

Genomic analysis reveals fifty years of antimicrobial resistance evolution in husbandry *Escherichia coli* from China

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Abstract

Antimicrobial agents have been used in meat production for decades and its consumption is considered an key driver for the emergence and dissemination of antimicrobial resistance (AMR). However, large-scale studies on AMR changes in animal isolates since the introduction of antimicrobial usage remain scarce. We applied whole genome sequencing analysis to 982 animal-derived *Escherichia coli* collected in China from 1970s to 2019 and found increasing trends for the presence of numerous antimicrobial resistance genes (ARGs), including those conferring resistance to critically important agents for veterinary (florfenicol and norfloxacin) and human medicine (colistin, cephalosporins, and meropenem). Extensive diversity and increasing complexity of ARGs and their associated mobile genetic elements (MGEs) such as plasmids were also observed. The plasmids, IncC, IncHI2, IncK, IncI, IncX and IncF played a key role as highly effective vehicles for disseminating ARGs. Correlation analysis also revealed an association between antimicrobial production and emergence of ARGs at a spatial and temporal level. Prohibiting or strictly curtailing antimicrobial use in animals will potentially negate the current trends of AMR as the bacterial genome is highly changeable and using different drugs of the same class, or even unrelated classes, may co-select for MGEs carrying a plethora of co-existing ARGs. Therefore, limiting or ceasing antimicrobial use in animals to control AMR requires careful consideration.

Introduction

Antimicrobial resistance (AMR) has become a global health and development threat. Currently, at least 700,000 people die annually of infections caused by antimicrobial-resistant organisms, and this number is projected to rise to at least 10 million with accumulated costs of approx. \$100 trillion by 2050¹. This relentless increase in AMR is exacerbated by global antibiotic consumption (AMC), which increased by nearly 70% during the 2000s with much of the AMC occurring in animal-production². In the USA, for example, over 70% (by weight) of antimicrobial agents assigned to classes that are medically important in human medicine, such as β -lactams, tetracyclines, macrolides and colistin, are sold for use in animals³. Such extensive AMC has driven the rapid emergence of multidrug-resistant (MDR) pathogens in both humans⁴ and animals⁵. Whilst horizontal gene transfer via plasmids and transposons may account for inter-bacterial dissemination⁶, the interconnections between animals and humans either through the food chain, direct contact or environmental routes, also contribute to the widespread of AMR⁷.

Monitoring longitudinal AMR data is not only essential for analysing trends, but also for the early detection of emerging resistance traits i.e. globally coordinated surveillance studies have been conducted since the 1990s⁸, of which *Escherichia coli*, a commensal bacterium, is considered as an excellent AMR indicator⁹. Moreover, *E. coli* is the most frequently reported clinical pathogen worldwide¹⁰. In 2015, WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) to facilitate AMR surveillance in bacteria causing critical infections and to assess AMR trends across WHO regions¹¹. However, no comparable framework exists for AMR surveillance in animals, and few country-specific monitoring or surveillance studies have been conducted in Europe¹² and the United States⁹. In low-middle countries (LMIC)s, the OIE are liaising with the Fleming Fund to leverage such data but is restricted to 24 countries^{13,14}. These programs show large AMR variations across countries, which may be due to a myriad of reasons including technical e.g. antimicrobial susceptibility testing (AST) methods. One of the earliest (2000-2018), large LMIC AMR studies on bacteria including *E. coli* from animals revealed an AMR increase among pig and chicken isolates¹⁵. AMR surveillance of *E. coli* from infirm chickens in China between 1993 and 2013 showed an increasing trend in MDR¹⁶. Most AMR surveillance studies on bacteria from animals are limited in their spatial and temporal sampling, and mostly focus on phenotypic data only. Moreover, an association between AMR and animal AMC has seldom been investigated.

In China, antimicrobial agents have been used as feed additives in the animal farming industry since the 1970s¹⁷. The annual per capita consumption of meat has increased from 10.3 kg in 1978 to 57.6 kg in 2019¹⁸, and this dramatic

increase has resulted in a correspondingly rapid increase in animal AMC; thereby, enflaming the AMR problem. However, despite these mitigating factors, changes in animal AMR levels remain poorly understood. Herein, we investigated the AMR trends of animal-derived *E. coli* in China from 1970s to 2010s. The evolution and transmission of ARGs alongside the plasticity of ARG-carrying plasmids were also evaluated, focusing on specific AMR markers to clinically important antimicrobial agents used in animals¹⁹ and humans²⁰.

Results

Dynamics of *Escherichia coli* populations from animals in China

Altogether, 982 isolates (450 from our laboratory, 532 from the NCBI database as of December 2019) were derived from animals or their breeding environments in 26 Chinese provinces (Fig. 1, Table S1). Most were collected from South and East China (n=572), where the meat production industry is mainly concentrated²¹. Isolates were collected from chickens (n=567) and pigs (n=258), sheep (n=62), cattle (n=46), ducks (n = 4), goose (n=1), and also 44 *E. coli* isolates from flies within poultry-farm environments. All isolates were chronologically divided into four groups: 1970s–1980s (n=113 isolates), 1990s (n=33 isolates), 2000s (n=169 isolates), and 2010s (n=667 isolates).

The phylogenetic tree of the 982 *E. coli* isolates showed substantial diversity within the population and was clustered into 9 lineages using Bayesian model-based population structures (BAPS) analysis (L1-L9, Fig. 1a). All lineages, except L2 and L5, comprised isolates from all temporal groups. The eight common phylogenetic groups (A, B1, B2, C, D, E, F, clade I), classified based on the Clermont *E. coli* phylo-typing method²², comprised 42.97%, 37.07%, 1.63%, 5.60%, 4.89%, 3.05%, 4.68% and 0.10% of the population, respectively (Table S1). Groups A (n = 422) and B1 (n = 364) accounted for 80.0% of the population. The representation of isolates from phylogroup C declined over time from 22.1% in the 1970-80s, 15.2% in the 1990s, 4.7% in the 2000s and 2.6% in the 2010s. Isolates from 1990s included more group B2 (15.15%) and group F (27.3%), which would more likely to be extraintestinal pathogenic *E. coli* (ExPEC)²³.

Analysis of the two clades (C1, n=31 and C2, n=32), with proportion of 62.5% and 38.7%, respectively, from 1970s-1980s samples, showed that the ARGs (*bla*_{CTX-M}, *bla*_{OXA}, *bla*_{NDM}, *oqxAB*, *qnrS*, *mcr*, and *floR*) and the plasmid replicons (IncHI2, IncI2, IncX1, IncX3, and IncB/O/K/Z) existed primarily in isolates from the later time groups in both C1 and C2 clades (mean prevalence of these ARG and plasmid were 0% and 3% in the 1970s-1990s vs 80% and 70% in the 2000s-2010s, see Fig. S1b).

MLST diversity and patterns

MLST of 982 isolates demonstrated significant diversity (Fig. S2), with isolates assigned to 226 distinct sequence types (STs), except for 67 isolates with novel STs. More than half of the STs (54.0%, n=122) were represented by a single isolate. ST10 was the most populated and contained 79 isolates (8.0%) across all four chronological datasets. Other major STs included ST101 (n=38, 3.9%), ST156 (n=36, 3.7%), ST48 (n=36, 3.7%), ST155 (n=28, 2.9%), ST224 (n=22, 2.2%), ST46 (n=20, 2.0%) and ST88 (n=20, 2.0%). The diversity of STs was higher in the 2010s data set, although this may reflect the larger number of isolates in this data set. The prevalence of ST48 increased in the years following 2010 (Fig. S3). Conversely, ST88, ST5086, ST351, ST90, ST345, ST1, ST100 and ST56 were more prevalent before 2000, while ST156, ST354, ST224, ST101 and ST46 were mainly observed after 2000 (Fig. S3). STs that were more prevalent in the most recent groups generally had relatively higher ARG presence (Fig. S4) e.g. ST156 and ST354 isolates had a mean ARG count of 23.9, which was significant higher than the overall mean of 15.0 ($p < 0.01$, Table S1).

Pan-genome analysis

Pangenomic analysis of the 982 *E. coli* isolates revealed an open pangenome of 66,767 genes and showed no evidence of reaching a plateau (Fig. S5a), indicating that genes were expanded as the sample size increased. Contigs were classified

as most likely originating from chromosomes or plasmids according to the prediction results from mlplasmids²⁴. The chromosomal core genome was predicted to contain 1,724 genes using a 99% threshold, and 57,434 accessory genes were identified with mean 2,837 genes per genome (Table S1). The isolates from the first three chronological groups (1970s–2000s inclusive) were found to be more conserved based for their chromosomal pangenome accumulation curves (Fig. S5b), while group 4 (2010s) appeared to have a more open pangenome, which may account for the larger spatial and host representation. The accessory gene counts per genome varied over time, ranging from 2,311 to 3,731. Isolates collected in the 1970s–1980s and 1990s had more chromosomal accessory genes (mean=2,223), compared to those from 2000s and 2010s (mean=2,152, $p < 0.001$), whilst the plasmid accessory genes increased from mean 193 in isolates from 1970s–1980s to mean 292 in isolates from 2010s ($p < 0.001$, Table S1).

Plasmid replicon marker analysis also indicates a slight increase in the number of plasmids over time with a mean count of 10.0 in isolates collected from the 1970s–1980s to 12.5 in isolates from 2010 ($p < 0.001$, Fig. S6a). The prevalence of several ARG-associated plasmids, such as IncX3, IncX4, IncI2 and IncHI2, were significantly higher in 2010s than in the 1970s–1980s (Fig. S8). The IncX3, IncI2 and IncB/O/K/Z plasmids were rarely detected between 1970s and 1990s (0%–6.06%), but increasingly presented in isolates from the 2010s (14.54% - 31.78%, Fig. S8). The plasmid profile was generally similar across isolates from different sectors. However, comparing isolates from pigs, the isolates from chickens had a significant higher prevalence of IncC (8.99% vs. 2.33%), Inc B/O/K/Z (14.46% vs 2.33%), IncK (14.46% vs. 2.33%), IncI1 (25.04% vs. 15.50%) and IncHI2 (20.99% vs. 13.95%), and a significant lower prevalence of IncX1 (16.75% vs. 23.64%), IncX3 (8.82% vs. 14.73%), IncX4 (2.29% vs. 7.36%) and IncY (14.11% vs. 22.87%) type plasmids.

Temporal changes of AMR, ARG and virulence gene (VG) profiles

Antimicrobial susceptibility testing of 450 *E. coli* isolates and ARG screening of 982 *E. coli* genomes revealed an increasing trend in AMR across the near-50 years time span (Fig. 2 a-c). We observed an increase in resistance to more recently approved and/or less commonly used veterinary drugs ($p < 0.01$), such as florfenicol, apramycin, norfloxacin, and ceftiofur, and the drugs exclusively used in human medicine, such as aztreonam and meropenem (Fig. 2a). Similar trends were observed in their corresponding ARGs in the full genome set (Fig. 2a and c). In contrast, resistance to antimicrobial agents commonly employed since the 1970's, e.g. tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol, remained relatively high throughout the 1970s–2010s (48.67%–79.78%, Fig. 2a, Table S2). Correspondingly, the presence of ARGs also remained high in the full genome set (Fig. 2c). Porcine isolates had a relatively higher prevalence of ARGs conferring resistance to colistin and fluoroquinolones (65.5% vs. 44.9% in other hosts, $p < 0.001$), whilst sheep isolates had the lowest prevalence of ARGs (mean count of 5.97 vs 15.57 in others, $p < 0.001$, Fig. 2c).

We specifically examined the resistance to β -lactams and phenicols which are key antimicrobial agents for both human and/or veterinary medicine. The rates of resistance to penicillins (ampicillin) and penicillins + β -lactamase inhibitors (amoxicillin/clavulanic acid), remained low in the 1970s–1980s (24.39% and 13.41%, respectively), but rose rapidly since 1990s (67.86% and 71.43%, respectively) and remain at a high-level (72.86% and 53.77%, respectively, in 2010s). Resistance to cephalosporins and aztreonam have increased over time (from 1.22% and 1.22% to 52.24% and 34.17%) and resistance to meropenem was most noticeable in the 2010s (1.01%, Fig. 2a, Table S2). The average counts of ESBL genes per genome for each chronological group in the full genome set were 0.24, 0.73, 0.83 and 1.16, respectively (Fig. 3a); 70.37% of the isolates ($n=691$) were found to contain at least one ESBL gene and the percentage in each time group increased from 22.12% in 1970s–1980s to 69.70%–77.66% in 1990s–2010s. Among the ESBL-positive isolates, 28.08% were also positive for *bla*_{NDM} ($n=194$). Although the use of chloramphenicol in food-producing animals was banned in China in 2002²⁵, florfenicol, a fluorinated thiamphenicol derivative, is still in use. The prevalence of chloramphenicol resistance has remained relatively high with a slight decline in the 2000s (60.71%–43.26%, Fig. 2a) and the most prevalent chloramphenicol resistance genes have changed from *catA1* to *catB3*, *cmlA* and *floR* (which mediates bacterial resistance to both chloramphenicol and florfenicol) (Fig. 2c, Table S3). Genetic context analysis of phenicol resistance genes indicate

that *catA* is linked with only a few ORFs encoding hypothetical proteins and likely associated with an IncP plasmid, while *catB*, *cmlA* and *floR* genes were found to be associated with IncHI2 plasmids along with other ARGs (Figs. 5 and 6).

Four fluoroquinolones (norfloxacin, pefloxacin, lomefloxacin, and ofloxacin) were banned from usage in food-producing animals by 2016²⁶, and colistin was prohibited for use as an animal growth promoter in April 2017²⁷. We therefore used these two time points to further group isolates in order to investigate how these restrictions may have affected AMR and ARG profiles. Resistance to norfloxacin increased rapidly from the 1990s to the 2000s before declining in the 2010s (Fig. S11a). Correspondingly, the average counts of PMQR genes for each chronological group were 0.02, 0.18, 0.45 and 0.73, respectively (Table S3). The prevalence of *oqxAB* and *qnrS* increased; *qnrB* and *qepA* continue to remain low, with a slight decrease in *aac(6')-Ib-cr* (19.39%, 102/526 before 2016 vs. 17.02%, 24/141, after 2016). In contrast, the prevalence of colistin resistance and the gene, *mcr-1*, increased rapidly during the 2000s (Fig. S11b), but decreased from 44.27% (n=278/628) in 2010-2016 to 23.08% (n=9/39) in 2017-2019, after the ban of colistin as growth promoter was enforced.

For virulence genes profiles, most isolates were detected to be hypovirulent, with 769 VGs (Table S3) functionally clustering into 16 classes and 100 groups (Fig. S9). No obvious trends or changes in VGs were observed. Pig isolates had a lower prevalence of virulence genes associated with adherence, autotransporters, invasion and iron uptake compared to isolates from chickens. Sheep isolates harbored highest virulence genes but lowest number of ARGs (Fig. 3a).

Correlation analysis of the gross output value of veterinary medicine for ARGs and VGs

To understand the association between AMC and AMR, we used the antimicrobial production data as an alternative measure to veterinary drug usage, which was unavailable in China prior to 2018. In general, the gross output value of the veterinary drugs produced in China during the different chronological periods (Table S5) were positively correlated with the detected average ARG counts ($r_{\text{Pearson}} = 0.95$, $p = 0.004$; Fig. 4a), inversely correlated with the average virulence gene count ($r_{\text{Pearson}} = -0.86$, $p = 0.028$; Fig. 4c), and exhibited no correlation with the average plasmid-associated replicon count ($r_{\text{Pearson}} = 0.18$, $p = 0.733$; Fig. 4b). We observed no correlation between ARGs and VGs ($r_{\text{Pearson}} = -0.04$, $p = 0.243$) and moderate positive association between plasmid replicon number and ARGs ($r_{\text{Pearson}} = 0.35$, $p < 0.001$), and virulence genes ($r_{\text{Pearson}} = 0.30$, $p < 0.001$; Fig. S12).

The transfer of ARGs

We applied the DISCOVER method commonly used for testing the somatic alterations in cancer²⁸ as a statistical approach for analyzing the co-occurrence of ARGs and plasmid replicons to infer the transmission of resistance genes. Several plasmid replicons were associated with ARGs (Fig. 5), such as IncC with the ESBL-related gene *bla_{CMY}*, IncI2 with *mcr*, IncX3 with the carbapenem resistance gene *bla_{NDM}*, IncHI2 with seven resistance gene classes, among others. The co-occurrence of ARGs and plasmid replicons largely remained stable when the analysis was restricted to different chronological-groups, with the IncC plasmid replicon being the one exception which was associated with the trimethoprim resistance gene *dfrA23* in the 1970s-1990s, but then associated with the ESBL gene *bla_{CMY}* after 2000 (Fig. S13). ARG genetic context analysis was used to investigate clustering of ARGs in assembled contigs, and showed that isolates from the 1970s-1990s possessed smaller clusters with fewer links between ARGs, but those from the 2000 and 2010 group gradually possessed more densely populated ARG regions (e.g. the β -lactamase genes or aminoglycoside resistance genes, Fig. 6 and S14). For example, we found that *catB* and *cmlA* co-existed with the *bla_{OXA-1}*, *bla_{OXA-10}*, *bla_{NDM}*, *aph(3')-XV*, *ant(2'')-Ia*, *aadA*, *ant(3'')-Ia*, the PMQR gene *aac(6')-Ib-cr*, and the rifampicin resistance gene *arr* (Fig. 6a-c). Additionally, *oqxAB* gene was the first to be detected in category of PMQR during the 1970s-1990s, while the appearance of *acc(6')-Ib-cr* in the 2000s was the only PMQR that co-existed with other ARGs such as *bla_{OXA-1}* and *arr*. The *mcr-1* gene was always associated with the IS30 family transposase, IS*Ap1*, but the flanking regions have diversified over time.

Discussion

Previous studies investigating AMR in animals in China have been limited at both a spatial and/or temporal level²⁹⁻³³, while our study presents a nation-wide surveillance report encompassing isolates from different food-producing animals from the 1970s, a time when antimicrobial agents seldom used in veterinary medicine and have since largely replaced traditional Chinese herbs³⁴, to 2019. Worldwide, China is now one of the largest users of veterinary antimicrobial agents²¹. In this study, we observed a significant association between veterinary antimicrobial production and the enrichment of ARGs, further demonstrating that the long-term use of antimicrobials in animals is one of the key drivers of AMR. As AMC in animals increased over the five decades, so the evolution of *E. coli* from animal origins with expanded AMR profiles continue to expand via antimicrobial selection.

To address this critical issue, the Chinese government announced a series of directives aimed at reducing AMR in animals. Concurrently, several programs were launched for AMR monitoring^{35,36} with several antimicrobial classes banned (chloramphenicol in 2002, four fluoroquinolones in 2016) and colistin prohibited as growth promoter in April 2017²⁵⁻²⁷. The overall prevalence of AMR profiles and corresponding ARGs; however, did not decline in the 2010s, but exhibited divergent trends (Fig. 3). The observation, that the dissemination of colistin resistance in *E. coli* dramatically reduced after the withdrawal of colistin, was consistent with our previous study³⁷. This reduction may have been mediated by the following: 1. the *mcr*-mediated target modification on lipid A moiety of LPS is the sole transferable mechanism of colistin resistance in *E. coli*³⁸. 2. *mcr-1* expression can impose a considerable fitness cost for *E. coli* carrying *mcr*-bearing plasmids³⁹⁻⁴². 3. The ban of colistin as a growth promoter significantly reduced the selection pressure for *mcr-1* which would have previously occurred. 4. The observation that *mcr* genes are seldom found together with other ARGs on the same mobile genetic elements (Fig. 6) reduces the likelihood for co-selection by other antimicrobial agents.

In contrast, the ban of chloramphenicol and the four fluoroquinolones from use in animals did not result in a phenotypic AMR decrease or a reduction in their corresponding ARGs. This might be attributable to the following: 1. Other antimicrobial agents within the same class, including florfenicol (phenicols) as well as ciprofloxacin, danofloxacin, enrofloxacin, and sarafloxacin (fluoroquinolones) were still used in animals and may have provided selection pressure for particular genotypes. 2. The co-selection by other antimicrobial agents might have contributed to the persistence and continual spread of resistance to phenicols and fluoroquinolones e.g. the PMQR gene *acc(6')-Ib-cr* is frequently found to be located in a class 1 integron with *catB*, *bla_{OXA}* and *arr*^{43,44}. Co-occurrence of these genes was observed in 64.1% (91/142) of our isolates that were positive for *acc(6')-Ib-cr*. 3. The immediate genetic environment have become increasingly complex due to the accumulation of additional ARGs via mobile genetic elements (MGEs) (Fig. 6). These genes are likely to be mobilised together and the exposure to any one of these corresponding antimicrobial agents could result in the co-selection of the entire gene cluster or plasmid. 4. Multiple mobile resistance genes mediating diverse resistance mechanisms relating to chloramphenicol resistance (*floR*-related efflux pump and *cat*-associated enzymatic inactivation, *cfr*-mediated target site modification) and fluoroquinolone resistance (*oqxAB* and *qepA*-related efflux pump, *acc(6')-Ib-cr*-related enzymatic inactivation and *qnr*-associated target protection) render the loss of these resistance traits more difficult than a single resistance mechanism e.g. *mcr-1*. 5. Fluoroquinolone resistance is more frequently mediated by mutations in specific chromosomal target genes, such as *gyrA*, *gyrB*, *parC* or *parE*. Such chromosomal mutations are very stable, especially under continuous selection pressure imposed by the use of fluoroquinolones in animals. Finally, fluoroquinolones can further induce mutations causing resistance to other classes of antimicrobials⁴⁵, which continue to strengthen their competitive positions.

Another difficulty hindering AMR control is the prosperity of horizontal gene transfer by MGEs. Acquisition of ARGs is greatly aided by a variety of MGEs, among which plasmids play a critical role⁴⁶. To date, 27 major plasmid incompatibility groups have been associated with ARGs in *Enterobacteriaceae*^{47,48}. The co-occurrence of ARGs and several plasmid replicon types was commonly detected within our isolates (Fig. 5). The IncI2, IncX3, IncHI2, IncC and IncF types were linked with ARGs, with some of these associations highlighted in previous studies⁴⁹. The IncF, IncC and IncX plasmid types were

recently identified to be the most prevalent types in carbapenem-resistant *E. coli*⁵⁰, while IncI2, IncHI2 and IncX4 types are commonly linked with *mcr-1* transmission⁵¹. As the increasing prevalence of ARGs was consistent with increasing detection of these plasmid replicons (Fig. 2c, S2b, S8), any AMR containment necessitates the mitigation of these multi-resistance plasmids and therefore strategies to inhibit their transmission should be further explored.

We acknowledge several limitations in this study. A few isolates (n=60) were collected several decades ago and their collection details (e.g., exact location) were no longer available. Apart from those downloaded from NCBI, all remaining isolates from the 1990s (n=28, 84.85%) were collected from a chicken farm in Jiangsu Province, and may represent a sampling bias. Additional sampling bias may also result from the available data from online database given the skew towards sequencing isolates with specific ARGs. However, given the extensive sources involved in this study, and the obvious huge diversity of these genomes, the bias is likely to be minor.

Stricter use of antimicrobial agents in animals is a strating to become a global trend; at least in theroy. As such, the European Union banned the use of antimicrobial growth promoters by 2006 (Regulation (EC) No. 1831/2003) and more recently, the Ministry of Agriculture and Rural Affairs, People's Republic of China has launched a regulation (No. 194) to stop growth-promoting medicines as feed additives after 1st July 2020⁵². It should be noted that antimicrobial agents are still irreplaceable for the treatment and prevention of infectious diseases caused by bacteria. It should also be noted that only healthy livestock animals provide high quality meat products for human consumption which is an increasing global trend. Thus, sick animals need an adequate treatment, which in the case of bacterial infections also involves the appropiate application of antimicrobial agents. However, fortified with the understanding that antimicrobial agents will select specific resistant genotypes, it is crtical to design AMR reduction stratgeies that considers the co-existence and co-transfer of ARGs i.e., interventions exploring separate classes of antimicrobials for humans and animals.

Materials And Methods

Sample collection and whole genome sequencing

A total of 450 *E. coli* isolates from animals in China collected since the 1970s were collated from our previous studies. Among which, 110 isolates were provided by the China Institute of Veterinary Drug Control⁵³, including 82 isolates collected from pig, cattle, sheep etc. in 1970s-1980s and 28 isolates collected from chicken in 1990s. The remaining 340 isolates were collected from our previous AMR epidemiological investigations on different regions⁵⁴⁻⁵⁶, which includes 84 from chicken in 2002-2006, 191 from chikcen in 2008-2013, and 65 from pig in 2008-2014. All of these isolates were collected with no preference. All isolates were recovered by identification on EMB medium (Land Bridge, Beijing, China) followed by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry). Of the 450 isolates, 169 have been sequenced and partly published (BioProject Accession number: PRJNA417344). Genomic DNA from the remaining 281 isolates was extracted for whole genome sequencing using the Wizard Genomic DNA Purification Kit (Promega, Beijing, China) following the manufacturer's instructions. DNA libraries were constructed using KAPA HyperPrep Kit Illumina platforms (Roche, Basel, Weitzerland) following standard protocols, and then sequenced on Illumina HiSeq X Ten platform (Annoroad, Beijing, China). Raw sequence data of clinical strains were assembled using SPAdes⁵⁷ v3.13.1 via the Unicycler⁵⁸ v0.4.7 assembly pipeline. Assemblies were deposited in NCBI (BioProject Accession number: PRJNA718052). Three isolates were selected for long-read sequencing, because they were collected from the early temporal group or were assigned to the clades in which we were particularly interested. MinION library preparation was performed using the Rapid Barcoding Sequencing kit (SQK-RBK004) according to the the manufacturer's (Oxford Nanopore, Oxford, UK) standard protocol. The constructed library was loaded into the Flow Cell R9.4 (FLO-MIN106) on a MinION device and run with the SQK-RBK004_plus_Basecaller script in MinKNOW1.5.12 software. De-multiplexing and basecalling were conducted using guppy 3.2.4. The long-read sequencing data was combined with their corresponding short-read Illumina data and used to generate *de novo* hybrid assemblies with Unicycler⁵⁸ v0.4.7. Additional genomes were

downloaded from the National Center for Biotechnology Information (NCBI) Pathogen detection database (<https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/>). There were n=1728 isolates retrieved with the search criteria "species_taxid:562" and "geo_loc_name: China*" (*E. coli* from China) on December 9th 2019. Among which, 606 isolates collected from food-producing animals (chicken, gallus gallus, pig, swine, duck, ovis aries, bos taurus, *et al.*) were selected. In addition to 74 genomes originating from our lab, 532 *E. coli* genomes collected between 1976-2019 were downloaded. The details of these projects involved in this study were listed in Table S6. About half of these isolates (n=243) were collected with no specific selection criteria, 5 isolates were submitted as virulent isolates, 5 isolates were related to specific phage, and 279 isolates were involved in AMR research. However, some of these resistant isolates were sequenced by our previous study and the other non-resistant isolates in the same epidemiological investigations have also been added. The large sample size of this collective dataset may account for a potential sampling bias to a certain extent.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the 450 *E. coli* isolates were determined using the agar diffusion method according to Clinical and Laboratory Standards Institute (CLSI) document M100, 30th ed.⁵⁹ and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard v10.0⁶⁰. Altogether, 16 antimicrobial agents from nine classes were selected based on their usage in the animal industry and/or in human medicine, and included gentamicin, apramycin, tetracycline, tigecycline, norfloxacin, florfenicol, chloramphenicol, ampicillin, amoxicillin/clavulanic acid, meropenem, ceftazidime, ceftiofur, trimethoprim/sulfamethoxazole, aztreonam and colistin.

Phylogenetic analysis

The full set of 982 genomes were used to generate a core-genome SNP alignment and construct a phylogenetic tree, using Parsnp v1.1.2 in the Harvest package⁶¹. The mid-point rooted phylogenetic tree was annotated in ITOL (<https://itol.embl.de/>). The Bayesian model-based population structures were identified with HierBAPS⁶² v6.0. Following initial phylogenetic analysis, two clades, C1 and C2, were selected for further analysis. A representative isolate from each clade was selected for long-read sequencing as detailed above, yielding high-quality complete chromosome sequences for use as reference genomes. Illumina data sets were aligned against the chromosomes of high-quality complete assemblies using Bowtie v2⁶³. Single nucleotide polymorphisms (SNPs) were identified, it for all of the sequenced genomes with SAMtools⁶⁴ v1.3.1 using RedDog v1beta.11 (<https://github.com/katholt/RedDog>) pipeline with default settings. For each of the two clades, core SNPs (conserved in >95% genomes) were used for subsequent phylogenetic analysis. Gubbins⁶⁵ v2.3.4 was used to identify and filter out putative chromosomal recombination imports within each lineage, before generating recombination-free maximum likelihood phylogenetic trees using FastTree⁶⁶ v2.1.10. The minimum spanning tree of MLST was generated in BioNumerics v7.6.

The gene content analysis

Illumina read sets were screened against the *E. coli* 7-locus multi-locus sequence type (MLST) database⁶⁷, ARGannot⁶⁸ database, PlasmidFinder⁶⁹ database and virulence factor database (VFDB, <http://www.mgc.ac.cn/VFs/>) using SRST2⁷⁰ v0.2.0 to assign sequence types (STs) and detect acquired AMR genes, plasmid replicons and VGs, respectively, in each genome. All the VGs were grouped according to the information from VFDB. The results were transformed into a binary table in R⁷¹ v3.6.0 to analyse the presence/absence of acquired AMR gene alleles and prevalence of each gene in different isolates grouped by temporal or host information. Contigs in each genome assembly were classified as chromosomal or plasmid sequences using mlplasmids²⁴ v1.0.0 the posterior probability cut-off at 0.5. The resulting contig sets (chromosomal vs. plasmid) were each annotated with Prokka⁷² v1.11 and subjected to a pan-genome analysis with Roary⁷¹ v3.6.0, separately. The resulting gene content matrix comprised 982 genomes and was used as input for plotting in R v3.6.3. Gene networks showing the linkage of genes within contigs were generated in Gephi⁷³.

Statistical analyses

Statistical analyses were conducted with R v3.6.3. The Pearson correlation analysis was used to determine the correlation between the gross output value of veterinary medicine and gene content, and the pairwise correlation between ARGs, VGs, and plasmid replicons. Differences between the numbers of ARGs/VGs/plasmid replicons per isolate in different decade-specific dataset (1970s-1980s, 1990s, 2000s, 2010s) were assessed by the Wilcoxon test. Gene prevalence differences between each decade-specific dataset were assessed with the χ^2 test ($n \geq 50$) or Fisher's exact test ($n < 50$).

We used the R package Discover (Discrete Independence Statistic Controlling for Observations with Varying Event Rates) v0.9.3, a novel statistical independence test, to identify the pairwise co-occurrence and mutual exclusivity of ARGs and plasmid replicons in the genomes²⁸. This package assesses pairwise co-occurrence and mutual exclusivity by counting how many genomes have both of the two genes and comparing this to the number of genomes expected to have such an overlap by chance if these genes were appeared independent. In addition, mutual exclusion can be detected, and the detection of mutual exclusion would be improved. In short, Discover identifies more significant co-occurrences and mutual exclusivities without increasing the false positive rate.

Declarations

Acknowledgements

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Figures

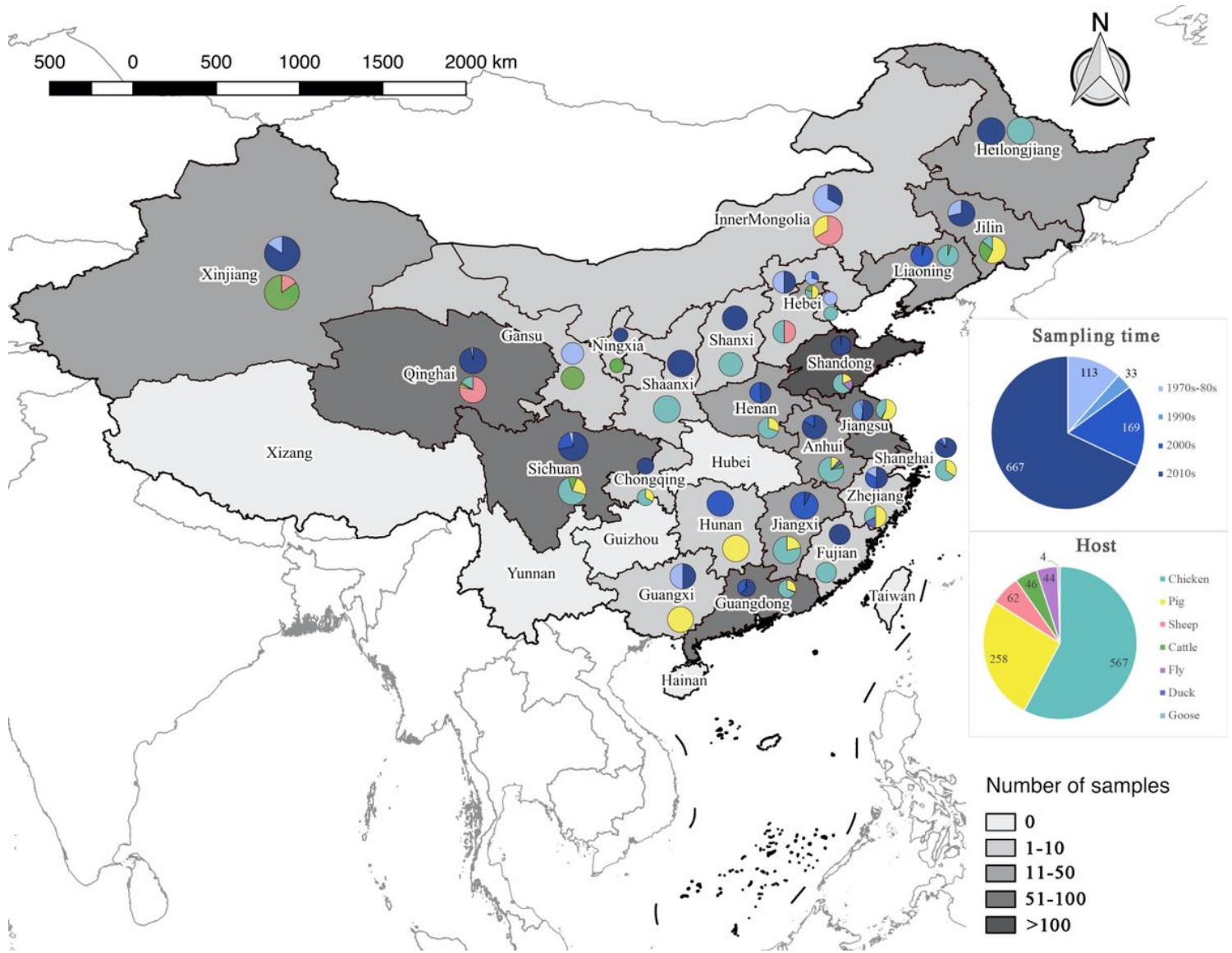


Figure 1

The origins of 982 *E. coli* isolates from 26 Chinese provinces. All isolates were grouped by sampling time and host. The two sets of pie charts show the temporal and host distribution of isolates overall and for each province. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

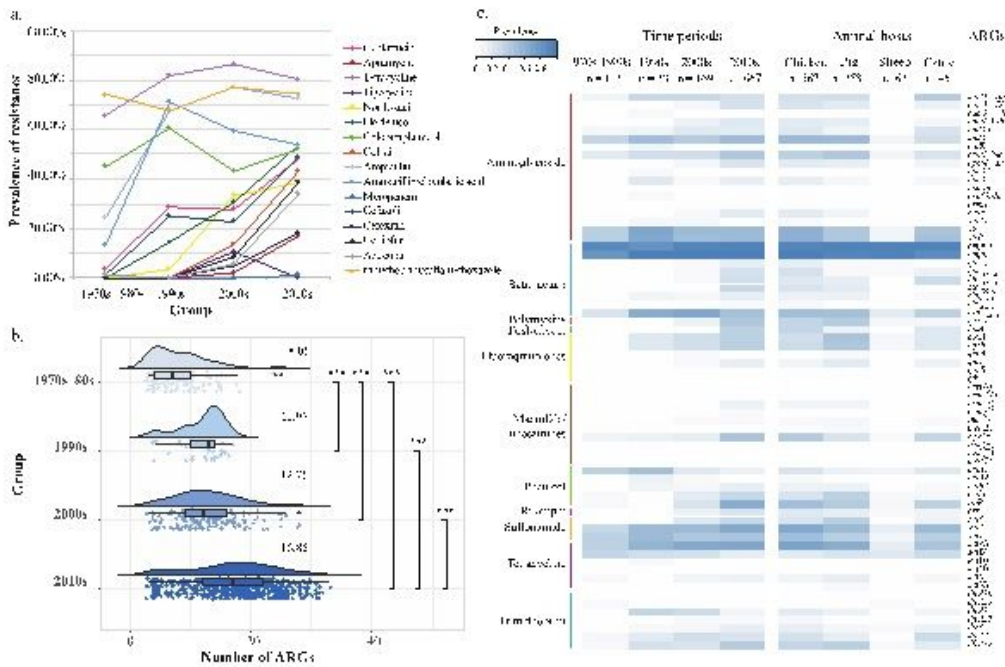


Figure 2

Temporal changes in AMR. a. Temporal changes in AMR phenotypes (n=450). Different colors represent the different antimicrobial agents. The values were presented in Table S2. b. The distribution of numbers of ARGs per genome in different sampling time groups (n=982). The mean ARG count for each group is noted. The “*” on the right represent p-values of pairwise groups using Wilcox test. *: p < 0.05; **: p < 0.01; ***: p < 0.001. c. ARG prevalence grouped by sampling time and host (n=982). ARGs that were detected at >5% prevalence in any one of the sampling groups are shown. They were clustered by their resistance of different groups of antimicrobial agents and colored by antimicrobial classes on the left.

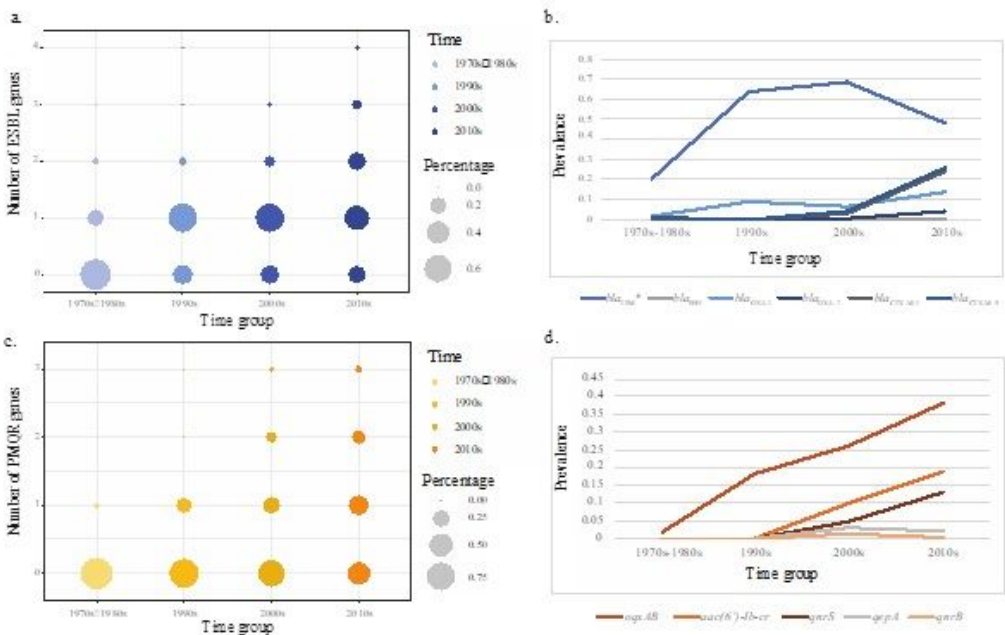


Figure 3

Temporal changes in ESBL and PMQR gene profiles amongst the full genome set (n=982). a. Number of ESBL genes per isolate over sampling time. b. Prevalence of ESBL genes over sampling time. c. Number of PMQR genes per isolate over sampling time. d. Prevalence of ESBL(b)/PMQR(d) genes over sampling time. The size of each circle in a and c corresponds to the proportion of isolates (percentage). blaTEM*: specify only ESBL producing blaTEM genes.

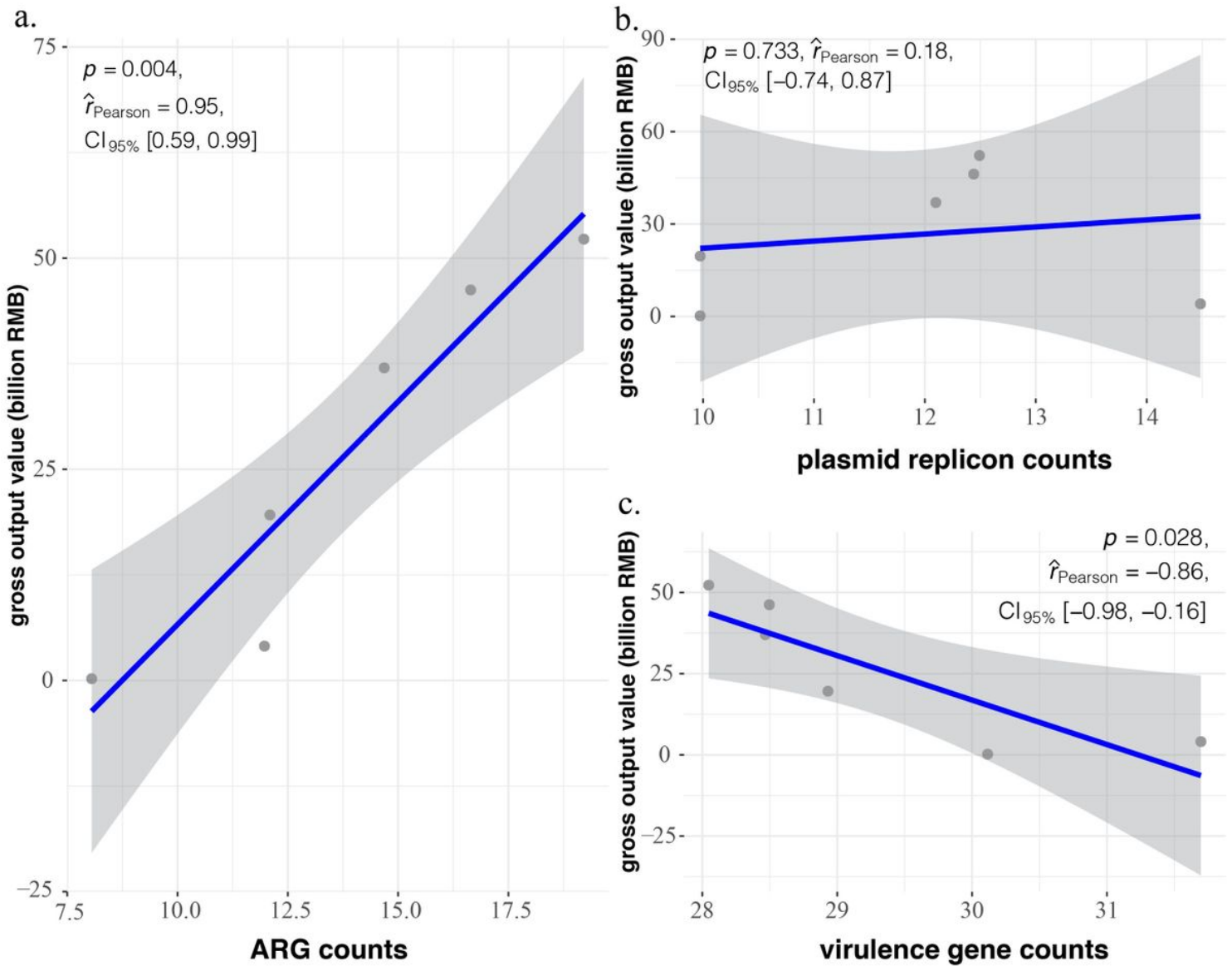


Figure 4

The correlation of antimicrobial production and resistance gene content. a. The correlation of gross output value of veterinary medicine and average count of ARGs detected from genomes. b. The correlation of gross output value of veterinary medicine and average count of plasmid replicons from genomes. c. The correlation of gross output value of veterinary medicine and average account of VGs detected from genomes.

ARG-plasmid

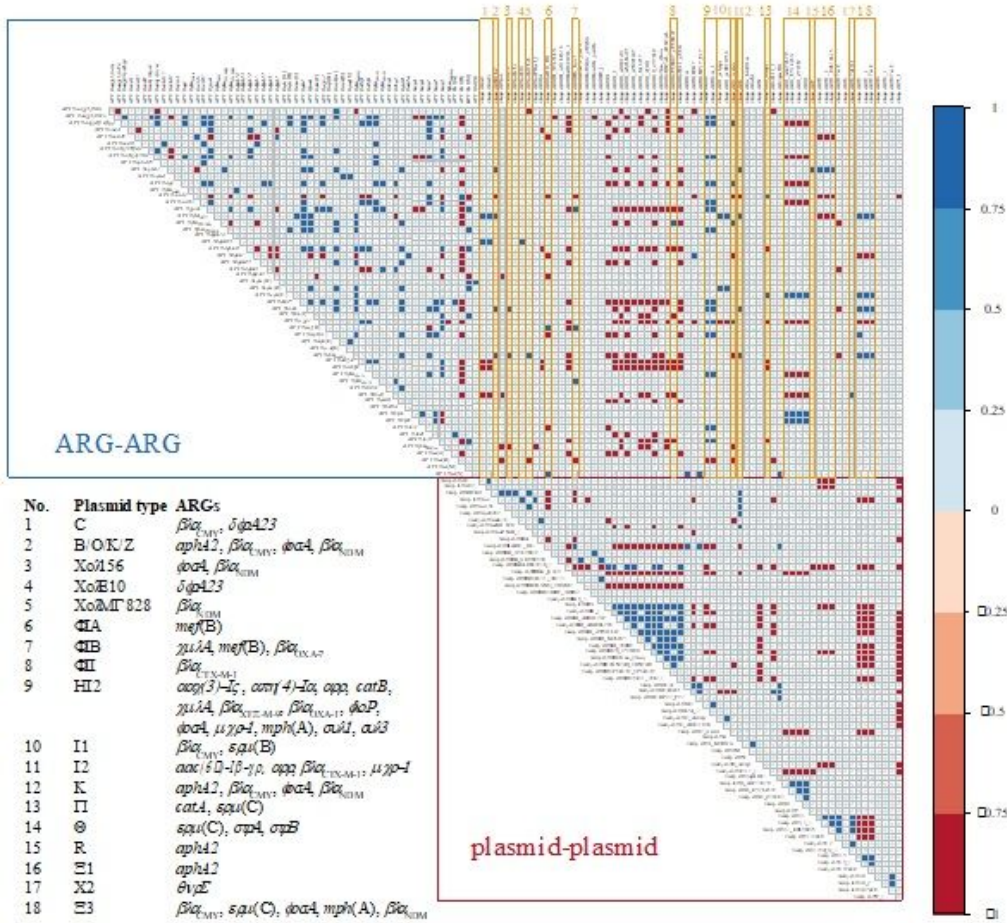


Figure 5

Co-occurrence of ARGs and plasmid replicons. We define the false discovery rate (FDR) threshold as 0.05 in this algorithm; significant pairwise values from 1 (blue) to -1 (red) (i.e. co-occurrence and mutual exclusivity) are shown. The blue box labelled “ARG-ARG” shows the pairwise co-occurrence and mutual exclusivity of ARGs, and the red box labelled “plasmid-plasmid” shows the pairwise co-occurrence and mutual exclusivity of plasmid replicons. The gold boxes labelled with numbers indicate the co-occurrence and mutual exclusivity between ARGs and plasmid replicons, and these are annotated on the bottom left

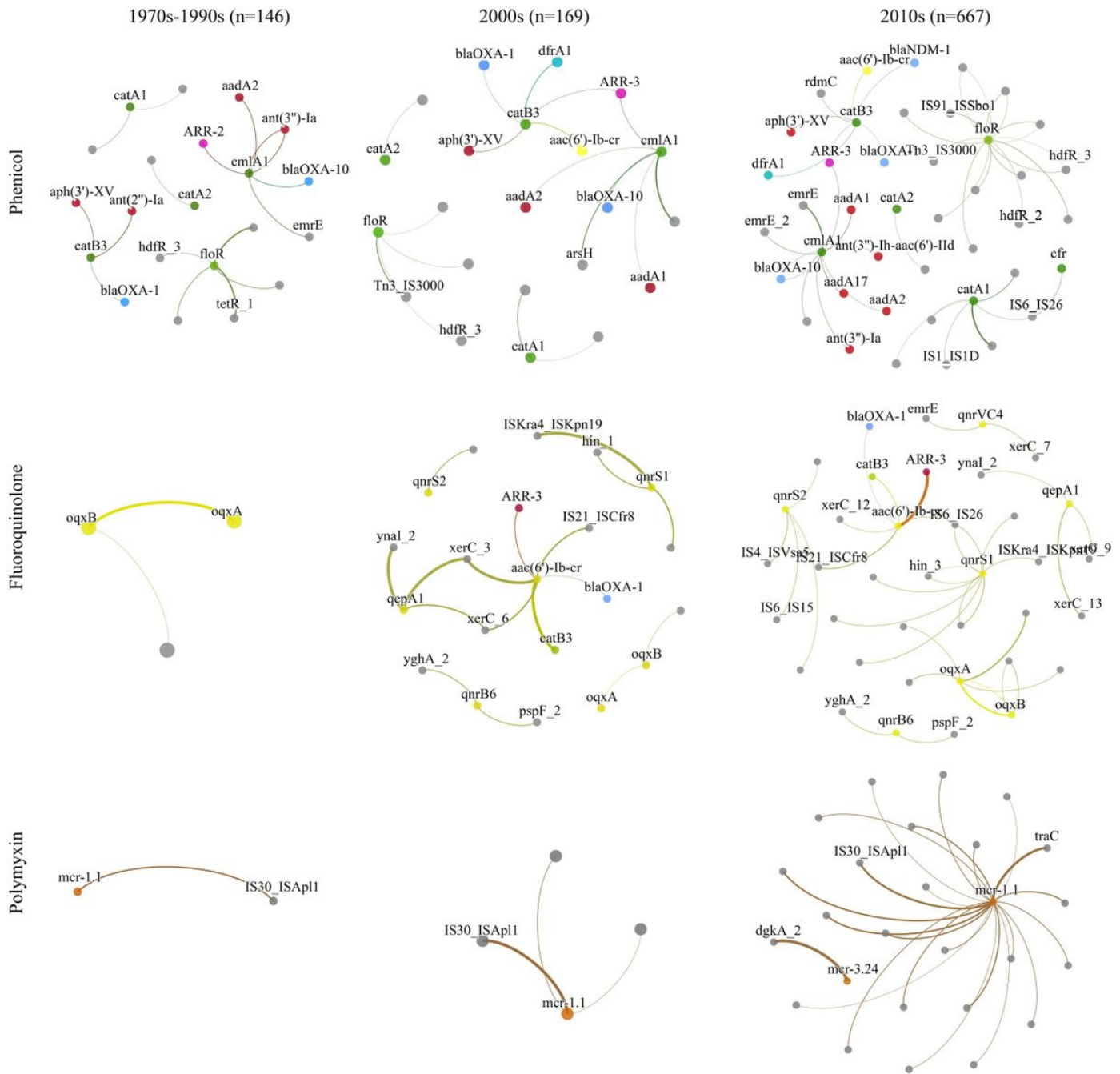


Figure 6

ARG co-occurrence networks of phenicol, fluoroquinolone and polymyxin resistance genes among isolates grouped by sampling time: 1970s-1990s (n=146), 2000s (n=169) and 2010s (n=667). Nodes represent genes and are colored to represent the antimicrobial class for ARGs; aminoglycoside: red; β -lactam: blue; trimethoprim: cyan; phenicol: green; rifampin: pink; fluoroquinolones: yellow; polymyxins: orange; grey: non-ARG. Unlabelled nodes were annotated as hypothetical protein. Edges are coloured according to the nodes they connect. Edge widths represent connection weight calculated by Roary.

Supplementary Files

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- [TableS1S3.xlsx](#)

- [EvolutionofAMRinanimalSupplementary.docx](#)