# Comparative metagenomics reveals poultry and swine farming are hotspots for multidrug and tetracycline resistance☆

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## ARTICLE INFO

## ABSTRACT

Antibiotic misuse in livestock is a major threat to human health, as bacteria are quickly developing resistance to them. We performed a comparative analysis of 25 faecal metagenomes from swine, poultry, cattle, and humans to investigate their resistance profiles. Our analysis revealed that all genes conferring resistance to antibiotic classes assessed except tetracyclines were more prevalent in poultry manure than in the remaining species. We detected clinically relevant antibiotic resistance genes, such as *mcr-1* which confers resistance to polymyxins. Among them, extended-spectrum β-lactamase *bla*CTX-M genes were particularly abundant in all species. Poultry manure was identified as a hotspot for multidrug resistance, which may compromise medical treatment options. Urgent actions in the livestock industry are imperative to hamper the emergence and spread of antibiotic resistance.

## 1. Introduction

Antimicrobial resistance (AMR) has become one of the greatest threats to global public health because the growing emergence of antibiotic-resistant bacteria is compromising the effectiveness of antimicrobial therapy. In fact, 4.95 million deaths in 2019 were estimated to be associated with AMR, including 1.27 million deaths directly attributable to AMR (Murray et al., 2022). Although AMR occurs in nature and has an ancient origin, overuse of antibiotics –and particularly their misuse as a prophylactic and growth promoter in livestock (van Boeckel et al., 2017)– has enhanced the emergence of AMR in the environment, where they may exert a selective pressure among bacterial communities for antibiotic resistance genes (ARGs), even at low concentrations (Sun et al., 2020). In fact, livestock manure is considered a hotspot for AMR, as well as the main source of antibiotics in the environment, particularly upon its use as fertilizer (Kim and Cha, 2021). Moreover, the acquisition and spread of ARGs in environmental settings may be facilitated by mobile genetic elements (MGEs), such as plasmids and integrative conjugative elements, as they mediate horizontal genetic transfer among different bacterial species (Igrejas et al., 2016; Partridge et al., 2018).

There is evidence of the environmental transmission of ARGs and their bacterial hosts among livestock and humans, which can spread via contaminated derived food, dust and manure, and wastewater discharge. Indeed, human chronic exposure to livestock-associated environments, such as farms, may be a key transmission route of AMR among animals and humans (Sun et al., 2020). Given that AMR represents a major global health concern, a greater understanding of the mechanisms and factors that promote this phenomenon is needed to implement appropriate strategies and mitigation programs at the human-animal-environment interface, according to the One Health approach (Walsh, 2018). Moreover, clinically relevant ARGs are frequently originated in other ecological niches different than clinical settings (Kim and Cha, 2021; Zhuang et al., 2021). Because diverse antibiotics used in livestock are of paramount importance in human medicine (World Health Organization, 2018), an exploration of their ARGs is demanding, including the MGEs that favour their acquisition and spread.

Among the 38 different animal species of livestock in the world, poultry, cattle, and swine are the most common worldwide with up to 33.1, 1.5 and 1.0 heads kept globally in 2020 (DESTATIS, 2022). Indeed,

of the main food products provided by livestock (meat, milk, and eggs), the global meat production from poultry, swine, and cattle in 2020 was respectively 133, 110, and 68 million tonnes; while the remaining species together produced only 26 million tonnes (DESTATIS, 2022).

This study aimed to uncover patterns of AMR among the most relevant animal species in livestock industry (i.e., swine, poultry, and cattle) and humans, to provide novel insights into the contribution of livestock farming to the evolution and spread of AMR in the environment and eventual threats to human health. We performed a comparative analysis in 4 human faecal and 21 animal manure metagenomes retrieved from peer-reviewed literature to investigate: (i) the occurrence and abundance of ARGs; (ii) differences among the resistomes of every species; and (iii) the presence and abundance of MGEs.

## 2. Materials and methods

A literature search was conducted on several scientific journals and databases using the key terms 'Metagenomes', 'Animal manure', 'Livestock', and 'Human'. Metagenomes from animal manure (including poultry, swine, and cattle) and human faeces were selected and downloaded from public repositories. Supplementary Table S1 summarises the publicly available datasets used for this study and the main aspects of the metagenomes retrieved. Animal manure and human faeces corresponding to the metagenomes assessed, were collected in different regions of China between 2015 and 2019. For instance, sampling of poultry and swine manure were conducted in up to 18 and 7 different provinces (e.g., Qinghai, Guandong, Guangxi, Hubei, Shanxi, Guizhou, Anhui, Shandong, Fujian, Sichuan, and Henan), while cattle manure was collected in Shandong and Sichuan provinces. On the other hand, human metagenomes studied were originated from faeces collected in Shanghai and Shandong provinces between 2018 and 2021. Metagenomes originated from animal manure collected in other regions of the world were available in public repositories as well. However, the present study aimed to narrow down the metagenomes assessed to the comparison among animal and human species. Therefore, the assessed metagenomes corresponded to animal species and humans located in the same region (i.e., China), thereby preventing misinterpretation of results due to specific characteristics of the location studied, such as antibiotic administration to livestock and human consumption Metagenomic reads assessed were obtained from Illumina sequencing technology (Illumina, Inc., San Diego, CA, USA) and corresponded to specimens without any specific treatment, considered as controls in their respective original studies. Metagenomes selected were downloaded as compressed FASTQ files and filtered (Q score ≥ Q20 in ≥90% sequence length) using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Filtered reads were then compared to the HMD-ARG-DB_v5 ARG database (Li et al., 2021) using DIAMOND (Buchfink et al., 2014). The alignment was restricted to the best match of the sequences with a minimum identity of an ≥90% over ≥90% sequence length. In order to obtain a detailed taxonomic classification and inference of bacterial abundance in the samples analysed, results were compared to a database of 16 S ribosomal RNA (rRNA) sequences using the program METAXA2 (Bengtsson et al., 2011).

The resistance profile of each animal/human species was investigated. To this, reads assigned to a particular antibiotic resistance class were counted and normalised by the total amount of 16 S rRNA detected in each metagenome so that values could be compared between the different animal species assessed. Reads for ARGs relevant for the study were selected as those more prevalent among the species investigated and genes conferring resistance to 'critically important antimicrobials' (World Health Organization, 2018). ARGs selected were classified based on the following criteria: (i) respective gene family according to the CARD database; (ii) conferring resistance to a given antibiotic class; (iii) products belonging to the same protein complex. Likewise, filtered reads were aligned to a database of MGE markers using DIAMOND against in-house database, as previously described (Gionchetta et al., 2022).

Identical restrictions to the analysis of ARGs were applied for the MGEs annotation. In both cases, relative abundance data were normalised to 16 S rRNA in each replicate. ANOVA with post-hoc Tukey HSD Test was performed for statistical analysis and comparison of the resistance profile among species. Heatmaps were used to display the abundances of the most frequent ARGs and MGEs using the ClustVis tool (Metsalu and Vilo, 2015).

## 3. Results

The selected most relevant ARGs observed in the metagenomes analysed were classified in up to 11 antibiotic families (Fig. 1). Antibiotic resistance profiles varied significantly among animal species. Poultry was the most abundant in total ARGs and significantly higher than the rest of species. Multidrug resistance was greatly prevalent in all species being the most abundant in poultry. Instead, tetracycline resistance was the most prevalent in the remaining species. Other relevant antibiotic groups were aminoglycosides, being highly abundant in poultry and swine, and MLS with high prevalence in poultry, swine, and humans.

Loads of ARGs and types were diverse among animals/human. Poultry manure was notably abundant in multidrug efflux genes (e.g., *acrB*, *cfr*, and *mdtB/C/F*) and *mcr-1* gene encoding resistance to polymyxins (Fig. 2). Likewise, ARGs belonging to β-lactam (e.g., *bla*CTX-M, *bla*PER, *bla*OXA, *bla*SHV, and *bla*TEM), fluoroquinolone (*mfd*), and fosfomycin (*fosB*) families, were mostly prevalent in poultry species. Relative abundance of genes conferring resistance to tetracyclines (e.g., *tetA*, *tetL*, *tetM*, *tetO*, *tetQ*, *tetW*, *tet40*, and *tet44*) and aminoglycosides (encoded by *acrD*, *APH(3')-IIIa*, *ANT(6)-lb*, *B2K_23630* and *rpsL*) were high in poultry and swine species, with *tetW* being the most prevalent. Instead, genes encoding for bacitracin (*bacA*), elfamycin (*tufAB*), and MLS (*macB*), were remarkably abundant in all metagenomes.

MGEs identified in faecal metagenomes (Fig. 3) belonged to (i) integrons (integron integrase), (ii) insertion sequences (IS*CR* elements and IS family transposases) and (iii) transposons (Tn family transposases), which may move within or between bacterial species; and (iv) plasmids (MOB relaxases), which are able to transfer between bacterial cells (Partridge et al., 2018). MGEs relative abundance and type varied among animal/human species. Excluding the MOBQ family belonging to MOB relaxases (marker for mobilizable plasmids) and the IS*1182*-family transposase, MGEs detected were mostly prevalent in poultry metagenomes. Such difference was particularly notable for MOBF/H/P relaxases, which were highly abundant in poultry compared to the other species. It is important to note that relative abundance of MOBV/T relaxases was the highest in human faeces.

## 4. Discussion

Results point out poultry manure as an important reservoir of AMR compared to cattle, swine, and human faeces. In line with previous findings (Qian et al., 2018; Shi et al., 2021; van Boeckel et al., 2019), the highest ARG abundance was observed in poultry metagenomes. However, poultry manure was predominantly enriched in multidrug resistance genes –accounting mainly on *acrB* and *mdt-B/C/F* multidrug efflux genes–, which are most typically detected in clinical settings rather than in livestock-associated environments (Zhuang et al., 2021).

Furthermore, clinically relevant genes conferring resistance to polymyxins (i.e., *mcr-1*) and β-lactams (including *bla*CTX-M belonging to the extended-spectrum β-lactamase gene family) were also the most prevalent in poultry manure. Our results support other studies reporting that *mcr1-9* and *bla*CTX-M genes may occur from animal, human, and environmental sources (Khedher et al., 2020). Indeed *bla*CTX-M is the most abundant extended-spectrum β-lactamase gene family in manure (Ejaz et al., 2021) and particularly enriched in poultry manure (Shi et al., 2021). The fact that many *mcr1-9* and *bla*CTX-M genes are plasmid borne has enabled their dissemination across several habitats (Khedher
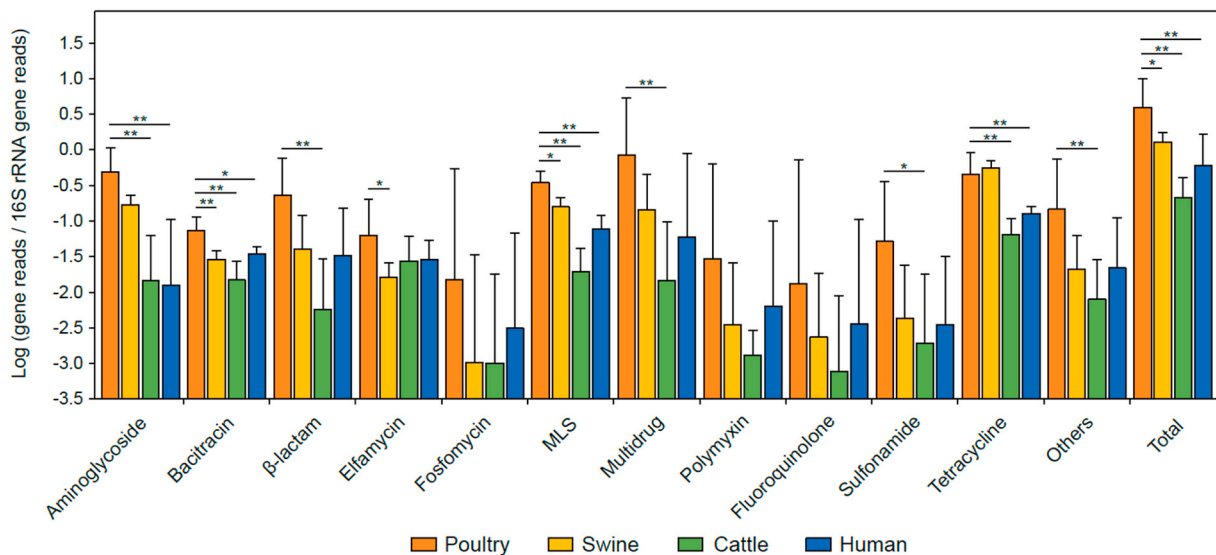
**Fig. 1.** Comparison between poultry (orange colour), swine (yellow colour), cattle (green colour) and human (blue colour), antibiotic resistance profiles. Total number of ARGs found among species is deployed according to their antibiotic classes. In both cases, data was normalised to the total number of reads annotated as 16 S rRNA genes and represented as log scale. *, p < 0.05; **, p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
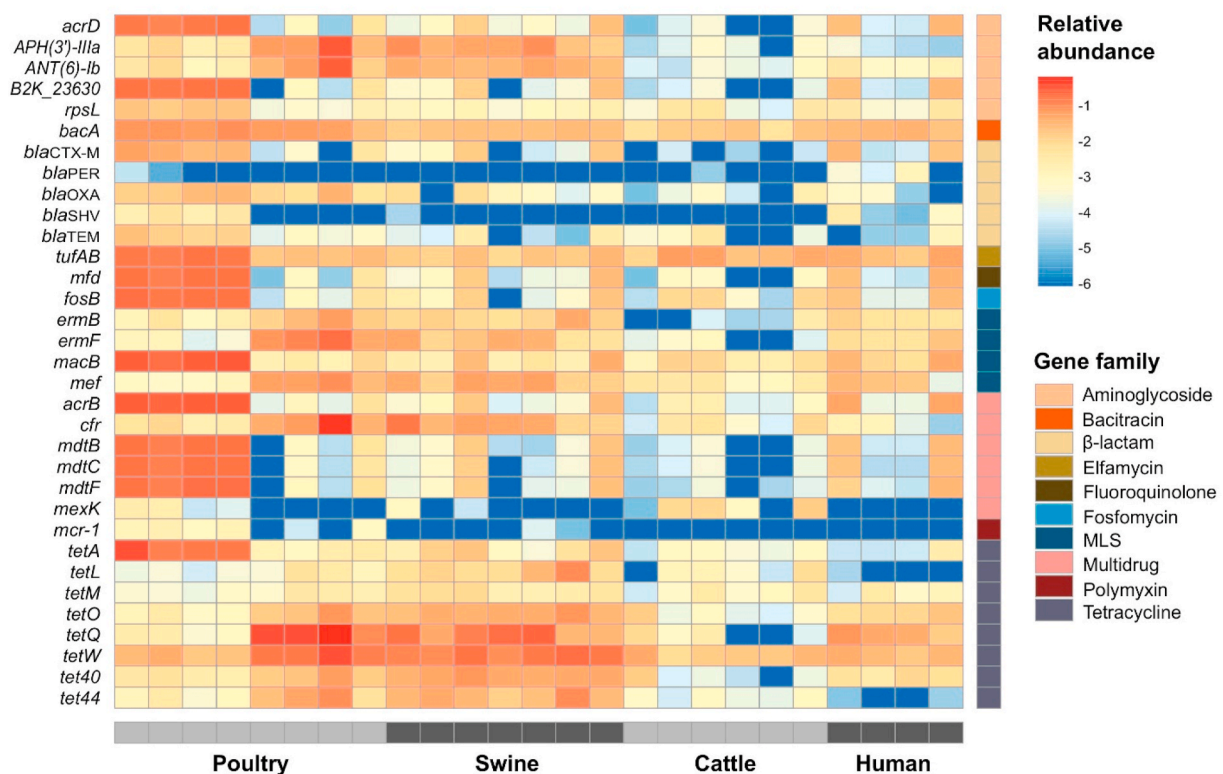


**Fig. 2.** Heat-map describing the relative abundance (log scale) of selected ARGs, normalised to the total number of reads annotated as 16 S rRNA genes in all metagenomes.

et al., 2020; Zhuang et al., 2021). Such mobility has boosted their occurrence in almost all common pathogens (Khedher et al., 2020), compromising the reliability of colistin (polymyxin E) and broad-spectrum β-lactams as last-resort treatment in multidrug resistant infections (World Health Organization, 2018; He et al., 2020).

Following environmental occurrence trends, ARGs conferring resistance to tetracycline, aminoglycoside and MLS were identified in all animal resistomes (He et al., 2020; Qian et al., 2018; Zhuang et al.,

2021). Such ubiquity is probably due to their extensive use, both in clinics and veterinary worldwide, for the past decades (He et al., 2020), by which perspectives to decrease in the near future are challenging (van Boeckel et al., 2017).

Interestingly, the metagenomic analysis evidenced that diversity of ARGs belonging to a certain antibiotic family selected is specific to every species assessed. Such specificity was already pointed out in a previous study (Qian et al., 2018). For instance, multidrug resistance encoded by
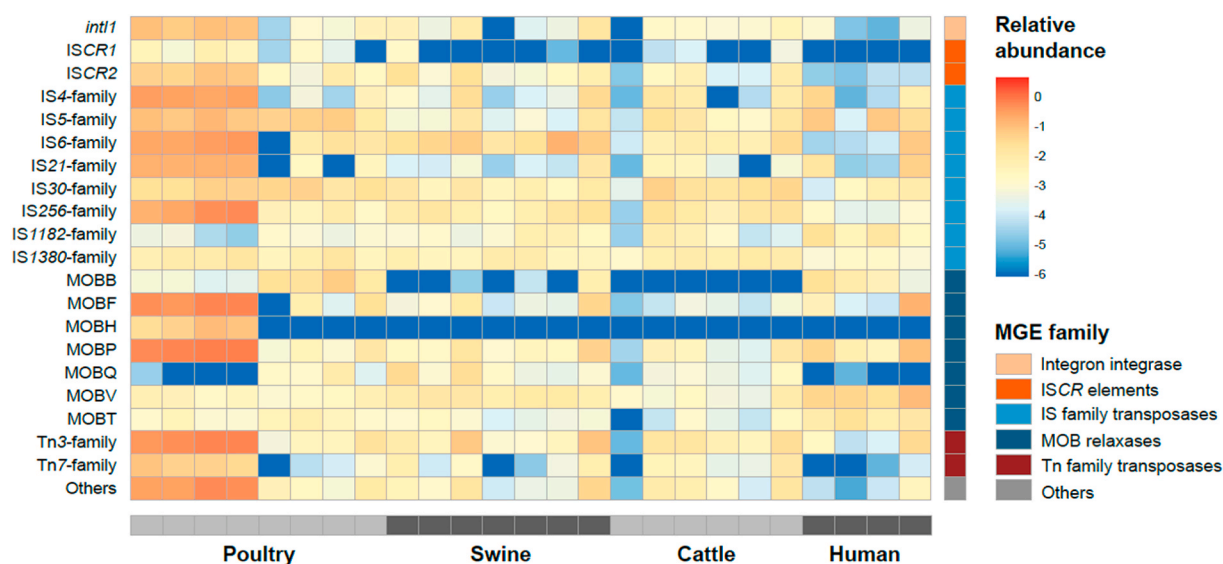
**Fig. 3.** Heat-map depicts the relative abundance (log scale) of selected MGEs in all metagenomes.

*acrB, mdtB, mdtC* and *mdtF*, is characteristic of poultry manure, whereas *cfr* and *mexK* are accountable for multidrug resistance in swine and cattle, respectively. Similarly, aminoglycoside resistance in poultry manure can be explained by *acrD*, while it can be attributed to *APH (3')-IIIa* enrichment in swine samples.

Importantly, selected MGEs co-occurred with the most relevant ARGs in all animal/human species. The co-occurrence and highest prevalence of MGEs and ARGs enhances their mobility to spread throughout different habitats, thereby increasing their potential transmission to human pathogens via HGT (Qian et al., 2018). In fact, many MGEs –and plasmids, particularly– were observed in the human metagenomes analysed at relatively high abundance, pointing out the notable burden of antibiotic resistance to human health.

## 5. Conclusions

We evidenced that poultry manure in livestock-associated environments is a hotspot for multidrug resistance as well as a reservoir of critically important ARGs for human medicine. Moreover, the highest prevalence of MGEs turns poultry manure into a pivotal breaking point of antibiotic resistance spread. Prevalence and ubiquity of multidrug, β-lactam and polymyxin resistance in poultry manure raises a particular concern for eventual effects on human health, as these are crucial antibiotics to treat multi-resistant bacteria. Agri-food consumption and exposure to livestock-associated habitats (Walsh, 2018) –and other sectors to which ARGs may disseminate– reshapes the human microbiome (Sun et al., 2020), rendering antibiotics ineffective in the clinic (He et al., 2020). Hence, our findings claim for actions to take on antibiotic use in livestock production industry in order to preserve human safety from a one health perspective (Walsh, 2018). These actions are particularly urgent in the case of poultry farming, as its production increased from 14.4 to 33.1 billion in only 20 years (2000–2020, DESTATIS, 2022) compared to the expansion of cattle (from 1.3 to 1.5 billion) and swine (from 0.9 to 1.0 billion). Regarding the uses of antibiotics in livestock: (i) last-resort and critical antibiotics should be restricted only for human treatment; (ii)non-therapeutic use i.e. sub-therapeutic doses administered through animal feed products for growth promotion, should be banned; and (iii) prescriptions identifying the antibiotic used and the doses administered to every livestock head should be properly registered in a public database. As for the activity in the farms/livestock facilities: (iv) animals receiving medical treatment should be isolated from the herd until complete elimination of antibiotic residues in their organism; (v) farmers should follow strict hygienic protocols and minimize contact with treated animals and their excretions (vi) livestock waste from animals undergoing antibiotic treatment should be properly cleaned applying advanced treatment technologies to eliminate antibiotic residues and the potential presence of ARGs and AMR; (vii) intensive farming facilities which may foster the spread of ARGs should be eliminated; and (vii) the re-use of manure as organic fertilizers in agricultural crops should be rigorously controlled, avoiding fertilization with manure from treated animals. Most of these recommendations have been already put in practice in Europe as a result of new strategies of the European Green Deal (European Commission, 2019). However, since the spread of AMR does not stop at borders, measures and actions to fight such a critical public health threat should be implemented at a global level, engaging all sectors affected from industry to management authorities and eventually citizens.

## Author contributions

ASM and JLB conceived the idea with input of VO. ASM performed the metagenomic analysis with scientific guidance of JLB and supervision of VO. All authors contributed to preparation of results and discussion of work. VO prepared the manuscript with contribution of JLB and ASM.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data used for this work is detailed in supplementary information.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121239.

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