment approach on AMR in the food chain.

A qualitative assessment of the risk posed by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae was undertaken, focused on hazard identification and exposure assessment. Exposure assessment modelling was carried out regarding the presence of ESBL-producing *E. coli* in pork meat products within the European Union (EU). A systematic literature review was conducted where the *Scopus* database was used for the article search. The extracted data were latter used for building an exposure assessment model. Software (@Risk and Crystal Ball) were used for the simulation part. Apart from the literature review conducted related to the prevalence of ESBL-producing *E. coli* on pork meat, the data from EFSA Comprehensive European Food Consumption Database (EFSA, 2020) were also taken into account in order to perform the exposure assessment.

Objective 3: Phenotypic and genotypic characterisation of a collection of isolates from FPEs.

The fellows characterised a wide collection of isolates from FPEs with classical microbiology tools and NGS (Figure 1). These included culture-based methods -pure culture isolation, determination of resistance to antibiotics (growth in media supplemented with antibiotics (chromogenic media)), and molecular-based methods (PCR). In more details, the two fellows isolated pure cultures of Enterobacteriaceae*, Enterococcus* spp. and *Staphylococcus* spp. from samples obtained from FPEs stemming from 30 industries from different food sectors. Over 500 pure isolates were screened on chromogenic media. Pure isolates were verified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF). Pure isolates were obtained and preserved. DNA isolation was performed for all the isolates which exhibited resistance in the chromogenic media. PCR was performed for Enterobacteriaceae isolates to determine presence of $bla_{CTX\text{-}M}$ and bla_{SHV} genes. Regarding the *Enterococcus faecalis* and *Enterococcus faecium*, the presence of the *vanA* and *vanB* genes was examined. 15 *E. coli* and 45 *E. faecalis* and *E. faecium* isolates were screened against a wide array of antimicrobials following the broth microdilution method. The most interesting isolates were analysed through whole genome sequencing (WGS), specifically using the Illumina HiSeq system. The acquired whole genome sequences were analysed by bioinformatic tools. In Figure 1, the flow chart followed for the experimental part is depicted.

Objective 4: Integration of metadata provided in Objective 3 into risk assessment models.

Main hazards were identified along the food production chain in different food industries. Analyses of a questionnaire on hygiene and sanitation practices filled by food producers were performed. Correlations between different variables were assessed, binary logistic regression, as well as some statistical analyses were performed. Regression analyses were conducted in order to investigate any possible correlations between the industry status in relation to the occurrence of Enterobacteriaceae and *Enterococcus* spp., respectively and the metadata on the food safety practices employed by each industry. The FSSP software was used to perform the analyses.

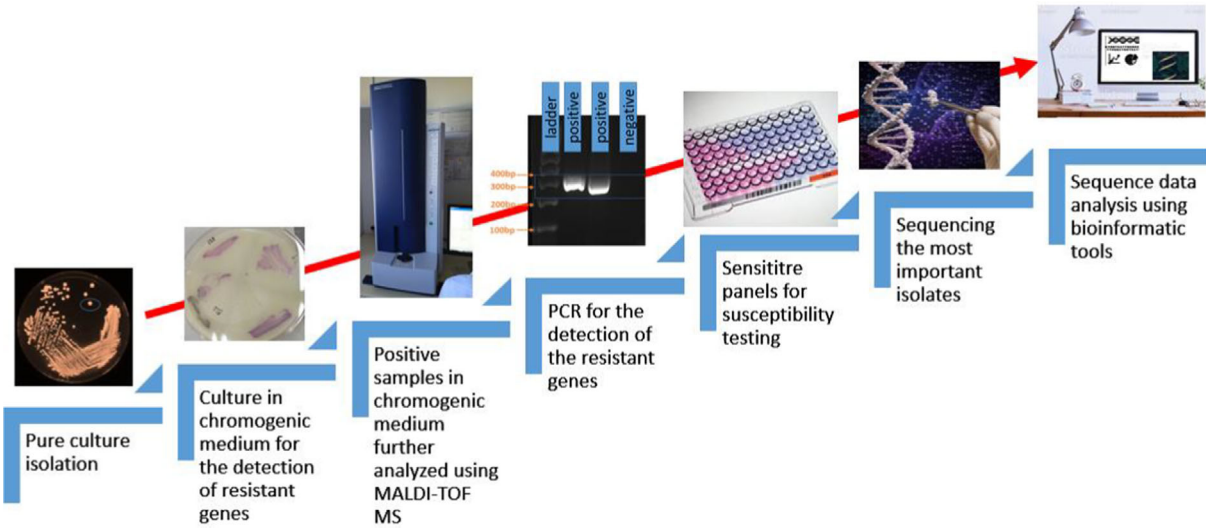

Figure 1: Flow chart of the experimental work undertaken under objective 3 of the work programme

Objective 5: Communication, dissemination and outreach activities.

The fellows participated in dissemination and outreach activities. Two review articles with the following titles 'A review on Extended Spectrum β-Lactamase (ESBL) producing *Escherichia coli* in pigs and pork meat in the European Union' and 'Rapid methods for antimicrobial resistance diagnostics' will be sent for publication during September in peer reviewed journals. The former review covers the analysis of the prevalence of ESBL-producing *E. coli* in pork meat and pigs in the EU, and dissemination pathways of these bacteria along the pork production chain. The latter review article epitomises the newly introduced rapid methods such as NGS, Fourier-transform infrared (FTIR) spectroscopy, MALDI-TOF and lab-on-a-chip (LoC) technologies addressing the detection of AMR in a fast and reliable manner. In Table 1, the courses, conferences and seminars attended by the fellows are summarised.

Fellows were also actively participating in outdoor activities hosted by members of the laboratory and by the work programme supervisors.

Table 1: Summary of courses, conferences and seminars attended by the fellows

Course/seminar	Type of presentation/ courses	Topic	Location
Seminar	Oral presentation, both fellows	Research interests and background	ICTAL, Leon, Spain. November 2019
International Scientific Symposium, Science To Strengthen Sustainable And Safe Food Systems	Poster presentation with previous EU-FORA fellow (Janis Ruško)	EU-FORA fellows of Latvia	BIOR, Riga, Latvia, 30–31 January 2020
	Oral presentation, by Georgia Kaprou	Integrated, fast, cost effective, semi-automated Lab on a Chip for foodborne pathogen detection	
MALDI-TOF instrument as a tool to identify bacterial species	On site courses organised by the Head of the Microorganism culture collection group Laura Alksne	MALDI-TOF use to identify bacteria species and AMR protein fingerprint detection	BIOR, Riga, Latvia, 4 February 2020

Course/seminar	Type of presentation/ courses	Topic	Location
Antimicrobial resistance theory and methods	Online courses	Antimicrobial action and resistance, antimicrobial susceptibility testing and interpretation, quality assurance	DTU, February–March 2020
WGS of bacterial genomes, tools and applications	Online courses	Typing of bacteria, use of WGS for surveillance of bacterial pathogens and AMR, MLST typing, phylogenetic tree building	DTU, February-March 2020
SPSS training on data interpretation	Online courses organised by E.A. Alexa, fellow from the 1st EU-FORA cohort	Chi-Square Test, One-way Anova and Binary logistic regression analyses	University of León, León, Spain. 18 May 2020
WGS analyses	Online courses organised by Jose Francisco Cobo-Diaz, bioinformatician at the hosting site	Assembling raw reads of sequences, single nucleotide polymorphism analyses, AMR gene detection, serotype identification	University of León, León, Spain. 16–17 June 2020
Monte Carlo simulation (distribution functions) using @Risk & Crystalball software	On site and online courses organised by Miguel Prieto Maradona	Data integration in existing models and building a model on ESBL-producing *E. coli* in pork meat	University of León, León, Spain. February–August 2020
Annual Scientific Meeting (ASM) of the One Health European Joint Programme (OHEJP)	Online event	Food-borne zoonoses, antimicrobial resistance and emerging threats	27–29 May 2020

MALDI-TOF: matrix-assisted laser desorption/ionisation time-of-flight; AMR: antimicrobial resistance; WGS: whole genome sequencing; MLST: multilocus sequence typing; ESBL: extended spectrum β-lactamase.

3. Conclusions

Both fellows have successfully fulfilled the objectives and tasks of the proposed work programme. Activities performed allowed the fellows to acquire skills related to the execution of risk assessments and the communication and dissemination of results through various actions (seminars, seminars, publications). More specifically, one review article is going to be submitted in the peer-reviewed *Journal of Antibiotics* (impact factor 3.893) and the second one in the *Journal of Microbiological Methods* (Impact Factor: 1.707). Moreover, Fellows were familiarised with risk assessment and Monte Carlo simulation software for data processing. Furthermore, short trainings were performed regarding NGS analyses as well as MALDI-TOF apparatus use.

Certain objectives were slightly amended due to the COVID-19 pandemic; however, the fellows successfully fulfilled the main objectives and tasks of the work programme. Apart from the two review papers which are going to be submitted during September, the fellows are planning to continue collaborating in the following months in order to publish the results stemming from the activities regarding the objectives 2 and 3; more specifically, the results of the analysis on the occurrence of AMR from FPEs and the WGS analyses of the most interesting isolates, as well as those from the exposure assessment modelling for ESBLs in pork products. The results of this ongoing effort will very likely lead to 2–3 more publications.

3.1. Future goals

The close cooperation between the fellows and the hosting site personnel will be maintained. Hopefully, research grant proposals between sending and hosting institutions will be drafted. Moreover, close collaboration between the hosting site and the home institution of the fellows is envisioned in order to further proceed with the outcomes of the work programme and finalise the remaining publications.

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Abbreviations

AMR	antimicrobial resistance
ARGs	antibiotic resistance genes
ASM	Annual Scientific Meeting
BIOR	Institute of Food Safety, Animal Health and Environment
CDC	Center for Disease Control and Prevention
DTU	Technical University of Denmark
ESBL	extended-spectrum β-lactamase
EU-for a	European Union Food Risk Assessment
FPE	food processing environment
FTIR	Fourier-transform infrared spectroscopy
ICTAL	Institute of Food Science and Technology
LoC	lab-on-a-chip
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
MLST	multilocus sequence typing
NGS	next-generation sequencing
OHEJP	One Health European Joint Programme
PCR	polymerase chain reaction
WGS	whole genome sequencing
WHO	World Health Organization