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RESEARCH ARTICLE

Exploring the Diversity and Dissemination Dynamics of Antimicrobial Resistance Genes in Enterobacteriaceae Plasmids Across Varied Ecological Niches

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A B S T R A C T

Antimicrobial resistance (AMR) among the Enterobacteriaceae poses a major public health threat as it encompasses several clinically significant microorganisms responsible for 30% of bacterial human infections limiting treatment options for bacterial infections. Therefore, the presence of plasmid-borne resistance genes in these bacteria in various ecosystems raises significant concerns. In this study, we investigated the distribution of antimicrobial resistance genes (ARGs) in plasmids isolated from human feces, wild birds, and aquaculture environments. Comprehensive antibiotic resistance database (CARD), ResFinder and NCBI databases were used to detect ARGs. The transferability of plasmids was assessed using *oriTfinder,* and phylogenetic tree based on MOB was generated using FastTree. A total of 453 (wild birds=266, 78=aquaculture, 109=human feces) plasmid sequences were identified and classified into 29 Enterobacteriaceae species while a total of 159 (35%) plasmids harbored the ARGs distributed into 23 species led by *Escherichia coli* and followed by *Klebsiella pneumoniae* and *Salmonella enterica.* Moreover, we detected 197 different ARGs conferring resistance to 13 different classes of antimicrobial agents. Plasmids from *E. coli*, *K. pneumoniae* and *S. enterica* harboring several ARGs were found in all niches investigated. Moreover, we detected that plasmid were classified into 36 different plasmid replicon types that were distributed among all three ecosystems. Almost 60% of the plasmids that were conferring resistance to at least one antibiotic has transfer potential and among them 40.8% were conjugative while 18.8% were mobilizable. Phylogenetic analysis revealed that Enterobacteriaceae plasmids co-evolve in nature, with widespread ARG dissemination across different ecosystems, highlighting their role in driving AMR within the One Health framework through human-animal-environment interactions.

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INTRODUCTION

Antimicrobial resistance (AMR) is a rising global threat that makes it harder to treat serious and life-threatening infections, due to the inaccessibility of treatment choices, subsequently leading to economic and public health losses (Weist and Hogberg, 2016) According to the Centers for Disease Control and Prevention (CDC), the annual cost in terms of AMR prevention and control is estimated at approximately USD 5.5 million (Dadgostar, 2019). In the last few years, there has been a rapid increase both in the actual number and extent of microbes, especially bacteria exhibiting multidrug-resistant properties.

Leading public health organizations, including the World Health Organization (WHO), the European Center for Disease Prevention and Control (ECDC), and the CDC, recognize infections caused by multidrug-resistant (MDR) pathogens as a perilous global health threat. (Roca *et al.*, 2015). A recent high-profile report by the United Kingdom government (The O'Neill Report) assessed that antimicrobial resistance is responsible for around 700,000

deaths each year which will cross 10 million annually by 2050 (O'Neill, 2016). AMR is particularly significant in the Enterobacteriaceae family as it incorporates an enormous number of clinically significant Gram-negative microorganisms that are responsible for 30% of bacterial human infection cases (Ibrahim and Hameed, 2015).

Genera such as *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia* are widespread in terrestrial and aquatic environments, and commonly inhabit the gastrointestinal tracts of humans and animals as commensals. However, they are also significant etiological agents of both human and animal diseases (Octavia and Lan, 2014) as the commensal microbiota of the gut, and also important from clinical and veterinary perspective as causative agents of several diseases (Kang *et al.*, 2018). Moreover, the emergence of AMR in Enterobacteriaceae especially resistance to last-resort antibiotics including colistin and tigecycline leads to millions of deaths due to treatment failure in case of infectious diseases. For instance, *E. coli* and *K. pneumoniae* were listed among the six deadliest pathogens linked with global mortality due to AMR (Murray *et al.*, 2022a). Moreover, according to WHO carbapenems and third-generation cephalosporins-resistant Enterobacteriaceae are classified as critical priority pathogens regarding to discovery of new drugs and alternative treatment options (Tacconelli *et al.*, 2018).

The rising antibiotic resistance patterns observed in Enterobacteriaceae is largely accredited to their ability to acquire and disseminate genetic material, including antimicrobial resistant genes (ARGs) through horizontal gene transfer (HGT) (Carattoli, 2009). In the event of HGT the vital role in the dissemination of ARGs between MDR isolates is played by conjugative plasmids that harbor the ARGs and pass on in the microbial community leading to the formation of extensively drug-resistant bacteria (XDR) superbugs. Notably, plasmids harboring ARGs show high transmissibility, between diverse bacterial species, even across phylogenetic boundaries, enabling their propagation within or across the ecosystems (Dolejska and Papagiannitsis, 2018).

Moreover, MDR bacterial isolates carrying conjugative plasmids are likely to be propagated between humans, the environment, and animals (food and companion animals) owing to their proximity via direct or indirect means or through farm runoff (Samtiya *et al.*, 2022). However, in addition to domestic animals, wild and migratory birds play a significant role in the dissemination of ARGs (Ahlstrom *et al.*, 2021). Their ability to travel long distances within short periods makes them potential carriers of resistant bacteria (Boto, 2010). These resistant strains are often acquired during migration from human-impacted environments such as polluted lakes, rivers, and ponds or through HGT during interactions with other avian species sharing similar ecological niches (Dunning, 2012).

The concurrent existence of multiple ARGs including genes encoding resistance to tetracycline and aminoglycoside among humans and other species in the same or different ecosystems has been documented (Ma *et al.*, 2016). In many bacterial species HGT is largely driven by mobile genetic elements (MGEs) that carry accessory genes with adaptive functions (Partridge *et al.*, 2018). Among the most prevalent MGEs are conjugative plasmids and integrative conjugative elements (ICEs), which play a central role in gene dissemination (de la Cruz *et al.*, 2010). Plasmids serve as major vectors of HGT, facilitating the spread of ARGs. Gaining a comprehensive understanding of plasmid mobility is therefore essential for developing strategies to curb the spread of ARGs, especially in light of the growing incidence of treatment failures and infection-related mortality in developed nations (Smillie *et al.*, 2010). Despite their significance, a systematic and large-scale analysis of plasmids has not been thoroughly conducted to date providing the motivation for the present study and we hypothesized that identified ARGs would mostly be harbored on conjugative/mobilizable plasmids, subject to selective pressures driven by irrational antimicrobial usage. The aim of this study is to investigate the distribution and diversity of antimicrobial resistance genes (ARGs) among *Enterobacteriaceae* plasmids across diverse ecological niches, including human feces, wild birds, and aquaculture environments. Furthermore, this study seeks to advance our understanding of the role of mobile genetic elements in facilitating the dissemination of antimicrobial resistance across both anthropogenic and natural reservoirs.

MATERIALS AND METHODS

Plasmid sequences extraction from the database: Plasmid sequences of the Enterobacteriaceae family were retrieved from the PLSDB-A plasmid database (https://ccb-microbe.cs.uni-saarland.de/plsdb2025/) (Schmartz *et al.*, 2021) in FASTA format using the keywords "Enterobacteriaceae*"* along with "wild and migratory birds", "Fishes and aquaculture" and "human feces, human stool, and human fecal samples". The average plasmid size in *Gammaproteobacteria* is approximately 58.7kb, with most plasmids exceeding 4 kb (Shintani and Kimbara, 2015). So, in this study plasmids smaller than 4kb have been excluded to avoid the analysis of incomplete sequences. No temporal restrictions were applied during data retrieval; all available plasmids meeting the keyword criteria were included in the analysis regardless of their date of deposition.

Database classification: Plasmid characterization included size, guanine-cytosine (GC) content, number of coding sequences (CDSs), and plasmid multi-locus sequence typing (pMLST). CDS annotations were primarily obtained from the NCBI database; for plasmids lacking this information, open reading frames were predicted using GeneMarkS v4.28 (Besemer *et al.*, 2001). GC content was calculated using an online genomics tool (https://www.sciencebuddies.org/science-fair-projects/references/genomics-g-c-content) (Dvorak *et al.*, 2019). pMLST profiles were determined via the "Plasmid Typing Database" hosted on the PubMLST platform (https://pubmlst.org/bigsdb?db=pubmlst_plasmid_seqdef) (Jolley *et al.*, 2018). Statistical comparisons of average plasmid size, GC content, and CDS count across different ecosystems (human, avian, aquaculture) were conducted using the Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. All statistical analyses were conducted using the BioRender web-based platform (https://BioRender.com).

Identification of ARGs: ARGs in plasmids were identified using abricate tool with three ARG databases: the Comprehensive Antibiotic Resistance Database (CARD), ResFinder, and NCBI AMRFinderPlus. Plasmid replicon typing was performed with abricate v1.0.1 using PlasmidFinder database (Bortolaia *et al.*, 2020; Carattoli and Hasman, 2020). The presence of ARGs was considered present in the plasmid if it was detected spotted in at least one ARG database with a minimum of 90% gene identity and coverage. To unify the nomenclature of the detected ARGs, ResFinder nomenclature was applied to genes however the genes that are not identified via ResFinder their nomenclature was attributed to NCBI AMRFinder Plus.

Classification of ARGs in different ecosystems: A Venn diagram was used to visualize the distribution of ARGs identified in Enterobacteriaceae plasmids across three ecological sources: human feces, wild birds, and aquaculture environments. The Venn diagram was generated via online tool available on the Bioinformatics and Evolutionary Genomic website (https://bioinformatics.psb.ugent.be/webtools/Venn/). Furthermore, a gene web was constructed from *E. coli, K. pneumoniae,* and *S. enterica* to reveal the distribution of common ARGs in all ecosystems as these three bacterial species were common in these ecosystems. The gene network was created with the help of Cytoscape version 3.10.2 (Shannon *et al.*, 2003).

Transferability of Enterobacteriaceae plasmids: To explore the potential transferability of the plasmids across different ecological niches, the oriTfinder online platform was used to identify key mobilization elements such as origin of transfers (*oriT*), relaxases genes, type-IV coupling proteins (T4CP), and the type-IV secretion system (T4SS) in DNA sequences of bacterial MGEs (Li *et al.*, 2018). Based on the presence or absence of these elements, plasmids were classified into three categories:

i) Conjugative plasmids: those encoding all four essential elements (*oriT*, relaxase, T4CP, and T4SS);
ii) Mobilizable plasmids: those possessing the *oriT* site but lacking one or more of the remaining components;
iii) Non-transmissible plasmids: those lacking the *oriT* element entirely.

This classification allowed for systematic assessment of plasmid-mediated horizontal gene transfer potential across ecosystems.

Mobility Protein-Centric Phylogenetic Reconstruction: The amino acid sequences of Mob proteins were used to reconstruct the phylogenetic tree of analyzed plasmids. A web-based tool oriTfinder was used to find the relaxases genes in plasmids (Li *et al.*, 2018) while GeneMarkS v4.28 was used to identify using open read frames (ORFs) encompassing the amino acid sequences for relevant relaxases genes (Besemer *et al.*, 2001). To classify relaxes based on mob gene amino acid sequences Relaxes were used as input in the online bioinformatic tool MOBscan (Garcillán-Barcia *et al.*, 2020). Muscle v5 was used to align these sequences and FastTree were used to construct the phylogenetic relationship (Price *et al.*, 2009) while iTOL v6 was used to visualize and annotate the tree (Letunic and Bork, 2021).

RESULTS

Characteristics of Enterobacteriaceae plasmids: The exploration of plasmids retrieved from Enterobacteriaceae recovered from human feces, aquaculture, and migratory wild birds in the PLSDB led to the identification of 453 (wild birds=266, 78=aquaculture, 109=human feces) plasmid sequences that were distributed in 29 different bacterial species from 30 different geographical locations in the worlds. The overall prevalence of plasmids harboring ARGs were observed in China (~53%) followed by Australia (~6.4%) and (~4.5%) in USA and Thailand (Fig. 1a). The average size of the Enterobacteriaceae plasmids did not diverge significantly among all the ecologies (Fig. 1b). Moreover, plasmids also exhibit significant variability in size within the ecosystems as plasmid size ranges from 18.9 kb to 407.7 kb in aquaculture, 5.9 kb to 311.6 kb in wild birds and 4.6 kb to 294 kb in human feces. The frequency of CDS numbers of plasmids followed the same profile in accordance with size, with the exhibiting lowest average of CDS in plasmids of wild birds (7 kb) and the highest in aquaculture (407.4 kb) (Fig. 1c). The average number of coding sequences (CDS) showed no significant variation ($P>0.05$) across ecosystems. The GC content of plasmids from various ecosystems ranged from 40 to 62.5% (Fig. 1d). Among all the analyzed plasmids, a total of six distinct pMLST profiles including were identified in this study. Notably, 45.2% of plasmids could not be classified using the pMLST scheme, highlighting limitations in typing resolution for a substantial subset of vectors. Of the typed plasmids, only 23% exhibited complete concordance of all loci with their assigned pMLST profile, while the rest of them harbored partial locus matches to their designated type. Incompatibility (Inc) group profiling revealed IncF and IncHI2 as the predominant replicon types, detected in 48 and 19 plasmids, respectively in wild birds and human feces (Fig. 1e). In contrast, aquaculture-associated plasmids exhibited all six pMLST types identified in this study, though IncF plasmid replicon type was the most prevalent one, followed by IncHI2 and IncA/C.

Prevalence and dispersion of ARGs in Enterobacteriaceae from diverse ecological niches: The characterization of ARGs in plasmids using ResFinder, CARD, and NCBI bring about the discovery of accumulatively 197 ARGs causing resistance to 13 different antibiotic classes and disinfectants. A total of 159 (32= aquaculture, 56=human feces and 71=wild birds) out of 453 plasmids (35%) harbor these ARGs distributed into 23 Enterobacteriaceae species led by *E. coli* and followed by *K. pneumoniae* and *S. enterica* from 22 different countries (Fig. 2). Moreover, we also calculated the relative abundance of ARGs in plasmids from all ecosystems under this study that confer resistance to multiple antibiotic classes including aminoglycosides, beta-lactams, polymyxins, fosfomycin, tetracycline, and results showed that ARGs conferring resistance to aminoglycosides and beta-lactams are most abundant in all ecosystems followed by phenicol's and fluoroquinolones while plasmids from human feces show highest abundance of ARGs conferring resistance to diaminopyrimidines. Moreover, ARGs conferring resistance to tigecycline and lincomycin are

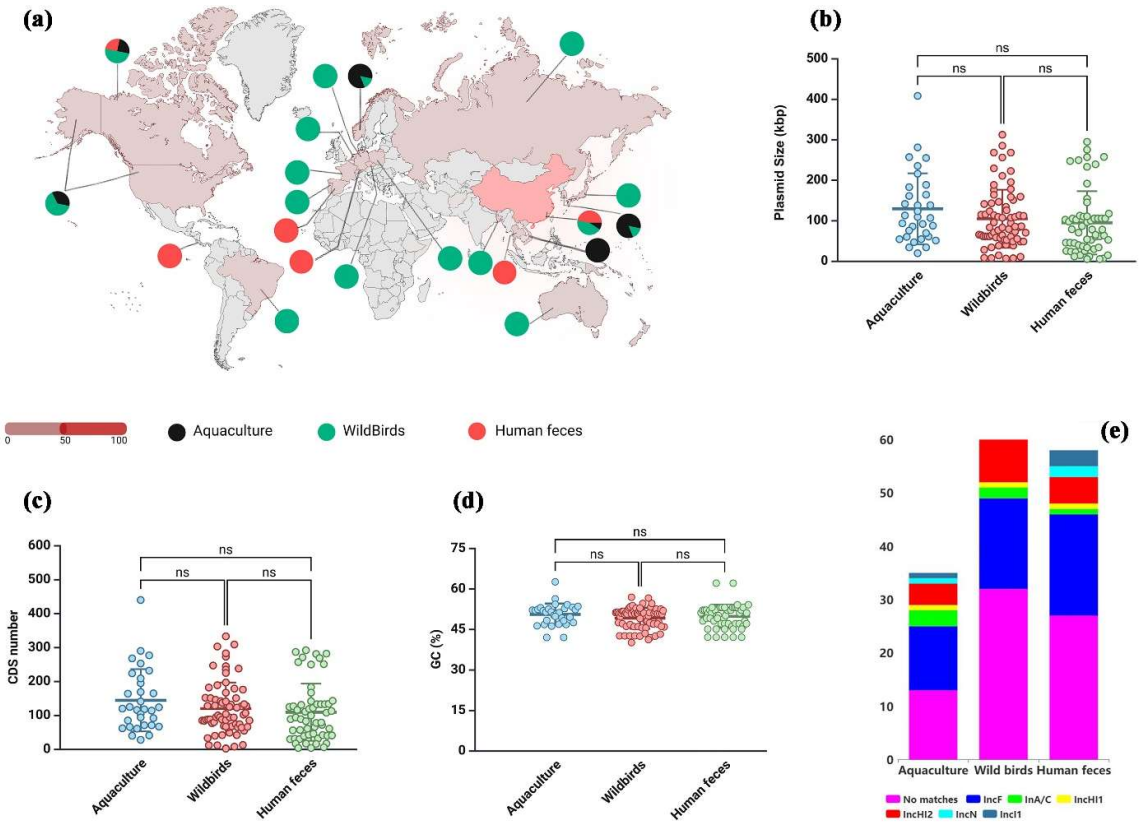

Fig. 1: (a) Global distribution of publicly available Enterobacteriaceae plasmids by country. The geographical heatmap was plotted based on the number of plasmid sequences among countries. The source distribution of the plasmids by country is represented by pie charts color-coded as indicated in the legend. (b) Plasmid size comparison (in kbp) across ecosystems. Each dot represents a plasmid, and the distributions show no statistically significant differences (*ns*) among aquaculture, wild bird, and human feces sources (c) Number of coding sequences (CDS) per plasmid from each ecosystem. Although there is variation, no significant difference was observed in CDS numbers among the three source groups (d) GC content (%) of plasmid sequences from different sources, showing similar distributions and no significant variation across ecosystems (e) Distribution of plasmid replicon types identified in each source group. The bar plot shows the prevalence of major incompatibility (Inc) groups including IncF, IncI1, IncN, IncA/C, IncHI1, and IncHI2, along with a large proportion of plasmids with no matches in current databases, indicating unclassified or novel replicons.

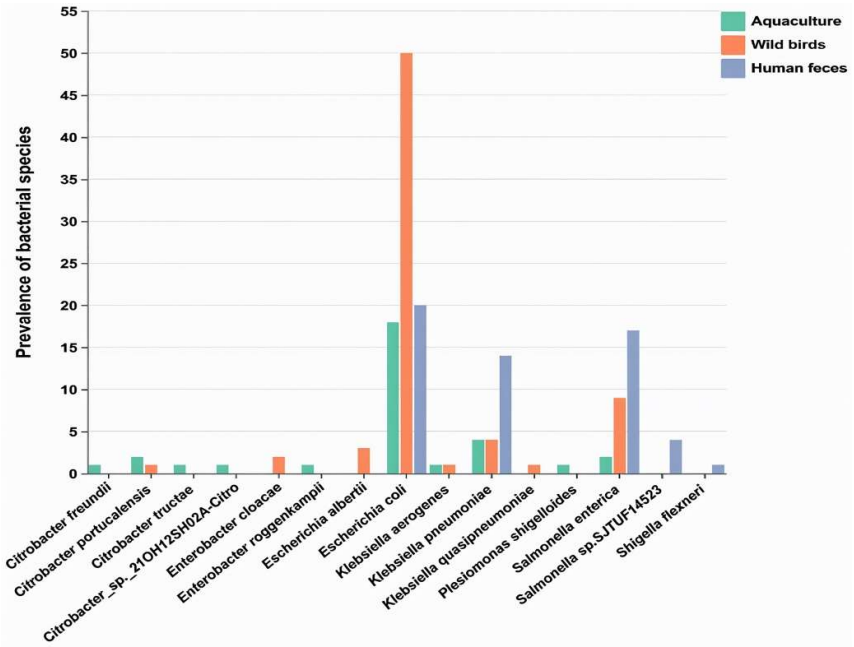

Fig. 2: Relative prevalence of bacterial species among different ecosystems: Bar plot depicting the normalized abundance of species across three plasmid sources aquaculture, wild birds, and human feces. The colored bars represent the different Enterobacteriaceae species identified in various ecosystems including human feces, wild birds, and aquaculture. While the bar height represents the prevalence of the species; the greater the height the more prevalent the species in different ecosystem.

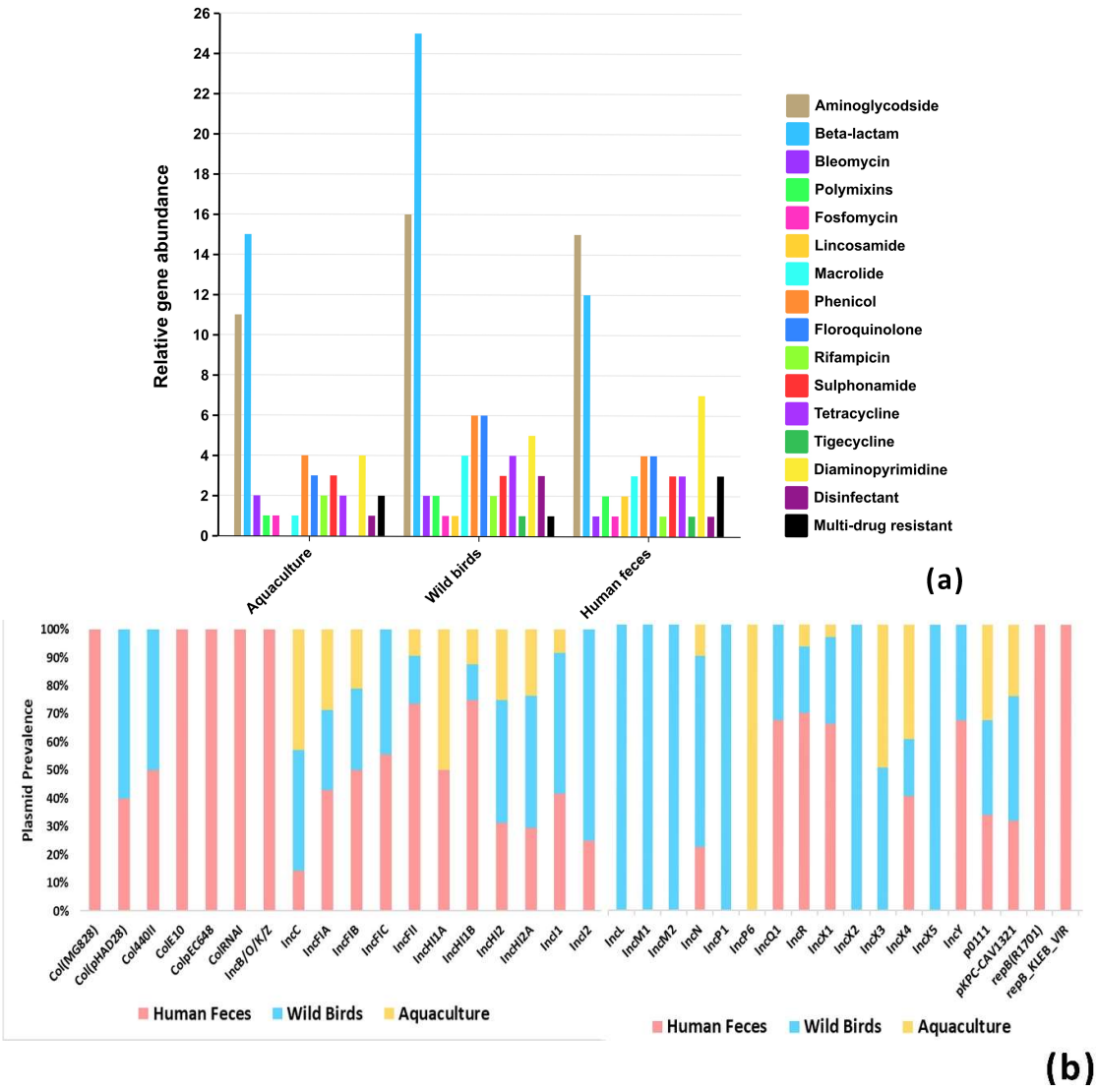

Fig. 3: Distribution of ARGs and plasmid replicon types across different ecological niches (a) *Relative abundance of ARGs in plasmid sources*: Bar plot depicting the normalized abundance of antimicrobial resistance genes across three plasmid sources aquaculture, wild birds, and human feces. Bar height corresponds to the relative abundance of ARGs, with taller bars indicating higher abundance. (b) *Prevalence of plasmid replicon types:* Bar plot showing the frequency of plasmid replicon types detected in three ecosystems: wild birds, aquaculture, and human feces. Values represent the proportion of samples within each ecosystem where specific replicon types were identified.

only present in humans and wild birds (Fig. 3a). However, Enterobacteriaceae plasmids from wild birds and human feces carried a total of ~111 different ARGs conferring resistance to all 13 antibiotic classes. Maximum number of AMR genes detected (*n*=51) conferred resistance to beta-lactams, followed by genes conferring resistance to aminoglycosides (*n*=42), diaminopyrimidine (*n*=16), phenicol (*n*=14), fluoroquinolones (*n*=13). In contrast, polymyxins (*n*=6), fosfomycin (*n*=3), and tigecycline (*n*=2) were the antibiotics with the lowest number of ARGs. The highest number of genes were detected in wild bird plasmids (*n*=82) followed by human feces (*n*=63) and aquaculture (*n*=52). However, Enterobacteriaceae plasmids from wild birds, human feces carried a total of ~145 ARGs conferring resistance to all 13 antibiotic classes. The identification of plasmid replicon types using PlasmidFinder resulted in the detection of collectively 36 replicon types harboring ARGs in three ecosystems. Among them, IncP6 plasmid type is only prevalent in aquaculture while IncI, IncM1, IncM2, IncP1, IncX2, and IncX5 plasmid replicons are only prevalent in wild birds. In contrast to aquaculture and wild birds Col (MG828), ColE10, ColpEC648, IncB/O/K/Z, and repB are the most prevalent replicon types detected in human feces. In addition to that IncHI1A was detected in humans and aquaculture while IncX3 was only detected in aquaculture and wild birds (Fig. 3b). A total of 27 out of 197 ARGs were shared across all three ecosystems wild birds, human feces, and aquaculture. Additionally, 14 ARGs were common between human and wild bird plasmids, 12 between wild birds and aquaculture, while only 3 ARGs were shared between human and aquaculture sources, indicating a weaker association between the latter two. (Fig. 4a). Additionally, a *E. coli* IncHI2 plasmid from aquaculture harbored 20 different ARGs, followed by a wild bird-derived *E. coli* IncHI2 plasmid carrying 19 ARGs. In human feces, a *K. pneumoniae* conjugative plasmid classified as IncFIB(K) harbored 18 ARGs. Given

their high prevalence across aquaculture, wild birds, and human fecal sources, *E. coli*, *K. pneumoniae*, and *S. enterica* were selected for focused ARG analysis. These species not only dominated the plasmid datasets but also represent major Enterobacteriaceae pathogens of significant public health concern, underscoring the importance of their comprehensive evaluation. The results exhibited that seventeen ARGs were identified in plasmids of *E. coli* from all ecosystems, including *tet*(A), *aph(3)-Ia*, *ant(3)-Ia*, *floR*, *tet*(X4), *cmlA1*, *aadA2*, *qnrS*, *bleO*, *qac*H, *aph(3)-Ib, dfrA12, sul2, dfrA14, aac(3)-lld, bla*$_{SHV-12}$,*bla*$_{TEM-1B}$ (Fig. 4b). In addition to *E. coli K. pneumoniae* harbor nine different ARGs common in all three ecosystems including *tet*(A), *aadA2*, *qnrS1 aph(3)-Ib*, *dfrA12, sul2*, *bla*$_{TEM-1B}$, *sul1* and *mph(A)* (Fig. 4c). However, *S. enterica* harbors only six ARGs in common detected from all ecosystems including *ant(3)-Ia*, *qnrS1, sul3, mph(A), aph(6)-ld, bla*$_{CTX-M-55}$ (Fig. 4d).

Transfer potential of MDR Enterobacteriaceae plasmids: Key genetic elements linked with plasmid mobility, including the origin of transfer (*oriT*), relaxases genes, type IV secretion system (T4SS), and type IV coupling proteins (T4CP). These features collectively determine the plasmid's potential for horizontal gene transfer (Alvarez-Rodriguez *et al.*, 2020). The computational analysis of plasmids collected in this study revealed 59.7% of the plasmids in this study are transmissible of which 40.8% were conjugative carrying all the key elements including *oriT*, relaxses, T4CP and T4SS while 18.8% were mobilizable, lacking one of the key elements except *oriT*. Apart from that 31.4% of plasmids carrying all genes required for the conjugation process except *oriT* and were declared as non-transmissible (Fig. 5). The most of the conjugative and mobilizable plasmids were detected in *E. coli, S. enterica, Salmonella flexneri, Citrobacter truncatae, Citrobacter portucalensis, K. pneumoniae and Klebsiella aerogenes* (Fig. 6a). Most of the conjugative plasmids were detected in the wild birds 17% (28/159) followed by human feces and aquaculture which account for 13.8% (22/159) and 9.4% (15/159), respectively. Moreover, similar patterns regarding the detection of mobilizable and non-transmissible plasmids were observed in all ecosystems (Fig. 6c). In addition to that the average size of plasmids according to their potential transferability was assed and reveal that the size of conjugative plasmids is larger than the mobilizable and nontransmissible plasmids (Fig. 6b).

Phylogeny of plasmids from diverse species and ecosystems: The phylogenetic analysis based on relaxases sequences revealed a clustering pattern predominantly aligned with relaxases classification (Fig. 7). Cluster annotation demonstrated that bacteria from diverse generic groups coalesced into multiple clades, highlighting the conservation and homology of relaxases sequences across genera within the Enterobacteriaceae. Furthermore, the relaxases associated with bacterial plasmids from various ecological niches examined in this study were broadly distributed across the phylogenetic tree that highlights the widespread evolutionary dissemination of these relaxases among plasmids from different ecological niches.

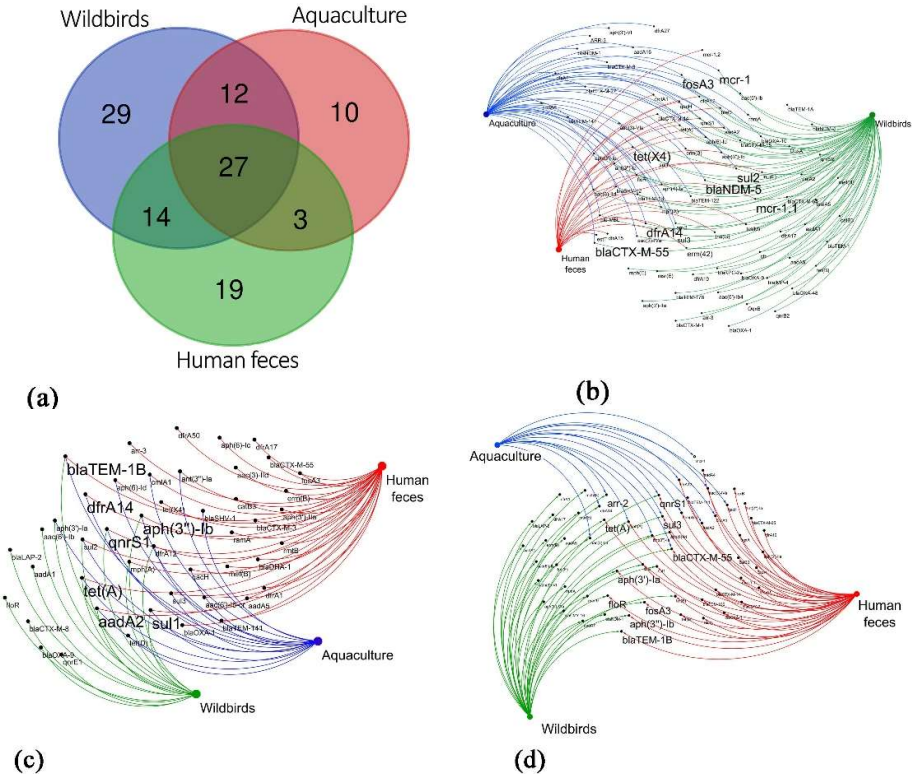

Fig. 4: (a) Venn diagram represents the sharing of ARGs among three different ecosystems including human feces, aquaculture, and wild bird's plasmids while (b, c & d) represents the network analysis of ARGs found in *E. coli, K pneumoniae* and *S. enterica* plasmids from aquaculture, wild birds and human feces respectively. Genes are denoted by black dots and the colored lines connecting the genes with their corresponding source of origin. The greater font size of ARGs represent their abundance in the respective species among different ecological niches.

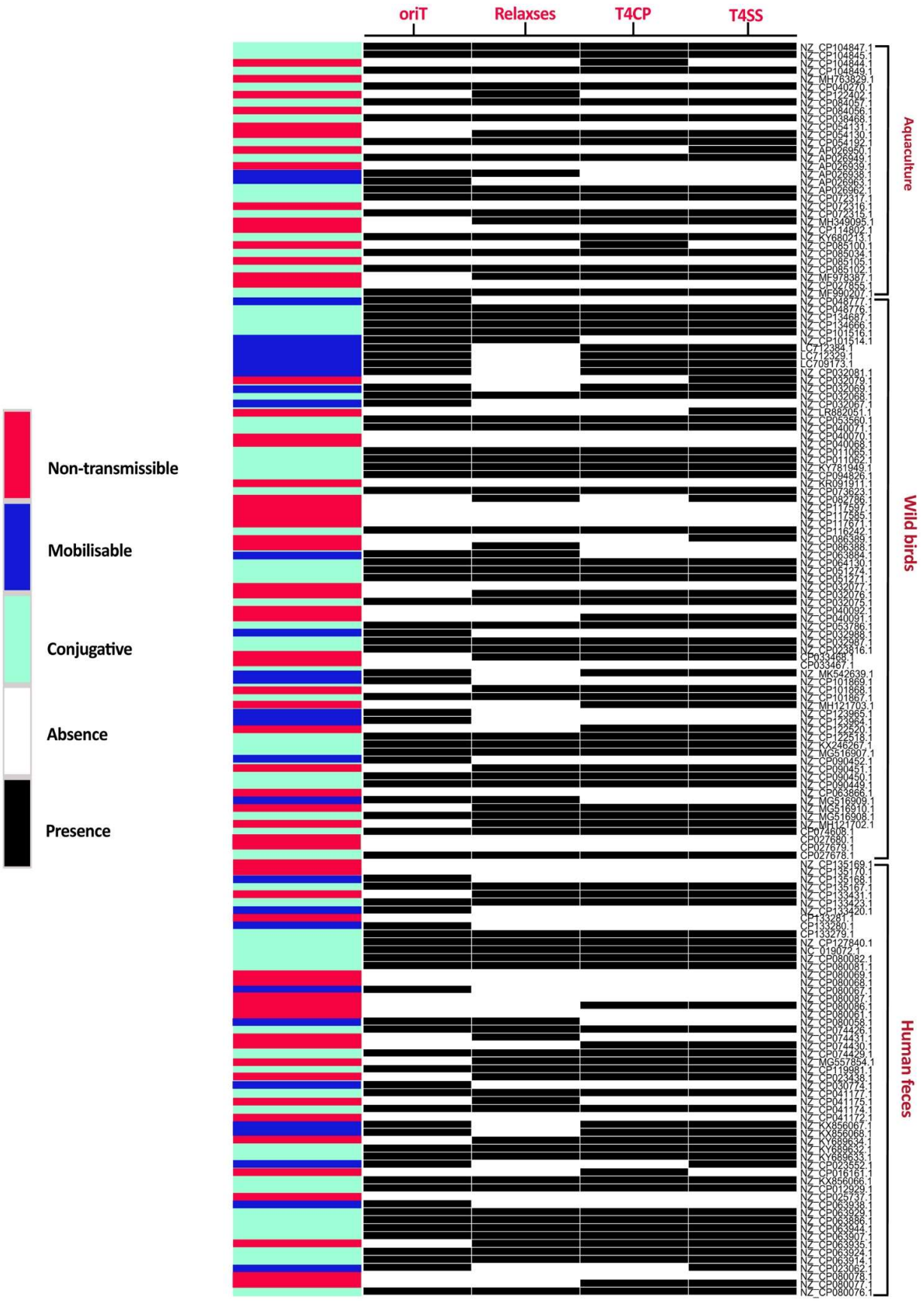

Fig. 5: The conjugative capacity of the Enterobacteriaceae plasmids is scrutinized in this work. The presence of genes related to the conjugative machinery is shown in black tiles and the absence of the respective genes is represented in white tiles. The classification of the plasmids as conjugative, mobilizable, and non-transmissible is shown in light blue, dark blue, and red respectively.

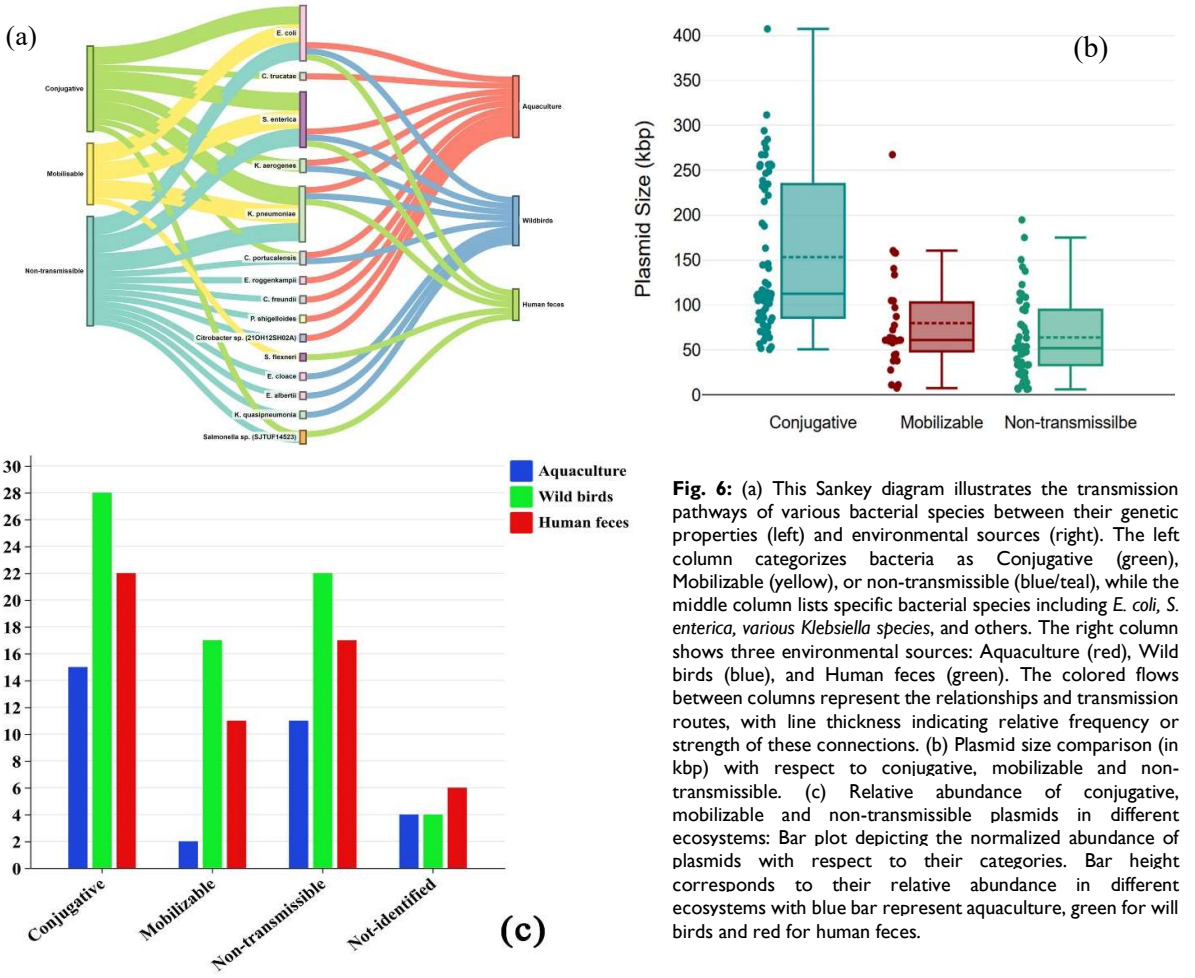

Fig. 6: (a) This Sankey diagram illustrates the transmission pathways of various bacterial species between their genetic properties (left) and environmental sources (right). The left column categorizes bacteria as Conjugative (green), Mobilizable (yellow), or non-transmissible (blue/teal), while the middle column lists specific bacterial species including *E. coli, S. enterica, various Klebsiella species*, and others. The right column shows three environmental sources: Aquaculture (red), Wild birds (blue), and Human feces (green). The colored flows between columns represent the relationships and transmission routes, with line thickness indicating relative frequency or strength of these connections. (b) Plasmid size comparison (in kbp) with respect to conjugative, mobilizable and non-transmissible. (c) Relative abundance of conjugative, mobilizable and non-transmissible plasmids in different ecosystems: Bar plot depicting the normalized abundance of plasmids with respect to their categories. Bar height corresponds to their relative abundance in different ecosystems with blue bar represent aquaculture, green for will birds and red for human feces.

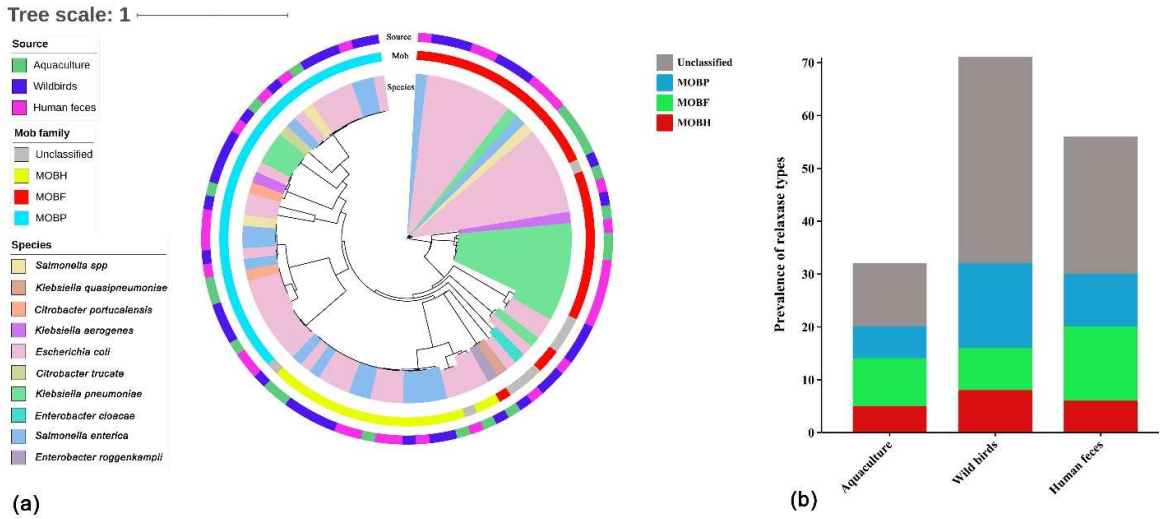

Fig. 7: (a) Phylogenetic tree of Enterobacteriaceae plasmids reconstructed using Mob relaxase sequences. Clades are colored by the bacterial genera associated with the plasmids. The outermost ring represents the source of each plasmid, while the middle ring categorizes plasmids based on their MOB (mobilization) family, including MOBP (blue), MOBF (red), MOBH (yellow), and unclassified (grey). The innermost ring shows the species from which each plasmid was recovered, color-coded as indicated in the species. (b) Prevalence of relaxaes types represents the abundancy of MOB family in different ecosystems including aquaculture, wild birds and human feces.

DISCUSSION

In the present study, we detected numerous ARGs dispersed in Enterobacteriaceae plasmids oriented from human fecal samples, wild birds, and aquaculture. Although the dataset encompasses diverse ecological sources, the dominance of plasmid records from well-studied species such as *E. coli* and *K. pneumoniae* in public databases may skew the results and limit the generalizability of our findings across the broader Enterobacteriaceae family. The majority

of ARGs encountered conferred resistance to beta-lactams, diaminopyrimidines, phenicol, tetracyclines, fluoroquinolones, and aminoglycosides. These antimicrobials are widely utilized for treating diverse bacterial infections in both clinical and veterinary medicine (Caneschi *et al.*, 2023). Antibiotic residues, excreted via feces and urine, can enter the soil by manure and employ selective pressure on soil microbiota. For instance, tetracyclines, incompletely absorbed or metabolized by hosts, contribute to environmental contamination, potentially influencing microbial communities (Wu *et al.*, 2013).

The most prevalent ARGs, including *aph(3')-Ib*, *tet*(A), $bla_{\text{CTX-M-55}}$, and *sul2*, were common among Enterobacteriaceae across all ecosystems examined. Moreover, these genes have been reported in dung and the environment and are often linked with MGEs (Heuer *et al.,* 2011; Lima *et al.,* 2020; Zalewska *et al.*, 2021). However, previous studies have stated that this co-occurrence of resistance genes to multiple antimicrobial classes on plasmids (Compain *et al.*, 2014; Mutuku *et al.*, 2022; Tsilipounidaki *et al.*, 2022), which strengthen the findings of present study, where *aph(3)-Ib* and *aph(6)-Id* were frequently detected alongside *tet*(A) and *sul2* on Enterobacteriaceae plasmids across investigated plasmids. Notably, *aph(3')-Ib* and *aph(6)-Id*, which encode aminoglycoside-modifying phosphotransferases, were among the most frequently detected genes (Ramirez and Tolmasky, 2010) thereby reducing the efficacy of aminoglycoside antibiotics.

The *tet*(A) gene, encoding a tetracycline efflux pump and frequently located on mobile genetic elements, is widely disseminated. It has also been reported in Enterobacteriaceae from diverse sources, including clinical settings (Akiyama *et al.*, 2013), food-producing animals, and the environment (Zhang *et al.*, 2009; Zhuang *et al.*, 2021). Similarly, *sul2*, a plasmid-mediated genes conferring sulfonamide resistance (Radstrom *et al.*, 1991) has been detected in various environmental contexts including water sources, food market and manure (Jiang *et al.*, 2019; Wang *et al.*, 2014; Zhao *et al.*, 2022). These genes are of particular concern, especially the tetracycline-resistant *tet*(A) gene as their mutant also triggers resistance to a last-resort antibiotic such as tigecycline (Chiu *et al.*, 2017; Hentschke *et al.*, 2010; Xu *et al.*, 2021). The detection of *tet*(A) variants in this study, consistent with Xu et al. (2021) who observed tigecycline resistance in 71.4% of *K. pneumoniae* harboring the tetracycline resistance *tet*(A) gene, provides evidence that mutations in the *tet*(A) gene can directly lead to tigecycline resistance. This linkage suggests that widespread tetracycline use in agriculture and human medicine exerts selective pressure favoring *tet*(A) mutants capable of resisting tigecycline, posing a significant threat to the efficacy of this last-resort antibiotic in Enterobacteriaceae.

Furthermore, the presence of other emerging ARGs resistance genes on Enterobacteriaceae plasmids emphasizes the multifaceted nature of AMR in these bacteria that confer resistance to last-resort antibiotics, including such as *mcr-1*, *mcr-1.2* and *mcr-9* imparting resistance to colistin (Wu *et al.*, 2024; Borjesson *et al.*, 2020; Mmatli, Mbelle, and Sekyere, 2022; Di Pilato *et al.*, 2016) bla_{NDM}, bla_{KPC} , $bla_{\text{OXA-48}}$ encoding resistance to several beta-lactams (Nordmann, 2014), *fosA*3 conferring resistance to fosfomycin (Ito *et al.*, 2018) and *tet*(X), which encodes resistance to tigecycline (He *et al.*, 2019). Among all investigated plasmids *E. coli*, *K. pneumoniae,* and *S. enterica* were the species detected in all the ecosystems. This is likely due to the more extensive datasets existing for these strains or plasmids in public databases, revealing their importance in clinical and veterinary contexts.

AMR is particularly significant in the Enterobacteriaceae family as it incorporates an enormous number of clinically significant Gram-negative microorganisms that can cause around 30% of bacterial disease (Ibrahim and Hameed, 2015). As example of importance of these species can serve fact that in 2019 MR *E. coli* was responsible for more than 0.8 million mortalities globally and therefore was declared as the leading pathogen related to deaths due to AMR (Murray *et al.*, 2022b). This observation was attributed primarily to the resistance of *E. coli* against last-resort antibiotics. In the present research, several genes including bla_{TEM}, bla_{NDM}, and *floR* were recorded in high frequency in plasmids originating from *E. coli*. In addition to that, genes conferring resistance to tetracyclines, aminoglycosides, and sulfonamides were also prevalent in *E. coli*.

Notably, our findings demonstrate that multiple Enterobacteriaceae species, including *S. enterica*, *K. pneumoniae*, *C. freundii*, *C. portucalensis*, and *E. coli*, harbor MDR plasmids, highlighting their potential as significant reservoirs and drivers of antimicrobial resistance in natural environments. Furthermore, we observed instances of these bacteria carrying more than 10 ARGs on a single plasmid such MDR bacteria belonging to *Enterobacterales* imposes a serious risk to human health globally by playing key role regarding nosocomial infections led to deaths and economic losses (Nordmann, 2014; Friedman *et al.*, 2016; Vrancianu *et al.*, 2021).

These MDR plasmids can propagate from one bacteria to another via several means, i.e. conjugation, transformation, and transduction (Soler and Forterre, 2020). In this study, we assessed the transferability of plasmids harboring ARGs and found that the majority are self-transmissible, indicating a significant potential for dissemination. Phylogenetic reconstruction of plasmid lineages presents significant challenges due to the dynamic nature of these mobile genetic elements, which undergo frequent structural rearrangements, including sequence insertions, deletions, and horizontal recombination events. Consequently, whole-plasmid sequence alignments often fail to resolve evolutionary relationships with fidelity. To circumvent this limitation, recent studies have employed relaxases enzymes (Mob proteins) key mediators of plasmid mobility as molecular markers for phylogenetic inference (Coluzzi *et al.*, 2022; da Silva *et al.*, 2022). These proteins exhibit evolutionary trajectories that, while occasionally punctuated by phylogenetic incongruences (e.g., horizontal transfer or modular recombination), demonstrate sufficient conservation to serve as proxies for plasmid lineage delineation. This approach aligns with the broader hypothesis that relaxases phylogenies reflect co-evolutionary dynamics between plasmids and their host genomes, despite occasional discordances arising from mosaic plasmid architectures (Smillie *et al.*, 2010).

Furthermore, in this study, we examined the phylogenetic relationships of conjugative plasmids from

different ecosystems. The extensive diversity of incompatibility groups, coupled with the mosaic structure shaped by frequent recombination events, underscores the intricate nature of plasmid phylogeny (Rozwandowicz *et al.*, 2018; Pesesky *et al.*, 2019). In response to these complexities (Fernandez-Lopez *et al.*, 2017; Smillie *et al.*, 2010) proposed the use of mobilization (Mob) proteins as phylogenetic markers to trace plasmid evolution and co-evolution, particularly in the context of mobile genetic elements (MGEs). Our Mob-based phylogenetic analysis exposed those plasmids from diverse species and ecological niches harbor relaxases with significant sequence similarity, proposing potential phylogenetic relationships between these plasmids. Although clear evidence for the persistence of individual plasmids within Enterobacteriaceae strains over time is lacking, our findings strongly suggest a common origin for many conjugative/mobilizable plasmids carrying multiple ARGs.

Conclusions: Our comprehensive analysis reveals widespread dissemination of ARGs conferring resistance to critically important antibiotics including tigecycline and colistin across Enterobacteriaceae plasmids from human feces, wild birds, and aquaculture environments. The high prevalence of conjugative and mobilizable plasmids (59.7%) underscores their significant role in HGT of resistance determinants while the Mob-based phylogenetic analysis demonstrates evolutionary relationships between plasmids from diverse ecological niches, suggesting common ancestral origins despite their current ecological separation. Notably, *E. coli*, *K. pneumoniae*, and *S. enterica* emerged as key species harboring MDR plasmids across all ecosystems, highlighting their potential as major reservoirs of AMR. The detection of identical ARGs in bacteria from diverse sources reinforces the interconnected nature of AMR transmission within the One Health framework. These findings emphasize the vital need for harmonized surveillance policies that surpass traditional ecological boundaries, implementation of targeted interferences to mitigate the spread of MGEs carrying resistance genes, and practical antimicrobial stewardship across human, animal, and environmental domains. Such holistic approaches are essential to efficiently address the global challenge of antimicrobial resistance.

List of abbreviations

AMR	Antimicrobial resistance
ARG	Antimicrobial resistant gene
CARD	Comprehensive antibiotic resistance database
CDC	Centers for Disease Control and Prevention
E. coli	*Escherichia coli*
ECDC	European Centers for Disease Control and Prevention
XDR	Extensively drug-resistant bacteria
GC	Guanine cytosine
HGT	Horizontal gene transfer
K. pneumoniae	*Klebsiella pneumoniae*
MDR	Multi drug resistant
MGE	Mobile genetic element
CDS	Coding sequences
oriT	Origin of transfer
pMLST	Plasmid multi-locus sequence type
S. enterica	*Salmonella enterica*
WHO	World health organization

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