Impact of Contrasting Poultry Exposures on Human, Poultry, and Wastewater Antibiotic Resistomes in Bangladesh

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Abstract

Interactions between humans, animals and the environment are considered critical foci for addressing antimicrobial resistance (AMR). However, One Health data on AMR in low- and middle-income countries are presently scarce. Using metagenomics, we investigated whether and how the antibiotic resistomes of humans are influenced by exposure to intensive and non-intensively reared poultry within contrasting settings of urban wet markets and rural households in Bangladesh. We also consider poultry and wastewater resistomes in these settings. We found that occupational poultry exposure did not significantly alter the human faecal resistome. In contrast, macrolide-lincosamide-streptogramin and streptothricin antibiotic resistance genes (ARGs) were enriched in poultry from urban wet markets relative to rural household chickens. Wastewater had the highest ARG richness, though this was only partially explained by poultry caecal and human faecal sources. Wastewater also contained clinically significant carbapenem ARGs. This study therefore provides critical insight into the distribution of ARGs in Bangladesh.

Introduction

The faecal carriage rate of extended-spectrum beta-lactamase producing Enterobacterales (ESBL-E) has been increasing globally with the highest prevalence rates in South Asian countries (Bezabih et al., 2021). Intestinal colonisation with antibiotic resistant organisms in humans poses an elevated risk of subsequent infection with resistant organisms. Besides, colonised humans and animals shed ESBL-E through faeces which are often disposed into the environment due to poor sanitation infrastructure. Therefore, reduction of community carriage of antimicrobial resistance (AMR) has been considered as a major step in combating AMR (Maillard et al., 2020). There are many drivers for drug resistant infections in low- and middle-income countries (LMICs), including unregulated sales of antibiotics; misuse of antibiotics in clinical medicine and agriculture; poor sanitation and sewerage infrastructure; and overall poor governance in health care.

Unregulated use of antibiotics in intensive farming of food-producing animals and in aquaculture has become a common practice in many LMICs (Van Boeckel et al., 2015). More alarmingly, antibiotics critical for human health are often used in animal and fish farming, resulting in development of resistance to clinically important antibiotics among bacterial pathogens of concern for human health (Myers et al., 2022). It has been suggested that transmission from animals to humans of bacteria and/or mobile genetic elements carrying ESBL-encoding genes may contribute to human infection with ESBL-producing *Escherichia coli* (ESBL-Ec), however existing evidence suggests this occurs infrequently (Madec et al., 2017; Nguyen et al., 2019). Nonetheless, in a community-based survey in Bangladesh, we found that 67.5% of healthy adults and 68.0% of poultry were colonised with ESBL-Ec whilst 92.5% of wastewater samples tested positive for ESBL-Ec with similar prevalence rates in rural and urban settings (Rousham et al., 2021).

Small scale commercial poultry production in Bangladesh makes heavy use of antibiotics which are added routinely to water or poultry feed (Masud et al., 2020). The close proximity of humans and animals in these farms provide opportunities for bidirectional transmission of

antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) between hosts. Moreover, the lack of sanitation infrastructure, waste management and waste treatment in both rural and urban areas has led to widespread environmental contamination by faecal bacteria, resistance genes and antibiotic residues. Small-scale commercial poultry farmers and the sellers of live poultry in urban markets in Bangladesh also face direct exposure to animal tissues, waste products and associated ARB and ARGs because they use little or no protective clothing, gloves or masks.

The contribution of different sources to AMR in the environment is considered an important gap in current understanding (Larsson et al., 2018). Although ARB have been shown to be prevalent in humans, animals and environmental samples in Bangladesh, the diversity and abundance of ARGs and the extent of sharing between different hosts and environmental compartments is less studied.

Only a few studies in LMICs have assessed all three domains (human, animal and environment) of the One Health paradigm for AMR surveillance (Rousham et al., 2018b). Importantly, there is a lack of data on sharing of microbiome and antibiotic resistomes between humans, domestic/farmed animals and environments within similar ecological units in LMIC settings, where humans and livestock often live in close proximity, and sanitation is often inadequate. Metagenomics-based surveillance makes it possble to compare resistomes and bacterial population structures within and between different ecological settings. Organisms that are significant contributors of ARGs within a population, including culturable, non-culturable and under-studied organisms, can be detected by metagenomic analysis. Recently sewage and wastewater surveillance for AMR using metagenomics has gained traction due to its advantages over traditional population-based surveillance, which is resource intensive for many LMICs (Pruden et al., 2021; Prieto Riquelme et al., 2022). Paradoxically, many LMIC settings that are considered as hotspots for AMR have the sparsest data on metagenomic-based AMR surveillance.

In this study, we aimed to understand the dynamics of antimicrobial resistance transmission in Bangladesh. Specifically, to determine whether the human gut resistome can be explained by exposure to poultry and wastewater in both poultry intensive and non-intensive settings. Additionally, we investigated the impact of intensive antibiotic exposure among poultry on the composition of the poultry gut resistome and its contribution to downstream environmental contamination through direct disposal of wastewater. We achieved this by leveraging systematically collected samples from humans, poultry and the surrounding wastewaters in urban wet markets and rural villages in Bangladesh as part of a purposefully-designed One health AMR surveillance study (Rousham et al., 2018a) and analysed them for whole metagenomic profiling.

Methods

Sampling strategy

The samples discussed in the present work were collected as part of a One Health surveillance study on ESBL-Ec in Bangladesh (Rousham et al 2018; Rousham et al 2021). Human faecal, poultry ceca and wastewater samples were collected between February and October 2017, from urban wet markets in Dhaka city and from households in a rural area in the Tangail district. The current work focusses on a subset of 40 samples which were subsequently processed for metagenomic sequencing. Specifically, these include 20 human faecal samples where we use the terms high and low exposure to refer to the extent of exposure to poultry (rural householders with backyard poultry *n* =2; rural households without backyard poultry *n* =5; poultry slaughterers in urban wet markets *n* =6, people with other professions in urban wet markets *n* =7), 10 poultry caecal samples (backyard poultry from rural households *n* = 4; broilers from urban wet markets *n* = 6) and 10 wastewater samples (rural households *n* =4; urban wet markets *n* = 6). Wastewater samples were collected from the outlet of the main wastewater drain at each urban market and rural household. Wastewater in urban market outlets typically contain waste from all parts of the market including large and small animals, fish, fresh produce etc. More detailed metadata are

available in Supplementary Table 1. We aimed to analyse shared and distinct resistomes according to sample origin (human, poultry or wastewater), occupational exposure to poultry and setting (urban wet markets versus rural households).

Sample collection

Human faecal samples were provided by study participants using a sterile stool sample container supplied by field staff. All faecal samples were stored on ice within 2 hours of collection. For poultry caeca samples, chickens were slaughtered, and the skin removed on-site by the owner following their usual procedures. The carcass was placed in a sterile bag, sealed immediately and placed in a cool box on ice for transportation. Wastewater samples were collected by taking approximately 150 mL of wastewater from three locations along the runoff drain adjacent to the selected household or market, by dipping a sterile container into the drain. Wastewater samples were then pooled by location in a sterile 500-mL plastic bottle (Nalgene, New York USA) and placed on ice for transportation.

All samples were transported to the laboratory within 5 hours of collection maintaining the cold chain, refrigerated on arrival, and processed within 18 hours of collection. In the laboratory, caeca samples were taken from the chicken carcass aseptically by cutting the keel bone, identifying, and excising the cecum with sterilized scissors and extracting the caecum contents.

Ethical considerations

Written and verbal information about the study was provided, and participating volunteers gave written informed consent. Ethical clearance was obtained from icddr,b, Bangladesh (PR-16071) and Loughborough University, UK (R17-P037). Local authorities were informed prior to wastewater sampling in markets. Broiler poultry and backyard chickens were purchased on a commercial basis and slaughtered by the owner/vendor who had consented to participate. Owners followed their normal procedure for slaughtering animals as for domestic consumption or commercial sale.

Metagenomic Sequencing and Sequence Cleaning

DNA was extracted from wastewater samples using the MO Bio Power Water DNA isolation kit (MO BIO Laboratories Inc, Carlsbad, CA USA) the QIAamp DNA Stool Mini Kit (Qiagen, UK) was used for human faecal and poultry ceca samples. Short read metagenomic sequencing (Illumina NovaSeq 6000, 150bp paired end libraries) of extracted DNA was carried out by Novogene (Novogene Co. Ltd, Cambridge, UK). On average, sequencing yielded approximately 11GB of data per sample.

Removal of sequencing adapters and quality trimming was carried out with *Trimmomatic* v0.38 (Bolger et al., 2014) (settings: 2:30:10, leading:3, trailing:3, slidingwindow:4:15, minlen:36). Host and other non-bacterial reads were removed by mapping with *bowtie2* v2.3.5 (Langmead and Salzberg, 2012) using default settings. The read removal strategy was based on the biological context of samples. For human stool samples, the human genome (RefSeq GCF_000001405.40_GRCh38.p14) was used for reference mapping and read exclusion. For poultry ceca, the broiler chicken (*Gallus gallus*) genome was used for reference mapping (RefSeq GCF_016699485.2_bGalGal.mat.broiler.GRCg7b). Finally, for wastewater samples, human, broiler chicken and cattle (*Bos taurus*; RefSeq GCF_002263795.2_ARS-UCD1.3) genomes were used for reference mapping.

Two samples (DL_164_WW2 and DL_087_WW2) were shown to be heavily contaminated (>80% reads mapped concordantly) with broiler chicken genetic material. Both samples originated from urban wet markets; the high proportion of reads mapping to broiler chicken is therefore likely to be a consequence of poultry slaughtering and waste disposal practice.

Metagenome Assembly

Following quality control and non-target sequence removal, samples were assembled individually using *Megahit* v1.2.9 with 21, 29, 39, 59, 79, 99, 119, and 141 *k*-mer intervals (Li et al., 2015b).

Annotation of Antimicrobial Resistance Genes in Unassembled Data and Contigs

ARGs were annotated with a locally installed copy of *ARG-OAP* v2.0 (Yin et al., 2018) using an ARG sequence identity cut-off of 80% and minimum query alignment length >25 amino acids, expect-value $1x10^{-7}$ (Feng et al., 2018; Murray et al., 2019; Qian et al., 2021). Normalisation of ARGs by estimated genome number was carried out by *ARG-OAP* which uses *diamond* v2.0.15. (Buchfink et al., 2021) to identify a suite of 30 universal single copy genes.

Contigs were screened for ARGs based on protein homology using the RGI online platform (resistance gene identifier, v6.0.0), combined with the CARD database (v3.2.5) (Alcock et al., 2020). Only matches exceeding 90% identity and coverage of reference protein sequences were considered in analyses.

Taxonomic Classification of Unassembled Data and Resistance Gene-Bearing Contigs

After quality checking, taxonomic classification of short reads was carried out with *Kaiju* v1.7.1 (Menzel et al., 2016) in combination with the pre-built *nr_euk* protein database (downloaded from *Kaiju* webserver March, 2019). The database contains non-redundant protein sequences for Archaea, Bacteria, Viruses, fungi and microbial eukaryotes from the NCBI-BLAST database (Sayers et al., 2019). *Kaiju* was run in 'greedy' mode, allowing three mismatches.

For ARG-bearing contigs of particular interest, NCBI megaBLAST (Morgulis et al., 2008) was performed to assign putative taxonomy.

Data Exploration and Statistical Analysis

To evaluate whether the predicted resistome composition varied between sample origin (poultry caecal, human faecal and wastewater) and setting (rural households and urban wet markets), Principal Coordinates Analysis (PCoA) was first used to generate unconstrained ordinations of sample composition (Hellinger distances of bacterial cell normalised ARG category and subtype abundance). As PCoA indicated clustering, permutational multivariate analysis of variance (PERMANOVA) was used to establish if significant multivariate

differences were observed between groups, following testing for heterogeneity of multivariate dispersion using the PERMDISP test. We performed discriminant analysis using canonical analysis of principal coordinates (CAP) in Hellinger space. To avoid model over-parameterisation, we identified the optimal PCoA axes to employ in CAP by maximising a leave-one-out allocation success to treatments. Having established any clustering within the multivariate ordination, we determined the likely ARG categories associated with sample clustering using Pearson correlation coefficients to determine linear relationships between ARGs and clusters. PCoA, PERMDISP, PERMANOVA and CAP were all conducted using the *PERMANOVA*+ add on to *PRIMER* version 7.0.20 (Clarke and Gorley, 2006; Anderson et al., 2008). For all tests, probabilities were based upon 99,999 permutations (denoted p_{perm}). In cases where the number of observations was insufficient to allow at least 999 permutations for post hoc pairwise tests, Monte Carlo probabilities (denoted p_{MC}) were calculated based upon an asymptotic permutation distribution.

Based on PERMANOVA, univariate differential abundance of ARGs in rural and urban settings were only assessed in poultry caecal samples. Only ARG categories associated with poultry caeca having $r > 0.2$ were analysed, see vectors associated with CAP analysis (Fig. 1). To account for non-normal data distribution and heterogeneity of variance between sample data, Welch's unequal variances *t*-tests were combined with 99,999 Monte Carlo permutations to determine probabilities associated with *t*. The Benjamini-Hochberg procedure for false discovery rate adjustment was applied to the resulting probabilities (denoted p_{adj}).

ARG subtype diversity was assessed using Hill numbers implemented by the *iNEXT* v3.0.0 (Hsieh et al., 2016) package in R with 40 knots and confidence intervals bootstrapped 399 times.

Other exploratory visualisations included boxplots of genome-normalised abundances of the five most abundant ARG subtypes by sample origin and ARG categories associated with sample origin according to CAP ($r > 0.2$). Abundant phyla and genera were summarised by

sample origin in compositional plots produced with the R package *microbiome v1.18.0* (Leo Lahti, 2017) (Supplementary Figure 1).

Further investigation into associations between specific ARG subtypes, taxa and sample origins were guided by contigs of interest and initial data exploration. In doing so we sought to avoid data dredging. We focussed on the following objectives: i) identifying whether specific taxa could be shown to drive dominant features of the resistome in the three sample origins (human, poultry, wastewater), ii) identifying taxa associated with multi-drug resistance determinants (three or more different antibiotic categories) iii) determining the distribution of the WHO priority one antibiotic resistant bacteria of critical concern (WHO, 2017). Where appropriate, associations between the Centred Log Ratio (CLR) of taxa and ARG count data were tested using major axis regressions in R using *lmodel2* v1.7-3 (Legendre, 2018). Only contigs >1 kbp were used for assigning putative taxonomy.

Antibiotic resistome source contribution analysis was carried out with *FEAST* algorithm R package (Shenhav et al., 2019; Chen et al., 2023) where poultry, human and wastewater samples were collected from the same urban wet market site (*n* = 3). *FEAST* was supplied count data for ARG subtype and was run using default settings (EM iterations= 1000, coverage = minimal sequencing depth in sink and sources).

Results

Sample origin has the greatest impact on resistome composition; setting has a secondary influence on poultry and wastewater ARG carriage.

Genes associated with tetracycline resistance were the most abundant ARG category on average, regardless of sample origin (mean abundance,1.2 copies per bacterial genome [cpbg], ± 0.12 standard error of the mean). However, based on abundance data, poultry ceca samples were the most enriched with tetracycline resistance genes (1.79 ± 0.18 cpbg,) compared to both wastewater (1.07 ± 0.32 cpbg) and human faecal samples (1.05 ± 0.05 cpbg). The next most abundant ARG categories overall, were Macrolide-Lincosamide-

Streptogramin (MLS) resistance genes (0.63 ± 0.09 cpbg) and beta-lactam resistance genes (0.55 ± 0.06 cpbg); these were most associated with poultry ceca (1.10 ± 0.22 cpbg) and human faecal samples (0.80 ± 0.06 cpbg), respectively. These trends were evident in the CAP linear ARG vector overlay (Figure 1.) and ARG category normalised abundance boxplots (Figure 2b).

At the subgroup level, no single ARG was dominant across human, poultry or wastewater samples. On average, the tetracycline ARG *tet(Q)* was the most prevalent subtype in poultry samples, *tet (Q)* and the beta-lactam resistance subtype *cfxA6* in human faecal samples (0.51 ± 0.05 and 0.61 ± 0.06 cpbg respectively), whereas wastewater samples were marked by greater between-sample variability and therefore multiple similarly abundant subtypes were present (Figure 2a).

Around 15% of core ARG subtypes (here defined as subtypes detected in at ≥2 samples in one or more sample origin; total n =1047) were shared by all sample origins (Supplementary Table 2). However, analyses show that the overall resistome compositions associated with each sample origin were distinct. This is evident in the separation of poultry ceca, human faecal and wastewater samples by unconstrained PCoA and CAP of ARG subtype and category data (Figure 1, see Supplementary Figure 2 and 3 for PCoA). ARG subtype diversity was assessed using Hill numbers. There was a significant difference in median ARG subtype richness (^0D) between sample origin (human, poultry, wastewater) (Kruskal-Wallis test; $\chi^2 = 23.3$, two-tailed df = 3, $p = 3\text{x}10^{-5}$), but not setting (urban wet market versus rural households) Kruskal-Wallis test; $\chi^2 = 1.5$, df = 1, $p = 0.214$. (Supplementary Figure 4). Post hoc pair-wise comparisons of median ^0D indicated significant differences between wastewater (median ^0D = 526) and all other sample origins (smallest difference; wastewater and poultry caecum, median ^0D = 223, Mann-Whitney $Z_U = 3.6$, $p_{adj.} = 0.00074$). No significant differences in richness estimates were observed between poultry caecum or human faecal samples (median ^0D of human faeces from high or low poultry exposure were 137 and 171, respectively). However, a significant interaction was identified between setting

and origin; Kruskal-Wallis test; $\chi^2 = 27.5$, df = 5, $p = 4.573e^{-5}$. Despite this, group-wise testing indicated no significant differences after correction for multiple testing (Kruskal-Wallis tests: poultry caecal; $\chi^2 = 6.55$, df = 1, $p_{adj.} = 0.06312$; human faecal; $\chi^2 = 0.01$, df = 1, $p_{adj.} = 1.00$; wastewater; $\chi^2 = 0.73$, df = 1, $p_{adj.} = 1.00$).

For the multivariate test of ARG assemblages, no significant heterogeneity of multivariate dispersion was observed for either sample origin or setting for ARG type. For ARG subtype however, there was significant heterogeneity of multivariate dispersion associated with sample origin (PERMDISP, pseudo-$F = 5.9$, $p_{perm} = 0.0058$). For both ARG type and subtype, dispersion was greater for wastewater samples than either human faecal or poultry caecal samples. Testing the effect of sample setting (rural households, urban wet markets) and sample origin (human faecal, poultry caecal, wastewater) indicated both sample origin (PERMANOVA, pseudo-$F = 24.8$, $p_{perm} = 1x10^{-5}$) and setting (pseudo-$F = 3.7$, $p_{perm} = 0.0087$) exerted a significant influence upon ARG subtype assemblages. Significant interaction between the two factors was also identified (pseudo-$F = 3.0$, $p_{perm} = 0.001$). Post hoc pairwise comparisons indicated that while there was no significant difference between ARG subtype assemblages in faecal samples collected from human subjects with either high or low exposure to poultry, assemblages in poultry caeca, human faeces and wastewater were all significantly different; however, we cannot discount the fact that this may reflect differences in dispersion between the different environments. This pattern was the same for both rural households and urban wet markets. In addition, differences in ARG subtype assemblages were also observed between wastewater ($t = 1.9$, Benjamini-Hochberg adjusted $p_{MC} = 0.014$) and poultry caeca ($t = 3.3$, Benjamini-Hochberg adjusted $p_{MC} = 0.0007$) from rural households and urban wet markets. These trends were consistent with the effect of factors upon ARG type.

After log10 transformation, total normalised ARG abundance varied significantly between settings (Welch $t = -4.17$, df = 29.623, $p = 0.00024$), with urban wet market samples exhibiting higher mean total ARG abundance than rural households. Due to small sample

size, unequal variance, and non-normal distributions which could not be resolved by transformation, we did not carry out further statistical tests on interaction effects between sample origin and setting. However, we observed trends which suggest the difference between urban wet market and rural households is primarily driven by wastewater samples (Supplementary Figure 5).

FEAST source attribution where wastewater, human faecal and poultry caecal samples were collected from the same urban wet markets showed that the majority of the wastewater resistome could not be explained by either potential source (mean 75.87% ± 4.50 unknown source). In urban wet markets, the poultry resistome made a greater contribution to corresponding wastewater streams (21.39% ± 3.08) than human faecal sources (2.73% ± 0.01%).

MLS and streptothricin ARGs are enriched in broiler poultry ceca

The multivariate differences between rural backyard chickens and urban wet market broiler poultry resistomes indicated by PERMANOVA were further investigated. Of the three ARG categories shown to associate with poultry resistomes according to CAP vector overlay (Figure 1), MLS and streptothricin resistance genes were significantly enriched in urban broilers (Welch $t = 3.8$, $p_{adj.} = 0.0145$; Welch $t = 6.3$, $p_{adj.} = 0.0245$, respectively; Figure 3). However, tetracycline resistance genes were prevalent at similar levels in urban and rural bird ceca (Welch $t = 0.51$, $p_{adj.} = 0.567$). Given the small sample size and numerous ARG subtypes, statistical tests are not reported; nonetheless, heatmaps of MLS and streptothricin ARG subtypes are provided for poultry samples in Supplementary Figure 6. Several ARG categories were shown to be indicative of wastewater resistomes, however differences between urban and rural samples were not tested due to low statistical power.

Urban and rural wastewaters contain carbapenem resistance genes

A total of 1662 contigs contained at least one ARG. Of these, 1553 were ≥1 kbp in length and were selected for taxonomic profiling (poultry, $n = 363$; human faeces, $n = 640$; wastewater, $n = 550$).

According to the WHO Global Priority Pathogens List, priority one ‘critical concern’ antibiotic resistant pathogens include ESBL-E, carbapenem resistant *Enterobacteriaceae* (CRE), *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. While 21 contigs >1kbp contained ESBL/carbapenem resistance genes, after cross-validation of taxonomy, only three contigs unambiguously fulfilled the ‘critical concern’ criteria; all originated from wastewater samples (Supplementary Table 3).

Human faecal (high and low exposure), poultry, and wastewater contig libraries contained *Enterobacteriaceae* carrying TEM-family genes, however all were classified as broad-spectrum beta lactamases (BSBLs) TEM-1. Similarly, SHV genes in wastewater (SHV-27, SHV-110) and human faecal (SHV-11, SHV-27) contigs were determined to be non-ESBL variants. PER and RSA group ESBL genes were detected, however, they could not be confidently associated with *Enterobacteriaceae*.

OXA-group genes were the most frequently identified beta-lactamases on contigs across the entire dataset (n = 50, n = 49 >1kb) and were found in all sample origins. However, OXA ESBL/carbapenemase genes could not be definitively associated with *Enterobacteriaceae*. For example, one rural wastewater contig carried an OXA-372 family carbapenemase gene (Bonnin et al., 2021) (OXA-641-like); OXA-641 is known to be found in *Morganella* spp (Supplementary Figure 7a). Additionally, one chromosomal *Acinetobacter baumannii* rural wastewater contig encoded a variant (OXA-65) belonging to the OXA-51 family (Evans et al., 2008) (Supplementary Figure 7b). Another *Acinetobacter* spp. contig derived from urban wastewater contained OXA-58, another a potential carbapenemase (Poirel et al., 2005) (Supplementary Figure 7c).

VEB group ESBLs were also identified; these were only recovered from wastewater contigs and could not be categorically associated with *Enterobacteriaceae*. However, the longest VEB-bearing contig (7.7kbp) contained a class 1 integron cassette with the potential to confer multidrug resistance (Supplementary Figure 7d) across four different antibiotic categories (beta-lactam, phenicol, aminoglycoside and fluoroquinolone). NCBI-BLAST

indicated this contig shared high nucleotide homology with enterobacterial plasmids (*Klebsiella quasipneumoniae,* 99.69% identity, 100% query coverage [CP058135.1], and *Escherichia coli*, 99.98%, 100% query coverage [LC745731.1]) and non-enterobacterial chromosomes (*Aeromonas veronii;* 100% identity, 94% query coverage [CP054855.1]). Finally, three wastewater contigs contained genes from the GES carbapenemase family. Urban and rural wastewater contigs (1.3-1.5 kbp) containing *bla*GES-2 shared high homology with *Pseudomonas aeruginosa* chromosomes and enterobacterial plasmids (Supplementary figure 7e). Another urban wastewater contig (1.8 kbp) contained *bla*GES-5 with nucleotide homology among Gammaproteobacteria, including chromosomal sequences of *Pseudomonas aeruginosa* (99.90% identity, 70% query coverage [KY860573.1]) and *Klebsiella pneumoniae*-associated plasmids (100% identity, 70% query coverage [MN436715.1]) (Supplementary figure 7f, Supplementary Table 3).

Select ARGs are associated with specific genera and sample origin, while most are widely dispersed.

To investigate whether select ARGs are linked to specific bacterial hosts and sample origin, contigs were used to direct correlation analyses between short-read abundance data of ARGs and taxa. Since the prevalence of beta-lactam resistance genes were shown to distinguish human faecal samples from those collected from poultry caeca and wastewater (see Figure 1, 2b), dominant beta-lactam ARGs were identified. The principal beta-lactam ARG in human samples was *cfxA6*. Contig data suggested CFXA family genes were typically associated with *Bacteroides* and *Prevotella* spp. We found a significant positive correlation between the CLR of *Prevotella* spp. and *cfxA6* counts across the entire dataset (R = 0.95, t =18.954, p = 5.799111e^{-21}, Supplementary Figure 8) and positive trends held within each sample origin. However, correlations with *Bacteroides* spp. or phylum Bacteroidetes were inconsistent within samples of different origin. Although contig analyses show *cfxA* genes are present in both *Bacteroides* spp. and *Prevotella* spp., these findings indicate *Prevotella* spp. were the most consistent carrier of *cfxA6* in these data.

The abundance of several ARG categories was shown to distinguish wastewater samples from other environments, including multidrug, sulfonamide, trimethoprim and phenicol ARGs. Contig data suggests chloramphenicol (*cmlA5*) and trimethoprim (*dfrA14*) resistance genes may co-occur on either chromosomes or plasmids (Supplementary figure 7d). The co-occurrence of multidrug (*qacEΔ1*) and sulfonamide (*sul1*) resistance genes may similarly occur on both plasmids and chromosomes, and this configuration can be found in all sample origins, not only wastewater. These two genes form the 3'-Conserved Segment of class 1 integrons, which are distributed across a broad taxonomic range. Considering this, correlations between wastewater marker taxa and ARGs were not carried out.

Lastly, dominant tetracycline ARG subtypes in poultry did not consistently occur on contigs belonging to specific taxa below the order level. Abundance correlations between taxa and ARGs were therefore not tested.

Discussion

This study aimed to explore the sharing of antimicrobial resistomes between humans, poultry and wastewater, with and without exposure to intensive poultry production. We also assessed the influence of antibiotics fed to poultry on the composition of the poultry gut resistome and its contribution to environmental contamination via wastewater outlets.

We found that antimicrobial resistomes were largely characterised by sample origin (human faeces, poultry ceca or wastewater), although those of poultry ceca and wastewater were additionally influenced by setting: namely, wet market broiler versus backyard poultry, and urban wet market versus rural wastewater.

The primary separation of samples by source is not surprising. Existing resistome surveys illustrate that different sample origins harbour distinct ARG assemblages (Li et al., 2015a; Gatica et al., 2019). In certain cases, these differences may reflect the microbial taxa which are adapted to specific environments and their intrinsic or commonly acquired resistance genes. For example, the *cfxA* beta-lactamase gene family has been identified as a dominant

member of mammalian gut and faecal resistomes, including healthy humans (Hu et al., 2013) cattle (Zhou et al., 2016; Baker et al., 2022) and pigs (Li et al., 2015a; Lim et al., 2020). Gatica et al. (2019) also showed robust association of *cfxA* genes with bovine and human faecal samples, while demonstrating their comparative scarcity in environmental samples.

The *cfxA* genes are well-documented within the phylum Bacteroidetes, of which genera such as *Prevotella* spp. and *Bacteriodes* spp. are abundant within mammalian anaerobic niches: the gastrointestinal tract and oral cavity. Furthermore, Suriyaphol et al. (2021) found a positive correlation between *Prevotella* spp. and *cfxA6* in pig gut microbiomes. We found a positive correlation between *cfxA6* and *Prevotella* spp. across all samples in the current work.

However, in many cases it was not possible to define such clear associations between highly abundant ARGs and bacterial genera in wastewater and poultry samples. For example, contigs harbouring tetracycline genes such as *tetQ* and *tetW* could not be linked to specific taxa below the rank of order (Bacteroidales) or phylum (Firmicutes) in any sample origin. This likely corresponds to the decoupling of these genes from strict phylogenetic constraints by virtue of horizontal gene transfer. Indeed, *tetW* is known to have an extensive host range encompassing both Gram-positive and -negative bacteria, and can be integrated on conjugal transposons (Roberts, 2005). The mobility of *tetQ* is less well documented, although it has been found on plasmids in *Bifidobacterium* strains (Ma et al., 2015).

Wastewater samples had the highest ARG subtype richness (^0D) compared to human and poultry samples consistent with having the greatest multivariate dispersion. Furthermore, Hill number extrapolations indicate the true richness of some wastewater samples may be underestimated due to under-sequencing (Supplementary Figure 8). These findings are consistent with wastewater receiving varied inflow material, including washing detergents, cooking residues, human and animal waste, and residues from animal slaughter. The wastewater resistome is therefore not simply a combination of human and poultry ARGs.

FEAST source attribution also supports this assertion, since less than half of the wastewater resistome in urban wet markets could be explained by corresponding human and poultry samples.

The complexity and variability of the wastewater resistome is also likely to explain why several ARG categories discriminate wastewater from human and poultry samples (shown in Fig. 1). Another contributing factor could be co-localisation of genes conferring resistance to different categories of antibiotic compounds. Several contigs assembled from wastewater samples support this notion: aminoglycoside resistance genes were frequently identified alongside beta-lactam, phenicol, trimethoprim and fluoroquinolone resistance genes. Our results show examples of these contigs share homology with both the chromosomes of *Aeromona*s spp. and plasmids associated with *Enterobacteriaceae* (Supplementary Figure 7d).

The overall predominance of tetracycline resistance genes in the present study parallels a previous study showing these genes are prevalent in humans, goats and chicken faeces in Bangladesh (Swarthout et al., 2022). Tetracycline resistance genes have also been shown to dominate the resistomes of human, pig and poultry faeces in Chinese wet markets (Wang et al., 2019). Metagenomic studies on wastewater outlets in Bangladesh are limited, with most wastewater surveillance studies relying on cultivating select target organisms (Asaduzzaman et al., 2022). To our knowledge, existing metagenomic studies on water resistomes in Bangladesh only include surface water (McInnes et al., 2021). Since the wastewater outlets surveyed in the present work discharge directly into surface water, we discuss whether similar resistance genes were recovered in these compartments. McInnes et al. (2021) found differences in the resistome of surface water and sediment obtained from rural (Mymensingh, Shariatpur) and urban (Dhaka) sites in Bangladesh. Specifically, the authors showed that urban surface waters were most enriched with macrolide, sulfonamide, aminoglycoside and multidrug efflux resistance genes, relative to rural settings. In the current work, sulfonamide, aminoglycoside and multidrug resistance genes were also among

those prevalent in wastewater samples from urban wet markets (Figure 1). McInnes et al. (2021) suggested that human gut bacteria drive antibiotic resistance genes in surface water. Given surface water bodies in urban areas receive wastewater run-off from wet markets and mainstream sewage, both human and animal waste should be considered potential sources of these genes in the environment. Lastly, McInnes et al. (2021) found tetracycline resistance genes made a comparatively minor contribution to surface water bodies in urban areas, indicating that while they dominate wastewater in our study, bacteria carrying these genes (though apparently diverse) may be less capable of competing in the wider aquatic environment.

Our findings suggest there was no significant difference between the abundance of tetracycline resistance genes in broiler poultry and backyard chicken ceca (Figure 3). This was unexpected given that tetracycline is frequently used in commercial poultry farming (Hasan et al., 2011; Rousham et al., 2021). Our household survey indicated that none of the rural poultry had received tetracycline antibiotics in the 4 weeks prior to sampling (Rousham et al., 2021). Though vendors reported that they do not give antibiotics to broiler poultry at the point of sale, the small-scale commercial farms which supply bird markets regularly use antibiotics throughout the production cycle. In a survey of small-scale commercial broiler farmers, 65% reported using tetracyclines (Rousham et al., 2021) with no withdrawal period before supplying birds to retail markets (Masud et al., 2020). It has been shown that tetracyclines can have long half-lives in the environment compared to other commonly used antibiotics (Baker et al., 2022), although many factors influence their persistence (Cycoń et al., 2019). The high prevalence of tetracycline resistance in both rural backyard chicken and broiler poultry may therefore indicate widespread contamination of the terrestrial environment with tetracycline residues and the proliferation of resistant bacteria.

A salient finding of the present work is that the resistomes of rural backyard chicken ceca are distinct from those of commercially reared poultry sold in urban wet markets. This appears to be driven in part by MLS (macrolide-lincosamide-streptogramin) and

streptothricin (nucleoside) resistance genes, which were significantly enriched in wet market broilers relative to rural backyard chickens. It is likely that the higher stocking density, range, and frequency of antibiotic exposure in commercial broiler farms contribute to these differences. Although the wet market environment itself may play a role in shaping the resistome of broiler poultry, it is important to note that the surveyed birds spent less than 24 hours in the urban wet market prior to slaughter. Consequently, we contend that the rearing environment exerts a greater influence on the resistome of these animals. According to ordinations, the caecal resistome of rural backyard chickens were less distant from human resistomes than urban wet market broiler resistome compositions (Supplementary Figure 2 and 3). This finding likely corresponds with microbiome sharing facilitated through routine direct contact with owners and indirect exchange through mutual exposure within household settings.

MLS-bearing contigs indicated multiple MLS resistance gene families were responsible for category level enrichment in broiler poultry, with putative hosts among Firmicutes, Bacteroidetes and Proteobacteria. MLS genes were occasionally co-localised with tetracycline, aminoglycoside and beta-lactam resistance genes in various configurations. These included association with transposable elements, highlighting their mobility. Macrolide antibiotics are known to be widely marketed in Bangladesh (Orubu et al., 2021) for both human and animal use; they have also been used by farms supplying urban wet markets surveyed in this study. Streptothricin, on the other hand, has only been used outside human clinical practice for prophylactic growth promotion in animals (Webb et al., 2017). We found no evidence of its use within poultry operations in Bangladesh. Broiler poultry contigs containing streptothricin resistance *sat*-family genes were commonly co-localised with aminoglycoside resistance genes and distributed across phyla (Firmicutes and Proteobacteria).

To date, few studies have applied metagenomic techniques to examine caecal resistomes in poultry samples, in Bangladesh or elsewhere. One study used a qPCR array to compare the

faecal resistome of small-scale broiler chickens with backyard poultry in Ecuador (Guo et al., 2018). The authors found ARG richness in production chickens was significantly higher than that of household chickens; our metagenomic data indicated only a trend in Bangladesh (Kruskal-Wallis test; $\chi^2 = 6.5$, df = 1, $p_{adj.} = 0.06312$).

A recent publication by Swarthout et al. (2022) used long-read sequencing to compare faecal resistomes of humans, goats and chickens in urban and rural households in Bangladesh. There are several experimental and methodological differences between the aforementioned study and our current work. Firstly, only household backyard poultry (rural and urban) were sampled, whereas the current study sampled commercially reared broilers sold in urban wet markets and backyard poultry in rural households. Secondly, Swarthout et al. (2022) separated urban and rural samples and then pooled DNA extractions, limiting the ability to discern the level of variability between individuals within these two groups. Thirdly, the current work obtained samples from poultry ceca whereas Swarthout et al. (2022) sampled poultry faeces collected from the environment.

Despite these methodological differences, some high-level findings correspond across both studies. For example, Swarthout et al. (2022) did not report a significant difference between human faecal samples derived from urban and rural locations, which parallels our findings. However, Swarthout et al. (2022) did not specifically survey urban wet market workers. This is significant, since our work extends previous findings, implying that the resistome of urban wet market workers is not dramatically altered by regular occupational exposure to broiler poultry viscera. Another study collected faecal samples from broiler farm chickens, live poultry market workers and humans with low exposure to poultry in China (Wang et al., 2021). Their findings indicated that humans with low exposure to live poultry markets had significantly lower ARG diversity than live poultry market workers. Although we did not find significant differences between low and high exposure human faecal samples; this may relate to the widespread practice of keeping backyard chickens and/or general environmental contamination with antibiotic resistant organisms in Bangladesh.

549 Although the total load and richness of ARGs is an important consideration when identifying
550 potential areas to focus mitigation measures for AMR, specific combinations of ARGs and
551 bacteria can present an immediate threat to human health, reflected in the WHO prioritised
552 surveillance list (WHO, 2017).

553 We found GES carbapenemases in both rural and urban wastewater. These genes are of
554 particular interest as the contigs suggested possible carriage on *Enterobacteriaceae*
555 plasmids and *Pseudomonas aeruginosa* chromosomes, both 'critical concern' organisms. A
556 recent meta-analysis indicates that GES-2 genes are among the most widely distributed
557 carbapenemase genes in aquatic environments, including wastewater, freshwater and
558 sediment (Lin et al., 2022). Likewise, GES-5 is carried by bacteria abundant in aquatic
559 environments (Manageiro et al., 2014). Although Lin et al. (2022) shows limited association
560 of GES-2 with humans, culture-based analyses have previously identified this variant in
561 nosocomial outbreaks of ESBL-producing *Pseudomonas aeruginosa* (Poirel et al., 2002).

562 More broadly, the presence of carbapenemases (GES and OXA) indicates that while the
563 total load and richness of ARGs may be greater in urban wastewater, rural wastewater
564 should not be discounted as a source of ARGs critical to One Health initiatives.

565 It is noteworthy that previous real-time PCR studies have shown considerable prevalence of
566 CTX-M-1 ESBLs, and to a lesser extent NDM-1 CRE genes, in wastewater from urban
567 markets, poultry farms and rural households (Asaduzzaman et al., 2022). Detection of these
568 genes was limited across our metagenomic dataset. This may be a consequence of
569 insufficient sequencing depth and the lack of targeted amplification for these lower
570 abundance genes. Focussing on contigs allows more confident definition of taxon-ARG
571 associations and potential ESBL/carbapenemase activity (since the complete genes can be
572 screened against variant databases). However, the use of short reads can lead to
573 incomplete assembly and many fragmented contigs with partial genes present at contig
574 ends, which can lead to underestimated variant prevalence. Alternatively, future studies may

consider hybrid sequencing, or employing culture-enriched metagenomics to better study these clinically relevant genes (Zhang et al., 2022).

Conclusion

We provide in-depth contextualisation of resistomes associated with human faecal, poultry caecal and wastewater samples in Bangladesh. We demonstrate that the impact of environmental setting on the resistome can differ depending on sample origin. The resistome of faecal samples originating from humans with and without routine occupational exposure to poultry are not significantly different. However, broiler poultry from urban wet markets have a significantly higher abundance of MLS and streptothricin ARGs compared to rural backyard chickens. The ARG compositions of human faecal and poultry caecal samples are also distinct. Overall, wastewater samples have the highest ARG richness and were under-sampled in our campaign. Nonetheless, rural wastewater was identified as a source of 'priority one' antibiotic resistant organisms selected by the WHO, highlighting wastewater in both urban and rural settings are a concern for human and animal health in Bangladesh. Wastewater is an important, but poorly understood component of One Health studies on AMR in Bangladesh. Further studies using long-read/hybrid or culture-enriched sequencing of rural backyard and broiler poultry in farms and in urban wet markets would generate a more complete understanding of how the poultry-rearing practices of medium- and small-scale farms in Bangladesh determine the resistome as opposed to the contaminated environments where free range chickens roam. Finally, deeper sequencing is likely to reveal that wastewater in Bangladesh contains an even greater variety of ARGs than identified in the present work.

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Data availability: Genomic data are available from the European Nucleotide Archive; study accession PRJEB48068. Other data relating to the wider project are openly accessible at https://doi.org/10.5285/0239cdaf-deab-4151-8f68-715063eaea45 and https://doi.org/10.5285/dda6dd55-f955-4dd5-bc03-b07cc8548a3d.

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Competing interests: The authors declare no completing interests.

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Figures

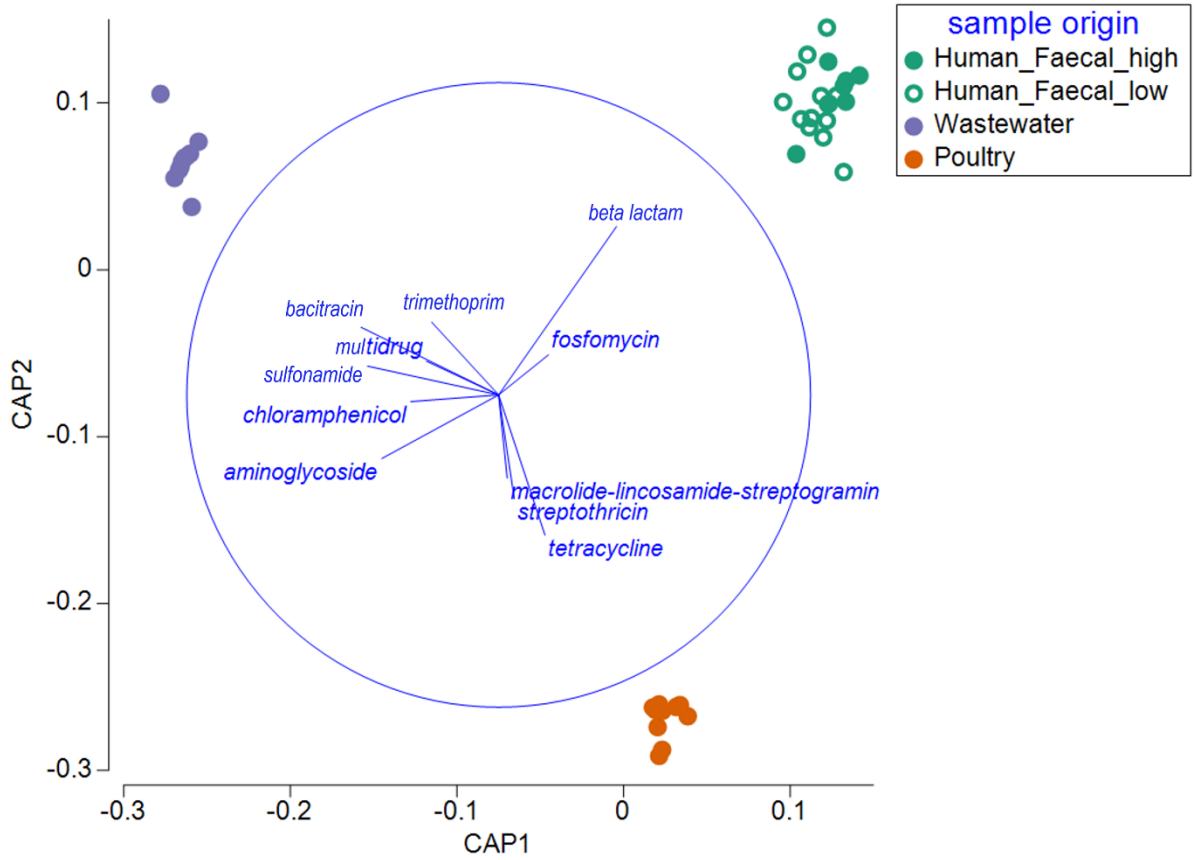

Figure 1. ARG category-contingent Canonical Analysis of Principal Coordinates (CAP) identifies significant separation of ARG assemblages between sample origins (trace statistic = 2.5, p_{perm} = 1x10^{-5}). CAP1 squared canonical correlation (δ_1^2) = 0.997, CAP2 δ_2^2 = 0.988. Cross validation based upon a leave-one-out allocation of observations to groups was associated with a 17.5% mis-classification error rate, resulting from mis-classifying human faecal samples with high and low poultry exposure. Vector overlays represent multiple partial correlations between CAP axes and ARG categories (r > 0.2). The circle has a radius of r = 1; the relative size and position of origin is arbitrary with respect to the underlying plot. Vector length and direction indicates the strength and sign, respectively, of association between each ARG category and the CAP axes.

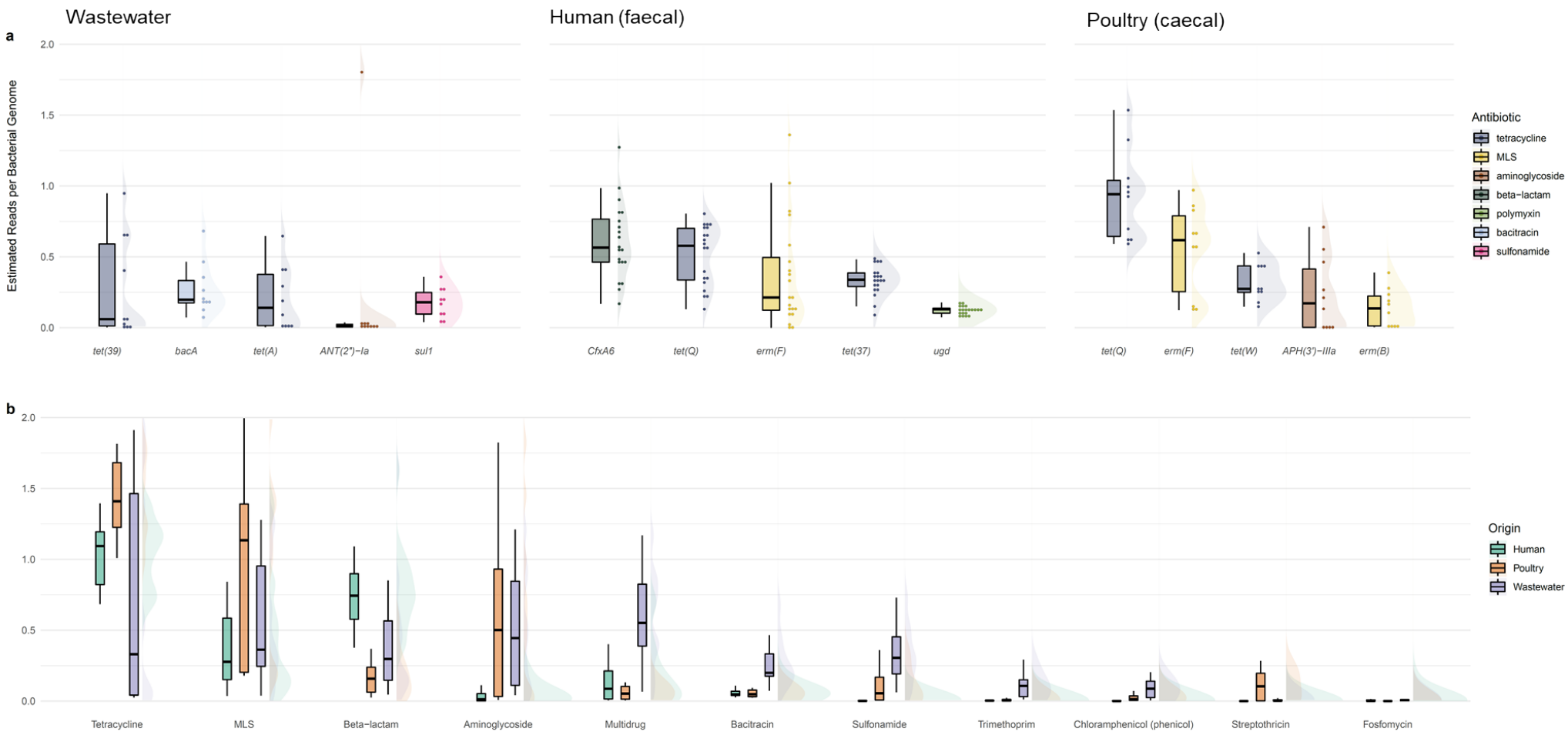

Figure 2. Summary of abundant antibiotic resistance genes in each sample origin a) Boxplots and half-violin plots with stacked dots showing the 5 most abundant ARG subtypes by mean in each sample source (wastewater, human faecal and poultry caecal) ordered by mean estimated reads per bacterial genome; **b)** Boxplots and half-violin plots of estimated ARG copies per genome for ARG categories highlighted by canonical analysis of principal coordinates (r >0.2) (compared by sample source).

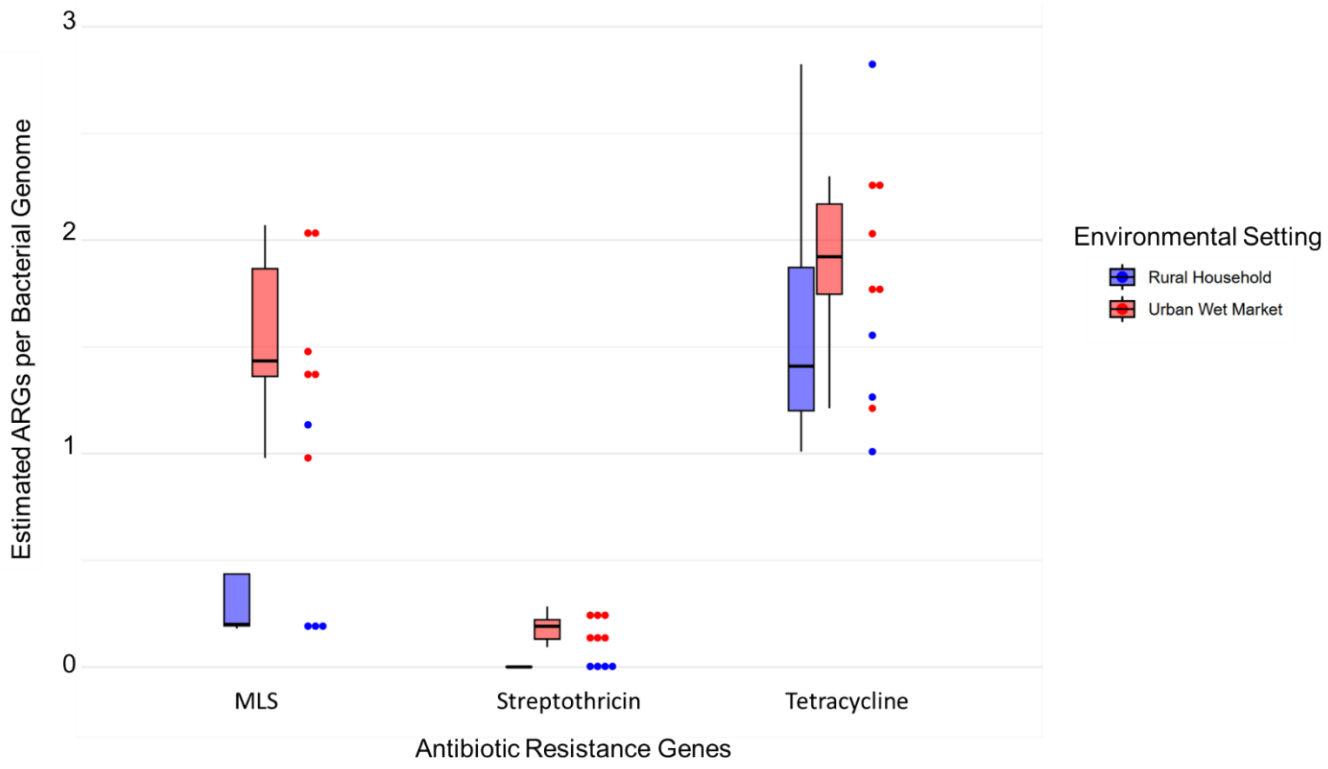

Figure 3. Differences in the abundance of select antibiotic resistance gene categories in poultry ceca samples collected from urban wet market and rural household chickens. Boxplots and stacked dot plots of MLS (macrolide-streptogramin-lincosamide), streptothricin and tetracycline resistance gene abundance are shown.