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Distribution and Transmission of Apramycin-Resistant *Escherichia coli* from Humans and Animal-Producing Sectors: A Multicenter, Cross-sectional, and One Health Study

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ABSTRACT

Apramycin, an aminoglycoside antibiotic used exclusively in veterinary medicine, has attracted growing interest for its potential clinical application owing to its low toxicity and potent activity against multidrug-resistant (MDR) bacteria. Despite the completion of two Phase I clinical trials, apramycin resistance dynamics across One Health interfaces remain poorly understood. This study, conducted from 2020 to 2023 in Chengdu, Qingdao, and Shanghai, China, collected 5160 non-duplicate samples from hospitals, broiler and pig farms and slaughterhouses, and markets. We identified 1394 isolates of apramycin-resistant *Escherichia coli* (*E. coli*) (AREC), with the highest detection rates in animal feces (58%, 700/1214), followed by animal carcasses (47%, 183/393), fresh meat (35%, 229/659), environments (21%, 127/593), human feces (7%, 103/1425), and clinical samples (5%, 42/876). Detection rates were higher in broiler-producing chains (57%, 742/1292) than in pig-producing chains (32%, 512/1609). Most AREC isolates (99.7%, 1390/1394) carried the *aac(3)-IV* gene, conferring resistance to apramycin, gentamicin, and tobramycin. Genomic analysis of 742 AREC isolates revealed sporadic clonal transmission events between animals and humans in Qingdao and Shanghai. Long-read sequencing of 66 representative isolates showed that *aac(3)-IV* genes were primarily located on IncHI2/IncHI2A plasmids, with high structural conservation across different sources. Temporal surveillance indicated a sharp increase in *aac(3)-IV* prevalence in livestock-associated *E. coli* following the adoption of apramycin in China. These findings demonstrate the rapid, plasmid-driven dissemination of apramycin resistance at the One Health interface, underscore the need for prudent veterinary stewardship and careful consideration of apramycin's clinical repurposing for human use.

1. Introduction

The World Health Organization (WHO) has highlighted the severe public health threat posed by the global spread of extensively drug-resistant (XDR) Gram-negative bacteria, particularly

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those exhibiting resistance to carbapenems, colistin, and tigecycline typified by the New Delhi metallo-β-lactamase gene bla_{NDM}, the mobile colistin resistance gene *mcr*, and the tigecycline-inactivating enzyme gene *tet*(X). The wide dissemination of these genes has compromised the efficacy of last-resort antibiotics in clinical settings [1–3]. This crisis is further exacerbated by the medicinal-chemical challenges in addressing the antibiotic pipeline [4]. Consequently, repurposing previously overlooked or veterinary antibiotics for human clinical use has become a medicinal necessity.

Apramycin, an aminoglycoside antibiotic discovered in 1967, has been used exclusively in food-producing animals for the treatment of Gram-negative bacterial infections [5]. Structurally, apramycin is characterized by a unique 4-monosubstituted deoxystreptamine core linked to an eight-carbon bicyclic dialdose moiety, which allows it to evade most aminoglycoside-modifying enzymes [6]. It binds to the 16S ribosomal RNA (rRNA) of the bacterial ribosome at a site distinct from N7-G1405, thus bypassing inhibition by most methyltransferases [7]. Consequently, apramycin exhibits high bactericidal activity against Gram-negative bacteria, with resistance occurring less frequently compared to other aminoglycosides. Reported the $MIC_{50/90}$ (minimum inhibitory concentrations inhibiting 50%/90% of isolates) values are 2–4/8–16 $\mu g \cdot mL^{-1}$ for clinical multidrug-resistant (MDR) Enterobacteriaceae, *Acinetobacter* spp., and *Pseudomonas aeruginosa*, and 0.5/1 $\mu g \cdot mL^{-1}$ for clinical *Mycobacterium tuberculosis* isolates [8–10]. Notably, apramycin also displays superior bactericidal activity against *Mycobacterium abscessus*—both *in vitro* and in a pulmonary mouse model—compared to amikacin [11], and shows synergistic effects with tigecycline against *tet*(X)-positive *Acinetobacter* spp. [12]. These unique properties, combined with the low toxicity observed in animal models [7,13], render apramycin a promising candidate for human clinical use. Recognizing this potential, the European Gram-Negative Antibacterial Engine (ENABLE) project selected apramycin as its first antibiotic candidate in 2018. Following the completion of a Phase I clinical trial (NCT04105205) in October 2020, apramycin showed safety and tolerability, further supporting its potential to treat XDR Gram-negative infections in humans [14]. A second Phase I clinical trial (NCT05590728), initiated in June 2023, has also been completed, although its results have not yet been publicly disclosed [15].

As apramycin progresses towards clinical application, emerging resistance in clinical pathogens poses a challenge to its therapeutic efficacy. Currently, no official breakpoints have been established for apramycin; therefore, apramycin is seldom included in comprehensive antibiotic resistance surveillance studies or systematic evaluations. Given the similar *in-vitro* and pharmacokinetics/pharmacodynamics profiles between apramycin and amikacin, the breakpoint for amikacin resistance (≥64 $\mu g \cdot mL^{-1}$) (Clinical Laboratory Standards Institute (CLSI) M100-S32) has often been applied as an interpretative cut-off value for apramycin [9]. Based on limited studies, apramycin resistance has been reported in 1% human clinical MDR Enterobacterales [9], 14% of *Escherichia coli* (*E. coli*) isolated from pig feces [16], and 21% of *E. coli* isolated from chicken respiratory tract infections [17], indicating a higher resistance rate in animal-derived isolates compared to clinical isolates. Although apramycin escapes most aminoglycoside resistance mechanisms, resistance has been associated with four specific genes: *aac(3)-IV* and *apmA*, which encode N-acetyltransferase that acetylate the N3 and N2' positions of apramycin, respectively, and *kamB* and *npmA*, which encode 16S rRNA methyltransferases that modify the N1-A1408 binding site on ribosomal 16S rRNA [18]. Among these, the *aac(3)-IV* is most commonly detected in animal-derived *E. coli* [17,19], and, although less frequent, has also been identified in human clinical isolates [18]. Due to the cross-resistance mediated by *aac(3)-IV* between apramycin, gentamicin, and tobramycin, the emergence of apramycin-resistant bacteria in clinical settings may be driven by selective pressure from other aminoglycosides [18]. From a One Health perspective, resistance genes are frequently exchanged among human, animal and environment sectors and exemplified by *mcr-1* and bla_{NDM} that can spread through animal-producing food chains [20,21]. Thus, the use of apramycin in livestock may exert selective pressure that favors resistant bacteria, which could be transferred to clinical settings. To date, only Zhang et al. [22] have reported *aac(3)-IV*-positive apramycin-resistant *E. coli* (AREC) in both farm animals and workers in northeastern China, with conjugation assays demonstrating that animal-derived plasmids could readily transfer to human isolates. However, comprehensive One Health-based evidence on the transmission of apramycin resistance is still lacking. Herein, we report the prevalence of AREC across diverse sources and elucidate its transmission dynamics through whole-genome sequencing and related genomic analyses.

2. Materials and methods

2.1. Study design

In this cross-sectional, multicentered, and One Health study, we conducted large-scale and widespread sample collections from hospitals, pig farms and slaughterhouses, broiler farms and slaughterhouses, and markets in and around Chengdu, Sichuan, from Oct 11, 2020 to March 15, 2021, Qingdao, Shandong, from Sept 22, 2021 to May 1, 2022, and Shanghai, from March 9 and Dec 2, 2023 (Fig. S1 and Table S1 in Appendix A). Sampling was designed to encompass the entire transmission chain, spanning from animals and animal carcasses to animal-derived food, and ultimately to humans. Public hospitals were selected as central points for the city population. *E. coli* isolates from clinical samples were collected through the routine screening from hospital microbiology laboratories. Human feces were collected from individuals undergoing physical examination, outpatients or those admitted to hospitals within 48 h. Patients with diarrhea or those residing in the intensive care units (ICUs) were excluded. All human samples were divided into two groups: human-infection and human-carriage. These samples were obtained from the China Antimicrobial Surveillance Network (CHINET) with approval from the Ethics Review Committee of Huashan Hospital Affiliated to Fudan University (ethics license number 2019-460 and 2022-700). Partner hospitals participating in the study included Sichuan Provincial Maternity and Child Health Hospital, the Affiliated Hospital of Qingdao University, Qingdao Municipal Hospital, Shanghai Children's Hospital, and Huashan Hospital of Fudan University.

Farms and slaughterhouses were managed by the same local large food-producing corporation, and were collected for animal feces, animal carcasses, environmental samples, and farm workers feces. Fresh meat samples were taken from markets selling pork and chicken from the same farms and slaughterhouses. Animal-derived samples were divided into six groups: pig-farm, pig-slaughterhouse, pig-market, broiler-farm, broiler-slaughterhouse, and broiler-market (Table S1). Animal samples were collected with verbal consent obtained from farms and slaughterhouses owners. Since this study did not involve any experimental procedures that could endanger the animals' life or health, the Laboratory Animal Welfare and Animal Experiment Ethical Review Committee of China Agricultural University approved an exemption from ethical inspection (exemption number: AWF03214202-2-01).

All AREC isolates derived from these samples were included in the subsequent study (Table S2 in Appendix A). Selection for whole-genome sequencing was based on quantitative differences, clinical relevance, and sample origin. All human-derived isolates

were sequenced. For animal- and environment-derived isolates, the number of isolates sequenced within each sample was determined by the total number of isolates: one-third were selected for sequencing, and all were included when only one or two isolates were available per sample. For food-derived isolates, one AREC isolate per sampling site was sequenced, if available.

To evaluate the development of AREC in China over the past five decades, additional *E. coli* genomes derived from chickens and pigs, collected from our own datasets and National Center for Biotechnology Information (NCBI) depositories, were incorporated as part of this study [23].

2.2. Bacterial isolation and identification

Human clinical samples and feces were collected by hospital staff. Other samples were collected by trained researchers and farm and slaughterhouse workers. All raw samples were enriched in Luria Bertani (LB) broth containing 32 μg·mL^{-1} apramycin and 30 μg·mL^{-1} vancomycin (selecting Gram-negative bacteria), and incubated at 37 °C for 18–24 h. In detail, for swabs, sewage, and water samples, 100 μL of the liquid was directly transferred to Eppendorf (EP) tube containing 1 mL of LB broth. For soil, fodder, bedding, and manure samples, 0.1–0.2 g of the specimens was transferred to LB broth. Meat products were soaked in 10 mL of LB broth in sterile sampling bags and gently agitated to ensure thorough mixing, followed by the transferring of 100 μL of the liquid.

Each of the above enriched samples were subsequently streaked onto CHROMagar™ Orientation agar containing 32 μg·mL^{-1} apramycin and 30 μg·mL^{-1} vancomycin, and incubated at 37 °C for 18–24 h. Dark pink to reddish colonies on CHROMagar™ Orientation agar were suspected to be *E. coli*, and were purified on MacConkey agar containing apramycin (32 μg·mL^{-1}). Single colonies on MacConkey agar were identified at the species level by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI–TOF MS; Autobio, Autof ms1000, China).

2.3. Antimicrobial susceptibility testing and resistance gene detection

All *E. coli* isolates were subjected to apramycin susceptibility testing using the agar dilution method. Detection of apramycin resistance genes was performed by polymerase chain reaction (PCR) with primers targeting the four main apramycin resistance determinants: *aac(3)-IV* (aac(3)-IV-F, 5'-TACGAATGGC GAAAAGCCGA-3'; aac(3)-IV-R, 5'-ATGACCGACTGGACCTTCCT-3'), *npmA* (npmA-F, 5'-TCAGCTTTGTATTGTTCGCTCA-3'; npmA-R, 5'-A CTCAAAGGAACAAAGACGGT-3'), *apmA* (apmA-F, 5'-CGTTTGCTTCG TGCATTAAA-3'; apmA-R, 5'- TTGACACGAAGCAGGGTTTC-3'), and *kamB* (kamB-F, 5'-AACCTGCTGTACCTGTGGG-3'; kamB-R, 5'-CAGT CGGCGAGCTTCCAC-3'). The sequenced strains were subject to gentamicin, tobramycin, and amikacin susceptibility testing using the agar dilution method and interpreted according to CLSI M100-S32 guidelines [24].

2.4. Whole-genome sequencing and bioinformatics analysis

Genomic DNA was extracted using the HiPure Bacterial DNA Kit (Magen, China). Prepared DNA libraries were sequenced on the Illumina NovaSeq 6000 platforms with 150-bp paired-end reads. Cleaned data was assembled by SPAdes v3.15.5 [25]. Genomes were annotated with Prokka v1.14.6 [26] and the rapid annotation using subsystem technology (RAST) server [27]. Antimicrobial resistance genes and plasmid replicon genes were identified by staramr v0.9.1 [28] using the Resfinder and PlasmidFinder databases. Phylogenetic tree was constructed by Parsnp v1.7.4 [29] and visualized by tvBOT [30]. Multi-locus sequence typing (MLST) was determined by mlst v2.23.0, and Minimal Spanning Tree (MST) was constructed by goeBURST Full MST algorithm with PHYLOViZ v2.0 [31] software. Transmission event analysis was performed according to the same protocols described by Thorpe et al. [32], using a single-nucleotide polymorphisms (SNPs) threshold of 25 as previously defined by Gorrie et al. [33]. Genome contexts of *aac(3)-IV* were aligned and visualized by clinker [34].

2.5. Long-read sequencing and plasmid analysis

Long-read sequencing was performed on 66 isolates representing different cities, sample sources, and *aac(3)-IV* genetic contexts. Sequencing was conducted using R10.4.1 flow cells (FLO-MIN114) for Nanopore MinION platform (Oxford Nanopore Technologies, UK) with the Rapid Barcoding Kit 24 V14. Hybrid assemblies were performed using Unicycler v0.5.0 [35]. Plasmid similarity was evaluated as described by Matlock et al. [36] and Evan et al. [37]. The Mash distance (a *k*-mers-based measure of overall sequence similarity), query coverage (the proportion of the reference plasmid aligned by the query), and SNPs were determined to evaluate the similarity between plasmids. Each pair of plasmids with Mash distance ≤0.005, query coverage ≥ 95%, and SNPs ≤ 15 per 100 kb were considered related. Plasmid maps were generated by SnapGene® v5.2 software. Plasmid similarity networks were visualized by Cytoscape v3.10.2 software. The antibiotic resistance regions (ARRs) of plasmids were identified by VRprofile2 [38] and visualized by clinker [34].

2.6. Statistical analysis

The 95% confidence intervals (CIs) for percentage of AREC were calculated using the exact binominal method. Due to the temporal discontinuity and variations in sample size, *E. coli* genomes from the past five decades were categorized into six groups: 1970–1989, 1995–1999, 2000–2004, 2005–2009, 2010–2014, and 2015–2019. The Cochran–Armitage trend test was employed to evaluate the prevalence trend of *aac(3)-IV*-positive *E. coli*. All statistical analyses were conducted using R v.4.3.0.

3. Results

3.1. AREC is prevalent across animal-producing sectors

We collected 5160 non-duplicate samples from Chengdu (n = 2066), Qingdao (n = 1737), and Shanghai (n = 1357), among which 1394 AREC isolates were identified (Figs. 1 and 2; Tables S1–S3 in Appendix A). The highest prevalence of AREC was observed in animal feces (58%, 700/1214), followed by animal carcasses (47%, 183/393), fresh meat (35%, 229/659), environmental samples (21%, 127/593), human feces (7%, 103/1425), and human clinical samples (5%, 42/876). Samples from broiler-producing chains—such as broiler feces (80%, 412/514), broiler carcasses (61%, 102/167), broiler farm and slaughterhouse environments (33%, 97/298), and chicken (42%, 130/306)—showed markedly higher detection rates of AREC than those from the pig-producing food chain, which included pig feces (41%, 288/700), pig carcasses (36%, 81/226), pig farm and slaughterhouse environments (14%, 40/295), and pork (28%, 99/353). Among these 1394 isolates, 1390 carried the *aac(3)-IV* gene and exhibited high-level resistance to apramycin (MICs ≥ 512 μg·mL^{-1}). The remaining four isolates were negative for the other known apramycin resistance genes (*npmA*, *apmA*, and *kamB*) and displayed MIC values of 64 to 128 μg·mL^{-1}.

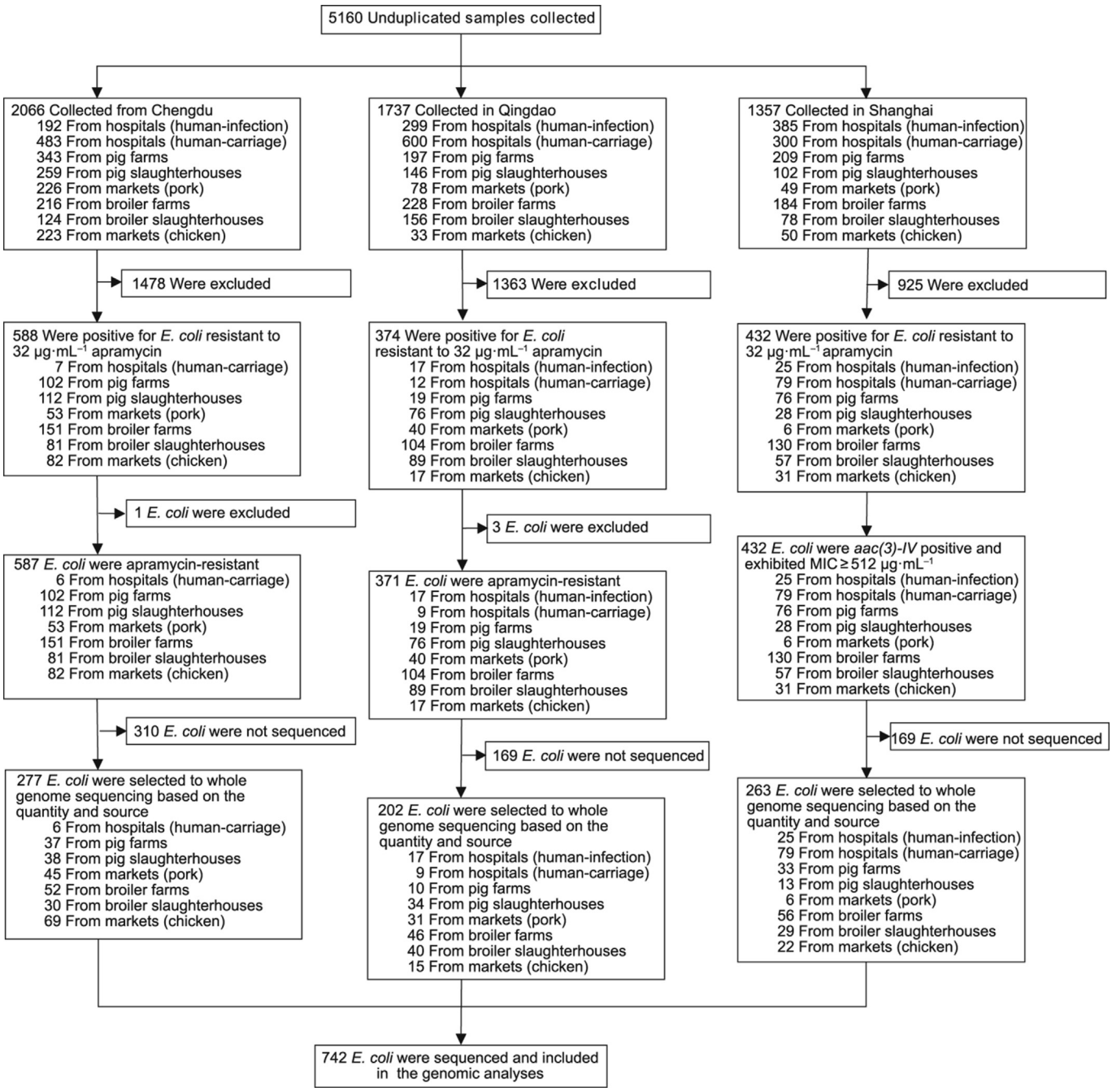

Fig. 1. Study profile.

3.2. Sporadic clonal transmission links animal and human reservoirs across production chains

Next-generation sequencing generated 742 high-quality genomes of representative AREC isolates selected based on their prevalence and source. Phylogenetic and MLST analyses revealed distinct genetic diversity, with no dominant sequence types (STs) observed (Figs. S2 and S3 in Appendix A). SNP-based comparisons indicated that while most transmission events occurred within single sources, sporadic isolates from different sources exhibited close genetic relatedness (Fig. 3). In Qingdao, two transmission pairs were identified between human-infection and broiler-market sources. In Shanghai, seven, three, and two transmission pairs were identified between human-carriage and pig-farm, pig-slaughterhouse and broiler-farm, respectively. Additionally, clear evidence of transmission of AREC within the pig- or broiler-producing chains was observed in all three cities (Fig. 3). However, no transmission events were detected between human-infection and human-carriage isolates in this study. Antimicrobial susceptibility testing revealed that the 742 AREC isolates also exhibited high resistance rates to gentamicin (99%, 733/742) and tobramycin (99%, 738/742), but interestingly low resistance to amikacin (5%, 39/742, Table S4 in Appendix A).

3.3. Three genomic backbones characterize the aac(3)-IV genetic environments

Among the 742 *aac(3)-IV*-carrying contigs, lengths ranged from 902 to 323 705 bp. Notably, 697 contigs (94%) exhibited three primary genomic backbone profiles: type A (136 contigs, 18%), type B

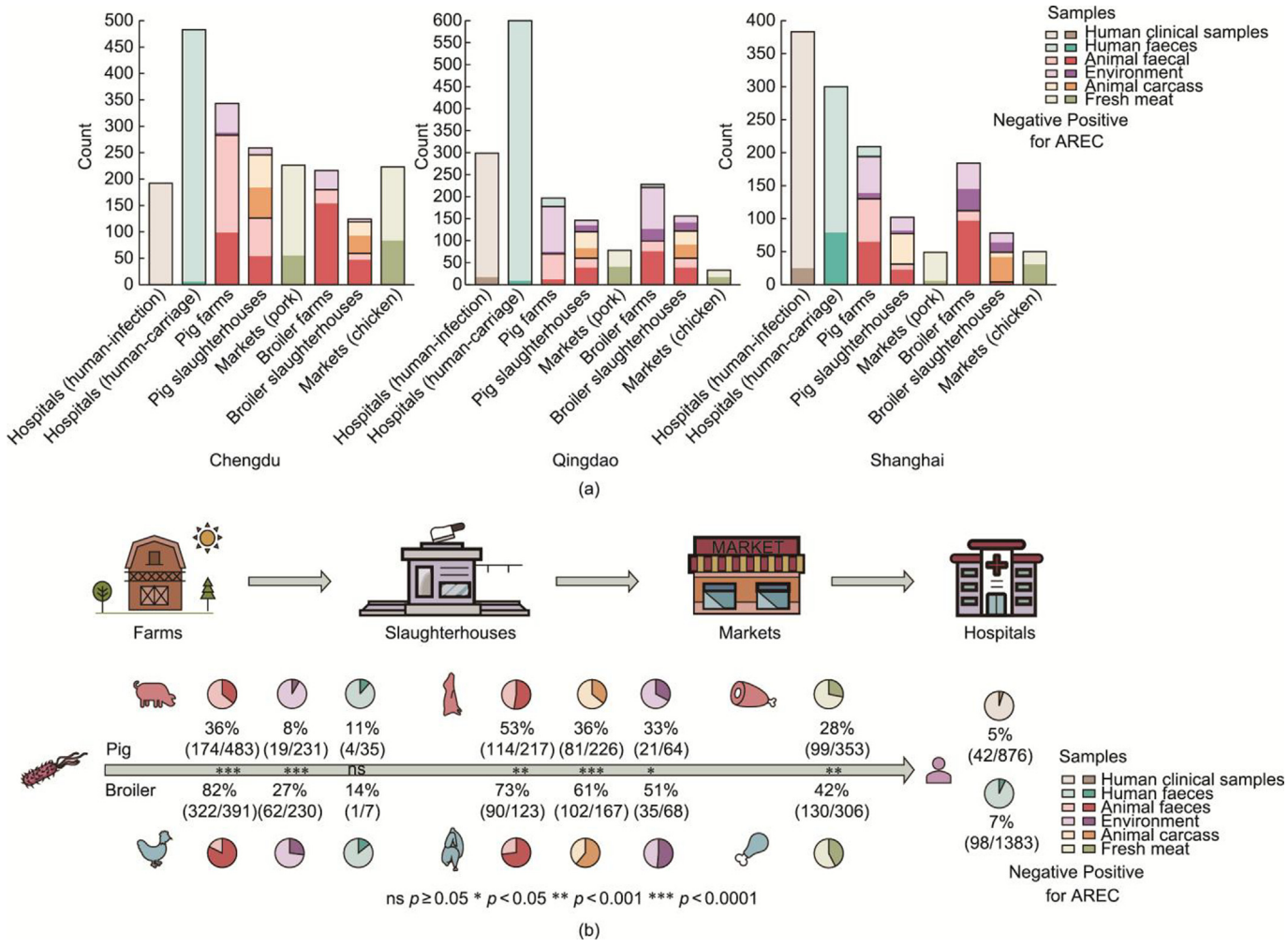

Fig. 2. Summary of sampling and sample processing results. (a) Stacked bar chart for the number of samples collected from various sampling sites in three cities. The colors of the bars represent different sample types, with dark and light shades indicating samples positive and negative for AREC, respectively. (b) The distribution of AREC isolates across diverse sampling sites and sample types.

(257 contigs, 35%), and type C (304 contigs, 41%) (Fig. S4 in Appendix A). These genomic backbones were identified across all sources, including human-infection (A, *n* = 4; B, *n* = 15; C, *n* = 20), human-carriage (A, *n* = 16; B, *n* = 43; C, *n* = 33), pig-farm (A, *n* = 7; B, *n* = 33; C, *n* = 33), pig-slaughterhouse (A, *n* = 15; B, *n* = 11; C, *n* = 43), pig-market (A, *n* = 22; B, *n* = 15; C, *n* = 34), broiler-farm (A, *n* = 32; B, *n* = = 59; C, *n* = 62), broiler-slaughterhouse (A, *n* = 21; B, *n* = 45; C, *n* = 30), and broiler-market (A, *n* = 19; B, *n* = = 36; C, *n* = 49). In the type A genomic backbone, *aac(3)-IV* was linked to a hygromycin B resistance gene *aph(4)-Ia*, IS*Ec59*, and ΔTn*5393*. These contigs were relatively short and represented the core regions shared by most *aac(3)-IV* gene environments, including those of types B and C. The type B genomic backbone contained an additional aminoglycoside resistance gene cluster (*aph(3')-Ia–aph(6)-Id–aph(3'')-Ib*) downstream of Tn*5393*. In the type C genomic backbone, the core region of *aac(3)-IV* gene was associated with the sulfonamide resistance gene *sul2*, and the florfenicol resistance gene *floR*, accompanied by diverse mobile genetic elements such as IS*CR2* and IS*1006*, as well as additional genes involved in cell wall synthesis (*glmM*), transcriptional regulation (*lysR*), and genetic transfer processes (*virD2*).

3.4. IncHI2 plasmids mediate cross-sector transmission of aac(3)-IV

Long-read sequencing of 66 selected AREC isolates revealed that the *aac(3)-IV* genes were located either on chromosomes (*n* = 18) or on plasmids (*n* = 50), with two isolates harboring two *aac(3)-IV*-positive plasmids each (Table S5 in Appendix A). The predominant *aac(3)-IV*-positive plasmid type was IncHI2/IncHI2A, accounting for 62% (31/50). Other plasmid types included IncY (4/50, 8%), IncFIB(AP001918)/IncFIC(FII) (3/50, 6%), IncFIA(HI1)/IncFIB(K) (2/50, 4%), IncFII (pCoo) (2/50, 4%), and IncN (2/50, 4%). Less common plasmid types, each representing 2% (1/50), included IncFIB(AP001918)/IncFIA, IncFIB(AP001918)/IncFII, IncFII (pHN7A8), IncX1, IncHI2/IncHI2A/IncY, and IncI(Gamma). Given that the *aac(3)-IV* genes were located on 32 IncHI2/IncHI2A-related plasmids, we further analyzed the carriage of IncHI2/IncHI2 plasmids in the broader dataset of 742 AREC isolates using next-generation sequencing. A total of 328 isolates (44%) carried IncHI2/IncHI2A plasmids, which were broadly distributed across different cities and sources. These findings indicate that IncHI2/IncHI2A plasmids serve as primary vehicles for the dissemination of *aac(3)-IV* (Fig. 4(a)).

For the 50 plasmid sequences acquired through long-read sequencing, we identified six potential similarity networks involving 32 plasmids across three plasmid types based on the Mash distances threshold (≤0.005). Network 1 consisted of 17 IncHI2/IncHI2A plasmids sourced from various origins across three cities: pig-farm (Chengdu, *n* = 2; Qingdao, *n* = 1; Shanghai, *n* = 1), pig-slaughterhouse (Chengdu, *n* = 1; Qingdao, *n* = 1; Shanghai, *n* = 1), pig-market (Qingdao, *n* = 1), broiler-farm (Qingdao, *n* = 1; Shanghai, *n* = 1), broiler-slaughterhouse (Chengdu, *n* = 1; Qingdao,

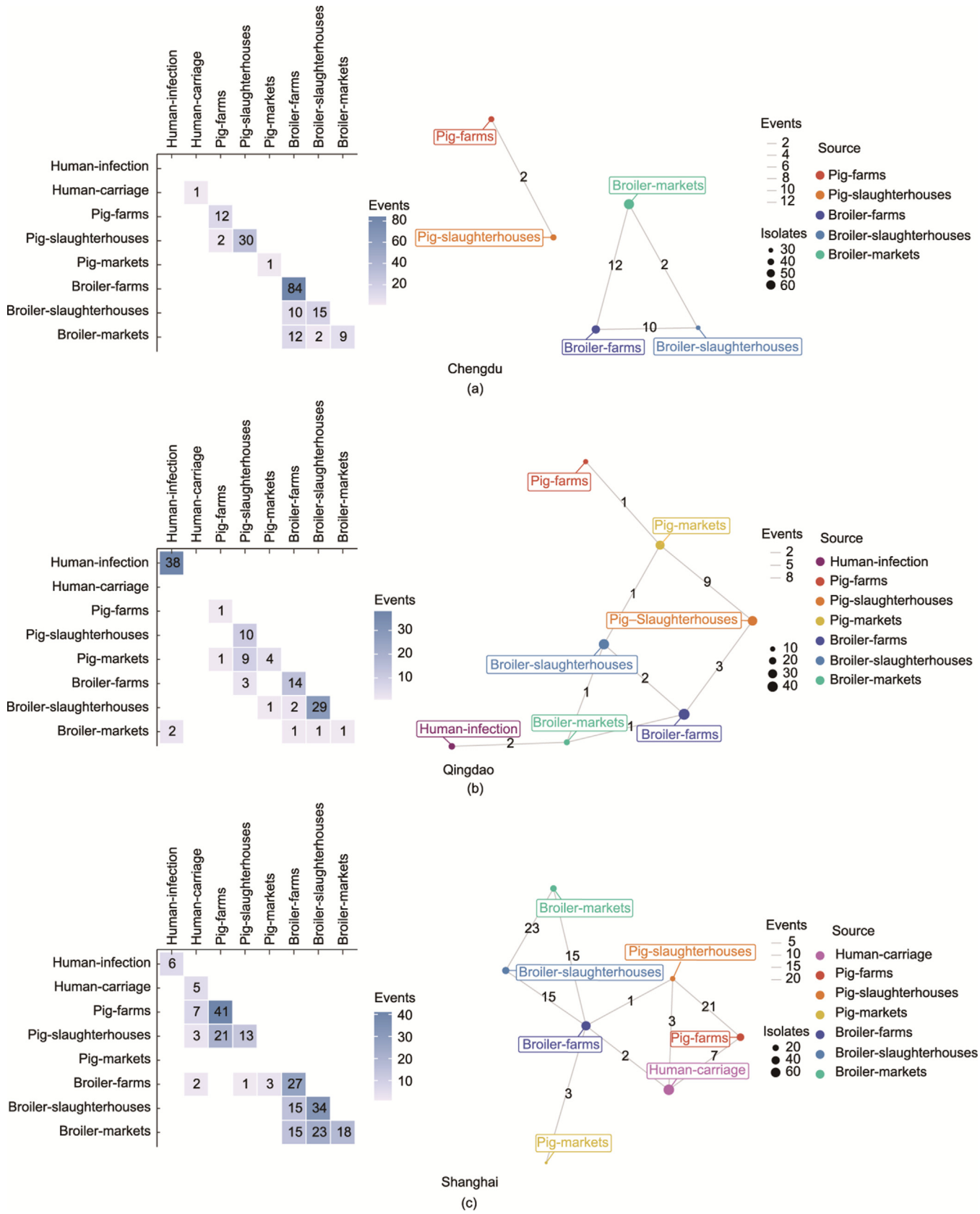

Fig. 3. Transmission heatmaps and networks of AREC. Heatmaps and networks showing the number of transmission events between each pair of sources in (a) Chengdu, (b) Qingdao, and (c) Shanghai, calculated with an SNPs threshold of 25. In the network diagrams, genomic relatedness between isolates from different sources is visualized: each node represents a distinct source category, with node size proportional to the number of isolates. Edges between nodes represent pairwise isolate connections with ≤25 SNPs, with thicker edges indicating a higher number of transmission events.

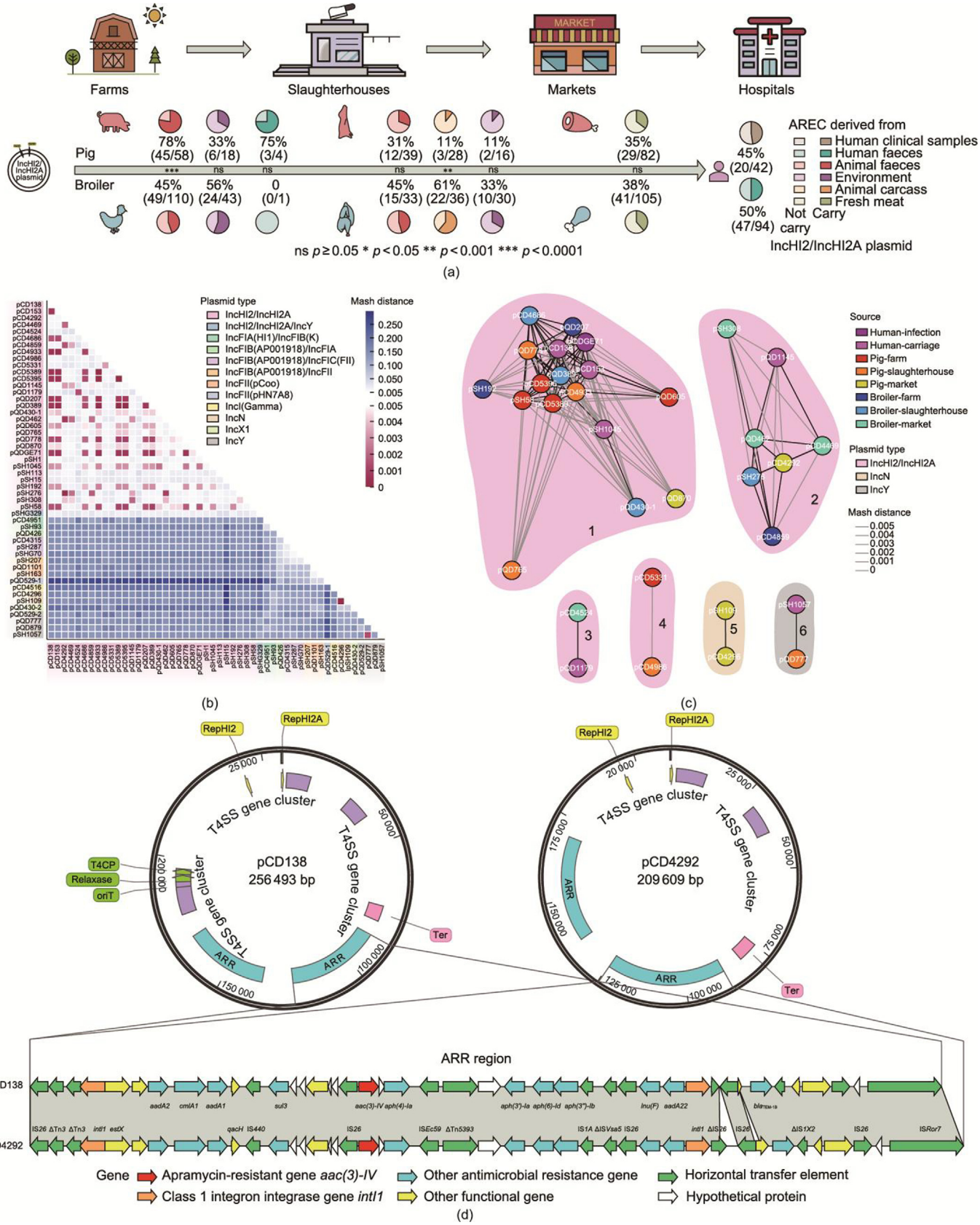

Fig. 4. Plasmid-mediated transmission of *aac(3)-IV*. (a) The distribution of IncHI2/IncHI2A plasmids in isolates from diverse sampling sites and sample types. (b) Heatmap showing pairwise Mash distances between *aac(3)-IV*-carrying plasmids. Mash distance reflects overall sequence similarity based on *k*-mer analysis; smaller values indicate higher similarity. Only distances <0.25 are shown. (c) Network showing plasmid relatedness based on Mash distance ≤0.005. Each node represents a plasmid, colored by source. Edges indicate pairs with high similarity (Mash ≤ 0.005); black edges highlight plasmid pairs with ≥95% query coverage and ≤5 SNPs per 100 kb, suggesting close genetic relatedness. (d) The structure of representative plasmids and their ARRs carrying *aac(3)-IV*. Arrows denote the transcriptional directions, with distinct colors assigned to different types of genes. Regions with ≥99.0% nucleotide sequence identity are shaded grey.

n = 2), human-carriage (Chengdu, *n* = 2; Shanghai, *n* = 1), and human-infection (Qingdao, *n* = 1). Network 2 comprised seven IncHI2/IncHI2A plasmids, derived from pig-market (Chengdu, *n* = 1), broiler-farm (Chengdu, *n* = 1), broiler-slaughterhouse (Chengdu, *n* = 1; Shanghai, *n* = 1), and broiler-market (Qingdao, *n* = 1; Shanghai, *n* = 1), and human-carriage (Qingdao, *n* = 1). The remaining networks (networks 3 to 6) were smaller, each involving only two plasmids: Network 3 and 4 contained IncHI2/IncHI2A plasmids, with network 3 including plasmids from human-carriage (Qingdao) and broiler-market (Chengdu), and network 4 comprising plasmids from pig-farm and pig-slaughterhouse sources (Chengdu). Network 5 and network 6 contained IncN and IncY plasmids, respectively, with network 5 including plasmids from pig-market (Chengdu and Shanghai) and network 6 including plasmids from human-carriage (Shanghai) and pig-slaughterhouse (Qingdao) (Figs. 4(b) and (c), Table S6 in Appendix A). Additionally, plasmid similarity thresholds established by Evan et al. [37] were used to further confirm that most plasmids within the same network exhibited high query coverage (≥95%) and close nucleotide sequence identity (≤15 SNPs per 100 kb), confirming the possibility that they are related.

We next compared the ARRs containing the *aac(3)-IV* gene across the 50 sequenced plasmids. With the exception of nine plasmids that exhibited rearrangements or deletions in the genetic context surrounding the *aac(3)-IV* gene, the ARRs of 41 plasmids could be grouped into two categories (Figs. S5–S7 in Appendix A). The first category corresponds to the type B genetic backbone, comprising 25 IncHI2/IncHI2A plasmids and one IncHI2/IncH2A/IncY plasmid (Fig. S5 in Appendix A). All plasmids from network 1 and 2 were assigned to this category. However, whilst pCD4292 (pig-market source, Chengdu) in network 2 showed only 80% query coverage to pCD138 (human-carriage source, Chengdu) from network 1, the ARRs in these two plasmids exhibited >98% query coverage and >99% nucleotide identity to each other (Fig. 4(d)). The second category corresponds to the type C genetic backbone, which includes a wider variety of plasmid types (*n* = 15), such as IncHI2/IncHI2A (*n* = 5), IncFIA(HI1)/IncFIB(K) (*n* = 1), IncY (*n* = 2), IncFIB(AP001918)/IncFIC(FII) (*n* = 3), IncFIB(AP001918)/IncFIC (*n* = 1), IncFIB(AP001918)/IncFII (*n* = 1), and IncN (*n* = 2) plasmids (Fig. S6 in Appendix A). All plasmids from network 3 to 6 were classified within this category.

3.5. Rising temporal trends in apramycin usage and resistance in livestock production

Over the past decade, China's annual consumption of apramycin has shown stable growth, reaching 405 t by 2019 (Fig. 5(a)) [39]. Approved in 2000 by the Chinese Ministry of Agriculture and Rural Affairs, apramycin was applied as a new veterinary drug for treating Gram-negative bacterial intestinal infections [40]. Subsequently, *aac(3)-IV* has emerged in *E. coli* isolates from chicken production and has shown a significant upward trend with the prevalence of *aac(3)-IV* increasing from 3% (2/71) in 2000–2004 to 35% (64/182) in 2015–2019 (Fig. 5(b)). Additionally, although the genomes data for *E. coli* isolates derived from pigs were limited due to the sample sizes from 1995–1999 and 2005–2009, we also

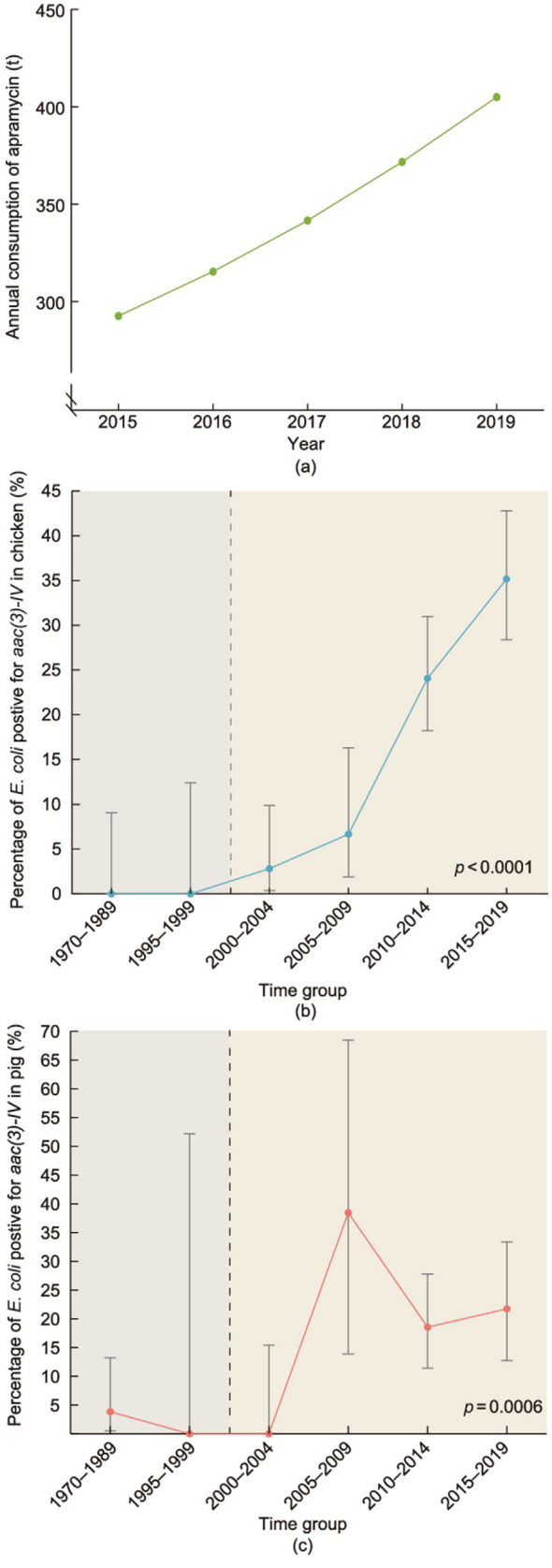

Fig. 5. Changes in annual consumption of apramycin and the prevalence of *aac(3)-IV* over time. (a) Annual consumption of apramycin from 2015 to 2019. The consumption data for 2019 is derived from forecasts based on the record in the first half of that year. (b) The increasing trend of *aac(3)-IV* gene in chicken-derived *E. coli* over decades. (c) The increasing trend of *aac(3)-IV* gene in pig-derived *E. coli* over decades. Dashed line denotes the temporal boundary for the use of apramycin in China's animal husbandry industry. The left side of the dashed line indicates the period prior to the use, while the right side indicates the period following its application. The Cochran–Armitage trend test was employed to evaluate the prevalence trend.

observed an increasing trend, with the prevalence of *aac(3)-IV* increasing from 0 (0/22) in group 2000–2004 to 22% (15/69) in 2015–2019 (Fig. 5(c)).

4. Discussion

Amid the rising challenge of drug-resistant infections in humans, apramycin has gained increasing attention for its impressive antibacterial activity against MDR Gram-negative bacteria, including ESKAPE pathogens and *E. coli* resistant to carbapenems, third-generation cephalosporins, and/or colistin [8,9,41]. Supporting this, Plattner et al. [18] reported the prevalence of apramycin resistance genes in genomes from human clinical isolates in the NCBI National Database of Antibiotic Resistant Organisms (NDARO), showing that the *aac(3)-IV* was present at a considerable low rate (averaging 0.7%) across 182 405 clinical isolates, and 0.8% in 37 283 *E. coli*/*Shigella* isolates. Furthermore, the prevalence of *aac(3)-IV* was only 2% in 21 195 carbapenem-resistant isolates and 5% in 1 022 carbapenem-resistant *E. coli*/*Shigella* isolates [18]. Additionally, apramycin-resistant genes other than *aac(3)-IV* were virtually absent in the human clinical isolates, with only two *Campylobacter jejuni* isolates carrying the *apmA* and two *Clostridium difficile* isolates carrying *npmA* [18]. These data, combined with our human-carriage (7%) and human-infection source (5%) data, indicate that AREC carriage and infection in humans are considerably lower than in the animal-producing sector (43%), further supporting a case for apramycin use in clinical applications. Moreover, the crystalline free base of apramycin, a clinical-stage drug candidate designated EBL-1003, has showed strong efficacy in preclinical studies, effectively treating *A. baumannii* murine lung infections and *E. coli* murine urinary tract infections, further validating its therapeutic potential and clinical utilization [13,42].

While apramycin resistance remains infrequently detected in human clinical isolates, its increasing prevalence in animals raises substantial concerns about potential transmission and emergence in humans. Our findings highlight the threat posed by AREC in the poultry-producing food chain, as evidenced by the following observations: ① a significantly higher prevalence of AREC in animal feces, carcasses, fresh meat, and environmental samples within the broiler-producing chain compared to the pig-producing chain (Fig. 2(b)); ② a notably rapid increase in the prevalence of *aac(3)-IV* among *E. coli* genomes derived from chickens compared to those from pigs (Figs. 5(b) and (c)); and ③ based on data from the China Veterinary Drug Association and Chinese Ministry of Agriculture and Rural Affairs, apramycin usage in the poultry industry is substantially greater (1.5 to 2.0 times higher) than that used in the pig industry [43]. Although the genomic relatedness analysis revealed considerable genetic diversity among AREC isolates, sporadic transmission events between humans and animal-producing chains still occurred. Given the strong selection pressure for apramycin resistance and the high prevalence of AREC in the animal-producing industry, the introduction of apramycin into human clinical practice is likely to mirror that of the animal sector and create a similar selective pressure, thereby promoting the emergence and dissemination of AREC in high-dependency hospitals wards where the drug is likely to be used.

Building on the risks associated with isolate transmission, an even greater concern lies in the spread of IncHI2/IncHI2A plasmids across human- and animal-derived isolates from the three cities investigated. IncHI2/IncHI2A plasmids have emerged as primary vectors for the *aac(3)-IV* gene, potentially accelerating the spread of apramycin resistance. Notably, these IncHI2/IncHI2A plasmids frequently co-harbor multiple antibiotic resistance genes, indicating that the spread of *aac(3)-IV* gene may also facilitate the co-mobilization of other resistance determinants, such as $bla_{\text{NDM-5}}$, which was identified in two sequenced IncHI2/IncHI2A plasmids in this study. The co-existence of these genes raises serious concerns about co-selection, which could further complicate treatment strategies and compromise the management of infections caused by MDR/XDR strains. Moreover, the genetic environment of *aac(3)-IV* remains highly conserved. Even when located on different plasmid backbones across isolates from diverse sources, the gene consistently retains a conserved genetic backbone, suggesting that its genetic context is readily mobilized across various plasmid types within *E. coli*, and potentially other members of Enterobacterales. Although the precise mechanism underlying the mobility of *aac(3)-IV* remains to be elucidated, IS*CR2*, an active transposon capable of mobilizing large segments of adjacent DNA via rolling-circle transposition, is located downstream of *aac(3)-IV* in the type C genetic backbone [44]. The transposition of IS*CR2* is oriented towards *aac(3)-IV*, and its activity has been demonstrated via inverse PCR (Fig. S8 in Appendix A), further supporting that IS*CR2* is highly active and likely to serve as an additional vector mediating the transfer of *aac(3)-IV* through insertion and recombination events [45,46].

Although apramycin shows considerable promise for clinical use in human medicine, its introduction into clinic practice raises concerns regarding the global stewardship of Watch or Reserve antibiotics currently used in farming. This conundrum was highlighted with the discovery of *mcr-1*, where its emergence prompted the rapid withdrawal of colistin from agriculture in China, followed by a rapid decrease in both *mcr-1* prevalence and colistin-resistant *E. coli* [2,47]. Even if apramycin were to be restricted or eventually banned in animal husbandry, cross-resistance with gentamicin and tobramycin mediated by *aac(3)-IV*, or the co-existence of *aac(3)-IV* with other antibiotic resistance determinants, may still drive or maintain apramycin resistance. For instance, *aph(3')-Ia*, which confers resistance to kanamycin, ribostamycin, and neomycin—another aminoglycoside used in animal husbandry—is frequently located downstream of *aac(3)-IV* on the same IncHI2/IncHI2A plasmids [48]. The use of neomycin in animal husbandry, together with gentamicin and tobramycin in clinical settings, could exert additional selective pressure on apramycin-resistant bacteria, thereby facilitating the spread of resistance between animals and humans [49]. Given the above, the use of apramycin in animal husbandry should be subjected to rigorous monitoring and regulation, and its clinical introduction must be approached with caution. More importantly, a clear distinction must be maintained between antimicrobials reserved for human use and those designated for veterinary medicine. Such separation is critical not only to preserve the efficacy of the limited antibiotic arsenal in human healthcare but also to prevent the cross-host transmission of antibiotic resistance genes.

This is the first One Health study on AREC; however, we acknowledge several limitations. Firstly, we did not collect fecal samples from community populations, and therefore our hospital-based fecal samples may not accurately reflect the fecal carriage of AREC in the general population. Secondly, we lacked access to detailed background information on the human clinical samples, such as infection sites, patient demographics (e.g., age and gender), and, most importantly, records of antibiotic exposure. The absence of clinical information hinders our ability to identify potential drivers contributing to the emergence of AREC in clinical settings and to determine whether the development of resistance is associated with specific drug use patterns or co-selection by other drug classes.

Although AREC has not yet emerged as a major concern in human clinical settings, our findings provide compelling evidence of its high prevalence within the animal-producing chain, particularly in the poultry industry. Moreover, AREC isolates have demonstrated the capacity to spread between the animal-producing chain

and humans. The IncHI2/IncHI2A plasmids serve as the primary vectors for spreading *aac(3)-IV*, further complicated by the presence of an active IS*CR2* element. Therefore, the clinical introduction of apramycin should be approached with caution, and the risk associated with its veterinary use require re-assessment. Additionally, the judicious use of apramycin, gentamicin, and tobramycin in human medicine is equally essential to prevent the selection and spread of AREC in clinical settings. Importantly, apramycin-resistant bacteria from both animals and human sources should be systematically incorporated into national and international antimicrobial resistance surveillance programs. From a broader antibiotic stewardship perspective, it is imperative to establish and enforce a clear separation between antibiotics designated for veterinary and human use. Such distinction is vital not only to preserve the efficacy of existing antibiotics in human healthcare, but also to mitigate the risk of resistance transmission across hosts—a concern that is particularly pressing for zoonotic infections, where the emergence of cross-resistant strains may further constrain the already limited treatment options.

CRediT authorship contribution statement

Yue Cao: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Dejun Liu:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Fen Pan:** Resources, Investigation, Data curation. **Zhenzhen Liu:** Resources, Investigation, Data curation. **Qin Zhang:** Resources, Investigation, Data curation. **Chengtao Sun:** Writing – review & editing, Supervision, Conceptualization. **Li Ding:** Resources, Data curation. **Siquan Shen:** Resources, Data curation. **Weishuai Zhai:** Methodology, Investigation. **Rina Bai:** Methodology, Investigation. **Zhiyu Zou:** Methodology, Investigation. **Yiqing Wang:** Methodology, Investigation. **Lu Yang:** Resources. **Zexun Lv:** Resources. **Bo Fu:** Investigation. **Shizhen Ma:** Investigation. **Yao Wang:** Methodology. **Ke Zhao:** Methodology. **Tingxuan Shi:** Methodology. **Yingbo Shen:** Project administration, Conceptualization. **Rong Zhang:** Writing – review & editing, Conceptualization. **Timothy R. Walsh:** Writing – review & editing, Validation, Supervision, Conceptualization. **Jianzhong Shen:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Fupin Hu:** Writing – review & editing, Validation, Resources, Conceptualization. **Yang Wang:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Congming Wu:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Data availability

The genomic data of AREC isolates have been submitted to NCBI under BioProject accession number PRJNA1204058. Long-read assembled plasmid sequences harboring *aac(3)-IV* have been uploaded to the figshare database.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2025.11.035.

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