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Training in metagenomics-integrated risk assessment for food-borne pathogens in the Slovenian and Spanish meat chain (METAMEAT)

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Abstract

Next-generation sequencing (NGS) has become an essential tool for antimicrobial resistance (AMR) surveillance, enabling comprehensive detection of AMR determinants in both bacterial isolates and complex microbial communities. Metagenomic sequencing enables culture-independent profiling of antimicrobial resistance genes (ARGs) in different environments, while whole-genome sequencing (WGS) is widely used in AMR surveillance laboratories to predict phenotypic resistance in major food-borne pathogens. AMR risk assessment usually considers factors such as the pathogenicity of the ARG-carrying bacterial host, the abundance of ARGs and their mobility potential inferred from association with plasmids or other mobile genetic elements that facilitate horizontal gene transfer. Clinical relevance of antimicrobials and the severity of clinical outcomes can further be implemented in AMR risk assessment. Exposure assessment contextualises hazards within real-world scenarios by estimating consumer exposure to AMR bacteria or their ARGs through food or other routes. Despite challenges in fully quantitative assessments, the integration of NGS-based surveillance with risk modelling represents a critical step towards proactive AMR risk management. In this study, broiler samples from different stages of a Slovenian and a Spanish slaughterhouse were analysed using conventional microbiology, shotgun metagenomic sequencing and WGS of isolates of selected pathogenic species. A modular, semi-quantitative risk assessment model was developed that combines (meta)genomic data with key risk factors and, where available, exposure assessment. This approach prioritises AMR risks in broiler meat processing and supports evidence-based decision-making in the areas of food safety and public health.

KEYWORDS

antimicrobial resistance, broiler meat processing chain, Illumina technology, metagenomics, next-generation sequencing, semi-quantitative risk ranking

CONTENTS

1 | INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing global public health crises of the 21st century. The rise of bacteria resistant to commonly used antimicrobials threatens to undermine decades of medical progress, making once-treatable infections potentially lethal (World Health Organization, 2022). The emergence and spread of AMR is driven by complex and dynamic interactions between pathogens, commensals and environmental bacteria that can exchange antimicrobial resistance genes (ARGs) through horizontal gene transfer (HGT). Successfully and comprehensively addressing AMR risks in interconnected human, animal and environmental systems requires a multi-method approach that is consistent with the One Health framework. Furthermore, there is a lack of science-based risk assessment frameworks that can both identify AMR-related hazards and transparently rank and quantify the associated risks. Such integration of (semi)quantitative data into AMR risk assessment models could be used to evaluate the impact of different hypothetical scenarios (targeted control measures) to combat AMR.

Next-generation sequencing (NGS), also known as high-throughput sequencing or massively parallel sequencing, has transformed the field of AMR detection, monitoring and characterisation. Whole-genome sequencing (WGS) of bacterial isolates enables the identification of known and putative novel AMR determinants in bacterial genomes. On the other hand, shotgun metagenomic sequencing of complex communities provides insight into the entire repertoire of AMR determinants within a given sample (i.e. the resistome) without the need for prior cultivation of AMR isolates.

In WGS, an entire genome of an isolate is sequenced, providing insight into the entire gene repertoire of a given isolate, including its ARGs and virulence genes. It has become essential in current AMR surveillance in public health, particularly for priority food-borne bacteria such as *Escherichia coli* and *Salmonella* Enterica. In these species, genotype–phenotype correlation of AMR profiles is high; therefore, prediction of phenotypic AMR profiles based on the presence of specific ARGs and AMR-associated mutations is highly accurate (Boolchandani et al., 2019; Clausen et al., 2016). Metagenomics bypasses the culturing step and enables culture-independent profiling of the entire resistome of complex microbial ecosystems such as microbiomes of the animal gut, soil, water, wastewater or food (Fitzpatrick & Walsh, 2016). Metagenomic approach can uncover potential hidden reservoirs of ARGs in non-pathogenic environmental and commensal bacteria that may serve as sources for the transfer of ARGs to pathogens through HGT. In addition, it captures the overall diversity and abundance of ARGs in all bacteria and offers insights into the environmental drivers and dissemination pathways of AMR that are not considered in isolate-based surveillance strategies. Consequently, it enables the tracking of resistomes across the One Health continuum (humans, animals, environment and food) and helps to identify AMR hotspots and transmission pathways (Martak et al., 2024).

While AMR surveillance identifies the presence and possibly the characteristics (class, type or group) of AMR-related hazards, risk assessment is the strategy that allows these hazards to be prioritised and resources to be allocated. Current AMR risk assessment frameworks that rely on NGS typically consider several interrelated key factors such as the pathogenicity of the bacterial host, the abundance of ARGs and their mobility potential. First, host pathogenicity is associated with the ability of the AMR host to cause disease. In other words, an ARG in a highly virulent and invasive pathogen poses a greater threat to human health than the same gene in a non-pathogenic environmental bacterium. It is usually measured by the number of known virulence or pathovar-associated genes. Second, a higher abundance of ARGs in a given sample or in the genome of the AMR isolate indicates a higher AMR risk. Third, ARGs can be transferred to other bacterial hosts through HGT, which increases the risk of AMR spread. The ability of an ARG to spread through HGT is influenced by its association with mobile genetic elements such as plasmids, transposons and integrons that facilitate such transfer (Partridge et al., 2018). In bioinformatics, ARG mobility is usually measured by their proximity to mobile genetic elements. Recently, long-read sequencing technologies such as Oxford Nanopore Technologies and PacBio SMRT sequencing have enabled the reconstruction of gap-free plasmids and the identification of ARGs associated with mobile genetic elements, improving the accuracy of predicting ARG mobility.

Two additional characteristics that can be included in the framework are the clinical relevance of the antimicrobial agent to which the bacterial host is resistant and the severity of the clinical outcome (EFSA and ECDC, 2025). AMR-related risk is of particular interest when it concerns antimicrobials critical for human medicine. In humans, resistance to last-resort antimicrobials such as extended-spectrum cephalosporins, carbapenems, fluoroquinolones and polymyxins (e.g. colistin) is of particular concern due to limited or no alternative treatment options, particularly because they can lead to increased mortality, prolonged hospitalisation, treatment failure and more severe disease outcomes (Zanichelli et al., 2023). In livestock, veterinary important antimicrobials for food-producing animals such as aminoglycosides, amphenicols, cephalosporins and macrolides can be prioritised (World Health Organisation for Animal Health, 2024).

A comprehensive farm-to-fork risk assessment model should include exposure assessment that estimates the likelihood and magnitude of human (or animal) exposure to AMR bacteria or ARGs through relevant pathways such as consumption of contaminated food/feed or water or transmission between individuals (EFSA BIOHAZ Panel, 2021; World Health Organization, 2021). Due to the currently limited data available, such an assessment is often semi-quantitative and based on several simplifications. Nonetheless, a farm-to-fork quantitative risk assessment model for cephalosporin-resistant *Salmonella* Heidelberg in broilers was developed (Collineau et al., 2020), which was used to assess the impact of different hypothetical interventions in broiler farms on the likelihood of human disease. Major gaps remain in the understanding of the pathogenicity, growth and survival of AMR strains compared with their susceptible counterparts.

Another challenge in reliable quantitative AMR risk assessment based on NGS data is the lack of standardisation of bioinformatic pipelines and databases for the detection and characterisation of ARGs. In addition, the interpretation of the

functional impact of novel ARG variants, the integration of different data sources (experimental, genomic, metagenomic, transcriptomic, epidemiological, clinical and consumption data) and the modelling of the complex transmission pathways are challenging. The integration of NGS data into AMR surveillance and risk assessment is a crucial step in fighting AMR. By providing insights into the genetic basis, distribution and mobility of ARGs, NGS facilitates the transition from reactive detection to a proactive risk management and mitigation.

Despite all the discussed technologies that have substantially strengthened AMR surveillance and hazard identification, there is still a need to translate (meta)genomic data into actionable, context-specific risk assessments that can guide decision-making and targeted interventions in the food chain. To address this, in this project, broiler neck skin and caecal samples were collected from different stages of a Slovenian and a Spanish slaughterhouse and analysed using both conventional microbiology and NGS to detect ARGs, mobile genetic elements and (putative) host pathogenicity. All broiler samples underwent shotgun metagenomic sequencing using Illumina technology. DNA from selected zoonotic pathogenic isolates was sequenced using Oxford Nanopore or Illumina technology. A modular, semi-quantitative AMR risk assessment model was developed to rank complex broiler samples and AMR isolates with respect to their AMR-associated risk, incorporating key factors such as host pathogenicity as well as the abundance and mobility of ARGs, while simultaneously weighting the clinical importance of antimicrobials and, in the case of selected AMR isolates, estimating consumer exposure through contaminated broiler meat.

2 | BACKGROUND AND TERMS OF REFERENCE

The European Food Risk Assessment Fellowship (EU-FORA) is a practical training program aimed at increasing the number of food safety risk assessment experts in Europe and promoting Member States' participation in risk assessment activities. The fellowship project, entitled 'Training in metagenomics-integrated risk assessment for food-borne pathogens in the Slovenian and Spanish meat chain (METAMEAT)', was developed through a partnership between the University of Ljubljana (Slovenia) as the sending institution of the fellow Dr. Bojan Papić, and the Technical University of Cartagena (Spain) as the hosting institution, with Dr. Enriqueta Garcia-Gutierrez and Prof. Pablo S. Fernández acting as supervisors.

3 | DATA AND METHODOLOGIES

3.1 | Sampling in Slovenian and Spanish broiler slaughterhouses

One Slovenian and one Spanish broiler slaughterhouse were included in the study, and the same sampling strategy was applied in both. Pooled neck skin samples were collected from three stages of the slaughter process, i.e. before and after evisceration and after the exit of the carcasses from the cooling tunnel.

Evisceration was chosen because it is one of the critical steps in the slaughter process for microbiological contamination of broiler carcasses. Namely, automated evisceration systems can rupture the intestine, leading to contamination of carcasses, equipment and surfaces (Gruntar et al., 2015).

Different slaughter batches (corresponding to different flocks) were analysed, and technical replicates were also analysed at each sampling point to ensure that potential significant differences between the study groups were reliably and consistently identified. Broiler caecal contents were also sampled.

3.2 | Sample preparation and microbiological examination

The neck skin samples (25 g) were first supplemented with 225 mL of buffered peptone water and homogenised for 2 min. Homogenised samples then underwent the following microbiological examinations:

- thermophilic *Campylobacter* count: direct cultivation on mCCDA agar (ISO 10272-2:2017);
- total *E. coli* count: TBX agar;
- total aerobic count: Petrifilm aerobic count plates.

In addition, non-selective pre-enrichment of the samples (37°C, 18 h) was performed, followed by cultivation on selective media:

- motile *Salmonella*: semi-solid MSRV agar for an additional selective pre-enrichment, followed by confirmation of *Salmonella* presence on chromogenic agars XLD and Rambach (ISO 6579-1:2017);
- Extended-spectrum beta-lactamase (ESBL)-producing *E. coli*: chromID ESBL agar;
- Carbapenemase-producing *E. coli*: chromidID CARBA SMART agar (CARB/OXA).

3.3 | DNA extraction and next-generation sequencing

Total DNA was extracted from neck skin and caecal samples using PowerSoil Pro kit (Qiagen). Shotgun metagenomic sequencing of the samples was performed using Illumina paired-end (2 × 150 bp) technology on a NovaSeq X Plus system.

Total DNA from the obtained bacterial isolates was extracted using DNeasy Blood & Tissue kit (Qiagen). WGS of isolates was performed using Illumina paired-end (2 × 150 bp) technology on a NovaSeq X Plus system or Oxford Nanopore's MinION sequencer.

3.4 | AMR risk assessment

For resistome risk ranking, we used a slightly modified model implemented in the MetaCompare 2.0 tool (Rumi et al., 2024). This model is intended for short-read NGS data generated with Illumina technology and takes into account the abundance of ARGs, their mobility and host pathogenicity. Originally, ARG abundance is represented by the number of ARG-carrying contigs weighted by the total number of contigs. ARG mobility is represented by the number of MGE-carrying ARG contigs weighted by the total number of contigs. Host pathogenicity is represented by the species-level taxonomic classification of contigs to a given list of bacterial pathogens. Each of these variables represents a dimension in a 3D (or 4D) hazard space, and the final risk score is calculated using the Euclidean distance of the sample to the maximum point in the 3D hazard space (Figure 1). It should be noted that this resistome risk ranking model does not include exposure assessment data but rather focuses on hazard identification and characterisation.

MetaCompare 2.0 defines two different resistome risk scores. Human health resistome risk focuses on clinically important (high-risk) antimicrobials and pathogens (e.g. ESKAPEE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *E. coli*). Ecological resistome risk focuses on all ARGs and all potential pathogenic hosts (Rumi et al., 2024).

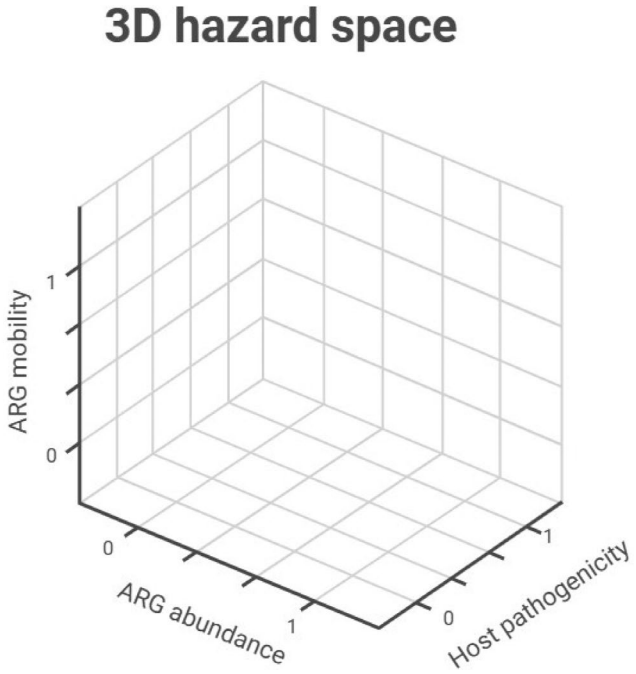

FIGURE 1 A concept of a 3D hazard space that is implemented in the MetaCompare 2.0 tool for resistome risk ranking.

A conceptually similar model was used in this training to rank the AMR isolates from Slovenia and Spain according to their AMR risk and broiler metagenomes according to their resistome risk. ARG abundance was estimated either by the number of ARGs (isolate-based AMR risk) or by the number of ARG-carrying contigs, normalised by the total number of contigs (resistome risk). ARG mobility was estimated as the number of MGE-carrying ARG contigs, normalised by the number of ARG-carrying contigs in resistome risk only. Host pathogenicity was assessed either through taxonomic classification of the ARG-carrying contigs to putative pathogen species (resistome risk) or by quantifying pathovar- or species-associated virulence genes (isolate-based AMR risk).

3.5 | Secondary scientific activities during the fellowship

The fellow participated (or will participate) in the following activities:

Invited speaker – conference:

- CESAR2025 (https://hmd-cms.hr/cesar2025/) – 22–25 September 2025, Zadar (Croatia), invited talk entitled 'Integrating (meta)genomic sequencing data into AMR risk assessment'.

Invited speaker – lecture:

- Seminar for the Advanced Techniques of Agrifood R&D PhD programme (TAIDA) entitled 'Integration of »omics« into risk assessment'.

Hands-on workshops:

- A course on microbial risk assessment using R, enhancing proficiency in modelling tools used in food safety risk assessment, delivered by Dr. Alberto Garre (UPCT, Spain).
- A wet-lab training on Oxford Nanopore sequencing, covering sample preparation, sequencing and bioinformatic workflows as well as troubleshooting, delivered by Mr. David Baker (Quadram Institute Bioscience, United Kingdom).

Webinars:

- ECDC GenEpi-BioTrain - Virtual training 14 - R data analysis and visualisation for beginners.
- ECDC GenEpi-BioTrain - Virtual training 18 - Empowering AMR Research through R: Analysis and Visualisation.

4 | ASSESSMENT

4.1 | Microbiological examination

Microbiological examination of broiler neck skin samples revealed different trends in microbial counts between the slaughterhouses examined. A linear mixed model showed that biological repetition (flock) had a significant influence on microbial counts in both slaughterhouses. In one slaughterhouse, *E. coli* counts increased after evisceration, indicating contamination from gut contents during the process; however, the counts subsequently decreased after chilling. These differences in microbial trends between slaughterhouses may be attributed to differences in meat processing technologies or hygiene practices. In addition, flock-level variation (e.g. gut microbiota composition, health status or on-farm management) likely also contributed to the observed differences, as shown by the significant influence of biological repetition in the model.

4.2 | Isolate-based AMR risk assessment

The risk assessment model was successfully applied to rank the obtained AMR isolates based on their AMR risk. It integrated the 3D hazard space concept (hazard characterisation) with national data on annual chicken meat consumption and portion size (exposure assessment). As a result, the model effectively differentiated between isolates of the same species based on their ARG repertoire and national consumption patterns.

4.3 | Resistome risk ranking

The resistome risk of caecal samples was very comparable between the biological repetitions. Resistome risk scores differed significantly between the analysed slaughterhouses, with the ecological resistome risk scores being higher than the human health resistome scores. In contrast, resistome risk ranking of the broiler neck skin samples differed markedly between the biological repetitions. A major methodological limitation in assessing the resistome risk of neck skin samples was the low abundance of detected ARGs, primarily due to the high (>98%) proportion of host (*Gallus gallus*) DNA in the obtained sequences. This finding suggests that a host depletion strategy should be employed to increase the proportion of microbial DNA in shotgun metagenomic sequencing. Potential approaches include neutrase–saponin pretreatment of food samples prior to DNA extraction, depletion of eukaryotic DNA post-extraction (e.g. using the NEBNext Microbiome DNA Enrichment Kit, NEB) or adaptive sampling during nanopore sequencing to reject reads that map to the host genome.

5 | CONCLUSION

The project confirmed the usefulness of metagenomic sequencing as a powerful tool for comprehensive AMR (resistome) surveillance in food systems, enabling high-resolution profiling of AMR determinants and supporting evidence-based risk modelling. The comparison between Slovenian and Spanish broiler processing chains revealed notable differences in ARG prevalence and microbial community structure, which may reflect national or flock-based differences in antimicrobial use, farming/slaughtering practices and biosecurity measures. The developed risk assessment model combines NGS data with public health considerations to produce a semi-quantitative framework for ranking AMR risks. Despite its semi-quantitative nature, this integrative approach improves the transparency and effectiveness of AMR risk prioritisation in the context of food safety and public health.

6 | RECOMMENDATIONS

We recommend the integration of metagenomic surveillance into routine AMR surveillance programmes in all EU Member States to complement conventional culture-based methods and enable comprehensive resistome profiling. We also recommend supporting the development and validation of modular, (semi)quantitative risk assessment tools that can be adapted to different food production and processing systems and countries to improve transparency and prioritisation in AMR risk management. AMR surveillance should prioritise ARGs conferring resistance to critically important antimicrobials, especially those associated with high prevalence, mobility potential and/or host pathogenicity, to better guide intervention strategies to protect public health. Finally, we aim to promote the open sharing of sequencing data, analytical pipelines and risk models to strengthen an integrated European AMR surveillance network and a collective risk assessment capacity. The investigation also revealed a critical lack of publicly available (genomic) data on antimicrobial susceptibility of food-borne pathogens from human clinical cases in national and joint EU reports. Accurate estimation of AMR profile occurrence across sectors would enable more precise attribution of human cases caused by AMR strains to broiler meat, especially when isolates from different sectors are typed using WGS.

ABBREVIATIONS

AMR	antimicrobial resistance
ARG	antimicrobial resistance gene
ESBL *E. coli*	extended-spectrum beta-lactamase-producing *Escherichia coli*
HGT	horizontal gene transfer
NGS	next-generation sequencing
WGS	whole-genome sequencing

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DISCLAIMER

The results of these studies are intended to be published in other scientific journals. To avoid copyright claims, they were described only very briefly in this report.

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