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Molecular epidemiology, phenotypic and genomic characterization of antibiotic-resistant enterococcal isolates from diverse farm animals in Xinjiang, China

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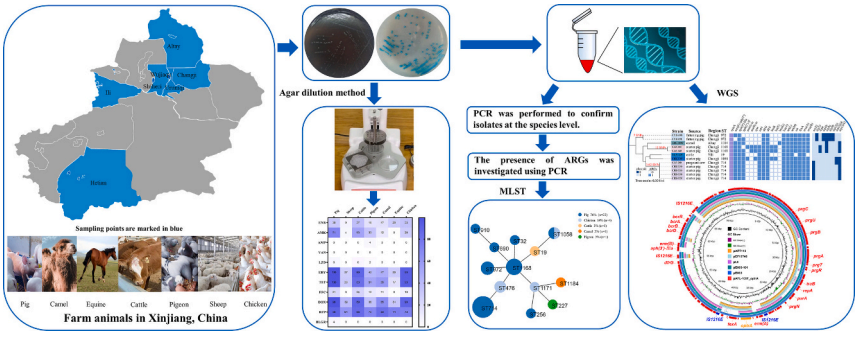
HIGHLIGHTS

- The prevalence of MDR *Enterococcus* (≥88 %) and four ARGs (≥75 %) are high in swine, cattle, and chickens.
- Enterococci have a high prevalence (>69 %) of resistance to antibiotics used previously as growth promoters.
- The regional epidemic of *optrA*-carrying ST714, ST1168, and ST972 *Enterococcus faecalis* within the intensive swine farm.
- Plasmids with multiple ARGs are important vectors for the *optrA* gene to circulate in humans and animals in different regions.

GRAPHICAL ABSTRACT



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ABSTRACT

Multidrug-resistant (MDR) bacteria in farm environments can be transferred to humans through the food chain and occupational exposure. *Enterococcus* infections caused by linezolid resistant enterococci (LRE) are becoming more challenging to treat as their resistance to antibiotics intensifies. Therefore, this study investigated the molecular epidemiology, phenotypic and genomic characterization of enterococci in seven species of farm animals (sheep, chicken, swine, camel, cattle, equine, pigeon) anal swab from Xinjiang, China by agar dilution method, polymerase chain reaction (PCR), whole-genome sequencing (WGS) and bioinformatics analysis. A total of 771 samples were collected, 599 (78 %) were contaminated with *Enterococcus* spp., among which *Enterococcus faecalis* (350/599) was dominant. Antimicrobial susceptibility testing showed that high resistance was observed in rifampicin (80 %), tetracycline (71 %), doxycycline (71 %), and erythromycin (69 %). The results of PCR showed the highest prevalent antibiotic resistance genes (ARGs) were *aac(6′)-aph(2″)* (85 %), followed by *tet*(M) (73 %), *erm*(B) (62 %), and *aph(3′)*-IIIa (61 %). Besides, 29 *optrA*-carrying *E. faecalis* isolates belonging to 13 STs (including 3 new alleles) were detected, with ST714 (31 %, 9/29) being the dominant ST type. The phylogenetic tree showed that *optrA*-carrying *E. faecalis* prevalent in the intensive swine farm is mainly caused by clonal

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transmission. Notably, *optrA* gene in *Enterococcus* spp. isolate from camel was first characterized here. WGS of *E. faecalis* F109 isolate from camel confirmed the colocalization of *optrA* with other five ARGs in the same plasmid (pAFL-109F). The *optrA*-harboring genetic context is IS*1216E-fexA-optrA-erm*(A)-IS*1216E*. This study highlights the prevalence of MDR *Enterococcus* (≥88 %) and four ARGs (≥75 %) in swine (intensive farming), cattle (commercial farming), and chickens (backyard farming) are high and also highlights that *optrA*-carrying *E. faecalis* of farm animals incur a transmission risk to humans through environment, food consumption and others. Therefore, antibiotic-resistant bacteria (ARB) monitoring and effective control measures should be strengthened and implemented in diverse animals.

1. Introduction

Antimicrobial resistance (AMR) is a growing worldwide threat to the healthcare of humans and animals with increased mortality and economic loss. AMR is not only a public health issue but also an agricultural and environmental problem, as an One-Health issue (George et al., 2022). Monitoring AMR is essential for understanding the changes of bacterial resistance to commonly used antibiotics and the emergence and prevalence of new antimicrobial resistance in various settings. Monitoring AMR acquired by *Enterococcus* is one of the key indicators in the One-Health surveillance system (Torres et al., 2018).

Enterococcus is ubiquitous in the gastrointestinal tract of humans and animals as well as in the environment. *Enterococcus* is a pathogen determined as one of the main causes of acquired infections in hospitals and communities (Almeida et al., 2020). It is involved in a variety of serious diseases such as urinary tract infections, infective endocarditis, and bacteremia (García-Solache and Rice, 2019). Over the last five decades, *Enterococcus faecalis* and *Enterococcus faecium* have emerged as leading causes of MDR infections (Xiong et al., 2022). They utilize plasmids, transposons, and insertion sequences to efficiently attain and transfer mobile resistance elements, facilitating the dissemination of resistance genes (O'Driscoll and Crank, 2015). Research has long shown that AMR of *Enterococcus* can be transferred among strains of the same species, different species within the same genus, and even across different genera (de Niederhäusern et al., 2004; Huys et al., 2004; Wang et al., 2023). The high-density herds and extensive use of antimicrobial agents in intensive farms create ideal circumstances for the emergence and dissemination of antibiotic-resistant *Enterococcus* and their resistance genes (Xuan et al., 2023). The spread of antibiotic-resistant *Enterococcus* has greatly increased the cost of antibiotics for intensive farms and affects the economic benefits of these farms. *Enterococcus* can be transmitted between animals, humans, and the environment through various means such as feces, sewage, and contaminated food (Bortolaia et al., 2016; Huijbers et al., 2015). The risk of transmission may vary across different modes of animal production. However, most current reports on the prevalence and antimicrobial resistance of *Enterococcus* species of animal origin only focus on a single production model (Alzahrani et al., 2022; Molechan et al., 2019; Xuan et al., 2023).

Enterococcus infections are often difficult to treat due to their inherent and acquired resistance to multiple antibiotics. The linezolid is being increasingly used to treat infections caused by the vancomycin resistant *Enterococcus* (VRE) (Sadowy, 2018); accordingly, the risk of nosocomial transmission of LRE is becoming a clinical concern. The primary oxazolidinone-resistance mechanisms include point mutations in the central loop of the domain V region of 23S rDNA, mutations or deletions in genes encoding the 50S ribosomal components L3, L4, and L22, and the expression of transferable oxazolidinone resistance genes such as *cfr*, *optrA* and *poxtA* (Antonelli et al., 2018; Torres et al., 2018). The *optrA* gene has been reported to play an important role in linezolid resistance (Cavaco et al., 2017a; Cavaco et al., 2017b; Freitas et al., 2017; Tamang et al., 2017). The *optrA* gene can lead to cross-resistance of *Enterococcus* to oxazolidinones and other veterinary antibiotics such as florfenicol. Although oxazolidinone antibiotics have not been used in treating farm animals for years, *optrA*-carried transferable plasmids have been detected in LRE isolated from animals (Cavaco et al., 2017a; Cavaco et al., 2017b; Freitas et al., 2017; Tamang et al., 2017). In 2018, the *poxtA* gene, a novel phenicol–oxazolidinone–tetracycline resistance gene was identified in a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. faecium* isolate in Italian successively (Antonelli et al., 2018; Papagiannitsis et al., 2019). In recent years, the dispersal of enterococci carrying *optrA* or *poxtA* in food-producing animals and retail meat has been frequently reported (Elghaieb et al., 2019; Hao et al., 2019; Wang et al., 2023; Yoon et al., 2020). The spread of *optrA* and *poxtA* poses a great challenge to veterinary and clinical medicine as well as a risk to public health. *E. faecalis* is one of the most common species of *Enterococcus* in human and animal intestines. However, there is currently limited data on the molecular epidemiology of *optrA*-carrying *E. faecalis* in farm animals in Xinjiang, where diverse animals existed.

It is important to determine the molecular epidemiology and resistance phenotype of *Enterococcus* in different modes of animal production for containment measures. Therefore, in this study, the AMR profiles and the prevalence situation MDR enterococci from animals in Xinjiang were systematically analyzed. Through this research, a roughly understanding of the possible health risks associated with the spread of antibiotic-resistant enterococci of animal origins can be obtained.

2. Materials and methods

2.1. Sample collection, isolation and identification of Enterococcus

A total of 771 samples from farm animals in Xinjiang province of China were collected. In 2015, 110 sheep (free-range farming) anal swab samples were collected in Shihezi; 42 chicken (backyard farming) anal swab samples were collected from Urumqi in 2016; 262 swine (intensive farming) anal swab samples were collected from Changji in 2019; 49 camel (commercial farming) anal swab samples were collected from Altay in 2019; 135 cattle (commercial farming) anal swab samples and 16 equine (free-range farming) anal swab samples were collected from Yili in 2019; 18 cattle (commercial farming) anal swab samples were collected from Wujiaqu in 2019; 100 pigeon (informal farming) anal swab samples were collected from Hetian in 2019; 20 camel (commercial farming) anal swab samples and 19 equine (free-range farming) anal swab samples were collected from Altay in 2020. All samples were stored in low temperature and transported to lab for bacterial isolation.

Enterococcus isolation followed the "one colony per sample" principle. The 1.5 mL Mueller-Hinton Broth (MHB) (Hope Bio-Technology Co., Ltd., Qingdao, China) centrifuge tube containing the sample was incubated at 37 °C for 2 h with shaking at 180 rpm. Then, 50 μL of each culture was inoculated into 1 mL of brain heart infusion broth (BHI) (Hope Bio-Technology Co., Ltd., Qingdao, China) supplemented with 6.5 % NaCl and incubated at 37 °C for 18 h with shaking at 180 rpm. All cultures were subcultured by streaking onto Bile Aesculin Azide agar (Hope Bio-Technology Co., Ltd., Qingdao, China). Plates were incubated for 24 h at 37 °C, and colonies of brown protrusions surrounded by black halos were considered presumptive enterococci. Identified colonies were further streaked onto CHROMagar™ Orientation (CHROMagar, Paris, France), and incubated at 37 °C to obtain pure colonies of blue-green. Presumptive enterococci were stored in 20 % glycerol stock solution at −80 °C until further analysis.

Stock cultures were grown on Mueller-Hinton Agar (MHA) (Hope

Bio-Technology Co., Ltd., Qingdao, China) plates incubated at 37 °C for 18 h. DNA was extracted using the heat lysis method as previously described (Englen and Kelley, 2000). The isolates were confirmed by using the polymerase chain reaction (PCR), which was performed to confirm bacterial species distinguished by specific genes ($ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ genes), according to the protocol described previously (Dutka-Malen et al., 1995) (Table S1). Specifically, the 30 cycles PCR with an annealing temperature of 55 °C was performed with Taq Polymerase on a T100 ™ thermal cycler (Bio-Rad, US). PCR products were purified using a TIAN quick Midi Purification Kit (TIANGEN Biotech (Beijing) Co., Ltd.), and then measured using the Qubit 2.0 Fluorometer (Invitrogen, CA), >100 ng/μL, and the using electrophoresis gel detected a single bright band. They were performed with Sanger sequencing (Sangon Biotech (Shanghai) Co., Ltd.), verified by homology comparisons of the specific genes ($ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ genes) with the nucleotide sequence database by using BLAST program (Altschul et al., 1990).

2.2. Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) for *Enterococcus* isolates and transconjugants were determined by the agar dilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) documents VET01-S2 (CLSI, 2013), and M100-S29 (CLSI, 2019). The MICs of 11 antimicrobials were tested, including gentamicin, enrofloxacin, florfenicol, vancomycin, erythromycin, amikacin, tetracycline, linezolid, doxycycline, rifampicin, ampicillin. All antibiotics were purchased from Shanghai Yuanye Bio-Technology Co., Lt. The MICs range (μg/mL) of antimicrobials used in this study were summarized in Table S2. As previously described, because standardized MIC breakpoints for florfenicol and enrofloxacin are not available for *Enterococcus*, we used the breakpoint for *Streptococcus suis* for florfenicol from VET01-S2 and the breakpoint for *Enterococcus* for ciprofloxacin as the breakpoint of enrofloxacin from M100-S29 (Liu et al., 2013). *E. faecalis* American Type Culture Collection (ATCC) 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains.

2.3. Detection of antibiotic resistance genes

The presence of ARGs was investigated using PCR, as previously described (Aarestrup et al., 2000; Hao et al., 2019; Kehrenberg and Schwarz, 2006; Wang et al., 2015; Woodford et al., 1993). The primers (Table S1) were designed by using Primer 6.0 and synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR amplification was performed according to the conditions shown in table S1. All reactions were carried out in a T100 ™ thermal cycler (Bio-Rad, US). Random selection of PCR products is further confirmed by Sanger sequencing (Sangon Biotech (Shanghai) Co., Ltd.). Both strands of the PCR products were sequenced with the forward and reverse primers (Table S1).

2.4. DNA extraction and MLST

Genomic DNA extraction was performed from overnight cultures of isolates grown in BHI at 37 °C under 180 rpm shaking conditions by using a FastPure® Bacteria DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Then, the extracted DNA was measured using the Qubit 2.0 Fluorometer (Invitrogen, CA), as per the manufacturer's instructions and the DNA was stored at −20 °C.

Refer to the methods recommended by the PubMLST database (https://pubmlst.org/efaecalis/), MLST of *optrA*-carrying *E. faecalis* isolates were determined using seven housekeeping genes (*gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*) (Ruiz-Garbajosa et al., 2006). Alleles and sequence types (STs) were assigned using the MLST database. The core genome allelic profiles of *E. faecalis* was built using PHYLOViZ (Ribeiro-Gonçalves et al., 2016).

2.5. Conjugation experiments

Transferability of *optrA* gene was determined by filter mating conjugation experiments using *optrA*-positive isolates as the donor strains and *E. faecalis* JH2–2 (fusidic acid and rifampicin resistant, ST8) as the recipient (1:3) at 37 °C as described previously (Li et al., 2014). The transconjugants were selected on MHA supplemented with 200 mg/L rifampicin, 25 mg/L fusidic acid, and 10 mg/L florfenicol. Colonies from the selective plates were confirmed by the detection of *optrA* gene using PCR, antimicrobial susceptibility testing, and MLST.

2.6. Genome sequencing and bioinformatics analysis

Genomic DNA of 12 *optrA*-carrying *E. faecalis* isolates was extracted according to the aforementioned methodology. WGS was performed with Illumina Hiseq 2500 platform and the paired-end reads were de novo assembled using SPAdes version 3.14.0. The less reported camel-derived *optrA*-carrying *E. faecalis* AFL-109F was selected for further sequencing by long-read Nanopore sequencing (the Oxford Nanopore Technologies MinION platform). Complete genome sequences were obtained using Unicycler version 0.4.8 with default parameters (Wick et al., 2017). The assembled sequences were automatically annotated through the RAST server (https://rast.nmpdr.org/) (Aziz et al., 2008) and manually checked by BLAST (www.ncbi.nlm.nih. gov/BLAST) (Altschul et al., 1990). ResFinder, PlasmidFinder, and ISfinder were used to detect the ARGs, plasmid replicon types, and insertion sequences (Bortolaia et al., 2020; Carattoli et al., 2014; Siguier et al., 2006). BRIG and Easyfig were used to display plasmid comparison maps (Alikhan et al., 2011; Sullivan et al., 2011).

2.7. Statistical analysis

The chi-square test was used to test the significant differences in the prevalence of enterococci between samples collected from different animals. P values <0.05 were considered statistically significant. Statistical analysis of the results was performed with Graph Pad Prism 8.0 software.

2.8. Nucleotide accession numbers

All the DNA sequences determined in this research were deposited in the GenBank database under the BioProject ID PRJNA889763.

3. Results

3.1. Sample collection and specie prevalence of enterococcal

As summaries in Table 1, a total of 599 (78 %) enterococcal isolates were isolated from the anal swabs of seven types of animals in Xinjiang. Molecular typing by PCR technique determined 58 % of the isolates as

Table 1
Distribution of *Enterococcus* species in seven farm animal species.

Sources	No. of samples	No. of *E. faecalis* isolates	No. of *E. faecium* isolates	No. of *Enterococcus* spp. isolates	Total of enterococcal isolates (%)[a]
Chicken	42	40	0	0	40 (95)
Swine	262	163	0	19	182 (69)
Cattle	153	55	20	33	108 (71)
Sheep	110	38	10	22	70 (64)
Pigeon	100	34	6	58	98 (98)
Equine	35	0	14	21	35 (100)
Camel	69	20	9	37	66 (96)
Total	771	350	59	190	599 (78)

[a] Enterococcal isolation rates = Total of enterococcal isolates / Number of samples.

E. faecalis (n = 350), 10 % as *E. faecium* (n = 59), and 32 % as other *Enterococcus* spp. (n = 190). The isolation rates of *E. faecalis* from chickens (95 %, 40/42) and swine (62 %, 163/262) were significantly higher than that of other animals ($P < 0.05$), and no *E. faecalis* were identified from equine.

3.2. Antibiotic resistance of the enterococcal isolates

The collected enterococci isolates were resistance to rifampicin (80 %, 479/599), doxycycline (71 %, 428/599), tetracycline (71 %, 423/599), erythromycin (69 %, 415/599), amikacin (40 %, 240/599), enrofloxacin (25 %, 151/599), florfenicol (15 %, 88/599), linezolid (3 %, 15/599), high-level gentamicin-resistant (HLGR) (1 %, 7/599) and ampicillin (1 %, 4/599), as indicated in Table S2. The prevalence of *Enterococcus* isolates that were not susceptible to florfenicol was 73 % (n = 437). Out of these isolates, 349 had a MIC = 4 μg/mL (intermediate according to CLSI VET01-S2). Although no VRE was detected, 37 % (n = 22) of *E. faecium* and 6 % (n = 12) of *Enterococcus* spp. showed not susceptible to vancomycin.

E. faecalis showed the highest prevalence of resistance to the following antibiotics: rifampicin (87 %, 306/350), erythromycin (87 %, 306/350), tetracycline (87 %, 305/350), doxycycline (87 %, 304/350), and amikacin (63 %, 222/350), as compared to the other isolates. *E. faecium* showed the highest prevalence of resistance to enrofloxacin (66 %, 39/59). In addition, 15 (3 %) linezolid resistant *E. faecalis* (LREfs) and 7 (1 %) HLGR *E. faecalis* isolates were detected.

The heatmap of AMR enterococcal isolates isolated from various animals revealed that isolates of swine origin were the highest (>70 %) prevalence of resistance to the antibiotics: erythromycin (100 %, 182/182), tetracycline (100 %, 182/182), doxycycline (98 %, 179/182), rifampicin (90 %, 164/182), and amikacin (71 %, 129/182). LRE was detected in enterococci isolated from swine (6 %, 11/182), cattle (6 %, 6/108), and pigeons (2 %, 2/98). Resistance to ampicillin was detected only in enterococci from pigeons (4 %, 4/98) (Fig. 1).

Multidrug-resistant analysis showed that 97 % (n = 581) of the enterococcal isolates were resistant to at least one antimicrobial, and 76 % (n = 457) were resistant to three or more antimicrobial classes. In addition, 38 % (n = 227) of isolates were resistant to six or more antimicrobial classes; among these, 3 % (n = 18) were resistant to eight antimicrobial classes, and 2 % (n = 12) were resistant to nine antimicrobial classes (Table 2).

3.3. Detection of antibiotic resistance genes

Detection of ARGs showed that except for *cfr* and *vanA*, other 8 ARGs were detected; and genes with prevalence >60 % included *aac(6′)-aph (2″)* (85%, 506/599), *tet*(M) (73%, 436/599), *erm*(B) (62%, 373/599), and *aph(3′)*-IIIa (61%,367/599). Tetracycline- and erythromycin-resistant isolates carried the *tet*(M) and *erm*(B) genes, respectively. The prevalence of phenicol-oxazolidinone resistance genes showed that

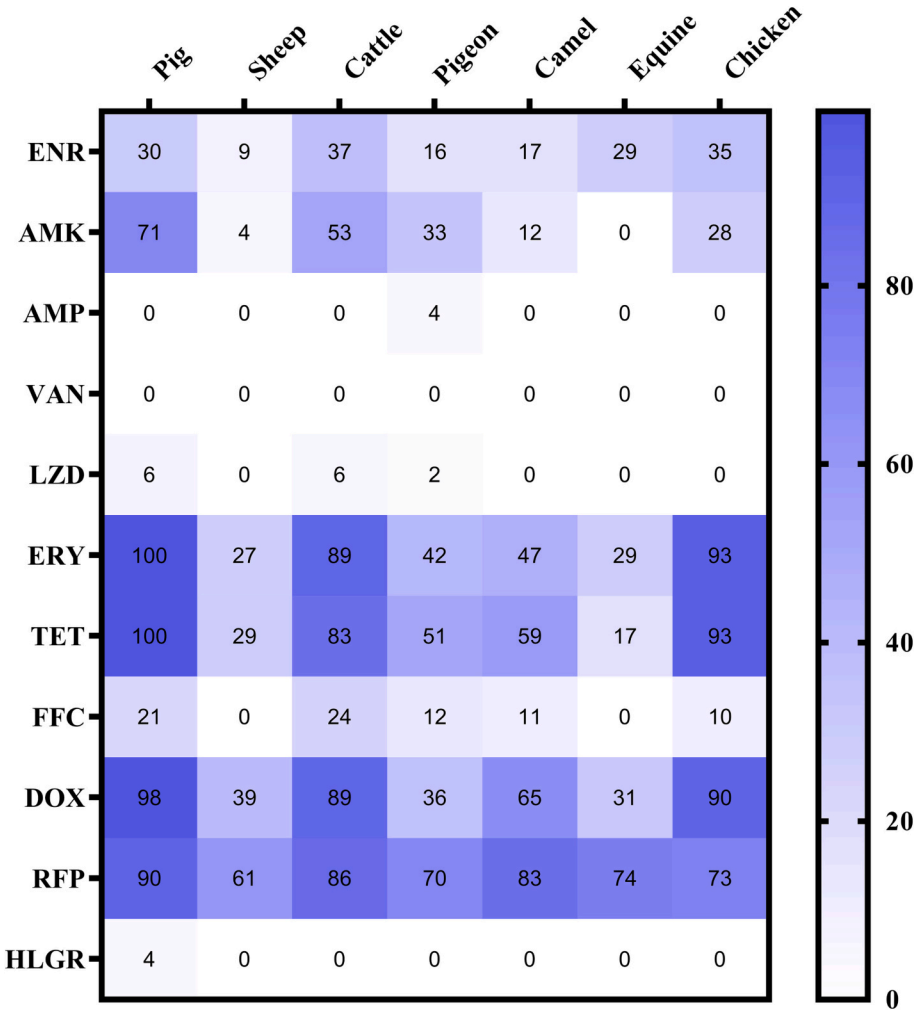

Fig. 1. The heatmap of antimicrobial resistance of the investigated enterococcal isolates according to seven farm animal species. The numbers in cells correspond to the percentage (%) of antimicrobial resistance isolates. Abbreviations: ENR: enrofloxacin; AMK: amikacin; AMP: ampicillin; VAN: vancomycin; LZD: linezolid; ERY: erythromycin; TET: tetracycline; FFC: florfenicol; DOX: doxycycline; RFP: rifampicin; HLGR: high-level gentamicin-resistant.

Table 2
Resistant or MDR prevalence of enterococcal isolates.

Antibiogram[a]	No. of *E. faecalis* isolates (%)[b]	No. of *E. faecium* isolates (%)[c]	No. of *Enterococcus* spp. isolates (%)[d]
0 - resistance	11 (3)	3 (5)	3 (2)
1 - resistance	10 (3)	2 (3)	38 (20)
2 - resistance	16 (5)	9 (15)	50 (26)
3 - resistance	15 (4)	6 (10)	26 (14)
4 - resistance	29 (8)	11 (19)	18 (9)
5 - resistance	86 (25)	10 (17)	29 (15)
6 - resistance	99 (28)	5 (9)	18 (9)
7 - resistance	64 (18)	7 (12)	8 (4)
8 - resistance	11 (3)	4 (7)	0
9 - resistance	9 (3)	2 (3)	0

[a] 0 - resistance means that resistance to zero of the antibiotics tested; 1 - resistance refers to the resistance of one antibiotic tested; 2 - resistance to 9 - resistance and so on.
[b] The prevalence of resistant or MDR isolates of *E. faecalis* = No. of *E. faecalis* isolates/Total of *E. faecalis* isolates (n = 350).
[c] The prevalence of resistant or MDR isolates of *E. faecium* = No. of *E. faecium* isolates/Total of *E. faecium* isolates (n = 59).
[d] The prevalence of resistant or MDR isolates of *E. faecalis* = No. of *E. faecalis* isolates/Total of *E. faecalis* isolates (n = 190).

poxtA was 2% (n = 10) and *optrA* was 7% (n = 43). Most *optrA*-carrying isolates also carried *fexA* and *erm*(A). The prevalence of *tet*(M), *tet*(K), *aph(3′)*-IIIa, *erm*(B), and *fexA* of *E. faecalis* was significantly higher than that of other species; however, no *poxtA* was detected, but 29 *optrA*-carrying isolates were detected. The *aac(6)/aph(2″)* prevalence of *E. faecium* (93.2 %, 55/59) was the highest, and four *optrA*- and *poxtA*-carrying isolates were detected. Eleven *optrA*-carrying isolates and five *poxtA*-carrying isolates were detected in other isolates of *Enterococcus* spp. (Table S3).

3.4. MLST of optrA-carrying E. faecalis

Seven pairs of housekeeping genes were sequenced for 29 *optrA*-carrying *E. faecalis* isolates, and then uploaded to the database for comparison. They belonged to 13 distinct STs (Table S4). The *xpt* gene of camel-derived AFL-109F was a new allele, which was assigned as xpt103 by the PubMLST database. AFL-109F was a new ST and assigned by the PubMLST database as ST1184. There are also two new STs assigned by the PubMLST database, including ST1171 and ST1168.

As shown in Fig. 2, 29 *optrA*-carrying *E. faecalis* isolates were isolated from swine, chickens, camels, cattle, and pigeons. Twenty-two isolates with eight distinct STs were isolated from swine. The two isolates of ST1058 were distributed in swine and chicken origin, and isolates from other animal origins belonged to different STs, namely, cattle ST19, pigeon ST227, and camel ST1184.

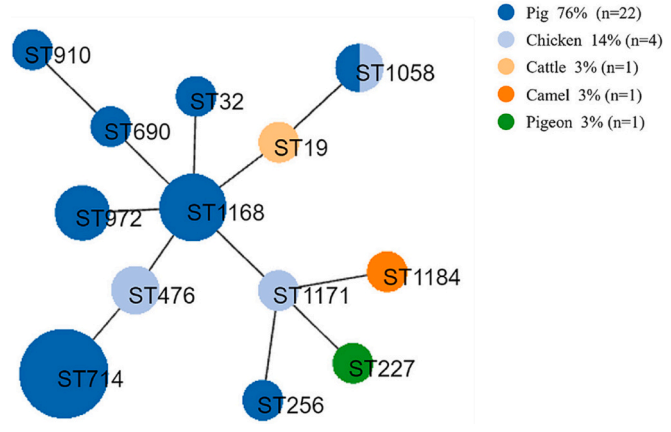

Fig. 2. Phylogenetic trees of *E. faecalis* MLST. Core genome MLST allelic profiles of *E. faecalis*. Swine origin *E. faecalis* has the most kinds of ST types.

3.5. Mobility of the optrA-carrying genetic elements

The *optrA* gene from 12 isolates was successfully transferred via conjugation to *E. faecalis* JH2-2. However, despite various attempts, the conjugation assays for the other 17 isolates failed to yield transconjugants, and the *optrA* gene was presumed to be located on the chromosome or other non-conjugative genetic elements. Antimicrobial susceptibility testing of these transconjugants revealed that the MIC value of 12 transconjugants increased 16-fold for florfenicol, and a 2-fold increase in the MIC value of linezolid in 6 of these transconjugants. The MIC value of five transconjugants increased >64-fold for erythromycin (Table S5).

3.6. Genomic epidemiology of optrA-carrying isolates

To further study the evolutionary relationship of the successfully transferred 12 *E. faecalis* isolates, we constructed the phylogenetic tree based on single nucleotide polymorphisms (SNPs) of core genomes (Fig. 3). The STs of *E. faecalis* were distributed across multiple farm animals. The 12 isolates were divided into four clusters with six branches. All the ST972 *E. faecalis* isolated from fattening swine are classified into one branch. Five clones belonged to the ST714 were isolated from various stages, including a pregnant swine (CJZ-280) and starter swine (CBZ-326, CBZ-329, CBZ-330, and CBZ-338). Two ST1168 *E. faecalis* isolated from starter swine were classified into the same clusters as the ST1184 *E. faecalis* from camels. The ST1058 *E. faecalis* isolated from starter swine and ST19 *E. faecalis* isolated from cattle were classified into the same cluster. The isolates with the same ST were clustered into the same branch, suggesting that there was a clonal correlation among the isolates.

In total, the 12 *optrA*-harboring plasmids were classified into seven plasmid replicon types. The rep22 and repUS43 type plasmid replicons were observed to be the most prevalent. The repUS43 type plasmids were detected in multiple swine production stages and camel. STs-based minimum spanning trees of *E. faecalis* isolates were constructed. We found that the STs of *E. faecalis* were distributed across multiple farm animals. The phylogenetic tree of the 12 *optrA*-carrying *E. faecalis* showed that the isolates with the same STs were clustered into the same branch on the phylogenetic tree, suggesting that there was a clonal correlation among the isolates.

3.7. Characteristics of the optrA-carrying plasmid pAFL-109F in E. faecalis strain

WGS showed that ST1184 *E. faecalis*, isolated from camel, carried two plasmids. We named one as pAFL-109F after detection of the *optrA* gene with a full-length sequence of 74, 497 bp and a GC content of 35 % that was consistent with the *E. faecalis* genome. In addition to *optrA*, it carried other ARGs including the trimethoprim resistance gene *dfrG*, the bacitracin resistance operon *bcrABDR*, the chloramphenicol/florfenicol resistance *fexA* gene, the macrolide–lincosamide–streptogramin B resistance *erm*(A) and *erm*(B) genes, and the aminoglycoside-modifying enzyme *aph(3′)*-IIIa gene. The other plasmid, with a full-length of 64, 400 bp, carried ARGs including the *erm*(B) gene, the tetracyclines resistance *tet*(L) and *tet*(M) genes, the chloramphenicol/florfenicol resistance *cat* gene.

The plasmid pAFL-109F shared similar DNA sequences of a ~68 kb region (91 % coverage and 99 % sequence identity) with the plasmid pE035 and pE006–101 (Table S6), which were isolated from the swine in the Henan province (Hao et al., 2019). Other plasmids shared significant homologies of the region with pL8 (human), pEF10748 (human), and pAPT110 (dog food) (Table S6 & Fig. 4a). All these plasmids were isolated from *E. faecalis* from various animals, and they also carried multiple sex pheromone response genes, including *prgA*, *prgC*, *prgT*, *prgU*,

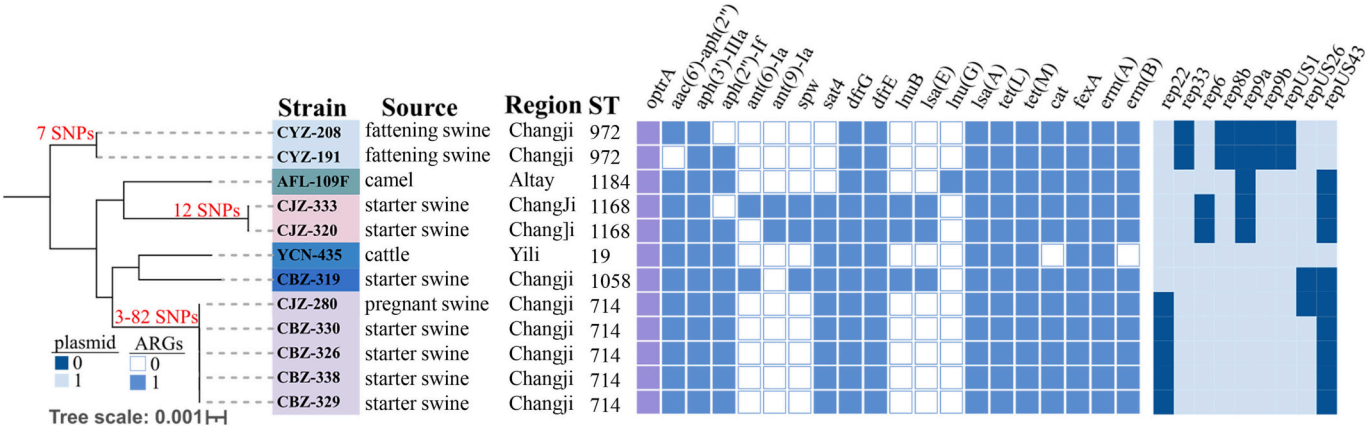

Fig. 3. Phylogenetic analysis and basic information for the 12 isolated *optrA*-carrying *E. faecalis*. A phylogenetic tree was constructed using Roary and FastTree based on core-genome SNPs.

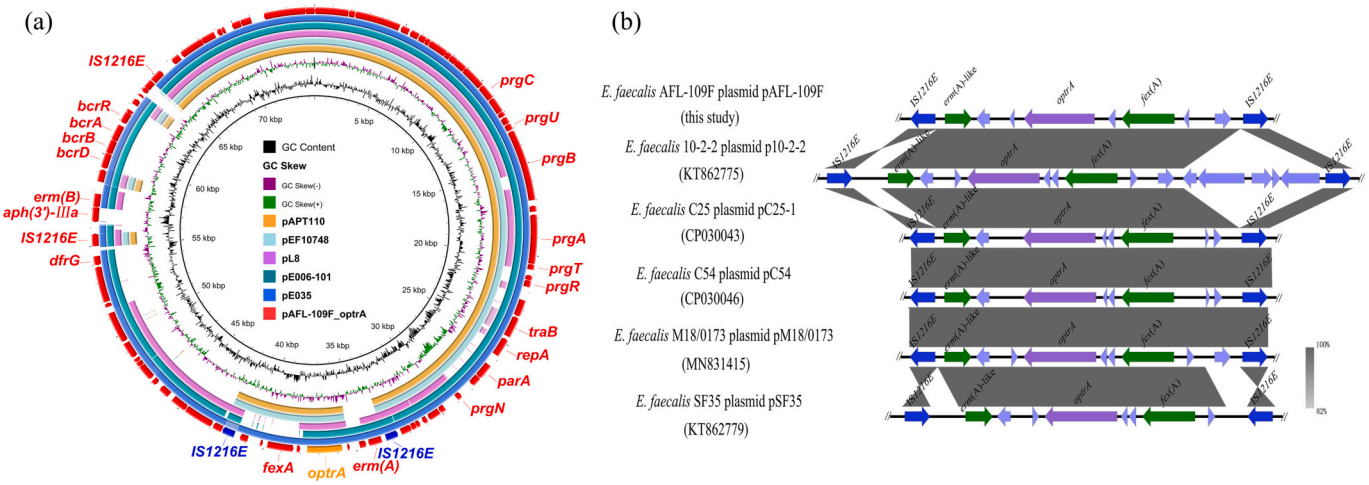

Fig. 4. Structures of the pAFL-109F and other similar plasmids. (a) Circular comparison between the *optrA*-carrying plasmid pAFL-109F and other plasmids closely related to them and deposited in GenBank. The positions and orientations of the genes of the plasmid pAFL-109F is indicated by arrows on the outermost circle. (b) Comparison of the *optrA*-carrying segments of plasmid pAFL-109F and other plasmids closely related to them in GenBank. The positions and orientations of the genes were indicated by arrows. The regions with ≥82 % homology between these structures are marked by grey shading.

prgN, and *traB*.

Within the plasmid pAFL-109F, an ~11 kb region carried the *optrA* gene, which was flanked with the *fexA*, *erm*(A)-like gene and two copies of IS*1216E* elements on the contrary orientation. The region was highly homologous (>99.9 % identity) to the plasmids pC25-1 (swine, Sichuan), p10-2-2 (swine, Guangzhou), pC54 (swine, Sichuan), pM18/0173 (human, Ireland), and pSF35 (Chicken, Henan) (Table S7 & Fig. 4b). All these plasmids were identified from *E. faecalis*.

4. Discussion

In this study, the overall isolation rate of enterococci from animals in Xinjiang was 78 %, higher than the previously reported isolation rate of enterococci of swine origin from eighteen provinces in China (27 %, 225/843) (Xuan et al., 2021). The isolation rate was consistent with that of enterococci of swine origin from Australia (Lee et al., 2021). This study suggests an overall predominance of *E. faecalis* isolates (58 %) followed by *E. faecium* (10 %) and undifferentiated species of *Enterococcus* spp. (32 %). In addition, the isolation rates (>63 %) of enterococci in the seven types of animals were all high, but the species-dominance had significant differences in different animals. Among porcine enterococci, *E. faecalis* was absolutely dominant, which was inconsistent with the previous report that there were more isolates of *E. faecium* in porcine enterococci from Australia (Lee et al., 2021) and Portugal (Gião et al., 2022). The isolation rates of enterococci of chicken origin were slightly higher than that reported in South Africa (Molechan et al., 2019). Although enterococci of other animal origins (camel, pigeon, sheep, etc.) have been rarely reported, this study and previous reports show that *Enterococcus* as intestinal symbiotic bacteria has a high isolation rate in animals, but the species-dominance in different animals and regions have disparity (Furuya et al., 2022; Gião et al., 2022; Lei et al., 2019; Smoglica et al., 2022).

The results of antibiotic susceptibility tests showed that enterococcal isolates in this study displayed the highest prevalence of resistance to rifampicin (80 %), tetracycline (71 %), doxycycline (71 %), and erythromycin (69 %). Partial results are consistent with China and other food-producing animal studies conducted in different countries (Kim et al., 2021; Lee et al., 2021; Liu et al., 2013; Molechan et al., 2019). Analysis of the reasons for the high rate of antibacterial resistance may be because the antibiotics of tetracyclines and macrolides as growth promoters in the food-animal industry approved by China previously. Previous studies have shown that colistin resistance and *mcr-1* abundance were significantly reduced in *Escherichia coli* from animal and human origins following the ban of colistin as feed additive in China (Wang et al., 2020). Fortunately, China has banned all drugs except traditional Chinese medicine as growth promoters in the food-animal industry in

2022 (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2019). The substantial use of rifampicin in human and veterinary clinics as a first-line antibiotic for tuberculosis, as well as its increasing use for treating Gram-positive bacteria such as MRSA (Tupin et al., 2010), may have contributed to the highest prevalence of enterococcal isolates that are resistance to rifampicin. Based on the drug investigation before sample collection, the not susceptibility of florfenicol can be related to the commonly used florfenicol to treat bacterial diseases in farms due to its cost-effective. The findings of the present study revealed that the great majority of enterococcal isolates (≥94 %) were susceptible to the clinically relevant antibiotics, vancomycin (94 %), linezolid (95 %), HLGR (99 %), and ampicillin (99 %). However, it is concerning that 76 % (n = 457) of isolates were resistant to three or more antimicrobial classes, displaying diverse antibiotic resistance spectrum. These results suggest the consumption of poorly cooked or raw animal-derived food contaminated with MDR bacteria may be a public health hazard and that it's a necessity to impose continuous monitoring of antibiotics.

This study indicated that the prevalence of ARGs of enterococcal isolates was high, and the resistance phenotype was consistent with the resistance genotype. Across all species, *aac(6′)-aph(2″)* (85 %), *aph(3′)*-IIIa (61 %), *tet*(M) (73 %), *erm*(B) (62 %) were frequently detected. This is consistent with previous reports from Alberta, Canada (Zaheer et al., 2020). *Enterococcus* shows resistance to aminoglycosides (gentamicin, amikacin, etc.) as a result of the presence of *aac(6′)-aph(2″)* and *aph(3′)*-IIIa. Although only one high-level gentamicin-resistant enterococci (HLGRE) was identified, it was previously reported that HLGRE might be mediated by *aac(6′)-aph(2″)* gene (Das et al., 2022; Rosvoll et al., 2012). Most enterococcal isolates resistant to tetracycline were positive for the *tet*(M) gene, this corresponds with other findings (Molechan et al., 2019; Zaheer et al., 2020). The *erm*(B) gene is the most prevalent gene found among the high-level erythromycin-resistant enterococci (Portillo et al., 2000). In addition, *erm*(B) can induce cross-resistance to streptogramin B (Isnard et al., 2013). A total of 44 *optrA*-carrying isolates were identified, and distributed in five animal species except for sheep and equine, and it is noteworthy that they were all free-range. In addition, 29 *optrA*-positive isolates showed linezolid MICs in the range of 2 to 4 μg/mL (categorized as susceptible according to EUCAST (http://www.eucast.org/clinical_breakpoints/) breakpoints and susceptible to intermediate according to CLSI M100-S29), consistent with the previous reports, probably leading to an underestimation of their actual incidence (Torres et al., 2018). Besides, ten isolates of *poxtA*-carrying enterococci were identified, including one strain from a swine and nine from cattle. The *optrA* and *poxtA* genes can mediate cross-resistance between florfenicol and oxazolidinones (Wang et al., 2015). Oxazolidinones are concerning considering that this is among the last resort antibiotics for the treatment of VRE (Bender et al., 2018). Therefore, the key ARGs need continuous monitoring and warrant effective control measures.

On the basis of the allelic profile, 29 *optrA*-carrying *E. faecalis* were divided into 13 STs, including three new STs. Of those isolates, the most prevalent was ST714 (31 %, 9/29), ST1168 (17 %, 5/29) was the second most prevalent clone. In this study, ST19, ST227, ST256, and ST476 *E. faecalis* carrying the *optrA* gene have been previously reported in swine origin in Sichuan Province (Huang et al., 2022). In particular, ST19 (McHugh et al., 2022) and ST476 (Freitas et al., 2020; Yoon et al., 2020; Zhang et al., 2018) *E. faecalis* have often been identified in humans, animals, and food. There was a possibility of cross-contamination between samples, resulting in a regional epidemic of *optrA*-carrying ST714, ST1168, and ST972 *E. faecalis* within the intensive swine farm. Besides, the same Inc. type plasmids could be identified not only from *E. faecalis* of the same STs but also from the different STs, which indicated the *optrA* gene may spread between different STs *E. faecalis* by horizontal transfer of plasmids.

To our knowledge, *optrA* gene in *Enterococcus* spp. isolate from camel was first characterized here. WGS of *E. faecalis* F109 isolate from camel confirmed the colocalization of *optrA* with almost all known sex-pheromone response genes, including the typical *prgA-prgB-prgC* cassette, in the same plasmid (pAFL-109F). Previous studies have shown that *optrA*-mediated linezolid resistance can be widely disseminated through sex pheromone plasmid transfer (Zou et al., 2020). Before 2020, plasmids with such gene organization have not been reported from any *optrA*-carrying *E. faecalis* isolate, but reports increased in the past three years (Freitas et al., 2021; Zou et al., 2020). The pAFL-109F contained two pseudo-compound transposon-like elements consisting of IS*1216E-fexA-optrA-erm*(A)-IS*1216E* and IS*1216E*-bcr*ABDR-erm*(B)-*aph(3′)*-IIIa-IS*1216E*, respectively. Besides, the genetic context IS*1216E-fexA-optrA-erm*(A)-IS*1216E* has been found in many diverse *optrA*-carrying plasmids globally, which indicated that IS*1216E-fexA-optrA-erm*(A)-IS*1216E* has a great transmission capability. (He et al., 2016; Kang et al., 2019). Notably, the plasmid has six ARGs and can mediate at least six types of antimicrobial resistance. This suggests that even without oxazolidinones, the plasmid pAFL-109F may be selected by other antibiotics and may spread between different bacterial species worldwide.

5. Conclusion

In conclusion, the enterococci isolates from swine, cattle, and chickens in Xinjiang exhibited high rates (≥88 %) of MDR, complex antibiograms, and a high prevalence of four ARGs (≥75 %). It is recommended to enhance the regulated utilization of antibiotics, as well as to improve the management of animal feces and sewage treatment systems. These measures are essential in mitigating the transmission of AMR. The *optrA* gene was detected in a variety of *E. faecalis* of animal origin in Xinjiang, among which swine origin was the most prevalent. *OptrA*-carrying *E. faecalis* clonal dissemination under florfenicol pressure played an important role in its widespread transmission. Plasmids with complete sex-pheromone response genes and multiple ARGs are important vectors for *optrA* gene to circulate in humans and animals globally. Effective and reasonable measures should be formulated to ensure the safety of animals and the environment.

CRediT authorship contribution statement

Wanzhao Chen: Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Qiaojun Wang:** Data curation, Formal analysis, Writing – review & editing. **Huimin Wu:** Investigation. **Panpan Xia:** Investigation. **Rui Tian:** Investigation. **Ruichao Li:** Writing – review & editing. **Lining Xia:** Validation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

This manuscript has not been published elsewhere and is not under consideration by another journal. All authors agree with submission to Science of The Total Environment. There are no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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