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Prevalence, transmission and control of clinically important antimicrobial-resistant bacteria/genes within one health framework

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Prevalence, transmission and control of clinically important antimicrobial-resistant bacteria/genes within one health framework

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First report of *Klebsiella pneumoniae* co-producing OXA-181, CTX-M-55, and MCR-8 isolated from the patient with bacteremia

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The worldwide spread of carbapenem-resistant Enterobacteriaceae (CRE) has led to a major challenge to human health. In this case, colistin is often used to treat the infection caused by CRE. However, the coexistence of genes conferring resistance to carbapenem and colistin is of great concern. In this work, we reported the coexistence of $bla_{OXA-181}$, $bla_{CTX-M-55}$, and *mcr-8* in an ST273 *Klebsiella pneumoniae* isolate for the first time. The species identification was performed using MALDI-TOF MS, and the presence of various antimicrobial resistance genes (ARGs) and virulence genes were detected by PCR and whole-genome sequencing. Antimicrobial susceptibility testing showed that *K. pneumoniae* 5589 was resistant to aztreonam, imipenem, meropenem, ceftriaxone, cefotaxime, ceftazidime, levofloxacin, ciprofloxacin, gentamicin, piperacillin-tazobactam, cefepime, and polymyxin B, but sensitive to amikacin. S1-pulsed-field gel electrophoresis (PFGE) and Southern blotting revealed the *mcr-8* gene was carried on a~138kb plasmid with a conserved structure (IS*903B-ymoA-inhA-mcr-8-copR-baeS-dgkA-ampC*). In addition, $bla_{OXA-181}$ was found on another ~51kb plasmid with a composite transposon flanked by insertion sequence IS*26*. The *in vitro* conjugation experiments and plasmid sequence probe indicated that the plasmid p5589-OXA-181 and the p5589-mcr-8 were conjugative, which may contribute to the propagation of ARGs. Relevant detection and investigation measures should be taken to control the prevalence of pathogens coharboring $bla_{OXA-181}$, $bla_{CTX-M-55}$ and *mcr-8*.

KEYWORDS

***Klebsiella pneumoniae*, OXA-181, CTX-M-55, MCR-8, bacteremia**

Introduction

As one of the significant challenges to global public health, bacterial resistance has attracted much attention in clinical treatment (Xiao et al., 2016; Lai et al., 2021). Especially the infection caused by carbapenem-resistant Enterobacteriaceae (CRE) puts pressure on the health care system in China (Zheng et al., 2018, 2019a; Tompkins and van Duin, 2021).

OXA-48, one of the most common carbapenemases, was first reported in a *K. pneumoniae* isolated from a patient in Turkey (Mairi et al., 2018). OXA-48, unlike the other major carbapenemases, is an ambler class D enzyme that shows low activity against carbapenems and spares extended-spectrum cephalosporins (Stewart et al., 2018). Therefore, it is challenging to detect $bla_{\text{OXA-48}}$-habouring bacteria clinically. Till now, OXA-48 has more than 10 variants, and OXA-181 is currently the second most common global derivative, which differs from OXA-48 by four amino acid substitutions (Messaoudi et al., 2021). Unlike the prevalence of KPC, NDM and IMP, OXA-181 mainly occurs in India, Europe and the South-East Mediterranean region (Nigg et al., 2019; Shanthini et al., 2019). The emergence of $bla_{\text{OXA-181}}$ in China has aroused concern extensively.

Extended-spectrum β-lactamases (ESBLs) are a class of enzymes that mainly confer resistance to beta-lactam antibiotics, including SHV, TEM, CTX-M and PER. Among them, the CTX-M has been reported to be the predominant type of ESBLs in various Enterobacteriaceae. Since the CTX-M-55 first appeared in India, it has been found in countries worldwide through the transmission of many mobile genetic elements. Recently, considering the increasing detection rate of $bla_{\text{CTX-M-55}}$ in China, many researches were performed about its characteristics.

Currently, colistin is widely used in clinical practice, mainly for treating infections caused by CRE (Durante-Mangoni et al., 2019). However, the mobile colistin resistance gene *mcr-8* significantly affects the therapeutic efficacy of colistin and the prognosis of patients with associated infections (Phetburom et al., 2021). In 2016, *mcr-8* was first identified in *K. pneumoniae* (Wang et al., 2018). Several *mcr-8* variants have been reported in *K. pneumoniae*, *Klebsiella quasipneumoniae*, *Raoultella ornithinolytica*, and *Enterobacter cloacae,* including *mcr-8.1 mcr-8.4* (Wang et al., 2019, 2022; Yang et al., 2019).

The spread of the *mcr* genes into CRE, which has been reported globally, is of great clinical concern, leading to the emergence of true pan-drug-resistant pathogens (Mediavilla et al., 2016; Zheng et al., 2017; Han S. et al., 2020; Chen et al., 2022). Meanwhile, the isolation and culture of such pathogen coharboring *mcr* and carbapenemase-encoding gene from the blood sample is rare. Accordingly, our work aims to describe the antimicrobial susceptibility, plasmid characteristics and genomic features of a *K. pneumoniae* strain co-producing OXA-181, CTX-M-55, and MCR-8 from China for the first time.

Materials and methods

Species identification and antimicrobial susceptibility testing

Isolates were collected from a tertiary hospital in Zhengzhou, Henan province, China, during our routine surveillance of CRE. Species identification was performed by matrix-assist laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen, Germany). The mobile colistin resistance genes *mcr-1* to *mcr-8* and the major carbapenemase genes, such as bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, bla_{VIM}, and bla_{IMP}, were identified using PCR, as described previously (Zheng et al., 2019b; Liang et al., 2021).

The susceptibility of *K. pneumoniae* 5589 and its transconjugants to antibiotics was tested using the agar dilution method, except for the polymyxins, which was performed using the broth microdilution method (Liu et al., 2021). The results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines. *K. pneumoniae* ATCC700603 and *Escherichia coli* ATCC25922 were used as the quality control.

Plasmid analysis and conjugation assay

The number and size of plasmids in *K. pneumoniae* 5589 were detected by S1-PFGE of total DNA (Chi et al., 2020). The locations of plasmids harboring the $bla_{\text{OXA-181}}$ and *mcr-8* were determined by Southern blotting and hybridization with digoxigenin-labeled specific probes. Furthermore, rifampin-resistant *P. aeruginosa* PAO1Ri was used as a recipient bacterium in transformation conjugation experiments to investigate whether the plasmids can transfer (Liu et al., 2021). The transconjugants which showed growth on Mueller-Hinton medium simultaneously containing 300 mg/L rifampicin and 2 mg/L meropenem were identified by MALDI-TOF/MS. The existence of $bla_{\text{OXA-181}}$ and *mcr-8* in transconjugants was detected by PCR and the antimicrobial susceptibility testing of transconjugants to confirm whether the plasmids carrying target genes were successfully transferred.

Whole-genome sequencing and analysis

The genome of *K. pneumoniae* 5589 was extracted using a specific bacterial DNA Kit (QIAGEN, Hilden, Germany). To better understand the genetic features, DNA sequencing was performed on the Illumina NovaSeq 6000 (Illumina, San Diego, CA, United States) and the Oxford Nanopore (Oxford Nanopore Technologies, Oxford, United Kingdom) platform (Bao et al., 2022). Then, the whole genome was annotated with Prokka. Additionally, the acquired ARGs were detected by ResFinder 4.1[1],

1 https://cge.food.dtu.dk/services/ResFinder/

TABLE 1 MIC values of antimicrobials for *Klebsiella pneumoniae* 5589, transconjugant 5589-PAO1Ri, and recipient strain PAO1Ri.

Antimicrobials	MIC values (mg/L)		
	K. pneumoniae 5589	5589-PAO1Ri	PAO1Ri
Aztreonam	>128 (R)	64 (R)	1 (S)
Imipenem	8 (R)	4 (R)	4 (I)
Meropenem	4 (R)	2 (R)	0.25 (S)
Ceftriaxone	>128 (R)	>128 (R)	4 (S)
Cefotaxime	>128 (R)	>128 (R)	8 (S)
Ceftazidime	>128 (R)	>128 (R)	1 (S)
Levofloxacin	>64 (R)	16 (R)	1 (S)
Ciprofloxacin	>64 (R)	1 (S)	1 (S)
Amikacin	2 (S)	2 (S)	2 (S)
Gentamicin	128 (R)	1 (S)	1 (S)
Piperacillin-tazobactam[a]	>128 (R)	8 (R)	1 (S)
Cefepime	32 (R)	4 (R)	1 (S)
Polymyxin B	8 (R)	4 (R)	1 (S)

[a]Tazobactam at a fixed concentration of 4 mg/L.

and the plasmid replicon type was identified by PlasmidFinder 2.1.[2] The transposon and insertion sequence were detected using the ISFinder database.[3] Finally, the circular comparison images of multiplex plasmids were generated by BLAST Ring Image Generator (BRIG). The linear comparison figures of multiple genomic loci surrounding the $bla_{OXA-181}$ and *mcr-8* were generated by Easyfig 2.0 software (Sullivan et al., 2011).

Results

Isolation of *Klebsiella pneumoniae* 5589 and antimicrobial susceptibility testing

Carbapenem-resistant *K. pneumoniae* 5589 was isolated from a blood sample of a patient who was hospitalized for myelodysplastic syndrome (MDS). During his hospitalization, the patient developed thrombocytopenia, high fever and groin infection. Subsequently, the patient's condition was controlled with a normal body temperature after the biapenem and tigecycline treatment.

The antimicrobial susceptibility profiles of *K. pneumoniae* 5589 and transconjugants were demonstrated in Table 1. *K. pneumoniae* 5589 was resistant to multiple antibiotics such as aztreonam, imipenem, meropenem, ceftriaxone, cefotaxime, ceftazidime, levofloxacin, ciprofloxacin, gentamicin, piperacillin-tazobactam, cefepime and polymyxin B, but remained susceptible to amikacin. Moreover, the transconjugants 5589-PAO1Ri showed a similarity antibiotic resistance profile to *K. pneumoniae* 5589 but was intermediate to imipenem and sensitive to ciprofloxacin and gentamicin.

Genomics features of *Klebsiella pneumoniae* 5589

The *K. pneumoniae* 5589 genome contains a 5,279,178 bp circular chromosome with an average GC content of 57.5% and three plasmids of different sizes from 51,479 bp to 290,720 bp (Supplementary Table S1). WGS revealed that *K. pneumoniae* 5589 was identified as ST273, which belongs to the clonal group 147. By researching the ARGs on ResFinder, 48 acquired resistance genes were detected (Supplementary Table S1). The chromosome of strain *K. pneumoniae* 5589 was found to harbor ARGs which confer resistance to beta-lactams (bla_{SHV-11}, bla_{SHV-67}), fosfomycin (*fosA*), chloramphenicol (*OqxA*, *OqxB*). Moreover, it carried multiple virulence genes such as coding for outer membrane receptor (*fepA*), transcriptional regulator (*fimK*), regulator protein (*ykgK*) and transcriptional activator (*mrkH*).

Characterization of plasmid bearing $bla_{OXA-181}$

S1-PFGE and southern blot results revealed that the resistance gene $bla_{OXA-181}$ was located on a 51,479 kb plasmid (p5589-OXA-181), which belongs to IncX3-ColKP3 with a GC content of 46% (Supplementary Figure S1). The plasmid carrying $bla_{OXA-181}$ was successfully transferred to a *P. aeruginosa* PAO1Ri recipient strain. The plasmid p5589-OXA-181 carries not only the $bla_{OXA-181}$ but also *qnrS1*, which enables the strain to be resistant to ciprofloxacin. According to the result of the BLAST search, plasmid p5589-OXA-181 was almost identical to pNIPH17_0036_1 (accession no: LC483179), pKBN10P04869C (accession no: CP026476), pABC264-OXA-181 (accession no: MK412917) and pBC947-OXA-181 (accession no: MK412920) with the similarity between 99% and 100% (Figure 1A). The genetic environment analysis showed $bla_{OXA-181}$ was located on a composite transposon surrounded by two copies of insertion sequence IS*26*. A similar region can be seen in *E. coli* plasmid pKBN10P04869C (accession no: CP026476), and *E. coli* plasmid pABC264-OXA-181 (accession no: MK412917). In plasmid p5589-OXA-181, the gene *repA1* is responsible for encoding ColKP3-type replication initiation protein, and the IS*Kpn19* fragment is located downstream of $bla_{OXA-181}$, while IS*3000* is located on the upstream (Supplementary Figure S2A). Many other functional genes such as encoding DNA topoisomerase (*topB*), proteasome-associated ATPase (*mpa*), type IV secretion system protein (*ptlH*, *virB9*, *virB8*), transcription antitermination protein (*rfaH*) are distributed on the backbone.

2 https://cge.food.dtu.dk/services/PlasmidFinder/

3 http://www-is.biotoul.fr/

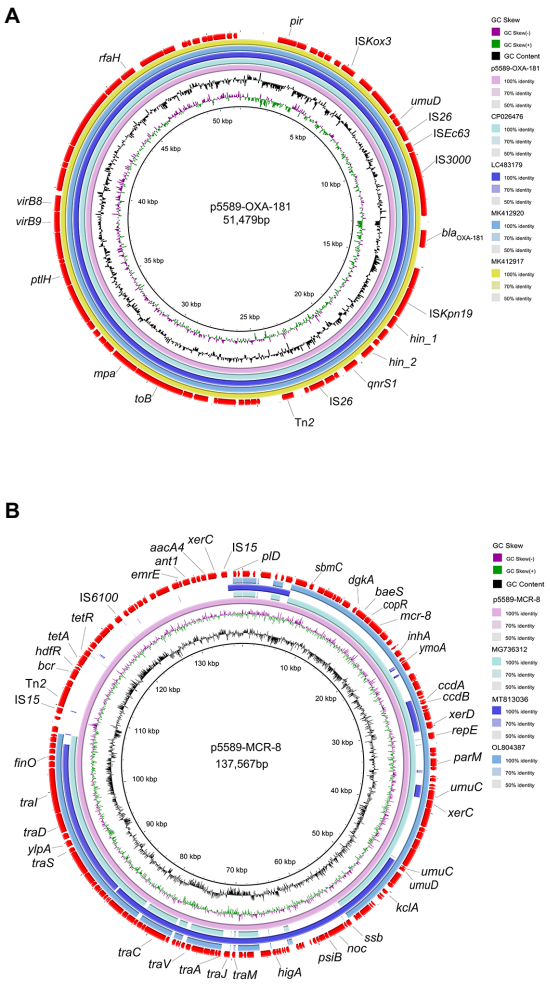

FIGURE 1
Comparative analysis of plasmids p5589-OXA-181 and p5589-mcr-8 detected in *Klebsiella pneumoniae* 5589. **(A)** Comparison of $bla_{OXA-181}$ bearing plasmid p5589-OXA-181 with pKBN10P04869C (GenBank accession no. CP026476), pNIPH17_0036_1 (GenBank accession no. LC483179), pBC947-OXA-181(GenBank accession no. MK412920) and pABC264-OXA-181(GenBank accession no. MK412917). **(B)** Comparison of mcr-8-carrying plasmid p5589-MCR-8 with pKP91(GenBank accession no. MG736312), pKQBSI104-1(GenBank accession no. MT813036) and pKP3(GenBank accession no. OL804387).

Characterization of plasmid bearing *mcr-8*

The *mcr-8* gene was carried by another plasmid of size 137,567 kb (p5589-mcr-8) with the replicon type of IncFIA-FII (Supplementary Figure S1). Meanwhile, we obtained the transconjugant harboring *mcr-8* successfully. The plasmid p5589-mcr-8 contained additional genes that make strain exhibit resistance to multiple antibiotics, such as bleomycin (*bleO*), spectinomycin (*aadA16*), ciprofloxacin (*aac (6′)-Ib-cr*, *qnrB91*), rifampicin (*arr-3*), azithromycin (*mph(A)*), trimethoprim (*dfrA27*), sulfamethoxazole (*sul1*), tetracycline (*tet(A)*), chlorhexidine (*qacE*), chloramphenicol (*floR*). On the other hand, the plasmid p5589_MCR-8 showed great similarity to pKP91(65%coverage and 99.70%identity; accession no: MG736312), pKQBSI104-1(52%coverage and 99.47%identity; accession no: MT813036), pKP3(65%coverage and 99.86%identity; accession no: OL804387; Figure 1B).

The inspection of the genetic regions revealed that *mcr-8* in this work was similar to that of the *mcr-8.1* gene in plasmid pK91 and pKP3. The upstream was the IS*903B* and other functional genes (*inhA*, *YmoA*). At the same time, the downstream of *mcr-8* were the transfer or transcription-associated genes (*copR*, *sasA dgkA*) and encoding β-lactamase gene (*ampc*) (Supplementary Figure S2B). The p5589-mcr-8 backbone carried regions responsible for toxin-antitoxin (TA) systems (*ccdA*, *ccdB*, *higA*, *ylpA*), replication (*repE*), mobilization (*tra*, *xerD*, *xerC*, *klcA*, *finO*) and stability (*parM*, *umuC*, *umuD*, *ssb*, *noc*, *psiB*). Other genes encode enzymes associated with DNA replication (*sbmC*, *ant1*, *aacA4*, *pld*), and proteins associated with the resistance and transport of multidrug (*bcr*, *tetA*, *tetR*, *emrE*) also can be found.

Discussion

OXA-181-producing Enterobacteriaceae have been reported in several countries, including Portugal, South Africa, and Singapore, but have rarely been described in China, where *Klebsiella pneumoniae* carbapenemase (KPC) is the major carbapenemase (Han R. et al., 2020; Chew et al., 2021). To our knowledge, OXA-181 has not emerged in China until 2015, and the report of $bla_{OXA-181}$ in China is still uncommon (Qin et al., 2018). Infections caused by OXA-181 in nonendemic areas were often associated with the travelling of patients to endemic areas (Chudejova et al., 2021). However, the identification of $bla_{OXA-181}$ in this work was from a patient without a history of foreign residence, which indicated its wider dissemination than previously anticipated.

So far, several studies have reported plasmids carrying $bla_{OXA-181}$ with different replicons, such as IncX3, IncA/C, ColE, IncT, IncN, IncFIIK and ColKP3 (Villa et al., 2013; Naha et al., 2021). But the most common plasmid in China is the IncX3 type which has a similar genetic environment to others (Liu et al., 2020). The similar $bla_{OXA-181}$ bearing IncX3 plasmid further highlights the role of IncX3-type plasmid as an irreplaceable vector of ARGs (Santos Tufic-Garutti et al., 2022). In p5589-OXA-181, $bla_{OXA-181}$ was found on a composite transposon which was considered to facilitate its horizontal transmission (Supplementary Figure S2A). Therefore, the absence of upstream mobile element IS*Ecp1* was detected in p5589-OXA-181, which was consistent with other studies (Naha et al., 2021). Generally, IS*Ecp1* plays an essential role in the transmission of ARGs; its absence may affect transposase activity and the maintenance of resistance genes on a plasmid (Potron et al., 2013; Naha et al., 2021).

The colistin resistance mechanism in Enterobacteriaceae is complicated and has not been wholly investigated (El-Sayed Ahmed et al., 2020). In general, the resistance to colistin can be acquired by intrinsic mutation or adaptation mechanisms and the horizontal transfer of *mcr* gene and its variants (Moffatt et al., 2019). Since the

initial report of *mcr-8*, this gene has been discovered in Enterobacteriaceae isolates from humans, animals and various environments worldwide (Anyanwu et al., 2020; Ngbede et al., 2020).

There have already been several studies about the genetic context analysis of *mcr-8* (Wu et al., 2020). The *mcr-8* gene was firstly recognized on a typical IncFII-type plasmid pKP91, with a conservative region flanked by IS*903B*, but in p5589-mcr-8, the IS*903B* located on the upstream was absent (Supplementary Figure S2B; Wang et al., 2018). The *mcr-8*-carrying plasmid in *Raoultella ornithinolytica* also harbored only one copy of IS*903B* located upstream (Wang et al., 2019). In addition, Farzana has reported the upstream IS*903B* of *mcr-8* in ST15 *K. pneumoniae* was replaced by IS*Kpn21* (Farzana et al., 2020). Thus, it was reasonable to speculate that the upstream IS*903B* surrounding *mcr-8* is unstable and replaceable. However, additional studies focusing on this are warranted.

K. pneumoniae 5589 in this work also carried $bla_{\text{CTX-M-55}}$, which was frequently found in *E. coli* (Zhang et al., 2014; Birgy et al., 2018; Feng et al., 2019). Hence, we could also pay more attention on $bla_{\text{CTX-M-55}}$-positive *K. pneumoniae* to better understand the molecular epidemiology of CTX-M-55 in China. Simultaneously, *K. pneumoniae* 5589 was assigned to ST273, which was recognized as the reservoir of many carbapenemase genes, including bla_{KPC}, bla_{VIM}, bla_{NDM} and bla_{IMP} (Chou et al., 2016; Liu et al., 2018). ST273 was divided into the specific clonal group 147, which had a high epidemic potential (Rodrigues et al., 2022). The original detection of ST273 was in Europe and had been gradually identified in Italy, Norway, and Russia, even causing the outbreak in Southeast Asia. Furthermore, whether the ST273 could influence the epidemiology of $bla_{\text{OXA-181}}$ and *mcr-8* remains unknown, and necessary attention should be paid to these sequence types to avoid epidemic outbreaks.

Previous studies have reported the co-producing of OXA-181 and other carbapenemases, such as co-harboring $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-5}}$ in Nepal, $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-1}}$ in French, and $bla_{\text{OXA-181}}$ and $bla_{\text{KPC-121}}$ in Italy (Sherchan et al., 2020; Gaibani et al., 2022). Remarkably, the spread of *mcr* gene into CRE resulting the accumulation of multidrug resistance genes. Nevertheless, the study about the co-carriage of $bla_{\text{OXA-181}}$ and *mcr-8* is limited, except for a report of an *Escherichia coli* co-producing OXA-181 and MCR-1 (Pulss et al., 2017). Identifying the isolate co-carrying $bla_{\text{OXA-181}}$ and *mcr-8* in *K. pneumoniae* reminds us that persistence detection and further exploration are needed to prevent the emergence and evolution of such MDR isolates.

Conclusion

In summary, our work firstly described the co-occurrence of OXA-181, CTX-M-55, and MCR-8 in *K. pneumoniae*. Our study also characterized the $bla_{\text{OXA-181}}$ and *mcr-8*-carrying plasmids, which contribute to exploring the transmission mechanism. The appearance of such clinical isolates producing carbapenemases and MCR narrows the therapeutic options and reveals the severe situation of antimicrobial resistance. Continuous observation and exploration are essential to control its spread.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

XG and BZ conceived and designed the experiments. HG, JQ, RC, CL, and JZ collected samples and performed the experiments. HX, RL, and XH analyzed the data. HG wrote the manuscript. BZ reviewed and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1020500/full#supplementary-material

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The variants of polymyxin susceptibility in different species of genus *Aeromonas*

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The aquatic environment is an important medium for the accumulation and dissemination of antibiotic-resistant bacteria as it is often closely related to human activities. Previous studies paid little attention to the prevalence and mechanism of polymyxin-resistant bacteria in the aquatic environment. As a Gram-negative opportunistic pathogen widely distributed in aquatic ecosystems, the antibiotic-resistant profile of *Aeromonas* spp. deserves much attention. In this study, we identified 61 *Aeromonas* spp. isolates from water samples in the section of the Yangtze River. The total polymyxin B (PMB) resistance rate of these strains was 49.18% (30/61), showing a high level of polymyxin resistance in *Aeromonas* spp. The MIC_{50} and MIC_{90} for PMB exhibited a significant discrepancy among different species ($p<0.001$). The MIC_{50} and MIC_{90} for PMB in the *Aeromonas hydrophila* were 128mg/L and above 128mg/L while in *Aeromonas caviae* and *Aeromonas veronii*, the MIC_{50} and MIC_{90} value were both 2mg/L. Only two *A. veronii* strains (MIC=2mg/L) and one *A. caviae* strain (MIC=0.5mg/L) were identified as carrying mobilized polymyxin resistant gene *mcr-3.42*, and *mcr-3.16*. All *mcr* genes were located in the chromosome. This is the first report that the downstream region of *mcr*-3.42 was the truncated *mcr-3*-like gene separated by the insertion sequences of IS*As20* (1,674bp) and IS*As2* (1,084bp). Analysis of epidemiology of *mcr*-positive *Aeromonas* genomes from GenBank database showed that the genus *Aeromonas* and the aquatic environment might be the potential container and reservoir of *mcr-3*. By the whole-genome sequencing and qRT-PCR, we inferred that the sequence differences in the AAA domain of MlaF protein and its expression level among these three species might be involved in the development of polymyxin resistance. Our study provided evidences of the possible mechanism for the variety of polymyxin susceptibility in different species of the genus *Aeromonas* and a theoretical basis for the surveillance of the aquatic environment.

KEYWORDS

aquatic environment, polymyxin resistance, *mcr*, MlaF, *Aeromonas*

Introduction

Antimicrobial resistance (AMR) has been a major challenge to global public health with the use of antibiotics over the decades. Antimicrobial resistance genes (ARGs) are considered as environmental pollutants as the result of the continuous release of residual antibiotics by human activities into the environment such as hospitals, livestock, and sewage treatment plant, which increased occurrence of antimicrobial resistant bacteria (ARB) (Pruden et al., 2006; Zurfluh et al., 2017). Moreover, the aquatic environment itself can serve as the sites of the spreading of AMR and the reservoir of ARGs, like *qnrA*, *qnrB*, *qnrS*, *mef*, and so on (Pei et al., 2006; Aedo et al., 2014; Nonaka et al., 2015). There are a large number of bacteria (> 100 million/liter of seawater) in the aquatic environment, from which bacteria such as *Aeromonas* spp., *Vibrio*, and *Pseudomonas* sp. are often isolated (Noonburg, 2005). Especially, *Aeromonas* spp., consisting of 30 species, is widely distributed in various kinds of natural water ecosystems (Chen et al., 2021). *Aeromonas* spp. are gram-negative, rod-shaped aerobes belonging to the *Aeromonadaceae* family. Among the members of *Aeromonas* spp., *Aeromonas hydrophila*, *Aeromonas veronii*, and *Aeromonas caviae* are the common zoonotic pathogens both for human and aquatic animals. In recent years, *Aeromonas* species have become the third most common enteric bacterial pathogens, with a clinical isolation rate of 56.73% (Yuwono et al., 2021). The antimicrobial susceptibility of *Aeromonas* has been reported in many studies, showing that *Aeromonas* were resistant to ampicillin, penicillin, and cephalosporins, tetracycline (Scarano et al., 2018; Jia et al., 2020; Sun et al., 2021).

Polymyxins, including polymyxin B (PMB) and polymyxin E (colistin), are a group of cationic polypeptides that disrupts membrane integrity by replacing cations like Mg^{2+} and Ca^{2+} in the outer membrane, which leads to the cell lysis. Due to the emergence of bacteria resistant to the most common-use antibiotic and the shortage of new antimicrobial agents to resist them, polymyxins have become the last line of defense against multiple drug-resistant gram-negative bacteria infections (Falagas and Rafailidis, 2008; Trimble et al., 2016). The most common mechanism of colistin resistance is the chromosomal mutations in genes involved in the synthesis and modification of lipid A of lipopolysaccharide (LPS). These genes include the *arnBCADTEF* operon which is regulated by the two-component system (TCS) PhoP/Q, PmrA/B, ParR/S, CprR/S, and ColR/S (Olaitan et al., 2014). In addition to that, the horizontal transfer of the plasmid-carrying gene *mcr* encoding phosphoethanolamine (PEtN) transferase which modifies the lipid A by addition of phosphoethanolamine has become another important cause of colistin resistance. Since the first plasmid-mediated colistin resistance gene *mcr-1* was identified in 2015, a total of 10 *mcr* gene variants (*mcr-1* to *mcr-10*) have been reported, mainly among the Enterobacterales (Hussein et al., 2021).

In *Aeromonas* species, *mcr-2*, *mcr-3*, *mcr-5*, and *mcr-7* have been reported, especially the variants of *mcr-3*, which were widely detected and frequently disseminated between Enterobacteriaceae and *Aeromonas* (Ma et al., 2018; Sun et al., 2018; Ragupathi et al., 2020; Wang et al., 2021). Beyond that, the *mcr*-positive *Aeromonas* strains showed the discrepancy of polymyxin-resistant phenotype, manifesting as a wide span of and the minimum inhibitory concentration (MIC) of polymyxin ranging from <0.5 mg/L to >128 mg/L. But the exact mechanism of polymyxin resistance in *Aeromonas* and reasons for the differences of polymyxin susceptibility in different *Aeromonas* species remains poorly understood and less attention has been paid to aquatic environments (Ling et al., 2017; Tuo et al., 2018; Ragupathi et al., 2020).

Here, we determined the polymyxin susceptibility in different *Aeromonas* species isolated from the section of the Yangtze River, investigated the prevalence of the *mcr* gene, and explored the connections between gene expression levels in the TCS involved in the synthesis and modification of lipid A and the polymyxin resistance. A total of 61 *Aeromonas* strains were detected and 30 strains showed polymyxin resistance, showing a high level of polymyxin resistance rate of 49.18% (30/61). Among different species in the genus *Aeromonas*, the polymyxin resistance rate showed a distinct discrepancy ($p < 0.001$). Two *A. veronii* strains were identified as *mcr-3.42* positive strains, and one *A. caviae* strain was identified as *mcr-3.16* while the rest strains remained negative of *mcr-1* to *mcr-10*. We inferred that the existence of *arnBCADTEF* and mutations in MlaF protein among different *Aeromonas* species could affect the function of phospholipid transportation and contribute to the resistance to polymyxins.

Materials and methods

Bacterial isolation and identification

To investigate the dissemination of *mcr* in the genus *Aeromonas* from the aquatic environment, 16 water samples were collected in sterile 1-L bottles from A section of the Yangtze River. All samples were stored at 4°C after collection and analyzed within 12 h. Luria-Bertani (LB), Mueller-Hinton (MH), and Salmonella Shigella (SS) culture mediums were used to perform the culture and isolation of bacteria. The whole isolates were screened for the presence of *mcr-1* to *mcr-10* by colony PCR (Woodman et al., 2016). The positive PCR products were verified by Sanger sequencing. Primer pairs used in this study were listed in Supplementary Table S1. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS identification and 16S rRNA sequencing were adopted for bacterial identification.

Antimicrobial susceptibility testing and microbiological assessment

Antimicrobial susceptibility testing (AST) was conducted by the broth microdilution method and agar dilution method.

Testing was performed according to Clinical and Laboratory Standards Institute guidelines (CLSI) (2021). The minimum inhibitory concentration (MIC) of PMB (Sigma, Shanghai, China), tigecycline (TGC), and tetracycline (TCY) (Solarbio, Shanghai, China) were determined by the broth microdilution method, and the MIC of common drugs for clinical therapy including amikacin, cefepime, ciprofloxacin, meropenem, aztreonam et al. (Solarbio, Shanghai, China) were determined by agar dilution method. The breakpoint of TGC was interpreted according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 10.0.[1] The other antimicrobial agents were interpreted according to the guidelines of the CLSI M100 (2021 version, 31st edition) and M45-A2 (2015 version, 3rd edition) documents. *Escherichia coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility testing.

Conjugation experiment and S1 nuclease pulsed-field gel electrophoresis analysis

The conjugation experiment was carried out to investigate the transferability of *mcr* gene. Procedures were performed according to the method described previously (Hadjadj et al., 2019). Sodium azide-resistant *E. coli* J53 (MIC of 200 mg/L) was used as recipient strain. The transconjugants were selected on the medium containing azide at 200 mg/L and PMB, at 1 mg/L was consistent with the MIC of the donor. Resultant colonies were screened for the presence of *mcr* by PCR.

S1-PFGE analysis of *mcr*-positive isolates was used to estimate the sizes of *mcr*-positive plasmids. *Xba*I-restricted DNA of *Salmonella enterica* serovar Braenderup H9812 was used as a DNA marker. Shortly, agarose plugs were made using the colonies in the medium of a single colony inoculated and digested with S1 nuclease (TaKaRa, Dalian, China). The DNA was separated using the CHEF-MAPPER PFGE system (Bio-Rad) under the following conditions: 14°C, 6 V/cm, and a 120° pulse angle for 16 h, with the initial and final pulses conducted for 2.16 s and 63.8 s, respectively. Then the dyed gel was visualized with the imaging system.

DNA extraction, whole-genome sequencing, and genomic analyses

Genomic DNA of *mcr*-positive isolates were extracted using Gentra Puregene Yeast/Bact. Kit (Qiagen, CA, United States), according to the manufacturer's instructions. Illumina Hiseq PE150 platform was used for the whole-genome sequencing (WGS). After read quality control, the reads from Illumina Hiseq platform were assembled using SPAdes 3.10.18 which is based on De-Bruijn algorithm and takes the appropriate k-mer values. Genetic predictions of assembly results were using the web platforms of the National Center for Biotechnological Information (NCBI) and ORFfinder.[2] The sequences were annotated using the RAST annotation server[3] and prokka 1.14.6.[4] Assembled contigs were analyzed *via* the Center for Genomic Epidemiology website[5] to screen for the presence of acquired AMR genes, plasmid incompatibility types, and Multi Locus Sequence Typing (MLST). Linear comparisons of *mcr* were generated using Easyfig. 2. Multiple alignments of nucleotide sequences were performed by using The European Bioinformatics Institute (EMBL-EBI) tools.[6] To investigate the distribution of *mcr*-positive strains of the genus *Aeromonas*, we retrieved the isolates in sequences deposited in GenBank by Genomes Browser,[7] (accessed 26 April 2022) and Isolates Browser[8] (accessed 26 April 2022).

Reverse transcription-quantitative PCR

Total RNA was extracted from mid-long phase (OD_{600} ~ 0.6) bacterial cultures using the Qiagen RNeasy Mini kit (QIAGEN, Alameda, United States), according to the manufacturer's instructions. Complementary DNA (cDNA) was generated from total RNA using the RT Premix. Supplementary Table S2 shows the sequences of transcript-specific primers used for qPCR. All samples were performed in triplicate, and the data were normalized to 16S rRNA levels and analyzed using the $2^{-\Delta\Delta CT}$ method to calculate the fold-change relative to the control. The difference between groups was analyzed by Welch's *t*-test.

Amino acids analysis and protein structure prediction

Multiple alignments of amino acid sequences were performed by using The European Bioinformatics Institute (EMBL-EBI) tools[9] and Jalview 2.11.1.4 was used to perform the multiple sequence alignment editing, visualization, and analysis. PROVEAN Protein tools[10] were calculated to evaluate whether amino acid alterations in PhoP/PhoQ, OmpR/EnvZ, and MlaF affected biological function. Moreover, the protein domains of PhoP/PhoQ, OmpR/EnvZ, and MlaF were subjected to SMART analysis.[11] The secondary structure and complex structure of MlaF

1 https://eucast.org/clinical_breakpoints/

2 https://ncbi.nlm.nih.gov/orffinder/
3 http://rast.nmpdr.org/
4 http://github.com/tseemann/prokka
5 http://genomicepidemiology.org/
6 https://www.ebi.ac.uk/Tools/msa/clustalo/
7 https://www.ncbi.nlm.nih.gov/data-hub/genome/
8 https://www.ncbi.nlm.nih.gov/pathogens/isolates/
9 https://www.ebi.ac.uk/Tools/msa/clustalo/
10 http://provean.jcvi.org/index.php
11 http://smart.embl.de/

TABLE 1 Characteristics of *mcr*-3 and *mcr*-negative *Aeromonas* strains isolated in this study.

Isolate	Species	PMB MIC (mg/L)	Isolate source	*mcr*	Accession
S461	*A. hydrophila*	>128	Yangtze River	*mcr*-negative	JANPYL000000000
S541	*A. hydrophila*	>128	Yangtze River	*mcr*-negative	JANKLR000000000
M894	*A. hydrophila*	>128	Yangtze River	*mcr*-negative	JANKLS000000000
L652	*A. hydrophila*	>128	Yangtze River	*mcr*-negative	JANKLT000000000
M621	*A. caviae*	1	Yangtze River	*mcr*-negative	JANKLU000000000
L7105	*A. caviae*	2	Yangtze River	*mcr*-negative	JANKLV000000000
L965	*A. caviae*	1	Yangtze River	*mcr*-negative	JANKLW000000000
L215	*A. caviae*	1	Yangtze River	*mcr*-negative	JANKLX000000000
S5183	*A. veronii*	2	Yangtze River	*mcr*-negative	JANKLY000000000
M194	*A. veronii*	2	Yangtze River	*mcr*-negative	JANKLZ000000000
L975	*A. veronii*	1	Yangtze River	*mcr*-negative	JANKMA000000000
L924	*A. veronii*	2	Yangtze River	*mcr*-negative	JANKMB000000000
S611	*A.* caviae	0.5	Yangtze River	*mcr*-3	JANKMC000000000
M694	*A. veronii*	2	Yangtze River	*mcr*-3	JANKMD000000000
M683	*A. veronii*	2	Yangtze River	*mcr*-3	JANKME000000000

protein were predicted by PSIPRED[12] and SWISS-MODEL,[13] respectively. The images of biomolecular structures of MlaF protein were generated by PyMoL (Seeliger and de Groot, 2010).

Nucleotide sequence accession numbers

The whole-genome nucleotide sequences of 15 *Aeromonas* strains have been submitted to DDBJ/ENA/GenBank under the accession numbers JANKLR000000000-JANKLZ000000000, JANKMA000000000-JANKME000000000, and JANPYL000000000 and were in a processing queue (BioProject: PRJNA864564). The accession numbers were listed in Table 1.

Results

Aeromonas spp. identification and the characteristics of antibiotic resistance

In order to investigate the distribution of polymyxin resistance in the genus *Aeromonas* bacteria isolated from the aquatic environment, we collected a comprehensive set of samples from the section of the Yangtze River, in Hubei province. In total, 163 gram-negative bacteria strains were isolated and identified by MALDI-TOF MS. Among them, 37.42% (61/163) of the isolates were identified as belonging to *Aeromonas* spp., namely *Aeromonas hydrophila* ($n = 29$), *Aeromonas caviae* ($n = 21$), and *Aeromonas veronii* ($n = 11$). Antimicrobial susceptibility testing was performed to investigate the drug resistance characteristics of 61 *Aeromonas* spp. isolates against fifteen common clinical drugs. 100% of *A. hydrophila* isolates exhibited resistance to PMB while 100 and 90.91% (10/11) of *A. caviae* and *A. veronii* isolates exhibited susceptibility to PMB. 96.72% (59/61), 93.44% (57/61), 90.16% (55/61), 93.44% (57/61), 55.74% (34/61), 65.57% (40/61), 73.77% (45/61), 96.72% (59/61), 50.82% (31/61), 77.05% (47/61), 75.41% (46/61), 83.61% (51/61) of *Aeromonas* spp. strains showed susceptibility to clinically important antibiotic drugs meropenem, imipenem, tigecycline, tazobactam/piperacillin, ciprofloxacin, levofloxacin, aztreonam, amikacin, gentamycin, cefepime, ceftazidime, cefoperazone/sulbactam, respectively. But for the sulfamethoxazole/trimethoprim and tetracycline, the majority of *Aeromonas* spp. remained resistant, with a resistance rate of 67.21% (41/61) and 60.66% (37/61) respectively (Supplementary Table S3). The results of the comparator antimicrobial drugs tested against 61 *Aeromonas* spp. isolates were listed in Table 2. And the MIC_{50} and MIC_{90} for PMB were 128 mg/L and above 128 mg/L in the *A. hydrophila* group. However, both the MIC_{50} and MIC_{90} were 2 mg/L in the *A. caviae* and *A. veronii* groups (Figure 1A). This result shows that the characteristics of PMB resistance in the genus *Aeromonas* vary in different species ($p < 0.001$).

Identification of *mcr*-positive *Aeromonas* from the aquatic environment

To investigate the prevalence of *mcr* in the genus *Aeromonas* bacteria isolated from the aquatic environment, colony PCR was conducted to screen *mcr* genes using primers as described in materials and methods. Only two *mcr-3* positive *A. veronii* strains (M683 and M694, MIC = 2 mg/L) and one *mcr-3* positive *A. caviae* S611 (MIC = 0.5 mg/L) were identified, while the rest of the 58 isolates were all negative of *mcr-1* to *mcr-10*, indicating that the prevalence of *mcr* in *Aeromonas* from the section of the Yangtze

12 http://bioinf.cs.ucl.ac.uk/psipred/

13 https://swissmodel.expasy.org/

TABLE 2 Comparator antimicrobial agents tested against 61 isolates of *Aeromonas* spp. collected in a section of Yangtze River.

Species (no. of isolates)	Antimicrobial agent	MIC (mg/L)			Percentage susceptible (%)
		MIC50	MIC90	MIC range	
Aeromonas spp., all (61)	MEN	0.06	0.25	0.015 to 4	96.72
	IPM	0.25	1	0.125 to 4	93.44
	PMB	2	>128	0.5 to >128	50.82
	TGC	0.5	2	<0.125 to 4	90.16
	TZP	2	16	≤1 to 128	93.44
	LVX	1	32	≤0.008 to >64	65.57
	ATM	0.125	16	≤0.06 to >64	73.77
	AMK	4	16	≤0.5 to >128	96.72
	FEP	0.06	32	≤0.015 to 64	77.05
	CIP	1	>64	≤0.004 to >64	55.74
	SXT	>8/152	>8/152	≤0.5/9.5 to >8/152	32.79
	CAZ	0.5	>64	≤0.06 to >64	75.41
	CSL	2	64	0.06 to >128	83.61
	GEN	4	>128	1 to >128	50.82
	TCY	16	128	0.5 to >128	39.34

MEM, meropenem; IPM, imipenem; PMB, polymyxin B; TGC, tigecycline; TZP, piperacillin/tazobactam; LVX, levofloxacin; ATM, aztreonam; AMK, amikacin; FEP, cefepime; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; CAZ, ceftazidime; CSL, cefoperazone/Sulbactam; GEN, gentamycin; TCY, tetracycline.

River is 4.92% (3/61). However, none of the three *mcr*-positive *Aeromonas* strains showed resistance to PMB (MIC <4 mg/L). Conjugation experiments showed that these three *mcr*-positive isolates were not able to transfer the polymyxin resistance phenotype to receipt strain *E. coli* J53. S1-PFGE and DNA hybridization were conducted to separate the plasmid of different sizes and locate the genetic position of *mcr-3*. No plasmid was found in the *A. caviae* S611. But a plasmid fragment of approximately 5.47×10^4 bp was found in *A. veronii* strains (M683 and M694). Southern blotting result suggested that the *mcr-3* was located in the chromosome (Supplementary Figure S1).

To investigate the characteristics of the epidemiology of *mcr*-positive *Aeromonas* strains, we retrieved all the details of isolates deposited in GenBank as described in materials and methods. The *mcr*-positive *Aeromonas* strains in the GenBank were reported to carry *mcr-1*, *mcr-2*, *mcr-3*, *mcr-5* as well as *mcr-7*, and *mcr-3* was the predominant type in these *mcr*-positive *Aeromonas* strains (47.70%, 352/738). Most of the *mcr*-positive *Aeromonas* were isolated from the United States (24.93%, 93/373) and China (22.25%, 83/373), and *mcr-3* was the main type (94.37%, 352/373) (Figure 1B). Nearly half of the *mcr*-positive *Aeromonas* were isolated from animals (42.63%, 159/373), while 24.40% (91/373) and 23.59% (88/373) of the positive samples were from the environment and clinic (Figure 1C). By the phylogenetic tree analysis, we found that the evolution of *A. caviae* S611 isolated from Yangtze River was closely related to *A. caviae* SCAc2001 (WUTZ00000000.1) isolated from clinical samples in China. The evolution distance of *A. veronii* M683 and M694 isolated from the aquatic environment was quite approximated and closely related to *A. veronii* MS-17-88 (GCA_003611985.1) isolated from animal samples in the United States (Figure 2).

Genome analysis of the *mcr*-positive *Aeromonas* isolates

To fully understand the genetic context of *mcr*-positive *Aeromonas* strains isolated from the section of Yangtze River, we perform WGS with three *mcr-3* positive isolates in this study (*A. veronii* M683, M694, and *A. caviae* S611) and the contigs were properly assembled.

In the *A. veronii* strains M683 and M694, ResFinder was used to reveal the antibiotic resistance genes, such as the aminoglycoside and fluoroquinolone resistance genes (*aac*, *aadA*, *ant*, and *qnrVC*), beta-lactam (*ampS*, *bla*CEPH-A3, *bla*OXA, and *bla*VEB-1), folate pathway antagonist (*dfrA*), quaternary ammonium compound (*qacL*), amphenicol (*cmlA*), and tetracycline antibiotic genes (*tet*(E)). In the *A. caviae* S611, the following antibiotic resistance genes were predicted, *aac*, *aph*, *bla*MOX, *bla*PER, *sul*, macrolide resistance genes (*mph*(A) and *ere*(A)), *cat*, *cmlA*, and *tet*(C) (Figure 2).

The nucleotide sequence of *mcr-3* in the *A. caviae* S611 showed 100% identity with *mcr-3.16* (NG_060517.1). A 1626-bp *mcr-3*-like gene lay downstream of the *mcr-3*. Its nucleotide sequence showed 83.03 and 67.32% identity to *mcr-3.16* and lipid A phosphoethanolamine transferase gene (*eptA*) in the *A. caviae* S611. Downstream region of the *mcr-3*-like gene lay *dgkA* encoding diacylglycerol kinase, a gene arrangement (*mcr-3-mcr-3*-like) that is often observed in many *mcr-3* positive *Aeromonas* strains. But *A. veronii* strains M683 and M694 were the exception. Their *mcr-3-mcr-3*-like segment was separated by the insertion sequence of IS*As20* (1,674 bp) and IS*As2* (1,084 bp) (Figure 3). This is the first report of the truncated *mcr-3-mcr-3*-like segment in the *mcr*-3 positive

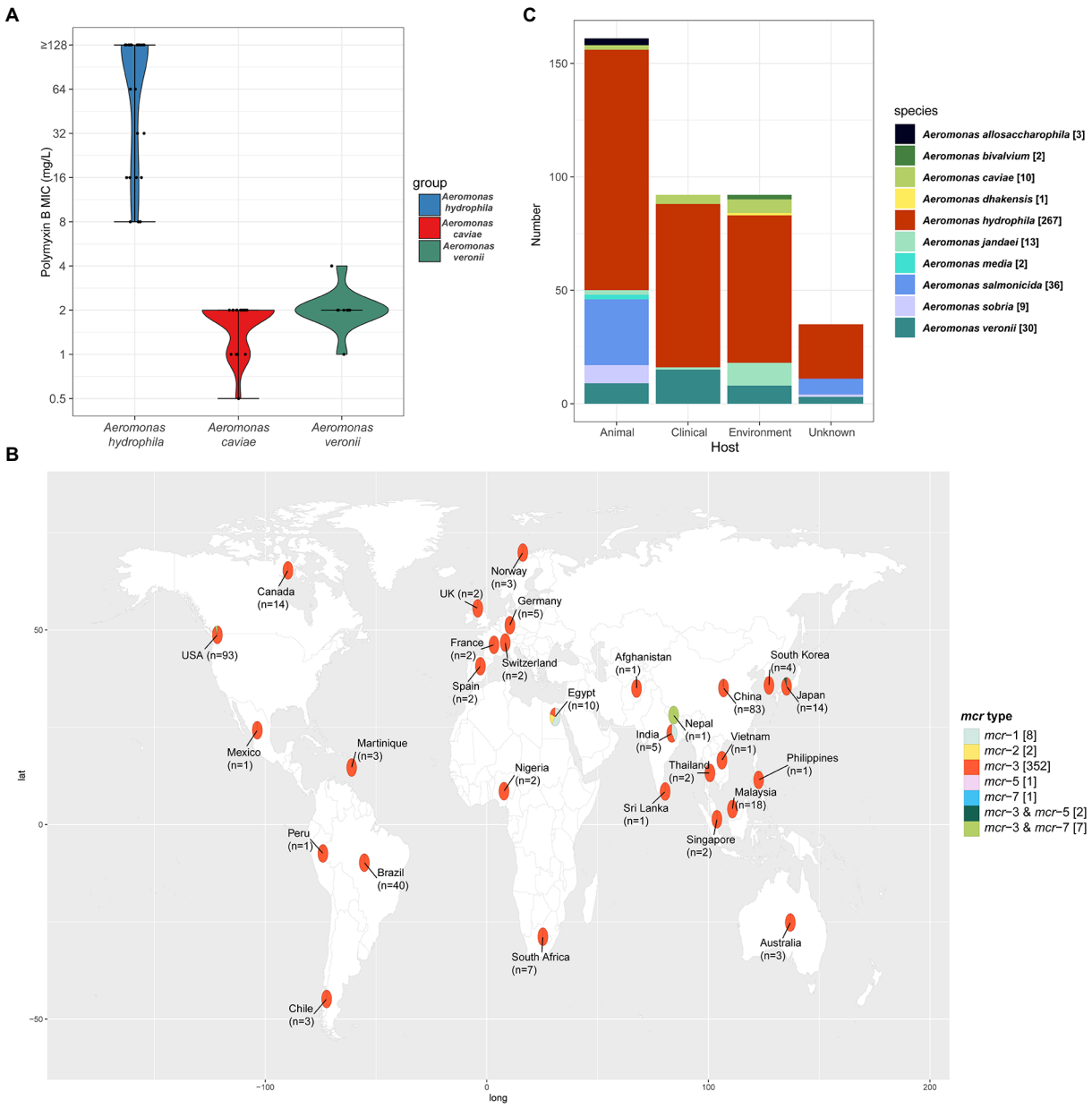

FIGURE 1
(A) The Violin-plot. The MIC of PMB in the three species of the genus *Aeromonas*. The black dot represents the dispersion of MIC value. And the shape of the violin represents the distribution of MICs in the corresponding group. Blue, red, and green indicated the species of *A. hydrophila*, *A. caviae*, and *A. veronii*. **(B)** Geographical distribution of the 326 isolates with definitive location, among all 373 *mcr*-positive *Aeromonas* isolates from the NCBI database. Different *mcr* types of the isolates were labeled with different colors and the number of each *mcr* type is given in square brackets. The number of all the *mcr*-positive isolates in each country was labeled on the world map under the country name. In addition, the location of the 47 *mcr*-positive isolates was still unknown. The world map was created using the corresponding map data with R package ggplot2 v3.3.5 (https://github.com/tidyverse/ggplot2). **(C)** The species and number of *mcr*-positive *Aeromonas* isolates from different sources. In total, 338 *mcr*-positive *Aeromonas* isolates were isolated and identified from the specific host and the sample sources of 35 isolates were still unknown. Different colors indicated the different species of the genus *Aeromonas*.

A. veronii strains. Both of the two 1,623-bp *mcr-3* variant genes of M683 and M694 exhibited 99.94% nucleotide sequence identity to the *mcr-3.32* (NG_070770.1), and the corresponding proteins exhibited 99.81% amino acid sequence identity to MCR-3.32 (WP_188331891.1). The two novel *mcr-3* variant genes of *A. veronii* M683 and M694 were termed as *mcr-3.42*. And the accession numbers were OP297669 and OP297670, respectively. The discovery of *mcr-3.42* genes in *A. veronii* was the first report about the *mcr-3* positive *A. veronii* strains isolated from the aquatic environment in China.

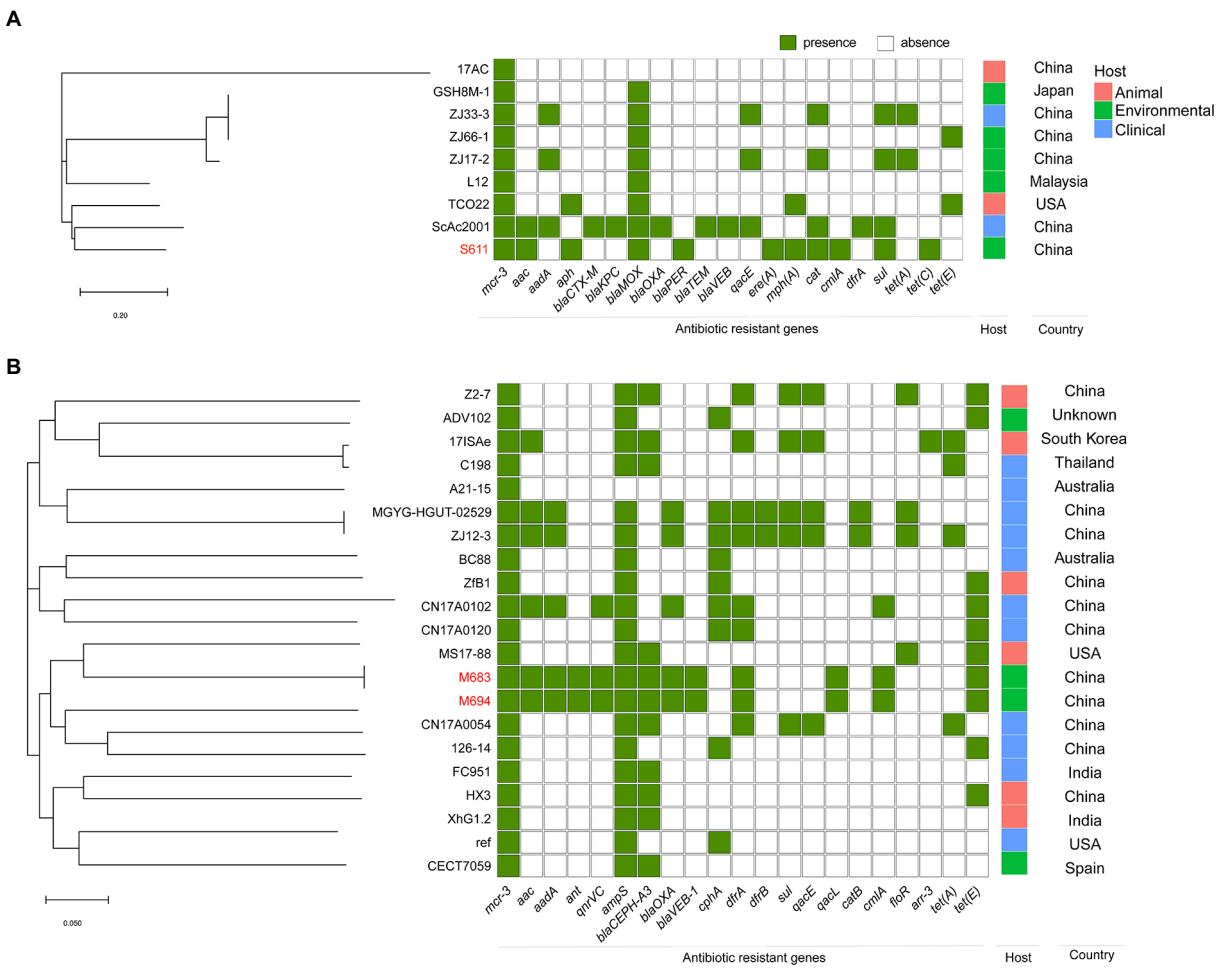

FIGURE 2
Phylogenetic tree generated from the core-genome sequences of the *mcr*-3-positive isolates identified in this study and other *mcr*-3-positive isolates within the same species randomly selected from GenBank database. Significant antimicrobial resistance genes, country, and host were shown. **(A)** Phylogenetic tree of *mcr*-3-positive *A. caviae*. **(B)** Phylogenetic tree of *mcr*-3-positive *A. veronii*.

The sequence variants of genes related to polymyxin resistance in different *Aeromonas* species

Although *mcr-3* gene were detected in some *Aeromonas* strains, these bacteria did not show high colistin resistance. And other *Aeromonas* strains with high colistin resistance did not possess *mcr* gene. Therefore, we suspected that the *mcr* gene might not be the primary cause for the colistin resistance diversity among *Aeromonas*. In order to investigate the possible reason for that, we screened 12 *mcr*-negative *Aeromonas* strains in three species with different levels of PMB sensibility (MIC of 4 *A. hydrophila* isolates was ≥128 mg/L, MIC of 4 *A. caviae* and 4 *A. veronii* isolates were ≤2 mg/L). Whole genome sequencing was performed to analyze the differences in the gene sequences of TCS such as PhoP/PhoQ, EnvZ/OmpR TCS, and MlaF in these 12 *Aeromonas* strains. And amino acids analysis and protein structure prediction was performed as described in materials and methods.

We compared the genome sequences of *A. hydrophila* with *A. caviae* and *A. veronii*, colistin resistance gene *arnBCADTEF* was only found in *A. hydrophila*, implying that *arnBCADTEF* operon might be the possible cause for the variety of colistin sensitivity among these species. We also screened the nucleic acids differences in the TCS PhoP/PhoQ, EnvZ/OmpR, and MlaF. Many base mutations were found in these genes in *A. hydrophila*, but most of the mutations did not change the amino acid sequences. For example, compared with *A. caviae* genome sequence, *A. hydrophila* possessed 12 site mutations in *phoP*, 53 site mutations in *phoQ*, 7 site mutations in *ompR*, 32 site mutations in *envZ*, and 32 site mutations in *mlaF*. While compared with *A. veronii*, there were 12, 93, 10, 55, and 5 site mutations in these genes (Supplementary Table S4). In order to evaluate whether these mutations would impact the protein function, PROVEAN software was used to predict the result of these mutations (Table 3). For *A. hydrophila*, PhoP amino acid substitution in Q39G, R69S/T, V153T/A, PhoQ in P82R, Y233F, OmpR in F30V,

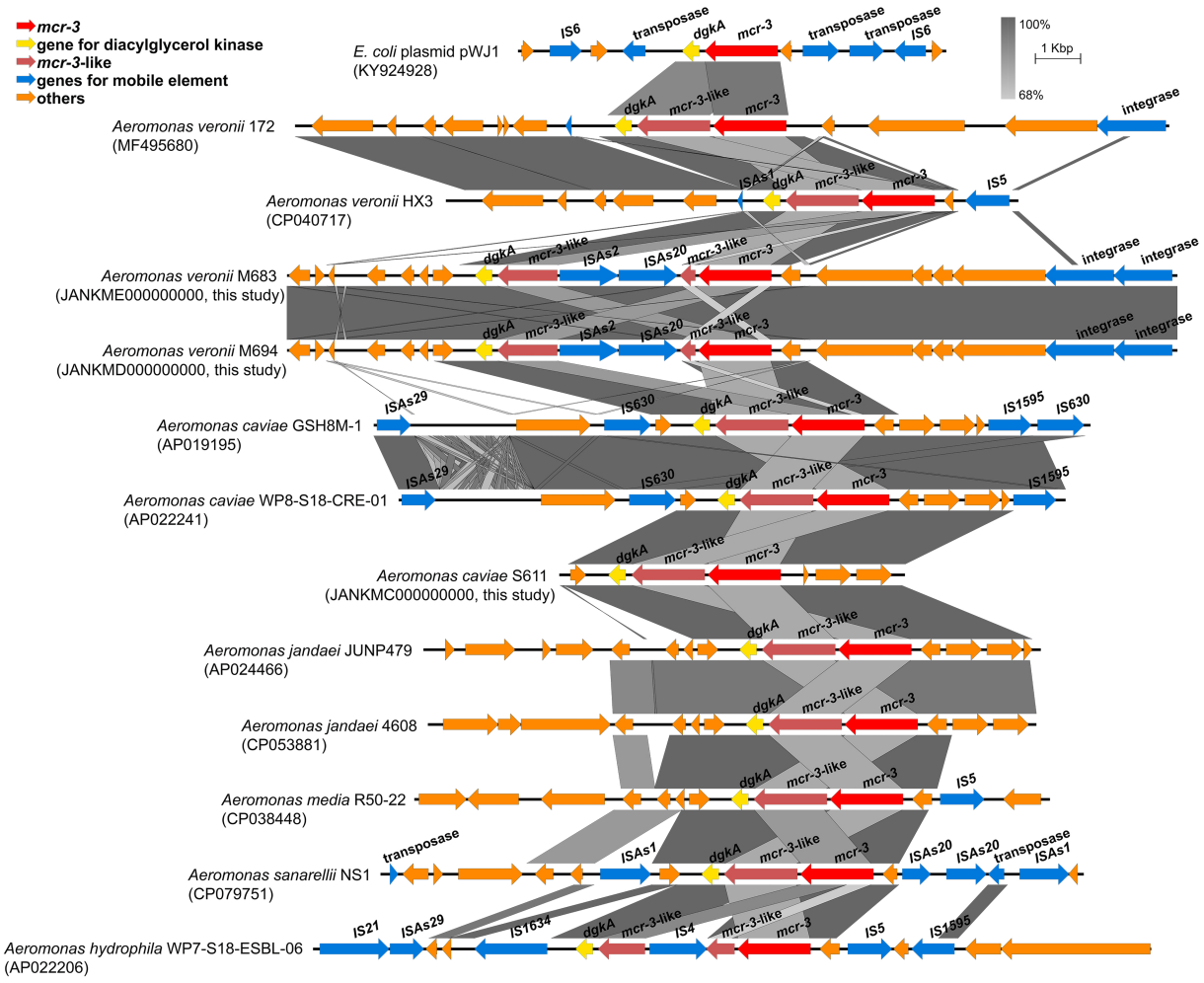

FIGURE 3
Schematic representation and comparison of the genetic environment of the *mcr*-3-flanking region in each genomic backbone type. Arrows indicate the direction of transcription of each gene, and different genes are shown in different colors. Regions of above 68% nucleotide sequence identify are shaded grey.

EnvZ in G108K, Q198L, Q302Y, L339Q, P369A, H407Y, and MlaF in S63F, A231E/D were supposed to cause disfunction. But secondary structure alignment showed that the AAA domain in MlaF of *A. hydrophila* (174aa) was shorter than that of *A. veronii* and *A. caviae* (198aa) (Figure 4). And protein structures predictions of MlaF based on the model proteins showed that MlaF of *A. hydrophila* lacked the C-terminal helix structure that exists in both MlaF of *A. veronii* and *A. caviae* (Figure 5).

In order to investigate whether expression levels of these TCS were involved with discrepancy of polymyxin resistance, we selected one strain as a representative strain from each of the three species with the fewest intraspecific mutations based on multiple alignments of TCS genes to perform qRT-PCR. Transcriptional levels of *phoP/Q*, *envZ/ompR*, and *mlaF* in *A. hydrophila* S541 (PMB MIC>128 mg/L), *A. caviae* L965 (PMB MIC = 1 mg/L), and *A. veronii* L924 (PMB MIC = 2 mg/L) were detected by qRT-PCR. *A. hydrophila* S541 was acted as the control. As shown in Figure 6, compared with *A. hydrophila* S541, transcriptional levels of *phoP*, *envZ*, *ompR*, and *mlaF* decreased in *A. veronii* L924, while only expression levels of *phoP/Q* decreased in *A. caviae* L965. We inferred that upregulated TCS PhoPQ in the colistin-resistant strain *A. hydrophila* S541 might participate in the diversity of colistin resistance among the genus *Aeromonas*.

Discussion

Water, especially the sewage regeneration system is the medium for the spread of *Aeromonas* spp. (McLellan et al., 2010; Pablos et al., 2011). The epidemiological study of *Aeromonas* spp. and sewage metagenomic studies have reported that *A. caviae* strains could be isolated from drinking water samples and diarrhea feces. Chen *et al* confirmed that horizontal gene transfer (HGT) was the dominant way to spread drug resistance in the water phase (Chen et al., 2019). The aquatic environment could be a reservoir for the transmission of polymyxin-resistant bacteria.

TABLE 3 Amino acid variations in functional domains of PhoP/PhoQ, EnvZ/OmpR, and MlaF in *Aeromonas*.

Species	MIC	Strain	PhoP			PhoQ	OmpR	EnvZ						MlaF		
			REC	Trans_reg_c		Unknown	REC	Unknown	HAMP	Unknown		HATPase_c		AAA	AAA/unknown	
			39	69	153	82	233	30	108	198	302	339	369	407	63	231
A. hydrophila	>128	L652	Q	R	V	P	Y	F	G	Q	Q	L	P	H	S	A
A. hydrophila	>128	M894	Q	R	V	P	Y	V	G	Q	Q	L	P	H	S	A
A. hydrophila	>128	S461	Q	R	V	P	Y	F	G	Q	Q	L	P	H	S	A
A. hydrophila	>128	S541	Q	R	V	P	Y	F	G	Q	Q	L	P	H	S	A
A. caviae	1	L215	Q	S	T	P	Y	F	K	Q	Q	L	A	Y	S	E
A. caviae	1	L965	Q	T	T	P	Y	F	K	Q	Q	L	A	H	F	E
A. caviae	2	L7105	Q	T	T	P	Y	F	K	Q	Q	L	A	H	F	E
A. caviae	1	M621	Q	T	T	P	Y	F	K	Q	Q	L	A	H	S	E
A. veronii	2	L924	G	R	A	R	F	F	G	L	Y	L	P	H	S	D
A. veronii	1	L975	G	R	A	R	F	F	G	L	Y	L	P	H	S	D
A. veronii	2	M194	G	R	A	R	F	F	G	L	Y	Q	P	H	S	D
A. veronii	2	S5183	G	R	A	R	F	F	G	L	Y	Q	P	H	S	D

Q, glutamine (Gln); G, glycine (GLy); R, arginine (Arg); S, serine (Ser); T, threonine (Thr); V, valine (Val); A, alanine (Ala); P, proline (Pro); Y, tyrosine (Tyr); F, phenylalanine (Phe); K, lysine (Lys); L, leucine (Leu); H, histidine (His); E, glutamic acid (Glu); D, aspartic acid (Asp).

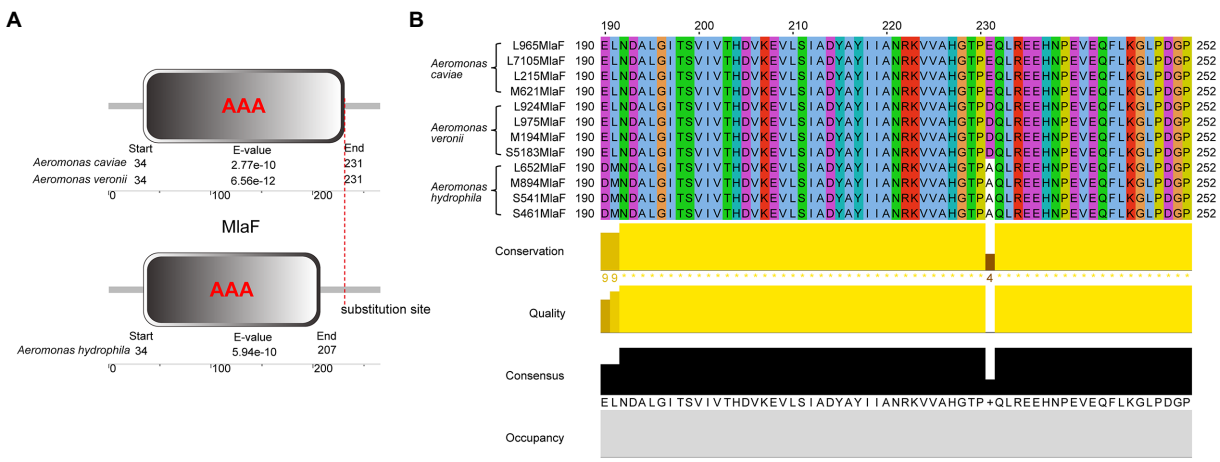

FIGURE 4
The structure and multiple sequence alignment of MlaF protein. **(A)** The structure of MlaF domain. **(B)** The multiple sequence alignment of MlaF from 225 position to the end in three species of the genus *Aeromonas*. The beautification of the image was used the Jalview 2.11.1.7 and the amino acid color was used Clustalx.

For example, the aquatic bacterium *Shewanella* and its associated aquaculture are the reservoir for the dissemination of *mcr-4* (Zhang et al., 2019a,b). It has been reported that *Aeromonas* may be the container for *mcr-3* and *mcr-7*, although it is not the inherent host of *mcr-3* (Parker and Shaw, 2011; Eichhorn et al., 2018; Shen et al., 2018). According to available research reports, it reveals that variants of *mcr-1* to *mcr-10* exhibit selectivity to some specific species. For example, *mcr-3* and *mcr-7* genes have been reported to locate on the chromosomes in the genus *Aeromonas* such as *A. hydrophila*, *A. veronii*, *A. caviae*, *A. jandaei* (Parker and Shaw, 2011; Ling et al., 2017). Our study proved this and showed that the genus *Aeromonas* and aquatic environment might be the potential container and reservoir of *mcr-3*.

Compared with abundant studies focused on polymyxin-resistant bacteria from clinics, less work has been carried out in the aquatic environment in China (Yang et al., 2017; Tuo et al., 2018; Pan et al., 2022). By phylogenetic tree analysis, we found that three *mcr-3* positive *Aeromonas* strains isolated from Yangtze River have a close evolution distance with clinical samples from China and animal samples from the United States, showing a wide spread of the *mcr-3* in *Aeromonas* strains. In this study, susceptibility of PMB between different species in the genus

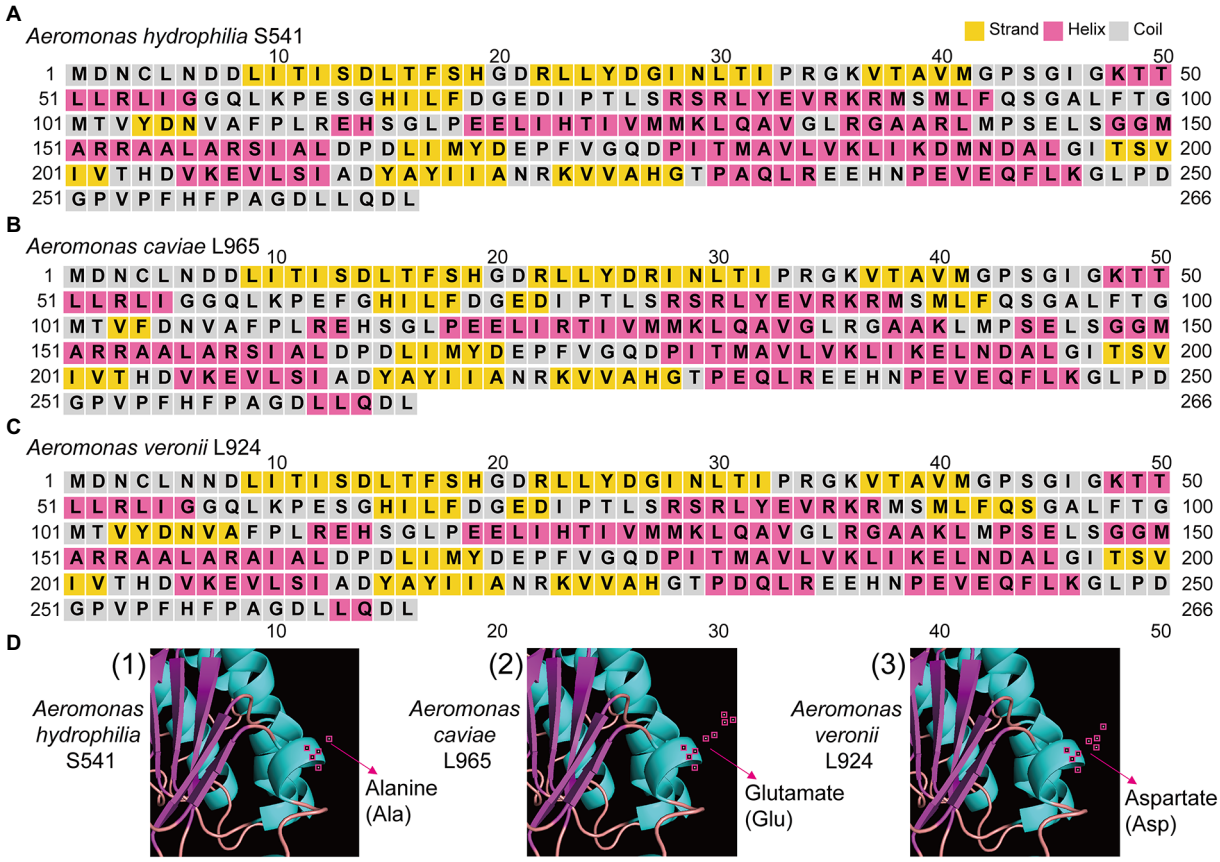

FIGURE 5
The structure of MlaF protein in three *Aeromonas* species. **(A)** The predicted secondary structure of MlaF protein in *A. hydrophilia* S541. **(B)** The predicted secondary structure of MlaF protein in *A. caviae* L965. **(C)** The predicted secondary structure of MlaF protein in *A. veronii* L924. **(D)** The predicted three-dimensional structure of MlaF protein in corresponding strains. The amino acid of 231 position of MlaF was pointed by red arrow.

Aeromonas exhibited a distinct discrepancy according to the results of MIC_{50} and MIC_{90} ($p < 0.001$). No *mcr* gene was found in *A. hydrophila*, but all the strains showed high-level resistance to PMB (8 mg/L ≤ MIC ≤128 mg/L). Three *mcr-3* positive strains were found in the *A. caviae* and *A. veronii* strains while most *A. caviae* and *A. veronii* isolates were susceptible to PMB (0.5 mg/L ≤ MIC ≤4 mg/L). Gene *mcr-3* in this study were all located on chromosomes as other studies have reported (Ling et al., 2017; Liu et al., 2020; Wang et al., 2021). This is the first report about the identification of *mcr-3.42* positive *A. veronii* strains isolated from the aquatic environment in China and was also the first report of the variant of polymyxin resistance phenotype in different species of the genus *Aeromonas*.

Genetic analysis in the surrounding gene–environment of the *mcr-3* positive isolates found that the *mcr-3*-like was truncated by IS*As20* (1,674 bp) and IS*As2* (1,084 bp) in two *A. veronii* isolates, leading to the separation of *mcr-3*-*mcr-3*-like segment. The truncated *mcr-3*-*mcr-3*-like segment is seldomly observed in the genus *Aeromonas*. Likewise, the *mcr-3*-like gene was also divided into two fragments by IS*4* in the *A. hydrophila* WP7-S18-ESBL-06. It indicated that the *mcr-3*-*mcr-3*-like segment was not so conserved that it could be divided by IS. In the upstream region of *mcr-3*, there also lied an IS. Wang *et al* have reported a novel transposon Tn6518 in the *A. veronii* w55, composing the genetic element IS*As2*-IS*Ahy2*-IS*As20*-*mcr-3.6*-*mcr-3*-like-*dgkA*-IS*As2* (Wang et al., 2020). In *A. veronii* FC951, *mcr-3.19* was inserted by an IS*As18* and become inactive, making the *A. veronii* FC951 susceptible to colistin (Ragupathi et al., 2020). Some IS family members, including IS*3*, IS*30*, IS*110*, IS*26*, and IS*CR1* elements, utilize circular DNA intermediates containing accessory genes to undergo gene translocation *via* copy-and-paste mechanisms. We supposed that the IS-gene-*mcr-3*-*mcr-3*-like-truncated-IS would be conducive to the spread of colistin resistance in different strains and leads to the dissemination of *mcr-3* in the aquatic environment. The three *mcr*-positive strains isolated from the Yangtze River were all susceptible to PMB. Among the reported *mcr-3* positive *Aeromonas*, most of the *A. veronii* and *A. caviae* were sensitive to polymyxins, while *A. hydrophila* were highly resistant to polymyxin (Ling et al., 2017; Shen et al., 2018; Hatrongjit et al., 2020; Huang et al., 2020; Wang et al., 2021). Therefore, we speculated that *mcr-3* might not be the main factor leading to the resistance to polymyxin among *Aeromonas*. One

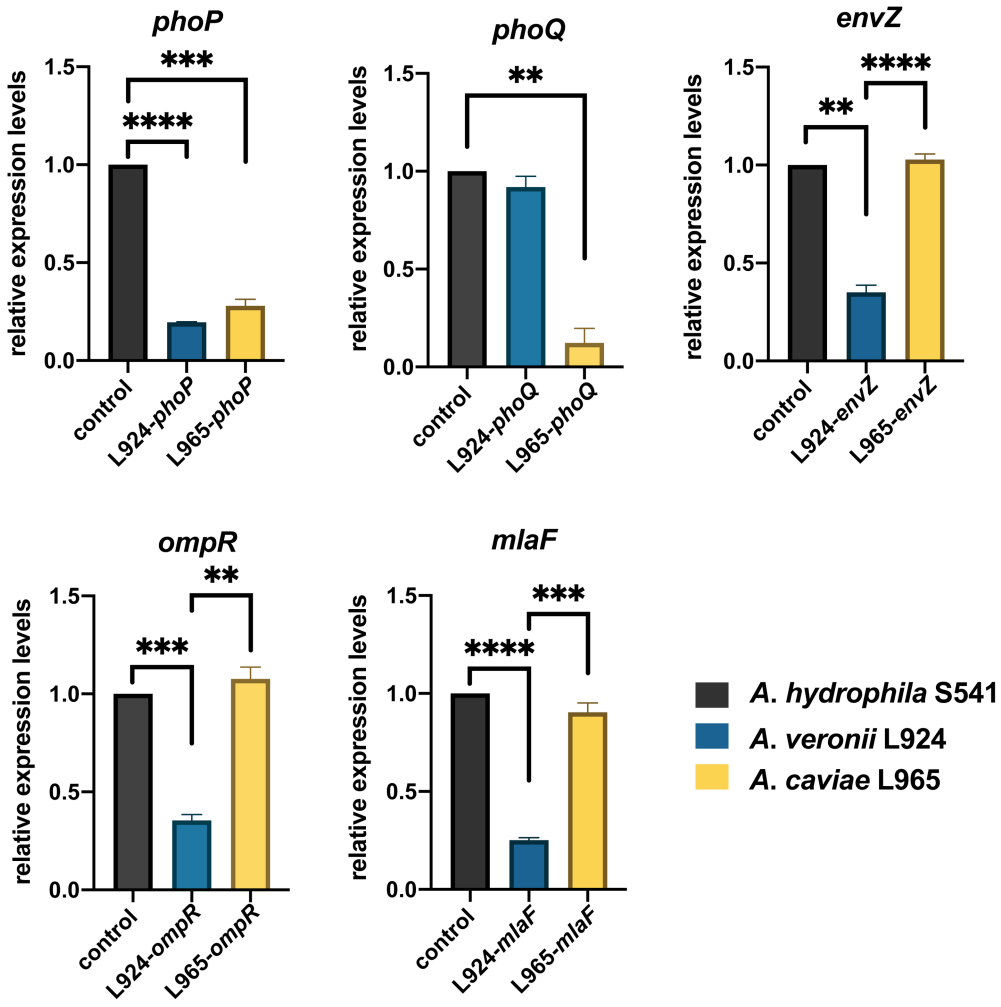

FIGURE 6
Gene expression of *A. hydrophila* S541, *A. veronii* L924, *A. caviae* L965. Relative expression levels of genes were determined using the $2^{-\Delta\Delta ct}$ method. Error bars represent the standard deviation of three biological replicates. $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$.

possible reason could be that the expression of *mcr-3* might not be activated due to the loss of expression elements during the process of the insertion of IS*As20* and IS*As2*.

Genome analysis of the isolates discovered that *arnBCADTEF* operon only existed in *A. hydrophila* genomes but not in *A. caviae* and *A. veronii* genomes, which might be a reason for the different phenotypes of polymyxin susceptibility among these *Aeromonas* strains. It has been reported that *A. hydrophila* could thrive in a complex colistin environment with the help of EnvZ/OmpR TCS and MlaF (Liu et al., 2021). We used these as the entry point and tried to investigate the possible cause for diversity in the colistin resistance in the *Aeromonas* genus by comparing gene sequences of PhoP/PhoQ TCS, EnvZ/OmpR TCS, and MlaF. Though some site mutations were found in PhoP/PhoQ and EnvZ/OmpR TCS, this nucleic acid substitution did not change the conformation structure of these proteins. Only nucleic acid changes in MlaF were predicted to impact the length of its domain. MlaF was one of the proteins of the Mla system that has played an essential role in phospholipid (PL) transport and constituted the inner membrane ABC transporter complex, MlaFEDB (Coudray et al., 2020). It is reported that MlaF was involved in the antibiotic resistance in *A. hydrophila* and the mutation in *mlaF* ($mlaF_{D173A}$) confers high-level colistin resistance *via* upregulation of the Mla system (Liu et al., 2021; Zhou et al., 2021). In this study, the substitution at the 231 position of the MlaF was predicated to be deleterious and this substitution may affect the biological function when the acidic amino acid that is E or D in the susceptible strains (*A. caviae* and *A. veronii*) is substituted for the neutral amino acid that is A in the resistant strains (*A. hydrophila*). We infer that the substitution at 231 position in MlaF in the susceptible strains (*A. caviae* and *A. veronii*) may alter the steric hindrance and may be related to the polymyxin resistance in different species. In addition, MlaF subunit possesses a unique ~25 aa C-terminal extension (CTE) forming a domain-swapped reciprocal 'handshake' that interacts with the adjacent MlaB to fulfill the function (Kolich et al., 2020). And results of the secondary structure alignment of MlaF showed that helix existed in

the susceptible strains (*A. caviae* and *A. veronii*, Figures 5B,C), not in the resistant strains (*A. hydrophila*, Figure 5A), which might have an influence on the interaction with MlaB, hence affecting the Mla system. In addition, the expression level of *mlaF* in *A. hydrophila* S541 and *A. caviae* L965 was much higher than that in *A. veronii* L924 (Figure 6). We supposed that the higher expression of *mlaF* might cause an increased level of PL and maintain the membrane homeostasis so as to withstand the polymyxin. Thus, we inferred that the amino acids differences in MlaF protein and its expression levels among different *Aeromonas* species could affect the function of transportation in the Mla system which is involved in the synthesis of membrane components and contribute to the resistance to polymyxins. In general, we speculated that the existence of *arnBCADTEF* and sequence differences in MlaF might contribute to the variety of polymyxin susceptibility in different species of the genus *Aeromonas*.

In sum, this is the first report describing the variety in the phenotype of polymyxin susceptibility in different species of the genus *Aeromonas*. And our study also identified two novel *mcr-3.42* positive *A. veronii* strains and one *mcr-3.16* positive *A. caviae* strain in the aquatic environment in China for the first time. We provided pieces of evidence of the possible mechanism for the different polymyxin susceptibility in different species of the genus *Aeromonas*, but the exact mechanism deserved further research.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

QL conceived and designed the experiments. LX, HF, and JF performed the experiments. YY, QL, and HF contributed to the reagents, materials, and analysis tools. LX wrote the original draft. QL and FW checked and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Mobile colistin resistance (MCR), extended-spectrum beta-lactamase (ESBL) and multidrug resistance monitoring in *Escherichia coli* (commensal and pathogenic) in pig farming: need of harmonized guidelines and clinical breakpoints

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Current data on antimicrobial resistance in pig production is essential for the follow-up strategic programs to eventually preserve the effectiveness of last-resort antibiotics for humans. Here, we characterized 106 *Escherichia coli* recovered in routine diagnosis (2020–2022) from fecal sample pigs, belonging to 74 Spanish industrial farms, affected by diarrhea. The analysis of virulence-gene targets associated with pathotypes of *E. coli*, determined 64 as pathogenic and 42 as commensal. Antimicrobial susceptibility testing (AST) performed by minimal inhibitory concentration (MIC) assay, was interpreted by applying breakpoints/cut-off values from the different standards EUCAST/TECOFF 2022, CLSI VET ED5:2020, and CASFM VET2020. Comparisons taking EUCAST as reference exhibited moderate to high correlation except for enrofloxacin, neomycin, and florfenicol. Of note, is the lack of clinical breakpoints for antibiotics of common use in veterinary medicine such as cefquinome, marbofloxacin, or florfenicol. AST results determined multidrug resistance (MDR) to ≥3 antimicrobial categories for 78.3% of the collection, without significant differences in commensal *vs* pathogenic isolates. Plasmid-mediated mobile colistin resistance gene (*mcr*) was present in 11.3% of 106 isolates, all of them pathogenic. This means a significant decrease compared to our previous data. Furthermore, 21.7% of the 106 *E. coli* were ESBL-producers, without differences between commensal and pathogenic isolates, and *mcr*/ESBL genes co-occurred in 3 isolates. Phylogenetic characterization showed a similar population structure (A, B1, C, D, and E), in both commensal

and pathogenic *E. coli*, but with significant differences for B1, C, and E (38.1 *vs* 20.3%; 19 *vs* 1.6%; and 7.1 *vs* 25%, respectively). Additionally, we identified one B2 isolate of clone O4:H5-B2-ST12 (CH13-223), positive for the uropathogenic (UPEC) status, and *in silico* predicted as human pathogen. We suggest that a diagnosis workflow based on AST, detection of *mcr* and ESBL genes, and phylogenetic characterization, would be a useful monitoring tool under a "One-Health" perspective.

KEYWORDS

***Escherichia coli*, MCR, ESBL, colistin, fluoroquinolones, swine, multidrug resistance, EUCAST**

Introduction

Currently, antimicrobial stewardship programs are being implemented in all environments under a "One Health" perspective, including hospital, community, and agricultural settings (McEwen and Collignon, 2018). Following the European Medicines Agency's (EMA) advice on the use of antibiotics, the Spanish Medicines and Healthcare Products Agency (AEMPS) launched in 2014 the National Plan against Antibiotic Resistance (PRAN) as a strategic action plan to reduce the risk of selection and dissemination of antibiotic resistance and, consequently, reduce the impact on human and animals' health, sustainably preserving the effectiveness of existing antibiotics. At the end of 2016, representatives of the national associations of veterinarians and professionals from the pig production sector signed, together with PRAN, the agreement for the voluntary Reduction of Colistin Consumption in the Pig Farming Sector ("REDUCE" program). The main aim of this alliance was to reduce the consumption of colistin to 5 mg/population correction unit (PCU) in 3 years and control the alternative consumption of neomycin and/or apramycin. Six years after the beginning of the program, the result is a reduction of almost 100% in the use of colistin in this sector, from 52 to 0.4 mg/PCU (European Medicines Agency, 2021).

Swine colibacillosis is one of the major challenges for the pig industry worldwide due to the high morbidity and mortality rates, and derived costs from prevention and antimicrobial treatment. This multifactorial syndrome caused by *Escherichia coli*, exhibits three main disease conditions (edema disease, neonatal and post-weaning diarrhea), which can be differentiated by the age range of the affected animals, pathogenesis, and *E. coli* pathotype. Among all, enterotoxigenic *E. coli* (ETEC) is the most prevalent pathotype, together with Shiga toxin–producing *E. coli* (STEC) and atypical enteropathogenic *E. coli* (aEPEC). Hybrid strains (ETEC/STEC) are also relatively common (Luppi, 2017; García-Meniño et al., 2018).

Our previous studies on antibiotic resistance in Spanish pig farming (2005–2017), revealed a high prevalence of colistin resistance (76.9%), associated with the presence of plasmidic *mcr* genes (mainly *mcr-4*, but also *mcr-1* and *mcr-5*), and multidrug resistance (MDR; >85%) within 186 ETEC, STEC and ETEC/STEC isolates implicated in post-weaning diarrhea (PWD) (García-Meniño et al., 2021). Remarkably, the potentially zoonotic of the extraintestinal pathogenic *E. coli* (ExPEC) lineage B2-ST131 (García-Meniño et al., 2018) was identified in 3.6% of 499 *E. coli* (18 ST131 isolates, including 7 *mcr* carriers) from diarrheal pigs, some of them showing genetic and genomic similarities with human clinical isolates (García-Meniño et al., 2018; Flament-Simon et al., 2020a).

We are currently facing big challenges in the use of antibiotics in veterinary medicine. On the one hand, in January 2022 the new legislation on veterinary medicines [Regulation (EU) 2019/6; Reglamento (UE), 2019] and medicated feed [Regulation (EU) 2019/4; Reglamento (UE), 2019] came into force, which, among others, limits the use of antibiotic prophylaxis and metaphylaxis. Besides, the EMA recently proposed a new categorization of antimicrobials, due to the risk of resistance development associated with use in animals and the potential impact on humans. This proposal establishes four categories, with Category A ("Avoid") including those antimicrobials not currently authorized in veterinary medicine in the European Union, such as fosfomycin or monobactams; and with Category B ("Restrict") including those restricted in animals to mitigate the risk to public health, namely, quinolones, broad-spectrum cephalosporins and polymyxins (EMA/688114/, 2020).

In the present study, we characterized a collection of 106 *E. coli* recovered in routine diagnosis (2020–2022) from fecal sample pigs, belonging to 74 Spanish industrial farms, affected by diarrhea, with two main aims: (i) to gain knowledge on the status of antimicrobial resistance in Spanish swine production; (ii) to analyze diagnosis and clinical difficulties in the veterinary sector when providing the best antibiotic therapy option in food production pigs.

Materials and methods

Samples and *Escherichia coli* collection

The study collection analyzed here, consisted of 106 swine *E. coli* isolates recovered from 93 stool samples of pigs reared on

74 farms, managed by 35 different pig companies. The farms involved here, belong to 19 Spanish provinces of those Autonomous Communities (10 out of 17) with the highest census of pig livestock in 2020, according to data from the Ministry of Agriculture, Fisheries and Food (Government of Spain): Aragon, Cataluña, Castilla y León, Andalucía, Región de Murcia, Extremadura, Castilla-La Mancha, Galicia, Comunidad Valenciana, Comunidad Foral de Navarra. Three production stage groups were included: lactation, transition, and fattening (38, 49 and 6 samples, respectively). The samples, either fecal swabs or isolation plates of MacConkey agar (LMAC, Oxoid), were received at the Spanish Reference Laboratory of *E. coli* (LREC, USC), and tested for routine diagnosis of enteric colibacillosis between September 2020 and April 2022. A brief voluntary questionnaire was performed about the use of specific drugs (ceftiofur, enrofloxacin/marbofloxacin, neomycin, colistin, sulfonamides, florfenicol, tiamulin) for infectious treatment in the farms involved in this study.

Briefly, the confluent growth from the fecal swabs plated on lactose MacConkey agar (LMAC, Oxoid), and incubated at 37°C for 18–24 h, was subjected to polymerase chain reaction (PCR) to detect the presence of ETEC, STEC, and EPEC using specific genetic targets encoding toxins (LT, STa, STb, and Stx2e), intimin (Eae) and fimbriae (F4, F5, F6, F18, and F41) as previously detailed (García-Meniño et al., 2021) (Supplementary Table S1). Additionally, confluents were also screened for *rbf*O25 associated with the pandemic lineage B2-ST131 (Clermont et al., 2008) (Supplementary Table S2). From PCR-positive confluents, different *E. coli*-like colonies (up to 10) were selected and plated on tryptone soy agar (TSA, Oxoid), which were in turn individually analyzed by PCR. Per sample, all *E. coli* colonies with different virulence-gene profiles were stored at room temperature in nutrient broth (Difco) with 0.75% nutrient agar (Difco) for further characterization. Likewise, from plates with negative confluents for all virulence targets, one *E. coli*-like colony was tested to confirm the status of non-pathogenic *E. coli* and conserved. PCR amplification of the β-d-glucuronidase-encoding gene (*uidA*) was routinely used to confirm the identity of the species *E. coli* (Gómez-Duarte et al., 2010) (Supplementary Table S2), which was further confirmed at the Laboratorio de Sanidade Animal de Galicia (LASAPAGA, Lugo, Spain) by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF; Bruker Daltonik, Bremen, Germany) after conventional culture. The phylogroup of the isolates was established according to the PCR-based method developed by Clermont et al. (2013, 2019) (Supplementary Table S3), which recognizes eight phylogroups belonging to *E. coli sensu stricto* (A, B1, B2, C, D, E, F, and G).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of the 106 *E. coli* isolates were determined at the LASAPAGA using the VITEK® 2 (bioMérieux, Inc., Hazelwood, MO, United States) systems for conventional AST (bioMérieux, Spain) with AST-GN96 test kit cards, following the manufacturer's recommendations. The following drugs and categories were included in the analysis: aminopenicillins (ampicillin); aminopenicillins in combination with beta-lactamase inhibitor (amoxicillin/clavulanic acid, ticarcillin/clavulanic acid); non-broad spectrum cephalosporins (cefalexin and cefalotin); broad-spectrum cephalosporins (cefoperazone, ceftiofur, and cefquinome); carbapenems (imipenem); fluoroquinolones (enrofloxacin, flumequine, and marbofloxacin); aminoglycosides (gentamicin and neomycin); tetracyclines (tetracycline); polymyxins (polymyxin B); sulfonamides, dihydrofolate reductase inhibitors, and combinations (trimethoprim, trimethoprim/sulphamethoxazole); amphenicoles (florfenicol). The florfenicol resistance was double tested for a representative group of 42 isolates using MIC and disk diffusion assays. In addition, and due to our previous data of high prevalence of resistance to chloramphenicol, susceptibility to this antimicrobial was determined by disk diffusion for the whole collection. AST results were interpreted following the recommendation of PRAN (AEMPS) and other European authorities (EMA, ESCMID, ECDC), which prioritizes the use of the clinical standard breakpoints established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2022) when available. As an alternative, epidemiological cut-off values (ECOFF) or tentative ECOFF (TECOFF) were applied (Table 1). ECOFFs (and TECOFFs) distinguish microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type, N-WT) to the agent in question. In addition, the veterinary microbiology laboratory standards from the Clinical & Laboratory Standards Institute (CLSI VET ED5:2020) (CLSI VET01SED5, 2020) and the veterinary recommendations from the Comité de l'antibiogramme de la Société Française de Microbiologie (CASFM VET2020) (CASFM Vétérinaire, 2020) were also visited (Table 1). Based on AST results, the isolates were classified as MDR if displayed resistance to a drug of ≥3 of the different antimicrobial categories (Magiorakos et al., 2012).

Screening and typing of *mcr* and ESBL genes

By PCR, the presence of *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5 genes was screened within the collection as described elsewhere (García et al., 2018). Likewise, genetic identification of the ESBLs was performed using the SHV, CTX-M-1 and CTX-M-9 group-specific primers followed by amplicon sequencing (García-Meniño et al., 2018, 2021) (Supplementary Table S4).

Whole genome sequencing, assembly, and *in silico* analysis

Since the prevalent global ExPEC lineages implicated in human and animal infections, such ST131, belong to phylogroup

TABLE 1 Antimicrobials, breakpoints/cut-off values and interpretation reviewed in the present study.

Antimicrobial	[a]Breakpoints CLSI VET ED5:2020			[b]Breakpoints CASFM (VET) 2020		[c]EUCAST 2022 *(T)ECOFF	[d]Breakpoints/cut-off values applied in this study		
	"R"	"I"	"S"	"R"	"S"	"N-WT" >	AST Standards	"R"	"S"
Ampicillin	≥32 (humans)	16	≤8	–	–	8	EUCAST 2022 CLIN	>8	≤8
Amoxicillin-Clavulanic Acid	≥32/16 (humans)	16/8	≤8/4	>16/8	≤4/2	–	EUCAST 2022 CLIN	>8	≤8
Ticarcillin/Clavulanic Acid	–	–	–	–	–	(16)	EUCAST 2022 CLIN	>16	≤8
Cefalexin (1[a])	≥32 (humans) from cefazolin	–	≤16	>32	≤8	(32)	EUCAST 2022 CLIN	>16	≤16
Cefalothin (1[a])	–	–	–	–	–	32	EUCAST 2022 ECOFF	>32	≤32
Cefoperazone (3[a])	–	–	–	>32	≤4	(1)	EUCAST 2022 TECOFF	>1	≤1
Ceftiofur (3[a])	≥ 8 (swine; respiratory; *S.* Choleraesuis)	4	≤2	>4	≤2	1	EUCAST 2022 ECOFF	>1	≤1
Cefquinome (4[a])	-	–	–	>4	≤2	–	CASFM 2020	>4	≤2
Imipenem	≥4 (humans)	2	≤1	–	–	0.5	EUCAST 2022 CLIN	>4	≤2
Flumequine	–	–	–	>8	≤4	2	EUCAST 2022 ECOFF	>2	≤2
Enrofloxacin	–	–	–	>2	≤2	0.125	EUCAST 2022 ECOFF	>0.125	≤0.125
Marbofloxacin	–	–	–	>2	≤2	–	CASFM 2020	>2	<=2
Gentamicin	≥16 (humans)	8	≤4	>4	≤2	2	EUCAST 2022 CLIN	>2	≤2
Neomycin	–	–	–	>16	≤8	8	EUCAST 2022 ECOFF	>8	≤8
Tetracycline	≥16 (humans)	8	≤4	>8	≤4	8	EUCAST 2022 ECOFF	>8	≤8
Polymyxin B / Colistin	–	–	–	>2	≤2	2	EUCAST 2022 CLIN	>2	≤2
Trimethoprim/Sulfamethoxazole	≥4/76 (humans)	–	≤2/38	> 8/152	≤ 2/38	0.5	EUCAST 2022 CLIN	>4	≤2
Florfenicol-MIC (N = 42)	≥ 16 (swine; respiratory; *S.* Choleraesuis)	8	≤4	–	–	16	EUCAST 2022 ECOFF	>16	≤16
Florfenicol-disk 30 µg (N = 42)	–	–	–	<19	≥19	–	CASFM 2020	<19	≥19
Chloramphenicol-disk 30 µg (N = 106)	≤12	13–17	≥18	<19	≥19	–	EUCAST 2022 CLIN	<17	≥17

[a]"R," resistant (comments on the species in which it is applied; pathological process; microorganism); "I," intermediate; "S," susceptible; –, not available.

[b]"R," resistant; "S," susceptible; –, not available.

[c]Epidemiological cut-off values (ECOFF) and tentative epidemiological cut-off values *(TECOFF); "N-WT," non-wild type; –, not available.

[d]EUCAST 2022 CLIN, results were interpreted, on a priority basis, according to the clinical (CLIN) breakpoints of the European Committee on Antimicrobial Susceptibility Testing (AST) EUCAST 2022; alternatively, ECOFF or TECOFF were applied; and if there was no option, CLSI VET ED5:2020 and CASFM VET2020 breakpoints were visited; "R," resistant; "S," susceptible. Numerical values are expressed in mg/l.

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B2 (Mora et al., 2013; Flament-Simon et al., 2020b), one isolate assigned by PCR to this phylogroup was further investigated by WGS. DNA was extracted and quantified as detailed in García-Meniño et al. (2019). Briefly, DNA was extracted with the DNeasey Blood and Tissue Kit (Qiagen, Hilen, Germany) according to the manufacturer's instructions. After extraction, the DNA was quantified by an Invitrogen Qubit fluorimeter (Thermo Fisher Scientific, Massachusetts) and assessed for purity using a NanoDrop ND-1000 (Thermo Fisher Scientific, Massachusetts). DNA sequencing was performed using Illumina technology with a NovaSeq 6,000 S4PE150 XP system to obtain 150 bp paired-end reads at Eurofins Genomics (Eurofins Genomics GmbH, Konstanz, Germany), after a standard library preparation (unique dual indexing, that has distinct, unrelated index sequences for each of the i5 and i7 index reads). The quality of the paired-end Illumina reads was evaluated using FastQC. The reconstruction of the genome and *in silico* analysis was performed as described elsewhere (García-Meniño et al., 2019). Briefly, the raw reads were assembled with the VelvetOptimiser.pl. script implemented in the "on line" version of PLAsmid Constellation NETwork (PLACNETw). PLACNETw is a tool implemented for the reconstruction of plasmids from next-generation sequence pair-end. It allows the manual pruning of the graph representation.[1] The assembled contigs, with genomic size 5.0 Mbp (Supplementary Table S5), were analyzed using the bioinformatics tools of the Center for Genomic Epidemiology (CGE)[2] as specified, and applying the thresholds suggested by default when required (minimum identity of 95% and coverage of 60%): for the presence of acquired genes and or chromosomal mutations mediating antimicrobial resistance (ResFinder 4.1.), for identification of acquired virulence genes (VirulenceFinder 2.0), plasmid replicon types (PlasmidFinder 2.1/pMLST 2.0), and identification of clonotypes (CHTyper 1.0), and serotypes (SeroTypeFinder 2.0). For the phylogenetic typing, two different MLST (2.0) schemes were applied. Additionally, cgMLSTFinder1.2 was applied for the core genome multi-locus typing (cgMLST) from the raw reads of the isolates. The bacteria's pathogenicity towards human hosts was predicted using PathogenFinder 1.1.

Statistical analysis

Pairwise comparisons were performed by a two-tailed Fisher's exact probability test,[3] namely: phylogroup distribution was correlated with production stage (lactation *vs* transition), with pathogenic status (commensal *vs* pathogenic group), and MDR. In addition, correlations between pathotypes and colonization factors with respect to the three stages of production, and differences between commensal and pathogenic isolates regarding antimicrobial resistance, *mcr*- and ESBL-production, were also compared. p values < 0.05 were considered statistically significant.

Results

In this study, 93 individual consecutive fecal samples from diarrheal pigs were received between September 2020 and April 2022, which were subjected to the routine diagnosis of colibacillosis at the LREC, USC. As a result, the confluent growth of LMAC plates from 42 individuals, as well as the isolated colonies (up to 10), tested negative by PCR for all virulence traits (toxins, intimin, and fimbriae) associated with diarrheagenic *E. coli* pathotypes. One *E. coli*-like of those negative colonies, classified as commensal *E. coli*, was included here for comparative purposes. On the other hand, 51 individuals (54.8%) tested positive on the confluent growth for any of the virulence traits analyzed here, with the recovery of 64 pathogenic *E. coli* (from 10 samples, up to 3 different isolates were detected based on their distinct virulence-gene profiles) (Table 2; Supplementary Table S6, columns A, B, and S–AK). From the voluntary questionnaire carried out on the use of antibiotic therapy, we learned that 93.2% of farms reported the use of fluoroquinolones (enrofloxacin/marbofloxacin), 63.5% ceftiofur, 44.6% neomycin, 29.7% sulfonamides, 10.8% colistin, 9.5% florfenicol, and 9.5% tiamulin (Supplementary Table S6; columns L–R).

Pathogenic *Escherichia coli*: Virulence profiles, phylogroups, and pairwise comparisons

ETEC was the most prevalent pathotype (78.1%) found among the 64 diarrheagenic isolates, positive for enterotoxin-encoding genes (*eltA* and/or *estA*, and/or *estB*). The remaining isolates were assigned to STEC pathotype (7.8%) positive for *stx2e* (Shiga toxin); aEPEC (6.2%) positive for *eae*; and ETEC/STEC (4.7%) positive for both Shiga toxin and enterotoxin-encoding genes. Additionally, two isolates did not fit the definition of any pathotype since tested positive only for F41 fimbriae. Regarding the other intestinal colonization factors, F18 was the most prevalent fimbriae detected (40.6%), followed by F4 (25.0%). Overall, the most common virulence profiles determined (> 5 isolates) were: STa, STb, LT, F18 (23.4%); STa, STb, F4 (14.1%); STb (14.1%) and STb, LT, F4 (9.4%) (Supplementary Table S6, columns T–AF; Table 2). Pairwise comparisons of pathotypes and colonization factors for the three stages of production, only showed a statistically significant association ($p < 0.05$) for F18 fimbria with the fattening and transition isolates.

By the quadruplex PCR described by Clermont et al. (2013, 2019), 5 phylogroups (A, B1, C, D, and E) were identified. Of them, phylogroup A was the most prevalent (48.4%) followed by E (25%) and B1 (20.3%). Prevalence differences were observed by origin of isolation, specifically within the isolates recovered from lactating pigs, where B1 was more prevalent (43.7%), followed by A (25%)

1 https://github.com/LuisVielva/PLACNETw
2 https://www.genomicepidemiology.org/services/
3 http://vassarstats.net/tab2x2.html

TABLE 2 Origin of samples, and main characteristics of the *Escherichia coli* collection analyzed in this study.

Samples and isolates		Commensal *E. coli*		Pathogenic *E. coli*: No. isolates and main characteristics		Pathotypes				Main colonization factors		
Production stage (approx. age range) No. animals sampled	**No. isolates**	**No. isolates**	**Phylogroups (No.; %)**	**No. isolates**	**Phylogroups (No.; %)**	**ETEC No. (%)**	**STEC No. (%)**	**ETEC /STEC No. (%)**	**aEPEC No. (%)**	**F4 (K88) No. (%)**	**F18 No. (%)**	**Other VF**
Lactation-pre weaning stage (1 day-4 weeks) n = 38	41	25	A (10; 40%) B1 (11; 44%) C (2; 8%) D (1; 4%) E (1; 4%)	16	A (4; 25%) B1 (7; 43.7%) D (2; 12.5%) E (3; 18.7%)	13 (81.2%)	0	0	1 (6.2%)	4 ETEC (25%)	1 ETEC (6.2%)	2 (F41)
Transition-growing stage (>4–9 weeks) n = 49	56	16	A (2; 12.5%) B1 (5; 31.2%) B2 (1; 6.2%) C (6; 37.5%) E (2; 12.5%)	40	A (21; 52.5%) B1 (5; 12.5%) C (1; 2.5%) D (1; 2.5%) E (12; 30%)	32 (80.0%)	4 (10.0%)	3 (7.5%)	1 (2.5%)	11 ETEC (27.5%)	22: 16 ETEC, 3 ETEC/STEC, 3 STEC (55%)	0
Fattening-finishing stage (>9 weeks) n = 6	9	1	A (1; 100%)	8	A (6; 75%) B1 (1; 12.5%) E (1; 12.5%)	5 (62.5%)	1 (12.5%)	0	2 (25%)	1 ETEC (12.5%)	3 ETEC (37.5%)	0
TOTAL	**93**	**106**	**42**	**B1 (16; 38.1%) A (13; 30.9%) C (8; 19%) E (3; 7.1%) B2 (1; 2.4%) D (1; 2.4%)**	**64**	**A (31; 48.4%) E (16; 25%) B1 (13; 20.3%) D (3; 4.7%) C (1; 1.6%)**	**50 (78.1%)**	**5 (7.8%)**	**3 (4.7%)**	**4 (6.2%)**	**16 (25.0%)**	**26 (40.6%)**	**2 (3.1%)**

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(Supplementary Table S6, column AK; Supplementary Table S7; Table 2).

Commensal *Escherichia coli*: Phylogroups and pairwise comparisons

The group of 42 *E. coli* negative for all virulence-associated genes with porcine diarrheagenic pathotypes exhibited 6 phylogroups (A, B1, B2, C, D, and E). Of them, B1 (38.1%) was the most prevalent, followed by A (30.9%) and C (19%) (Table 2). Pairwise comparisons regarding origin of isolation showed a significant difference ($p < 0.05$) in prevalence of phylogroup C (8% lactation *vs* 37.5% transition).

Overall, the commensal *E. coli* exhibited a similar phylogenetic structure to that of the pathogenic group, but with significant differences in the prevalence of B1, C, and E (38.1 *vs* 20.3%; 19 *vs* 1.6% and 7.1 *vs* 25%, respectively) (Supplementary Table S7). Besides, phylogroup B1 was significantly associated with the lactating group of isolates.

Antimicrobial susceptibility and genotypic characterization of ESBL and *mcr* genes

MIC values determined for the 106 *E. coli* isolates showed the highest levels of resistance (>50%) to ampicillin, tetracycline, enrofloxacin (non-wild type, N-WT), flumequine (N-WT), and trimethoprim/sulfamethoxazole (Table 3). Noticeably, we found resistant/N-WT isolates for all antibiotics, except for imipenem (category A of the EMA). Broad-spectrum cephalosporins and polymyxin resistances (category B of the EMA) were exhibited by 16–21.7 and 8% of isolates, respectively. Additionally, disk diffusion assay revealed that 50.9% of the 106 *E. coli* isolates were

TABLE 3 Antimicrobial resistances and pairwise comparisons.

[a]Antimicrobial	[b]EMA	[c]Breakpoints/cut-off values applied in this study	"R"	Total Analyzed ($N = 106$)		Commensal *E. coli* ($N = 42$)		Pathogenic *E. coli* ($N = 64$)		[d]Two-tailed *p* value
				No "R"	% "R"	No. "R"	%"R"	No. "R"	% "R"	
Ampicillin	D	EUCAST 2022 CLIN	>8	90	**84.9**	36	**85.7**	54	**84.4**	1.00
Amoxicillin–Clav. Acid	C	EUCAST 2022 CLIN	>8	3	2.8	2	4.8	1	1.6	0.56
Ticarcillin/Clav. Acid	C	EUCAST 2022 CLIN	>16	11	10.4	4	9.5	7	10.9	1.00
Cefalexin (1[a])	C	EUCAST 2022 CLIN	>16	23	21.7	13	31.0	10	15.6	0.09
Cefalothin (1[a])	C	EUCAST 2022 ECOFF	>32	24	22.6	13	31.0	11	17.2	0.15
Cefoperazone (3[a])	B	EUCAST 2022 (T)ECOFF	>1	17	16.0	9	21.4	8	12.5	0.28
Ceftiofur (3[a])	B	EUCAST 2022 ECOFF	>1	23	21.7	13	31.0	10	15.6	0.09
Cefquinome (4[a])	B	CASFM 2020	>4	17	16.0	9	21.4	8	12.5	0.28
Imipenem	A	EUCAST 2022 CLIN	>4	0	0.0	0	0.0	0	0.0	-
Flumequine	B	EUCAST 2022 ECOFF	>2	78	**73.6**	29	**69.0**	49	**76.6**	0.50
Enrofloxacin	B	EUCAST 2022 ECOFF	>0.125	81	**76.4**	30	**71.4**	51	**79.7**	0.36
Marbofloxacin	B	CASFM 2020	>2	32	30.2	16	38.1	16	25.0	0.19
Gentamicin	C	EUCAST 2022 CLIN	>2	33	31.1	10	23.8	23	35.9	0.21
Neomycin	C	EUCAST 2022 ECOFF	>8	41	38.7	7	16.7	34	53.1	**0.00**
Tetracycline	D	EUCAST 2022 ECOFF	>8	82	**77.4**	31	**73.8**	51	**79.7**	0.64
Polymyxin B	B	EUCAST 2022 CLIN	>2	8	7.5	0	0.0	8	12.5	**0.02**
Trimethoprim/ Sulfamethoxazole	D	EUCAST 2022 CLIN	>4	69	**65.1**	24	**57.1**	45	**70.3**	0.21
Florfenicol-MIC ($N = 42$)	C	EUCAST 2022 ECOFF	>16	13	31.0	4	21.1	9	39.1	0.32
Florfenicol-disk 30 µg ($N = 42$)	C	CASFM 2020	<19	16	38.1	7	36.8	9	39.1	1.00
Chloramphenicol-disk 30 µg ($N = 106$)	C	EUCAST 2022 CLIN	<17	54	**50.9**	18	42.9	36	**56.3**	0.23

[a]Minimal inhibitory concentrations (MICs) of the 106 *E. coli* isolates were determined for all antibiotics showed in the column with the exception of florfenicol, which was tested in parallel by means of MIC and diffusion disk assays for a representative group of 42 isolates. In addition, susceptibility to chloramphenicol was determined in the whole collection by disk diffusion.

[b]Categorization of antimicrobials proposed by the European Medicines Agency (EMA).

[c]"R," resistant breakpoint (EUCAST 2022 CLIN, CLSI VET ED5:2020, CASFM VET2020), or epidemiological cut-off value ((T)ECOFF); highlighted in bold, prevalences ≥ 50%.

[d]Two-tailed Fisher's exact probability test; *p* values <0.05 were considered statistically significant (in bold).

chloramphenicol resistant. Lastly, the parallel assay for florfenicol (MICs and disk diffusion) performed for a representative group of 42 isolates showed moderate discrepancies (31% *vs* 38.1% of N-WT/resistant isolates, respectively), affecting AST results close to the cut-off/breakpoint values (MIC > 16 N-WT; disk-diffusion $R < 19$, respectively) (Table 3; Supplementary Table S6, columns AL–BY). Comparing the two groups of commensal isolates *vs* pathogenic *E. coli*, only two significant differences were observed, namely, against neomycin (16.7 *vs* 53.1, respectively) and polymyxin B (0 *vs* 12.5%, respectively; $p < 0.05$).

Of the 106 isolates, 83 (78.3%) showed resistance to at least one agent in ≥3 antimicrobial categories, which were defined as MDR (Magiorakos et al., 2012), with no significant difference between commensal *vs* pathogenic isolates. However, MDR to ≥6 antimicrobial categories, were significantly associated with pathogenic *E. coli* (48.4%) *vs* commensal (28.6%) isolates ($p < 0.05$) (Table 4). Regarding phylogroup distribution between the groups of MDR (≥3 antimicrobial categories) *vs* non-MDR, the phylogroup C appeared significantly associated with non-MDR isolates (4.8% *vs* 21.7%, respectively; $p < 0.05$) (Supplementary Table S7).

Carriage of ESBL genes was determined in 23 isolates, 13 commensal (31%) and 10 pathogenic (15.6%) *E. coli* ($p > 0.05$), originating from the three stages of production (14 lactation, 8 transition, and 1 from fattening). These 23 isolates were phenotypically predicted as ESBL by the VITEK® 2 system, with resistant MICs for all non-broad spectrum cephalosporins (23 isolates) and for the broad-spectrum cephalosporins: ceftiofur (23 isolates), cefoperazone (17 isolates), and cefquinome (17 isolates). The ESBL-typing by sequencing revealed the presence of SHV and CTX-M genes in 6 and 17 isolates, respectively. Most of the CTX-M positive sequences were classified as group 1 (CTX-M-1, 2 isolates; CTX-M-15, 2 isolates; CTX-M-32, 7 isolates; CTX-M-55, 1 isolate; one could not be typed), and 4 belonged to CTX-M of group 9 (CTX-M-14) (Table 4; Supplementary Table S6, columns CB–CG).

The 106 isolates were also screened for the presence of *mcr-1* to 5 genes, which determined that 12 isolates (11.3%) were *mcr* carriers (8 *mcr*-1 and 4 *mcr*-4), all conforming the ETEC pathotype. These *mcr*-positive isolates were recovered from the lactation and transition subgroups (3 and 9 isolates, respectively). Interestingly, 2 *mcr*-1 and 1 *mcr*-4 isolates were also ESBL-carriers (CTX-M-32 and CTX-M14, respectively). Phenotypically, 8 out of the 12 *mcr*-positive isolates exhibited resistance to polymyxins (MICs > 2), and all 32 isolates, positive for ESBL and/or *mcr*, exhibited MDR (Table 4; Supplementary Table S6, columns AL–CG). Notably, 10 ESBL isolates (2 commensal and 8 pathogenic *E. coli*) displayed MDR to ≥8 categories, which were recovered from the three stages of production (3 lactation, 6 transition and 1 fattening) (Supplementary Table S6, columns BZ–CG).

Supplementary Table S8 compares AST results obtained by applying breakpoints/cut-off values from different standards (EUCAST 2022, TECOFF 2022, CLSI VET ED5:2020, and CASFM VET2020). Clinical breakpoints are missing within the three standards for veterinary antibiotics such as cefquinome, marbofloxacin, or florfenicol.

TABLE 4 Prevalence of MDR, ESBL and *mcr* within the 106 *E. coli* and pairwise comparisons.

Status	Total *E. coli* ($N = 106$)		Commensal *E. coli* ($n = 42$)		Pathogenic *E. coli* ($n = 64$)		[a]*p*
	No.	%	No.	%	No.	%	
MDR ≥ 3	83	78.3	32	76.2	51	79.7	0.81
MDR ≥ 6	43	40.6	12	28.6	31	**48.4**	**0.04**
ESBL	23	21.7	13	31.0	10	15.6	0.09
mcr	12	11.3	0	0.0	12	**18.8**	**0.00**

[a]Two-tailed Fisher's exact probability test. *p* values < 0.05 were considered statistically significant (in bold).

In silico characterization: O4:H5-B2-ST12 (CH13-223)

The PCR screening of *rfb*O25, performed to presumptively detect the pandemic ST131 clonal group of phylogroup B2, gave no positive result. However, this phylogroup was determined in one commensal isolate, which was further investigated by WGS to determine its virulence and potential zoonotic profile. Table 5 shows the analysis of the assembled contigs using the bioinformatics tools of the CGE (see footnote 2). Briefly, SeroTypeFinder predicted O4:H5 antigens, MLST tools assigned the sequence type (ST)12 of the Achtman 7-gene scheme and core genome ST (cgST) 44799 based on the Enterobase database, and CHTyper predicted the clonotype (CH)13–223, so that the clonal group eventually assigned to this isolate was O4:H5-B2-ST12 (CH13-223). The resistome analysis revealed the absence of chromosomal mutations but the presence of encoded mechanisms of acquired antibiotic resistance for beta-lactams (bla_{TEM}), aminoglycosides (*aph(3″)-Ib, aph(6)-Id*), sulphonamides (*sul2*) and tetracycline *tet*(A), which correlated with the phenotypic expression of resistance against ampicillin and tetracycline. VirulenceFinder revealed virulence traits associated with ExPEC lineages. In fact, the virulence profile predicted for this isolate conforms the status of extraintestinal pathogenic *E. coli* (ExPEC status ≥2 of these five virulence traits: *papA* and/or *papC*, *afa/dra*, *sfa/foc*, *iutA*, *kpsM* II) (Johnson et al., 2003), and the status of uropathogenic *E. coli* (UPEC status ≥3 of these four traits: *chuA*, *fyuA*, *vat* and *yfcV*) (Spurbeck et al., 2012). Furthermore, this genome was predicted as human pathogen (probability 88%) when analyzed with PathogenFinder. Regarding plasmid replicon types, PlasmidFinder identified an IncQ1 with 100% identity but with a coverage of 529/796. The PLACNET genome reconstruction (Supplementary Figure S1) showed a MOBQ relaxase within a 977 kb chromosomal contig, implying the presence of a potential integrative and conjugative element (ICE). Besides, a plasmid

TABLE 5 *In silico* characterization of the B2 commensal *E. coli*.

[a]ID code for isolate/ genome	[b]O:H antigens	[c]ST#1/ ST#2	[d]cgST	[e]CHType	[f]Acquired resistances	[g]Plasmid replicon	[h]Virulence genes	[i]Mobile genetic elements (and relation to AMR and virulence traits)	[j]Predicted as human pathogen (probability)
FVL196 / LREC_294	O4:H5	12 / 36	44799	13–223	*bla*$_{TEM-1B}$, *aph(3″)-Ib*, *aph(6)-Id*, *sul2*, *tet(A)*, *sitABCD*	IncQ1	*cea*, *chuA*, *clbB*, *cnf1*, *focC*, *focC/sfaE*, *fyuA*, *gad*, *hra*, *iroN*, *irp2*, *iss*, *kpsE*, *kpsMII*, *mchB*, *mchC*, *mchF*, *mcmA*, *ompT*, *papA_F11*, *papA_F14*, *papC*, *sfaD*, *sitA*, *tcpC*, *terC*, *usp*, *vat*, *yfcV*	IncQ1 (*aph(3″)-Ib*, *aph(6)-Id*, *sul2*), ISEc41 (*kpsE*, *terC*, *kpsMII*), ISEc40-ISEc13 (*mchB*, *mchF*, *sfaD*, *focC*, *mchC*, *cea*, *mcmA*, *iroN*), IS682-ISKpn37 (*cnf1*), MITEEc1 (*terC*), ISSen4 (*yfcV*),	Yes (0.88)

[a]Isolate and genome (LREC) identification.
[b]O and H antigen prediction with SerotypeFinder 2.0.
[c]Sequence types (ST#1 and ST#2) based on two different MLST schemes *E. coli* #1 and *E. coli* #2, respectively, and retrieved with MLST 2.0.4.
[d]Core genome ST obtained with cgMLSTFinder1.1. Software run against the Enterobase database.
[e–j]Clonotype, acquired antimicrobial resistance genes, plasmid replicon, virulence genes, mobile genetic elements associated with antimicrobial resistance, virulence traits, and the prediction of a bacteria's pathogenicity towards human hosts were also analyzed by using CHtyper 1.0, ResFinder 4.1, PlasmidFinder 2.1, VirulenceFinder 2.0, MobileElementFinder 1.03, PathogenFinder 1.1 online tools at the Center of Genomic Epidemiology (http://www.genomicepidemiology.org/services/), respectively.
[f]Resistome: Acquired resistance genes: beta-lactam: *bla*$_{TEM-1B}$, aminoglycosides: *aph(3″)-Ib*, *aph(6)-Id*; sulphonamides: *sul2*; tetracycline: *tet(A)*; peroxide: *sitABCD* (mediates transport of iron and manganese and resistance to hydrogen peroxide).
[g]Virulence determinants: *cea:* colicin E1; *chuA*: outer membrane hemin receptor; *clbB*: hybrid non-ribosomal peptide / polyketide megasynthase; *cnf1*: cytotoxic necrotizing factor; *focC*: S fimbrial/F1C minor subunit; *focC/sfaE*: S fimbrial/F1C minor subunit; *fyuA*: siderophore receptor; *gad*: glutamate decarboxylase; *hra*: heat-resistant agglutinin; *iroN*: enterobactin siderophore receptor protein; *irp2*: high molecular weight protein 2 non-ribosomal peptide synthetase; *iss*: increased serum survival; *kpsE*: capsule polysaccharide export inner-membrane protein; *kpsMII:* polysialic acid transport protein group 2 capsule; *mchB*: microcin H47 part of colicin H; *mchC*: MchC protein; *mchF*: ABC transporter protein MchF; *mcmA*: Microcin M part of colicin H; *ompT*: outer membrane protease (protein protease 7); *papA_F11*: major pilin subunit F11; *papA_F14*: major pilin subunit F14; *papC*: outer membrane usher P fimbriae; *sfaD*: S fimbrial/F1C minor subunit; *sitA*: iron transport protein; *tcpC*: Tir domain-containing protein; *terC*: tellurium ion resistance protein; *usp*: uropathogenic specific protein; *vat*: vacuolating autotransporter toxin, *yfcV*: fimbrial protein.

replication initiator protein (RIP) was identified in a node of 4.6 kb linked to plasmid and chromosomal references.

Discussion

Antibiotic resistance is of great public health concern since the antibiotic-resistant bacteria associated with the animals may be easily transmitted to humans via food chains, and widely disseminated in the environment via animal wastes (sludge-fertilized soils and manure). Intensive pig farming is one of the livestock activities widely recognized for its higher antimicrobial consumption (European Medicines Agency, 2021). The high prevalence of *E. coli* in swine enteric disease, their role as commensal, and their potential spread throughout animal-derived foods, makes this species key for the monitoring of AMR in industrial swine farming. In fact, *E. coli* is the main causative agent implicated in neonatal and PWD, and once the clinical signs appear, antimicrobials are the control solution for colibacillosis (Luppi, 2017). On the other hand, *E. coli* is commonly used as an indicator bacteria on the "One-Health" surveillance of antimicrobial resistance (AMR) by the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2019). On top of that, there is a global consensus on the need for efficient antimicrobial stewardship programs in pigs to reduce the selection of resistant bacteria (Verliat et al., 2021; Bosman et al., 2022; Vilaró et al., 2022). Here, we aimed to gain knowledge on the diagnosis and clinical difficulties in the veterinary sector when providing the best antibiotic therapy option. Another objective here was to obtain up-to-date information on antimicrobial resistance, and *E. coli* pathotypes in Spanish swine production. The protocol applied in the present study has been performed within the diagnosis service of the LREC, covering the same geographical areas of Spain for more

than 20 years. Thus, the results obtained here could be compared with our previous data (García-Meniño et al., 2021).

Antimicrobial susceptibility testing interpretation: challenges without clinical breakpoints

The goal of *in vitro* AST is to inform clinicians whether an antimicrobial is appropriate for the infection caused by a specific isolate. Furthermore, there is no successful option of empiric treatment for *E. coli* due to their heterogeneous MIC profile, so optimization to minimize resistance selection should be based on AST for each clinical case (Cortés et al., 2010; García-Meniño et al., 2019, 2021; Vilaró et al., 2022). However, we report here the lack of standardized breakpoints for many antimicrobials of common use in livestock. Currently, in the EUCAST reference method, there are no clinical breakpoints for cefalothin, cefoperazone, ceftiofur, flumequine, enrofloxacin, neomycin, tetracycline, and florfenicol. For these, we reviewed ECOFF (and TECOFF) values. Since cefquinome, marbofloxacin had neither clinical breakpoints nor cut-off values in EUCAST (nor in CLSI), we took them from CASFM VET2020, which were also applied for florfenicol-disk assay interpretation (Table 1). It is of note that the last version of CLSI and CASFM veterinary standards was reviewed in 2020. The comparison of results using the different standards (Supplementary Table S8), showed a moderate to high correlation between clinical breakpoints and cut-off values, except for enrofloxacin, neomycin, and florfenicol. The discrepancies observed for enrofloxacin and neomycin are explained by lower ECOFF values compared to CASFM VET2020 (> 0.125 *vs* > 2 and > 8 *vs* > 16, respectively). This observation is consistent with the fact that ECOFF values define a microorganism as non-wild type (N-WT) for a species by the phenotypical detection of an acquired or mutational resistance mechanism to the drug in question, while clinical breakpoints define a microorganism as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure. Nevertheless, it is outstanding the correlation obtained for flumequine applying CASFM VET and ECOFF (64.2% resistant and 76.4% N-WT, respectively), in comparison with data on enrofloxacin (31.1% resistant and 76.4% N-WT, respectively). Besides, resistance prevalence to marbofloxacin is expected to correlate with enrofloxacin (Vilaró et al., 2022). In our previous study (2005–2017), where the CLSI2020 clinical breakpoints were applied, a high percentage of isolates implicated in colibacillosis exhibited quinolone/fluoroquinolone resistance: 82.3% to nalidixic acid, 56.5% to ciprofloxacin and 48.4% to levofloxacin. To note, 93.2% of the farms involved in the present study reported the use of fluoroquinolones, and 44.6% reported the use of neomycin. Although applying ECOFF values would greatly restrict the therapeutic possibilities of neomycin and enrofloxacin, the high percentage of N-WT determined in both (> 70% commensal and pathogenic *E. coli* for enrofloxacin; 16.7% commensal and 53.2% of pathogenic *E. coli* for neomycin) would indicate an overuse, which might compromise therapeutic use soon. The ATS analysis revealed another critical point, which is the fixed range of drug concentrations provided by commercial kits for MIC determination. This greatly limits the flexibility of standard applications, as shown in Supplementary Table S8 for cefoperazone, and trimethoprim/sulfamethoxazole.

Regarding phenicols, we conducted a comparative florfenicol AST using MIC and disk diffusion in 42 isolates. In addition, susceptibility to chloramphenicol was determined in the entire collection by disk diffusion. By applying the clinical EUCAST breakpoint for chloramphenicol, we observed a similar high resistance prevalence (around 50%) compared to the previous study, with no discrepancies within standards. However, contradictory differences were observed in florfenicol resistance (50% "R" *vs* 31% N-WT, according to CLSI VET 2020 and ECOFF values, respectively). Phenicols are broad-spectrum antimicrobials used in veterinary medicine, although chloramphenicol was banned in EU in 1994 for its use in food-producing animals due to its toxicity and side effects for humans. The resistance level to chloramphenicol maintained over time (observed in this and previous study) can be probably due to different molecular mechanisms conferring resistance to both non-fluorinated (e.g., chloramphenicol) and fluorinated (e.g., florfenicol). Besides, genes encoding phenicol resistance (*cat*A1, *cml*A, and *flo*R) are often carried by plasmids together with other resistance genes. Co-selection under the selective pressure of non-phenicol antimicrobial drugs has been reported (Poirel et al., 2018). Florfenicol in pigs is indicated for the treatment of swine respiratory disease caused by *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. The CLSI 2020 references the breakpoint for *S. enterica* subsp. *enterica* serovar Choleraesuis "R" > =16, and the suggested ECOFF value for *E. coli* to detect an N-WT is > 16.

The high prevalence of MDR to ≥ 3 antimicrobial categories (> 75%) observed in our study is of concern. Furthermore, no differences were observed between the groups of commensal and pathogenic isolates, which would indicate that the therapeutic administration is exerting selective and permanent pressure on the commensal population (Zeineldin et al., 2019). On the other hand, the finding of MDR to ≥ 6 categories associated with pathogenic isolates, still implies a greater selective pressure on commensals which might incorporate resistance genes or could be displaced by resistant bacteria with the successful stabilization in the porcine microbiota. Strict control measures are necessary to avoid transmission (MDR bacteria or genes) to humans via food, or to the environment through animal wastes.

Phylogeny, *mcr* and ESBL genes: Monitoring tool

Since the *E. coli* phylogroups are not randomly distributed, the phylogroup assignment described by Clermont et al. (2013); Clermont et al. (2019) is a simple and valuable method to analyze

population changes, and even for clinical purposes. Different factors define the phylogeny, such as environment, physiology, diet, and maturation of the digestive tract, host status, or virulence-gene carriage of the bacteria (Escobar-Paramo et al., 2006; Cortés et al., 2010; Clermont et al., 2013). Thus, we apply this method in the diagnostic routine to monitor the evolution of pathogenic clones. The structure of *E. coli* in pigs is quite homogeneous worldwide, and within commensal pig *E. coli*, the predominance of phylogroup A, followed by B1 has been reported by different authors (Bok et al., 2013; Reid et al., 2017). Here, we found a similar phylogenetic population structure both in commensal and pathogenic isolates, which were assigned to A, B1, C, D, and E (additionally, one commensal isolate showed phylogroup B2). However, significant differences were detected in the prevalence of certain phylogroups associated with commensal (B1 and C) or pathogenic (E). Bok et al. (2020) reported wider diversity, including in addition the detection of F, or B2. The B2 phylogroup was determined by Bok et al. (2020) in 4.5% of piglets and 2.4% of sows. B2 is a phylogroup rarely reported in domestic pigs, which in our previous surveys was found in 3.6% of 499 pig isolates, associated with the pandemic clonal group ST131. In the present study, we did not recover any B2-ST131 isolate but the phylogroup B2 was determined in one commensal *E. coli*. The *in silico* characterization revealed the presence of clone O4:H5-B2-ST12 (CH13-223). The virulome analysis showed the carriage of various traits associated with extraintestinal virulence and, notably, its virulence profile conforms both, the ExPEC and UPEC status. Besides, it was predicted as human pathogen. ST12 is one of the global ExPEC lineages according to Manges et al. (2019), which is frequently implicated in extraintestinal disease, in both humans and domestic animals (mainly dogs, cats, and pigs) (Spindola et al., 2018; Flament-Simon et al., 2020b; Kidsley et al., 2020; Carvalho et al., 2021). The finding of this B2 clone, likewise our previously reported ST131 *mcr*-1 isolates (García-Meniño et al., 2018), deserves special attention. It would be indicative of swine as a potential reservoir of ExPEC not only for livestock but for humans too (Zhu et al., 2017; Bok et al., 2020; Flament-Simon et al., 2020b). Extraintestinal infections by *E. coli* such as urinary tract infections (UTI) also cause severe losses in the swine industry, mainly linked to antibiotic therapy, early disposal of breeding sows, or acute death when severely affected (Spindola et al., 2018). Therefore, specific clones such as O4:H5-B2-ST12 (CH13-223), or O25b:H4-B2-ST131 (CH40-374/161) of virotype D5 (García-Meniño et al., 2018; Flament-Simon et al., 2020a), should be monitored under a "One-Health" perspective.

Regarding the present collection of 64 pathogenic isolates, we found here a higher phylogenetic diversity, with a shift in prevalences compared to previous data. While the prior study (2005–2017) on pathogenic *E. coli* clearly showed the predominance of phylogroup A (85.5%), followed by E (12.4%) and B1 (2.1%), the present collection (2020–2022) exhibited a different distribution: phylogroup A (48.4%), followed by E (25%), B1 (20.3%), D (4.7%) and C (1.6%). These changes in prevalence, and therefore, in the predominant clones involved in swine colibacillosis, were already observed by us in Spain between the periods 2005–2017 and 1986–1991 (García-Meniño et al., 2021). This fact might be explained by the selective pressure derived from different antimicrobial therapies and vaccination programs applied over time.

An outstanding finding in the present study, is the important decrease in colistin-resistant *mcr*-bearing isolates, from 76.9% within 186 PWD isolates of the period 2005–2017 to 11.3% (present study, within 106 *E. coli*; $p < 0.05$). Besides, we have found *mcr* genes exclusively associated with pathogenic isolates (11.3% *vs* 0% of commensals; p value < 0.05), and the result is still significant if we compare data only from the group of diarrheagenic isolates: 12 isolates from 64 *E. coli*, 18.7% (2020–2022) *vs* 143 out of 186, 76.9% (2005–2017). Differently to our first study, where we observed co-occurrence of *mcr*-1/*mcr*-4, *mcr*-1/*mcr*-5, and *mcr*-4/*mcr*-5 in a significant number of pathogenic pig isolates (García et al., 2018; García-Meniño et al., 2019), the 12 *mcr*-positive isolates recovered in the period 2020–2022, show single *mcr* carriage (*mcr*-1 or *mcr*-4). Ours would be a unique study, analyzing not only *mcr*-1 but also other prevalent *mcr* genes in swine, in both commensal and pathogenic *E. coli*. The mandatory monitoring in the EU under Commission Implementing Decision (EU) 2020/1729 [Commission Implementing Decision (EU), 2020] is based on phenotypic susceptibility only, and it does not discriminate between different colistin resistance mechanisms. From our point of view, molecular testing is required to confirm the underlying mechanisms of resistance and to gain understanding on the epidemiology of *mcr*-positive *E. coli*. Besides, if we apply the standardized phenotypic assays and current clinical cut-off (>2 mg/l), many *mcr*-positive *E. coli* (especially those of the *mcr*-1 type) remain undetected (García-Meniño et al., 2020). This would be the case of 4 out of the 12 *mcr*-1 *E. coli* in the present study. Another limitation of the Decision (EU) 2020/1729 [Commission Implementing Decision (EU), 2020], is that surveys only refer to indicator commensal *E. coli*. We suggest the monitoring of pathogenic (invasive) *E. coli* in surveillance programs to accurately determine the persistence of colistin resistance. Our findings on the decrease in colistin-resistant *mcr*-bearing isolates would reinforce the conclusions of longitudinal studies on the *mcr*-1 abundance in food-producing pigs, and its relationship with the use of polymyxins (Wang et al., 2020; Miguela-Villoldo et al., 2022). Thus, Miguela-Villoldo et al. (2022) reported the decreasing trend of colistin resistance associated with *mcr*-1 gene in Spain, since the EMA and AEMPS strategies were applied in 2016 to reduce colistin use in animals. Likewise, Wang et al., 2020 showed a substantial reduction in sales of colistin in animals (2015–2018), after the withdrawal of colistin as growth promoter in China in 2017, which was rapidly followed by a significant reduction in the prevalence of *mcr*-1 in both the animal and human sectors. Despite the decrease of antibiotic selective pressure due to the colistin consumption reduction, the effect of co-selection by other antimicrobials, such as broad-spectrum cephalosporins, could be causing the persistence of colistin resistance, as recognized in the joint report of the ECDC, EMA and the European Food Safety Authority (EFSA; JIACRA III) (European Centre for Disease Prevention and Control (ECDC); European Food Safety Authority

(EFSA); European Medicines Agency (EMA), 2021). In the present study, 23 of the 106 isolates (21.7%) showed resistance to ceftiofur, and the 23 were positive for ESBL genes, without significant difference in commensal *vs* pathogenic groups (31% *vs* 15.6%; $p > 0.05$). The AST analysis of our previous study (2005–2017) determined that 17 (9%) isolates were phenotypically identified as ESBL-producers (including 11 *mcr*-positive isolates), which would mean a significantly lower figure to present data (12 of 64, 18.7% of diarrheagenic *E. coli*; $p < 0.05$) (García-Meniño et al., 2021). The resistant prevalences showed here, both for commensal and pathogenic strains, might be highlighting an increasing use of cephalosporins, in agreement with the voluntary survey, in which 47 (63.5%) out of 74 farms reported the use of ceftiofur.

Conclusion

There is an urgent need of harmonized guidelines, including standardized clinical breakpoints for all antimicrobials of common use in veterinary medicine. Our findings show a significant decrease in *mcr* isolates of porcine origin which would correlate with the drastic reduction in the use of colistin in Spain. We suggest that a diagnosis workflow based on AST, detection of *mcr* and ESBL genes, and phylogenetic characterization, would be useful as a monitoring tool for MDR and *E. coli* population shifts, under a "One-Health" perspective.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material. In addition, the nucleotide sequence of the B2-ST12 LREC_294 genome (FVL196/22 isolate) was deposited in the European Nucleotide Archive (ENA) with the following accession (ERR10033261) and BioSample (SAMEA110466098) codes, as part of BioProject ID PRJEB55192.

Author contributions

AMo, VGo, AMe, AA, and ER: conceptualization. VGa and IG-M: methodology. VGo, MJ-O, AMo, and AA: software. AMo, VGa, IG-M, VGo, and AMe: formal analysis. AMo, VGa and IG-M: investigation. VGa, IG-M, VGo, MJ-O, AMe, AA, ER and AMo: writing—review and editing. AMo: supervision, project administration, and writing—original draft preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AA and ER were employed by company Laboratorios Syva S.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1042612/full#supplementary-material

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MRSA compendium of epidemiology, transmission, pathophysiology, treatment, and prevention within one health framework

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Staphylococcus aureus is recognized as commensal as well as opportunistic pathogen of humans and animals. Methicillin resistant strain of *S. aureus* (MRSA) has emerged as a major pathogen in hospitals, community and veterinary settings that compromises the public health and livestock production. MRSA basically emerged from MSSA after acquiring SCC*mec* element through gene transfer containing *mecA gene* responsible for encoding PBP-2α. This protein renders the MRSA resistant to most of the β-lactam antibiotics. Due to the continuous increasing prevalence and transmission of MRSA in hospitals, community and veterinary settings posing a major threat to public health. Furthermore, high pathogenicity of MRSA due to a number of virulence factors produced by *S. aureus* along with antibiotic resistance help to breach the immunity of host and responsible for causing severe infections in humans and animals. The clinical manifestations of MRSA consist of skin and soft tissues infection to bacteremia, septicemia, toxic shock, and scalded skin syndrome. Moreover, due to the increasing resistance of MRSA to number of antibiotics, there is need to approach alternatives ways to overcome economic as well as human losses. This review is going

to discuss various aspects of MRSA starting from emergence, transmission, epidemiology, pathophysiology, disease patterns in hosts, novel treatment, and control strategies.

KEYWORDS

MRSA, epidemiology, pathophysiology, transmission, treatment, prevention, MRSA infections

1. Introduction

Currently, there are 81 species and numerous subspecies in the genus *Staphylococcus*. The majority of the genus's species are opportunistic pathogens or commensals of mammals. Numerous species have veterinary as well as medical significance. *Staphylococcus aureus* (*S. aureus*) is among the most prodigious and important staphylococcal species for human pathogenicity (Haag et al., 2019). The name *Staphylococcus* is derived from two Greek words, "staphyle," which means cluster or bunch, and "kokkos," means grapes, and so-called as "bunch of grapes" upon observation under microscope. The term "golden staph" is derived from the phrase "*Staphylococcus aureus*" which means "Golden Cluster Seed" (Ogston, 1881). *S. aureus* is a coccus-shaped, gram-positive, non-motile, non-spore-forming, opportunistic bacteria with biochemical profile as catalase, nucleases, lipases, coagulase, catalase, proteases, collagenases, and β-lactamase are the enzymes produced by *S. aureus*. It produces colonies in a variety of colors on various culture media such as pink colonies on chromogenic agar, golden or grayish-white colonies on blood agar, and yellow colonies on mannitol salt agar (Carter et al., 1995). Under microscope, *S. aureus* appears as rounded seeds arranged in bunches, demonstrating its growth in various planes. *S. aureus* is the cause of a wide spectrum of illnesses whose symptoms range from superficial to fatal manifestations. It may colonize diverse sites on both human and animal body surfaces due to its commensal as well as opportunistic characteristics. *S. aureus* is a common inhabitant of the skin, mucosa, urinary tract, gastrointestinal tract, and, in particular, the anterior nares of the respiratory tract (Cuny et al., 2010). *S. aureus* has the ability to produce a wide variety of virulence substances such as various types of proteins, enzymes, toxins, and other substances responsible for high pathogenicity. *S. aureus* produces fibronectin-binding protein and protein A, both of which contribute to the bacterium's ability to adhere to and colonize cell surfaces. The type of toxins produced by *S. aureus* are alpha, beta, gamma hemolysins, Panton-Valentine leukocidin (PVL) toxins, exotoxins, and enterotoxins. These all aid in the spread of *S. aureus* infection, which can cause severe blood stream and necrotizing infections in individuals (Gillet et al., 2002). This review article will cover the following aspects related to methicillin resistant strain of *S. aureus*; prevalence, transmission, pathogenesis, diseases, treatment, and prevention.

2. Emergence and types of MRSA

First time this bacteria was isolated from pus sample and given the name of "*S. aureus*" in 1881 (Ogston, 1881). Before the availability of penicillin, which was discovered by Radetsky (1996), an increased number of deaths were reported due to *S. aureus* infection that reached 90% case fatality rate which persisted up-to 19th century (Jevons, 1961). Later on production of β-lactamase enzyme by *S. aureus* makes the penicillin useless due to hydrolyzes of β-lactam ring of penicillin and *S. aureus* become resistant to penicillin soon after its discovery. Then, another antibiotic with the name of methicillin were discovered in 1950 which also found effective against *S. aureus* for long time. Unfortunately, the bacteria also acquired a significant resistance to this antibiotic and makes it ineffective anymore. The resistance to this antibiotic was reported at increased percentage which was name as methicillin resistant strain of *S. aureus* (MRSA). The molecular basis of MRSA was originated from a large mobile genetic element known as the staphylococcal cassette chromosome mec (SCC mec) genes such as *mecA* gene which are obtained by methicillin susceptible *S. aureus* (MSSA) through horizontal gene transfer among bacteria. The resistance was exhibited to all β-lactam antibiotics due to production of penicillin binding protein (PBP-2α) encoded by *mecA* gene (Vengust et al., 2006). In 1961, a study was conducted which noted among 50 staphylococci samples, 18 were found resistant to methicillin indicating high percentage (36.0%) of MRSA. These isolates were discovered to have the potential to keep both coagulase and hemolytic activity. MRSA detection was difficult in the early years of its discovery because methicillin resistance in *S. aureus* was diverse among different isolates and in a study, 5444 *S. aureus* samples were tested and only 3 isolates were diagnosed as MRSA (Barrett et al., 1968). As a result, heterogeneous strains mostly consist of bacterial cells that are both highly resistant and susceptible to methicillin. However, the addition of sugar or sodium chloride (NaCl) to the culture medium may promote the expression of phenotypic resistance in presence of β-lactam antibiotics (Datta et al., 2011).

From the long time, MRSA is considered as prototype of MDR and nosocomial pathogens which cause infection in hospitals and other healthcare settings. In the past three decades, the percentage of MRSA infections has significantly risen, and new strain of MRSA known as healthcare-associated (HA-MRSA) has spread and become endemic throughout industrialized nations as the leading cause of life-threatening infections like pneumonia, skin and bloodstream infections (Diekema and Pfaller, 2001). According to a US study, *S. aureus* infections are responsible for seven million hospitalizations of humans in the country, demonstrating the significant loss caused by hospital-acquired MRSA (HA-MRSA). The estimated yearly cost of these infections is $2.7 million, a considerable loss of 12,000 annual deaths, and sets the country's economy at financial stress of over $9.5 billion (Noskin et al., 2005). First, MRSA was primarily associated with healthcare settings, and the risk factors that contributed to its spread were well-known (Chambers, 2001). A new type of MRSA rapidly emerged toward the end of the 1990s in the community and was known as community-acquired (CA-MRSA), with very high level of pathogenicity and the potential to spread, making it capable of infecting young as well as healthy individuals (DeLeo et al., 2010). Recently, MRSA also reported as frequent colonizer among animals due to extensive and frequent use of antibiotics in animal production. This new MRSA strain called as livestock associated MRSA (LA-MRSA) is primarily identified in food animals such as pigs, cattle, sheep, and goat with having extensive zoonotic potential (Pantosti, 2012). These cases of MRSA infection in humans and animals show how animals can serve as a source for the transmission of the disease, thus posing a serious threat to the population (Cefai et al., 1994). Rising public concern over MRSA has led to monitoring recommendations, such as information on the prevalence of the infection in healthy dogs, cats, and humans (Noskin et al., 2005).

3. Prevalence of MRSA

The global emergence and transmission of MRSA is one the most important aspect in the epidemiology of MRSA. The spread of all types of MRSA have been reported from many countries as listed in **Table 1**. The spread of MRSA is known to occur by one of the two ways which are either spread of existing clones among humans, animals, from animals to humans or humans to animals and acquisition of SSC*mec* element through horizontal genes transfer (Lee et al., 2018). Currently, MRSA is known to be more endemic in hospital settings and is among the major nosocomial pathogens. According to the statement of CDC, MRSA is known to be a major threat to public health because of its increasing prevalence in hospitals, community and animals, transmission between humans and animals, infection rates, resistance, and therapeutic issues (Ferri et al., 2017). On an average, it was estimated the annual health cost due to MRSA infections accounts 3 billion dollars. CA-MRSA has been also emerging as a principle pathogen from the recent years. It is noted MRSA mostly causes skin and soft tissue infection leading to bacteremia which lead to higher mortality rates ranging from 15 to 60% (Lee et al., 2013).

Recent trend in the prevalence of HA-MRSA was noted varying among the countries for example it was noted higher 58.4% from Portugal in 2013 (Tavares et al., 2013), 46% from India in 2009 (Arora et al., 2010), 52% from Pakistan in 2017 (Siddiqui et al., 2017), 45% from China from 2015 to 2017 (Chen et al., 2022), and 38.9% from Norway from 2008 to 2016 (Enger et al., 2022). However, with the increasing prevalence of HA-MRSA in different countries, MRSA prevalence also noted lower in many such as 4.6% from Germany (Sassmannshausen et al., 2016), 25% from Texas (Davis et al., 2004), 19.1% from Mexico (Hamdan-Partida et al., 2022), 15.1% from Australia (Coombs et al., 2022), and 26% from Italy (La Vecchia et al., 2022) are summarized in **Table 1**. Similar increasing and decreasing trend of MRSA prevalence from different countries also noted for CA-MRSA such as 79% from Japan (Ogura et al., 2022), 84.9% from Australia (Coombs et al., 2022), 64.7% from India (Alvarez-Uria and Reddy, 2012), 61% from Norway (Enger et al., 2022), and 44.3% from Iran (Tabandeh et al., 2022) with lower prevalence from Egypt (16%) (Mostafa et al., 2022), China [1.7% (Bi et al., 2018); 24% (Chen et al., 2022)], 7.3% from Gerorgia (Hidron et al., 2005), and 12.8% from Switzerland (Harbarth et al., 2005). This decline in the prevalence of HA-MRSA and CA-MRSA may be linked with better implementation national prevention measures. This increase and decline in the prevalence of HA−MRSA and CA-MRSA may be linked with increasing acquisition of LA-MRSA from animal reservoirs to humans especially from food and companion animals. The predominant LA-MRSA strain which is also identified from human MRSA isolates belong to CC398 as illustrated in **Figure 1**. However, person to person transmission of LA-MRSA found to be uncommon. The prevalence of LA-MRSA from different studies also noted higher from different countries as mentioned in **Table 1**. Recent studies conducted in Pakistan detected 15.6% LA-MRSA from goat milk (Muzammil et al., 2021), 24.5% from cow milk (Lodhi et al., 2021), 30.4% from cats (Shoaib et al., 2020), and 33.9% from dogs (Shoaib et al., 2020). Another study conducted in Malaysia in 2018 detected 38.6% MRSA from cow milk (Aklilu and Chia, 2020). Furthermore, two studies from Switzerland reported 1.41% from cow milk (Huber et al., 2010), 2.9% from pig nasal swabs (Huber et al., 2010), and 1.6% from calf nasal swabs (Huber et al., 2010) (summarized in **Table 1**).

4. MRSA transmission between humans and animals

Transmission of MRSA among different hosts is primarily known to happen by physical contact with source. The capability of transfer of MRSA among different host species

TABLE 1 Prevalence of livestock associated MRSA (LA-MRSA), healthcare-associated MRSA (HA-MRSA), and community-acquired MRSA (CA-MRSA) in different countries.

Sr. No	Year of study	Prevalence	Sample size	Sample sources	Country	References
Livestock associated MRSA (LA-MRSA)						
	2021	24.59%	787	Bovine milk samples	Pakistan	Lodhi et al., 2021
	2021	15.6%	200	Goat milk samples	Pakistan	Muzammil et al., 2021
	2020	30.0%	100	Tracheal and nasal samples of quails	Portuguese	Silva et al., 2021
	2020	30.43% in cats, 33.91% dogs, 25% in humans, and 50% in environment	384	Swab samples from cats, dogs, and environment	Pakistan	Shoaib et al., 2020
	2018	38.6%	95	Dairy cattle milk and nasal samples	Malaysia	Aklilu and Chia, 2020
	2017	34.0%	900	Bovine milk samples	Pakistan	Aqib et al., 2017
	2014	47.6%	450	Cow milk samples	China	Pu et al., 2014
	2013	6.3% from milk samples, 4.7% from hand and nose samples, 1.2% from farm environment	1146	559 milk samples, 86 hand and nose samples, 501 farm environment samples	Korea	Lim et al., 2013
	2012	71.5%	500	Swab samples of turkeys	Germany	Richter et al., 2012
	2011	16.7%	280	Cattle milk samples	Germany	Spohr et al., 2011
	2011	78% farm level 28% animal level	2151	Nasal samples of veal calves	Netherlands	Bos et al., 2012
	2009	1.41%	142	Cattle milk samples	Switzerland	Huber et al., 2010
	2009	2.9% in pigs and 1.6% in calves	1100	Nasal swabs from pigs and calves	Switzerland	Huber et al., 2010
	2007	0.4%	595	Cattle milk samples	Hungary	Juhász-Kaszanyitzky et al., 2007
Hospital acquired MRSA (HA-MRSA)						
	2008–2016	38.9%	318	Nose, ear, blood, pus, wounds, abscesses, eyes, genital	Norway	Enger et al., 2022
	2015–2017	45.0% (658/1466)	1466	Hospital patients	China	Chen et al., 2022
	2017–2021	26.0%	76	Ear, rectal swabs, oropharyngeal, nose, skin, wound, conjunctiva, bone, urine, synovial and peritoneal fluid, lymph node	Italy	La Vecchia et al., 2022
	2011–2019	35.1%	210	Inpatients and outpatients	Japan	Hosaka et al., 2022
	2018–2019	21.0%	164	Blood and soft tissues	Japan	Ogura et al., 2022
	2014–2020	23.4%	565	Blood, pus, wound exudate, sputum	China	Wang et al., 2022
	2020	15.1%	456	Blood	Australia	Coombs et al., 2022
	2021	34.8%	295	Urine, sputum, wound swabs, nasal swabs, fomites	Nigeria	Ugwu et al., 2022
	2019–2020	55.7%	97	Wound, pus, CSF, blood, skin lesions, sputum, joint fluid, ear, nose, throat	Iran	Tabandeh et al., 2022
	2021	19.1%	47	Nose, pharynx, and mobile phone	Mexico	Hamdan-Partida et al., 2022
	2020	43.44%	200	Wound, nose, cerebrospinal fluid of patients	Pakistan	ur Rehman et al., 2020
	2019	50%	742	children	Uganda	Kateete et al., 2019
	2017	52%	180	Human blood	Pakistan	Siddiqui et al., 2017

(Continued)

TABLE 1 (Continued)

Sr. No	Year of study	Prevalence	Sample size	Sample sources	Country	References
	2016	4.6%	726	Health care workers	Germany	Sassmannshausen et al., 2016
	2013	58.4%	1487	Humans	Portugal	Tavares et al., 2013
	2009	46.0%	6743	Humans	India	Arora et al., 2010
	2004	25.0%	758	Human patients	Texas	Davis et al., 2004
Community acquired MRSA (CA-MRSA)						
	2008–2016	61.0%	318	Nose, ear, blood, pus, wounds, abscesses, eyes, genital	Norway	Enger et al., 2022
	2015–2017	24.0% (105/434)	434	Community settings	China	Chen et al., 2022
	2018–2019	79.0%	164	Blood and soft tissues	Japan	Ogura et al., 2022
	2019–2020	16.0%	25	Nasal swabs	Egypt	Mostafa et al., 2022
		31.2%	565	Blood, pus, wound exudate, sputum,		Wang et al., 2022
	2020	84.9%	456	Blood	Australia	Coombs et al., 2022
	2021	28.7%	295	Urine, sputum, wound swabs, nasal swabs, fomites	Nigeria	Ugwu et al., 2022
	2019–2020	44.3%	97	Wound, pus, CSF, blood, skin lesions, sputum, joint fluid, ear, nose, throat	Iran	Tabandeh et al., 2022
	2018	23.5%	152	Nasal swabs	Pacific Asia	Wong et al., 2018
	2017	2.8%	404	Pig ear swab	China	Bi et al., 2018
	2017	1.7%	753	Nasal sample	China	Bi et al., 2018
	2012	64.7%	178	Pus sample	India	Alvarez-Uria and Reddy, 2012
	2009	38.5%	120	Dialysis patients	United Kingdom	Johnson et al., 2009
	2005	12.82%	14253	Nasal and inguinal swabs	Switzerland	Harbarth et al., 2005
	2005	7.3%	726	Anterior nares culture	Atlanta Georgia	Hidron et al., 2005

including humans and animals is the characteristic feature of MRSA lineages. HA-MRSA is primarily acquired from hospital settings such as contaminated instruments, bedding, doors, and equipment's while CA-MRSA is primarily acquired by physical contact with infected or healthy person as *S. aureus* is a commensal bacterial in the nares of healthy individuals. LA-MRSA transmission to humans when the individual has physical contact with animal and environment (Pantosti, 2012). Firstly, LA-MRSA was restricted only to animals until 1961 before the Hungarian cow was reported to be the source of LA-MRSA transfer to its caretaker by testing throat swabs (Cefai et al., 1994). This was the first report of MRSA transmission from animal to human which proved the ability of MRSA horizontal transmission among animals and humans. Later on a number of reports were published by various authors from different regions of world from different animal's species such as poultry, pigs, cattle, sheep and goat, equines, and companion animals. These reports noted number of clonal complexes (CCs) such as CC5, CC8, CC9, CC59, CC1, CC30, CC45, CC22, CC130, CC97, and CC398 with multi-locus sequence types (STs) were found similar among human isolated MRSA strains and animal isolated MRSA strains. On the other hand, various HA-MRSA and CA-MRSA strains are also found similar to other LA-MRSA strains which are elaborated in **Figure 1**. A human clone ST1 was found in animals and is responsible of causing mastitis in bovines (Grundmann et al., 2010). Similarly, animals clone CC398 and lineage ST398 found among humans' which cause infections similar to HA-MRSA and CA-MRSA (Witte et al., 2007). Moreover, a worldwide clone of poultry ST5 also found among humans working at poultry farms (Lowder et al., 2009). Similarly, a clone of small ruminants CC130; ST 130 also recovered from humans (Guinane et al., 2010). The MRSA transmission from companion animals to humans is also well-documented in various studies for example a study conducted in USA and Canada documented 18% carriage rate of MRSA among the owners of companion animals (Faires et al., 2009). Another study in UK in nursing home documented similar strain in patient, hospital staff and nursing cat (Scott et al., 1988).

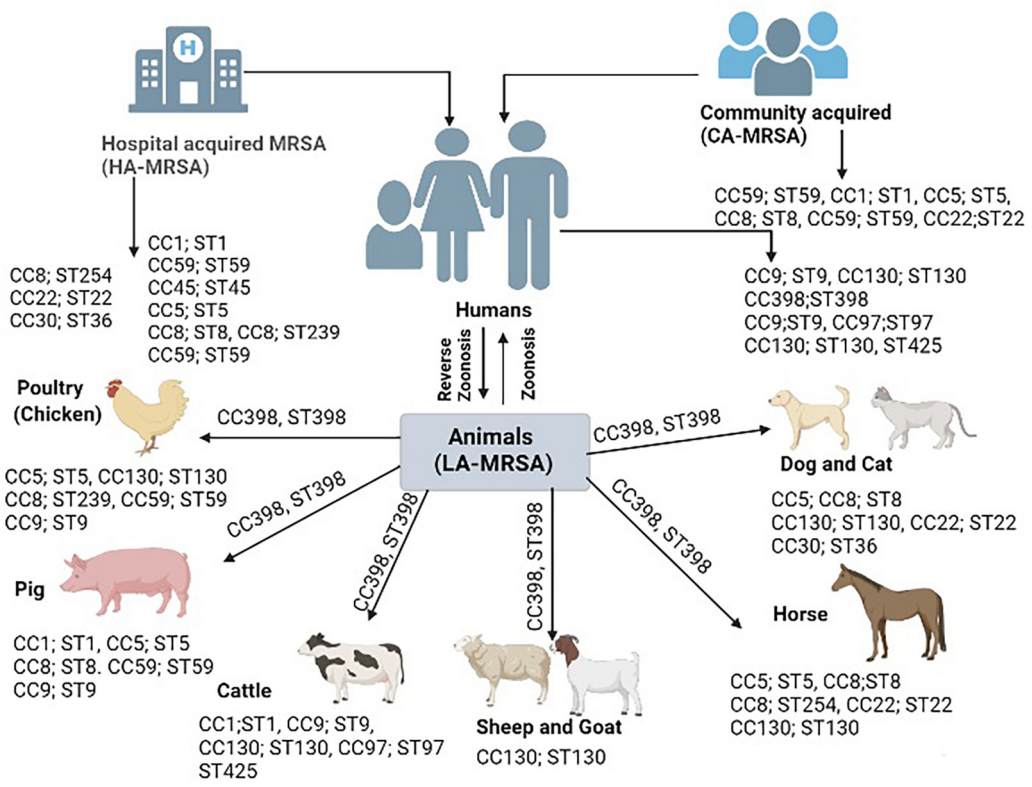

FIGURE 1
Bovine-adapted clonal complexes *Staphylococcus aureus* (*S. aureus* CCs) appear to have derived from human CCs and acquired bovine affinities through a series of spill-over events that resulted in the acquisition of various mobile genetic elements (MGEs). Several hosts have a high prevalence of the CC398 lineage. This lineage seems to have started in humans *via* reverse zoonosis, then it spread to pigs, then it returned to humans *via* pig zoonosis, and ultimately it spread to other species.

Similarly, a study conducted at veterinary hospital detected transmission of MRSA lineage ST22 from infected dogs to veterinary staff (Baptiste et al., 2005). However, pets also found to be colonized with human PVL positive CA-MRSA strain by a household member in Netherland (Van Duijkeren et al., 2005). The risk of getting MRSA in animal caretakers (1.7%) was higher compared to those who were not exposed to animals (Wulf et al., 2006). For 13 months, eleven horse patients were hospitalized at the veterinary hospital for various diagnoses and surgical procedures. After procedures, a strain of MRSA was identified. MRSA strain was also isolated from 3 of 5 and with 1 person found to be colonized with 2 biotypes of MRSA. The isolates of human MRSA appeared to be identical to those of horses. The results showed that the isolates of horses and humans are members of a very close group and, apparently come from a common source. According to the pattern associated with the infection, a special mode of transmission was still unknown, but it was assumed that the main cause of the infection is the staff of the veterinary hospital (Seguin et al., 1999).

A number of risk factors also play a significant role in the spread of CA-MRSA and HA-MRSA infections. Important risk variables for cellulitis were overweight, the existence of abscesses, and head-and-neck sores relative to infections produced by other microorganisms according to an analysis of individuals with the condition. The presence of abscesses and obesity were additional important risk variables for MRSA dermatitis (Khawcharoenporn et al., 2010). Significant correlations between MRSA colonization, skin infection in the previous 3 months, sharing soap, and MRSA skin and soft tissue infection (SSTI) against no SSTI were found, college education, knowing about "staph" before, taking bath daily, and the previous contact with a health care worker (Haysom et al., 2018). Further research compared those with MRSA to those with MSSA to identify risk variables for MRSA colonization. According to research, there are a variety of meaningful risk factors for MRSA infection, such as the involvement of family members under the 7 years of age, a smoking habit, and the consumption of antibiotics the year before. Additional characteristics including age, sex, married status, chronically sick patients, education, taking a daily shower, and family income, however, were not meaningful risk factors for MRSA colonization (Wang et al., 2009).

Except these risk factors in humans, a study also highlighted the risk factors associated with LA-MRSA mammary infection

in dairy animals. Among those risk factors are animal parity number, age, feeding status, body condition score, udder hygiene, hand or machine hygiene while milking were important factors associated with this infection while milking frequency was found non-significant risk factor for LA-MRSA infection (Aqib et al., 2017). Another study conducted by Shoaib et al. (2020) highlighted the risk factors associated with transmission of LA-MRSA from companion animals. Among those risk factors, animal health status, infection on body, long term antibiotic therapy, veterinarians, pet access to bedroom were found significant risk factors in transmitting MRSA to humans while the size of dog, owner's sex, and sample site were found to be non-significant risk factors associated with MRSA transmission. Another study conducted by Mulders et al. (2010) highlighted the possible risk factors of MRSA transmission from poultry to humans are farm workers, individuals having contact with live birds at slaughterhouse, type of slaughtering method, and slaughtering environment are significantly correlated with higher carriage of MRSA among humans.

5. MRSA pathophysiology

Staphylococcus aureus is a pathogenic and commensal bacterium that normally lives in the anterior nares of both humans and animals as well as in axillae, groin, and gastrointestinal tract are sites where it can also colonize. The major steps in pathogenesis of infection are colonization, virulence, initiation of infection, abscess formation, systemic infection, regulation and adaptation with the help of number of virulence factors. Colonization enhances the risk of bacterial infection when the host's defenses are compromised either by physical disruption or other diseases (Wertheim et al., 2005). As MRSA is the methicillin-resistant strain of *S. aureus*, the *S. aureus* by self-contain a number of potential virulence factors which includes several surface proteins called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs), which bind to fibrinogen, fibronectin, and collagen fibers of host cells to attack the host tissues. These factors may lead to infections of prosthetics, bones, joints, and endovascular system (Menzies, 2003). The ability of *S. aureus* to produce biofilm on both prosthetic surfaces and the host allows it to adhere to those surfaces by evading the effect of antimicrobials and host immune system. *S. aureus* also have the ability of produce small colony variants (SCVs) which have ability to cause persistent and recurrent infections. *S. aureus* also contain the anti-phagocytic microcapsule (type 5 or 8) which act as a primary defense mechanism. Moreover, due to internaction of Zwitter ionic capsule with Fc region of an immunoglobulin, the MSCRAMM protein A facilitate the protection of *S. aureus* from opsonization (Gordon and Lowy, 2008). Except these *S. aureus* contain a number of virulence factors such as adhesion proteins, chemotaxis inhibitory proteins, various enzymes such as proteases, lipases, hyaluronidase, staphylokinase, catalase, nucleases, lipases, coagulase, catalase, proteases, collagenases, β-lactamases, and elastases which help the *S. aureus* in causing infection in host. Except these virulence factors, MRSA also contain different mobile genetic elements (MGEs) in different animals' species as listed in **Table 2** that also aid and enhance its pathogenicity. Besides these, *S. aureus* produces various type of toxins such as exotoxins, enterotoxins, TSST-1, hemolysin toxins and PVL toxins as illustrated in **Figure 2**. Additionally, certain strains of *S. aureus* release superantigens that can also cause infections such as food poisoning and toxic shock syndrome (TSS) (Dings et al., 2000). Normal expression of *S. aureus* virulence factors plays a significant role in pathogenesis. Virulence factors express only according to the requirement of the bacterium to decrease unnecessary metabolic demands. Although secreted proteins like toxins are generated during the stationary phase, MSCRAMMs typically express during logarithmic growth phase. Early MSCRAMM protein expression helps in the initial colonization of tissue sites, while late toxin production helps in the dissemination of infection into the bloodstream. Mainly the *S. aureus* pathogenicity is regulated by the quorum-sensing accessory gene regulator (AGR) (Gordon and Lowy, 2008). *S. aureus*, in short, has a wide range of ways to cause disease and evade host defenses. However, the existence of some virulence factors is independent of the genomic structure and are related to clonal type (Peacock et al., 2002).

Hospital acquired methicillin-sensitive *S. aureus* (MSSA) is less dangerous pathogen than HA-MRSA, which increases the pathogenicity and mortality. Nonetheless, the specific pathogenicity mechanism is unknown. However, it is thought that the PBP2-α protein, which is associated with β-lactam antibiotic resistance and expressed by the *mecA* gene, directly contributes to immunopathology during MRSA infection. Poor peptidoglycan cross-linking with β-lactam antibiotic caused by PBP2-α results in increased survival of MRSA strains as compared to MSSA (Yao et al., 2010). Improved immune system evasion and *S. aureus*-exclusive toxin synthesis all contribute to CA-MRSA strains' enhanced virulence. Researchers have found that *S. aureus*'s PVL protein has dermonecrotic and leukocyte-lysing properties that lead to increased pathogenicity of CA-MRSA strains (Chini et al., 2006). Further investigations are required, since studies claim that the relationship between PVL and CA-MRSA virulence is complex (Wardenburg et al., 2007). Another study conducted by Wang et al. (2007) also showed that phenol-soluble modulin proteins which promote inflammation and impair the function of neutrophils in bacteremia patient and mice models were more abundant in CA-MRSA strains than HA-MRSA strains. The emergence of LA-MRSA and its transmission to humans and reports of humans strains in animals further increases the pathogenicity of MRSA as now MRSA have diversity of host species which results in genomic

TABLE 2 Methicillin resistant strain of *Staphylococcus aureus* (MRSA) mobile genetic elements (MGEs) associated host determinants in different species.

Disease	Host	MGEs	MGE-linked host determinants	References
Mastitis, skin infections	Ruminants	SaPIbov, enterotoxin gene cluster, SaPIbov4, Non-mec SCC, SCC-*mecC*	Sec, Seg, Seo, Sel, Sei, Sem, Sen, TSST-1, ssl07, ssl08, vWbp, and LPXTG surface protein	Viana et al., 2010; Wilson et al., 2011; Resch et al., 2013; Bar-Gal et al., 2015
Neonatal septicaemia, skin infections	Swine	Pathogenicity islands (SaPI-S0385) and plasmids	SSC*mec*, SaPI5, SaPIbov1, vWbp, resistance to heavy metals	Schijffelen et al., 2010; Richardson et al., 2018
Skin, thoracic and joint or bursal infections	Equine	ΦSaeq1, SaPIeq1	Immune modulators, Scn gene, *luk*PQ genes, vWbp	Viana et al., 2010; Garzoni and Kelley, 2011; Koop et al., 2017; De Jong et al., 2018
Skin and soft tissue infections	Pets	Plasmid SAP078A, SCC*mec* type IV, bacteriophage Φ2, Φ3, Φ6, rep_{10} plasmid, SaPI	Replication genes (rep_5, rep_{22}), heavy metal resistance genes (copB, arsR, cadC, arsA, mco, and cadA), host immune evasion genes (scn, sak, and chp),	Loeffler et al., 2013
Pododermatitis	Poultry	ΦAvβ, pAvX, pAvY, pC221, ΦAv1, SaPIAv, pUB112	ornithine cyclodeaminase, protease, ear-like proteins (ear pathogenicity islands such as SaPI1, 3, 5 and SaPImw2, Thiol protease ScpA, Lysophospholipase, Tetracycline and Chloramphenicol resistance	Ehrlich, 1977; Lowder et al., 2009; Murray et al., 2017
Lung infection, Bloodstream infections, Endocarditis, Osteomyelitis,	Human	ΦSa3 (β-hemolysin converting phage)	(clfB, isdA, fnbA, atlA, eap), genes involved in Wall Teichoic Acids (WTA) biosynthesis (tagO and tarK), cell surface dynamics/remodeling enzymes (sceD, oatA, atlA), immune-modulatory factors, TSST-1, PVL toxins and staphylokinase	Manders, 1998; Foster, 2002; Richardson et al., 2018

modification and increases the antibiotic resistance. SCC*mec* cassettes, particularly SCC*mec* IVa and SCC*mec* V, are present in LA-MRSA while other cassettes, such as SCC*mec* type XI, which includes *mecC*, have also been reported (Voss et al., 2005). According to several studies, the LA-MRSA CC398 misplaced human-associated virulence factors like exfoliative toxins and acquired the antibiotic resistance genes like *mecA*, *tetM*, TSS toxin I, and PVL genes (Ballhausen et al., 2017). The staphylococcal protein A gene (spa) in CC398 are also currently reported which aid in MRSA pathogenicity (Peeters et al., 2015).

6. MRSA infections in humans

Methicillin resistant strain of *S. aureus* (MRSA) is an emerging pathogen that can cause mild to serious infections in both animals and humans. Among humans, it mostly causes mild to life deadly infections such as skin and soft tissue infections which are staphylococcal scalded skin syndrome (SSSS), pustules, impetigo contagiosa, abscesses, and papules while deadly infections include TSS, pneumonia, or newborn TSS-like exanthematous disease in humans (Takahashi et al., 1998). Among the 100,000 cases of MRSA infections per year, 20% of the patients died (Klevens et al., 2007). Previously, CA-MRSA and HA-MRSA were two major types of developing infections in humans. HA-MRSA infections are seen in athletes, children, and in hospitalized individuals while CA-MRSA most offenly causes various types of SSTIs which range from mild such as fruncles, impetigo to deadly such as necrotizing fascilitis, and pneumonia infections. CA-MRSA causes less severe infections in animals and humans as compared to HA-MRSA (David and Daum, 2010).

6.1. Hospital-acquired MRSA

Significant risk factors associated with HA-MRSA infections are aged and immunosuppressive patients due to extensive usage of broad-spectrum antibiotics for a long time (Kurkowski, 2007). HA-MRSA shows resistance to almost all β-lactam drugs which is the reason for its spread among hospital-acquired infections (Lindsay, 2013). Compared to HA-MRSA, CA-MRSA may be found in healthy people and its prevalence may be high among the people working at day care centers, prisons, players, and among military personnel because they are living in close contact with each other (Daum, 2007). HA-MRSA being a multidrug-resistant organism causes many healthcare-associated infections in children and adults. The infection rate is high in people that are immune compromised and in patients having cuts on the skin that may a source of

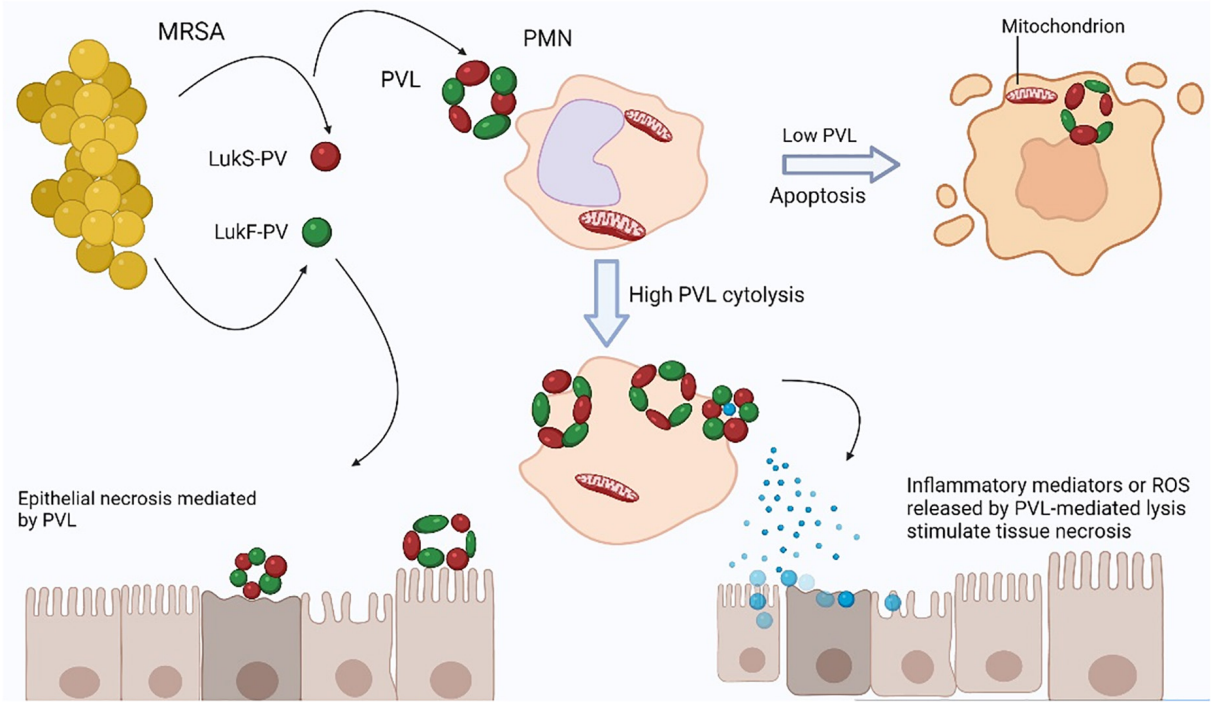

FIGURE 2
Tissue necrosis is caused by Panton-Valentine leukocidin (PVL). The two PVL components secreted by *Staphylococcus aureus*, LukS-PV, and LukF-PV, collectively form a pore-forming heptamer on the membranes of polymorphonuclear leukocytes (PMNs). Low PVL concentrations cause polymorphonuclear leukocytes (PMN) apoptosis through direct binding to mitochondrial membranes, whereas high PVL concentrations cause PMN lysis (Genestier et al., 2005). From lysed PMNs, reactive oxygen species (ROS) can cause tissue necrosis. Furthermore, the release of granules from PMNs that have been lysed may cause an inflammatory response that leads to tissue necrosis. PVL is unlikely to cause direct necrosis of epithelial cells.

spread of infection (Köck et al., 2010). HA-MRSA clones are dominant bacterial clones that are the major cause of these types of infections in humans and animals. These clones are distributed differently in their geographical areas. HA-MRSA epidemics start in the 1980s or 1990s and the major reason behind this was due to the development of novel clones of MRSA (Chambers and DeLeo, 2009). These clones circulate quickly among hospitals and lead to an increase in the death and morbidity rate. The important HA-MRSA clones include CC, spa type, sequence type (ST), PAGE type, and most simply by their lineage type. Among the CC includes CC30, CC5, CC45, CC8, and sequence type 239 (ST239) (Okuma et al., 2002; McDougal et al., 2003; Naimi et al., 2003; Klevens et al., 2007).

6.2. Community-acquired MRSA

Community-acquired MRSA (CA-MRSA) causes infections on several body parts, most commonly skin and soft tissues, but also in lungs, bone, joints, bloodstream, surgical sites, and urinary tract (Dantes et al., 2013). Although, CA-MRSA is not limited to the skin and soft tissues but also the major cause of septicemia and necrotizing pneumonia. CA-MRSA also causes bacteremia that's a complication that will lead to endocarditis and osteoarticular infections (Alzomor et al., 2017). CA-MRSA is becoming a global issue among infants, children, and adolescents (Stankovic and Mahajan, 2006). Researchers in Japan using techniques like agr typing, spa typing, coagulase typing, PCR assay for virulence genes, SCC*mec* typing, and multi-locus sequence typing (MLST) characterized the PVL-positive CA-MRSA in children in 2003 and similar strain was noted in athletes having cutaneous abscess (Takizawa et al., 2005). A rise in CA-MRSA infections was noted up-to 29.8%, and left 70.2% was due to unknown risk factors in Saudi Children Hospital. A study conducted in King Fahad Medical City, Riyadh, Saudi Arabia from 2005 to 2008 among outpatient children showed that 29.8% of cases were positive for CA-MRSA while the other 70.2% were due to unknown risk factors (Mermel et al., 2009). Initially, it was believed that CA-MRSA was a nosocomial strain that had been transmitted from hospitals to the population. However, unlike the HA-MRSA strains often identified in healthcare settings, CA-MRSA strains are paradoxically sensitive to non-β-lactam antimicrobials and showed clinical symptoms more resembling those of MSSA strains (Herold et al., 1998). The

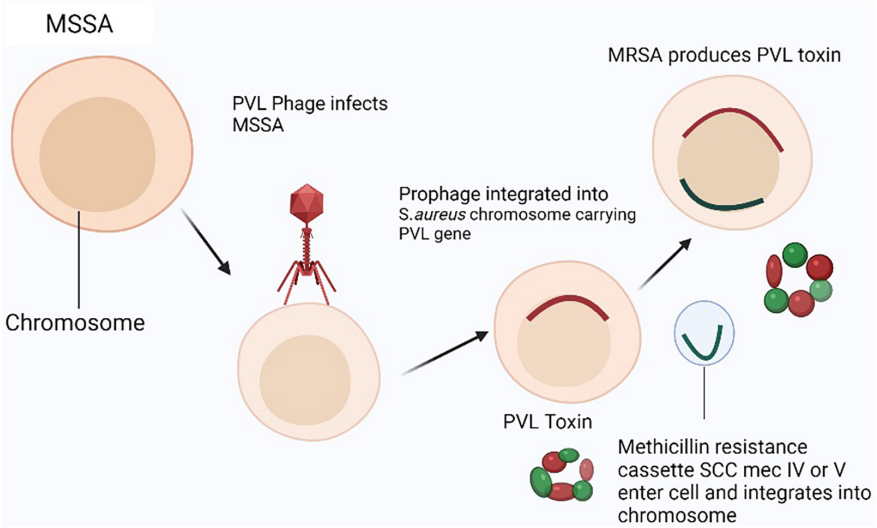

FIGURE 3
Panton-Valentine leukocidin (PVL) producing community-acquired–MRSA (CA-MRSA) model: In MSSA strain, two genes (pvl) encoding the methicillin-resistant phage virus (PVL) are infected and lysed by the phage (phiSLT) (Staphylococcal Leukocytolytic Toxin). Then, a horizontal transfer of a methicillin resistance cassette (SCCmec IV, V, or VT) carrying the mecA gene into the pvl-positive methicillin susceptible *Staphylococcus aureus* (MSSA) strain allows it to incorporate into the genome somewhere other than the phiSLT (Staphylococcal Leukocytolytic Toxin) integration site.

genetic lineage, genetic make-up of the methicillin resistance genes, and existence of PVL are the three main attributes that differentiate a CA-MRSA strain from a HA-MRSA strain. The current evidence indicates several distinct MSSA ancestral clones that are circulating in the world are incorporated by SCC*mec* especially SCC*mec* IV in CA-MRSA strains through gene transfer mechanism (**Figure 3**; Enright et al., 2002).

7. LA-MRSA infections in food and companion animals

Although the spread of MRSA infections in food and companion animals was initially thought to be slower, it is now becoming a serious problem for food animals and food industries too. LA-MRSA is an important cause of mastitis in cows and buffaloes resulting in a decrease or no milk production (Javed et al., 2021). LA-MRSA also causes the infections in poultry such as comb necrosis, chondronecrosis, and septic conditions (Fluit, 2012). Almost all the companion animals like dogs, cats, and horses, are potential sources of LA-MRSA transmission to humans having direct or indirect contact with these animals. Besides mammals, LA-MRSA colonization in foxes, roes, rabbits, wild boars, and wild animals (e.g., pigeons, pheasants, ducks, buzzards, gulls, and rocks) has also been reported (Smith et al., 2009).

7.1. MRSA infection in food animals

Mastitis is an important disease of dairy animals, which is linked to the maximum use of antibiotics responsible for huge economic losses. *S. aureus* is a chief pathogen of mastitis among all other causative agents throughout the world. LA-MRSA is also an important cause of pustular dermatitis in their milkers (Grinberg et al., 2004). Contrarily, all bovine MRSA clones are infrequently seen in dairy cows, responsible of subclinical mastitis in cattle (Aqib et al., 2018b; Abdeen et al., 2021). The first time MRSA was detected in Cattle in Belgium in 1972 in milk samples which thought to be spread from milker hands through contamination (Lee, 2003). MRSA infection of mammary gland in cattle leads to a decrease in milk production and may cause cessation of milk from mammary glands in severe cases which pose the dairy industry a big economic loss (**Figure 4**; Holden et al., 2013). Mammary gland inflammation results from cytotoxicity of *luk*MF9, a powerful virulence factor of LA-MRSA (Peton et al., 2014). *luk*MF9 has a high affinity for chemokine receptor CCR1 on bovine which results in significant inflammation and damage as a consequence of more accumulation of neutrophils in mammary gland (Fromageau et al., 2010; Vrieling et al., 2015). A tropical phage called wPV83, which is capable of hematogenous spread to *S. aureus*, carries the *luk*M and *luk*F genes (*luk*M-*luk*F-PV) which are closely associated with synthesis of *luk*MF9 (Yamada et al., 2005). A conformational change in the nucleotide of a gene produces a suppressor protein (*rot*) in *S. aureus* strains that block the activation of many toxin genes that exhibit large

quantities of the *luk*MF9. Genetic studies have linked strains overexpressing *luk*MF9 to the lineage ST479 (Hoekstra et al., 2018). Superantigens (*SAgs*) are a class of bacterial toxins that stimulate the immune system and are released by staphylococcal species, particularly *S. aureus*. They have the potential to cause an unsustainable cytokine cascade (Tuffs et al., 2018). However, SAgs seem to perform a crucial function in bovine mastitis, and the majority of cattle *S. aureus* isolated have five or more genes encoding *SAgs*, although the precise function of *SAgs* in mastitis is yet unknown (Wilson et al., 2018). *SAgs* disrupt the human autoimmune reaction, which makes them potentially significant in chronic infections (Ferens and Bohach, 2000). By preventing immune-regulatory activation, the multiplication of T cells and the production of interleukins may stopped by SAgs that might reduce the efficiency of the immune system response (Tuffs et al., 2018). The cow susceptibility island SaPIbov is present in lineages that are related to cattle, including CC151 and CC133 (Wilson et al., 2018). Numerous toxins, including the toxic shock syndrome toxin 1 (TSST-1), the staphylococcal enterotoxin-like protein, and bovine staphylococcal enterotoxin C are also stimulated by SaPIbov (Fitzgerald et al., 2001).

Pigs in various studies exhibit higher than expected percentages of MRSA. Highly pathogenic strains are also rising in the pig population. In a study, 10% of samples were positive for the ST398 strain of MRSA, and the overall presence of MRSA among the three studied farms was considerably high. These 10% ST398 strains of MRSA were further found related to spa type, t1793 and t034 and they were exhibiting a high level of resistance to multiple antibiotics. This is another clue for the new emerging zoonotic strain of MRSA in Europe among the pig population (Hasman et al., 2010). Moreover, LA-MRSA ST398 is not only confined to pigs in Europe but it also spreads to Canada and USA (Khanna et al., 2008; Smith et al., 2009). Except ST398 strain, pigs also harbor other LA-MRSA strains such as ST1, ST97 and ST9 but at a lower frequency. A study conducted by Wagenaar et al. (2009) in China identified LA-MRSA ST9 lineage type colonization in pigs as well as workers. Another study was conducted by identified the human clone ST1 in pigs which indicating human to pig transmission of clone called as reverse zoonosis as depicted in **Figure 1** (Monaco et al., 2010).

Methicillin resistant strain of *S. aureus* (MRSA) is a chief bacterium isolated from mastitis milk of goats which may infect individuals who are consuming contaminated milk. Contaminated milk may contain certain types of staphylococcal enterotoxins (SE), which is a major cause of food poisoning. All SE genes are capable of causing illnesses that are particular to their hosts, such as staphylococcal enterotoxin B causes infection in humans (Da Silva et al., 2005; Altaf et al., 2020). MRSA can also cause pyaemia which is a type of abscess which gain access to bloodstream and lead to sepsis (Webster and Mitchell, 1989). MRSA in sheep and goats is among the predominant cause of clinical and subclinical mastitis which leads to high somatic cell count in milk and make milk unfavorable to drink (Muzammil et al., 2021).

Livestock associated MRSA (LA-MRSA) has been also reported in cloaca and nares of healthy poultry birds. In poultry birds, it may cause pyoderma, omphalitis, urinary tract infection, arthritis, and otitis (Pickering et al., 2022). After performing different antimicrobial agents. MRSA was found spa type t011 and spa type t157. While ST398 is a new livestock-associated strain of MRSA that is also found in poultry (Nemati et al., 2008). Another investigation showed that all MRSA isolates of poultry origin were belonging to spa type t1456 (Persoons et al., 2009).

7.2. MRSA infection in companion animals

The companion animals mostly suffer skin and soft tissue infections predominately after surgical procedures. The United Kingdom observed 95/6519 MRSA-positive samples that were comprised of 24 cats, 69 dogs, 1 horse, and 1 rabbit (Boag et al., 2004). Molecular analysis of MRSA in dogs and cats using SCC *mec* typing and sequence typing of *ccrAB* gene (cassette chromosome recombinant gene AB), and established MRSA strains using MLST from cats and dogs similar to that of human SCC *mec* gene in staphylococci. A further cross-sectional study revealed the transmission of MRSA strains from humans to dogs especially MRSA strain ST239-III (Malik et al., 2006). A research finding at Irish Veterinary Hospital noted that 69.44% of canines, 22.22% of horses, and 2.78% of cats, rabbits, and seals were infected with MRSA (O'Mahony et al., 2005). Risk factors such as the site of infection, surgical history, medical history, intravenous catheterization, and use of previous antibiotics were inferred after 6 years of a controlled trial conducted in veterinary health care in the USA and Canada. The most prevalent sites of colonization were found skin and nares (Manian, 2003). MRSA also causes remarkable and life-threatening infections in horses and personals taking care of them. Horses may have septic arthritis, bacteremia, osteomyelitis, and inflammation of the skin and delicate tissues (Cuny et al., 2006), metritis, omphalitis, pneumonia, and infections related to catheters are a few examples of illnesses associated with implants. First case of MRSA in horses was noted in 1993 due to postoperative infections. Reverse zoonosis with CA-MRSA-5 (ST8) in horses has also been found with a wider range of antibiotic resistance, e.g., oxacillin, tetracycline, gentamycin, etc., (Weese et al., 2005).

8. Current and futuristic approaches to treat MRSA infections

Among individuals who have established MRSA infections, bacteremia that might result in mortality and could affect nearly 50% of the population (Nickerson et al., 2009). MRSA preventive

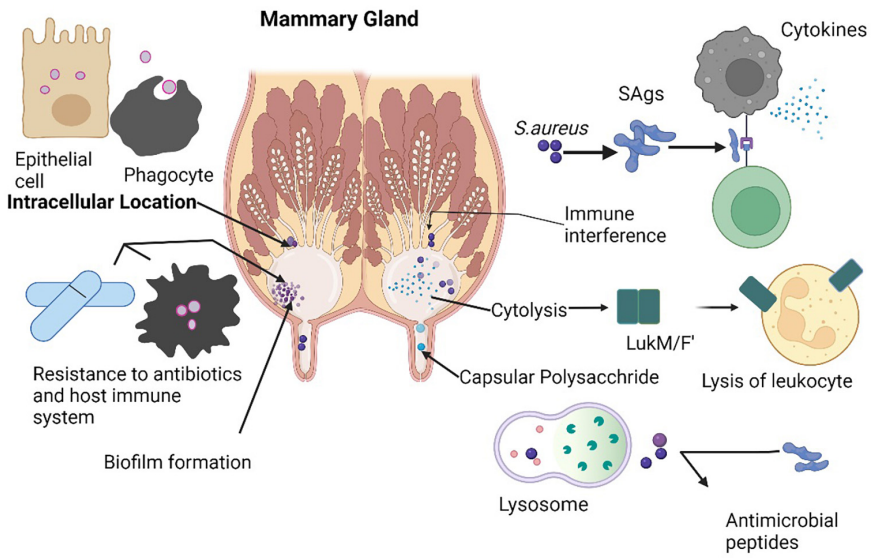

FIGURE 4
Different tactics used by *S. aureus* to survive inside mammary glands to cause infection.

measures and use of a recent antibiotic therapy alone and in combination may result in decrease MRSA infections (De Kraker et al., 2013). The ineffective disease management at the start of infection may be the reason of excessive death rate (Simor et al., 2016). Additionally, several virulence genes have been linked to increased mortality, such as acquisition of antibiotic resistance genes through horizontal gene transfer mechanisms are major cause of making antibiotics ineffective against MRSA infection (Albur et al., 2012). However, still there are antibiotics and futuristic approaches to treat MRSA infections which are discussed in further sections. The advent of several new antibiotics alone or in combination are available in the market, a hope for MRSA infected patients. Additionally, scientists now are switching from single-agent therapy to combination therapy, immunotherapy, and few latest alternative ways to combat MRSA infections such as phytochemicals, probiotics, nanoparticles, and bacteriophages as an antibiotic alternatives (Lee et al., 2016).

8.1. Antibiotics to treat MRSA infections

In comparison to MSSA infection, bacteremia caused by MRSA is more severe (Fowler et al., 2006). A prolonged duration of bacteremia may result in a more severe consequences (Fowler et al., 2003). A survey conducted in Australia noted that ceftaroline and cephalosporin resistance were found in 17% of MRSA cultures. Although several other new drugs have been approved for use, vancomycin remains the most effective (Abbott et al., 2015). Moreover, combining a β-lactam antibiotic with glycopeptide for example daptomycin to treat MRSA infections is recommended by the Spanish Society of Clinical Microbiology and Infectious Diseases (Gudiol et al., 2015). The mechanism of action of daptomycin somewhat different, it causes potassium and calcium ions to cross the plasma membrane, which causes apoptosis of the bacterial cell. Daptomycin inhibits the function of *fem* and *aux* genes, hence reducing the expression of the *mecA* gene. Daptomycin can reduce PBP-2α binding to its peptidoglycan moieties in the early phases of peptidoglycan synthesis (Rand and Houck, 2004). Daptomycin's ability to bind to β-lactam antibiotics is improved when used in combination (Dhand et al., 2011). However, mutation at the gene level may makes the daptomycin a resistant drug against MRSA. The major genes involved in daptomycin resistance include multi-peptide resistance (*mprF*) and the regulatory gene *walKR* (also called *yycGF*). This is because of polymorphism in single nucleotide sequences (Jiang and Peleg, 2015). Another type of mutation e.g., point mutation in genes such as *rpoB* and *rpoC* also associated with the development of resistance against daptomycin (Peleg et al., 2012).

Vancomycin was once thought to be the most efficient antibiotic for treating MRSA-related severe infections. The suitability of vancomycin's primary action is determined by gathering evidence of overall resistance, unachievable pharmacokinetic/pharmacodynamic (PK/PD) targets, and reduced effects (Holmes et al., 2012; Van Hal and Fowler, 2013). Vancomycin intermediate *S. aureus* (VISA) and hetero resistant (hVISA) are both commonly treated with glycopeptides. Vancomycin, therefore, begins to become resistant to them as well as losing sensitivity to glycopeptides, which causes the development of vancomycin-resistant *S. aureus* (VRSA). These isolates are more sensitive to other antibiotic classes,

particularly β-lactam antibiotics, despite having the *mecA* gene. The "seesaw effect" refers to the sensitivity of MRSA isolates to anti-staphylococcal β-lactam antibiotics caused by higher minimum inhibitory concentrations (MICs) of daptomycin and vancomycin. Research on the synergistic effects between vancomycin and β-lactam antibiotics presents great potential (Werth et al., 2013). When the β-lactam seesaw effect and cross-resistance among glycopeptides, lipopeptides, and lipoglycopeptides against MRSA strains were examined, the important factors responsible for developing the cross-resistance to daptomycin, vancomycin, and dalbavancin is the change in membrane lipid composition such as fatty acyl and ultimately resulted in resistant strains. Abundance of long-chain fatty acyl peptidoglycans in membrane has a negative correlation with β-lactam sensitivity and a positive correlation with cross-resistance (Hines et al., 2020).

The combined effect of β-lactam antibiotics and vancomycin is found synergistic in *in vitro* studies when checked through antimicrobial sensitivity assays. The synergistic effect is highly linked with MIC of vancomycin. An *in vivo* trial was conducted on rabbits infected with VISA strain. The efficacy of two drugs, vancomycin and nafcillin, was checked to compare their effects. The results were ineffective with a single administration of drugs but give curative effects with combination therapy by reducing the magnitude of infection up to 4.52 $\log_{10}$ CFU/g. The extent of synergism was highly correlated with the MIC of vancomycin (Climo et al., 1999). Another *in vitro* study evaluated combinations of vancomycin and cephalosporin showed synergistic effects against MRSA isolates (Seibert et al., 1992). These isolates were also checked against the combination therapy of vancomycin and imipenem under the synergistic effect (Silva et al., 2011). Many more combinations have also been inquired such as rifampicin and gentamycin; daptomycin and rifampicin therapy against biofilm-producing MRSA isolates (Rosenberg Goldstein et al., 2012). These therapies proved better results by comparing the daptomycin and cloxacillin in a rat infected with MRSA and indicated better results against rifampicin-resistant MRSA infections.

The fifth generation cephalosporins are the most effective against MRSA infections than other antibiotic generations. The most widely utilized drugs in health care facilities among cephalosporins are ceftaroline and ceftobiprole. Ceftaroline is a drug that is licensed to treat skin associated infections and community-acquired pneumonia (CAP) (Purrello et al., 2016). To compare the efficacy of ceftobiprole, ceftriaxone, and linezolid, multi-center clinical studies were carried out in 2006–2007 among patients in hospitals who exposed to CAP. A multi-center FOCUS-1 study, conducted by a researcher in 2008–2009, investigated the effectiveness of ceftaroline and ceftriaxone against CAP. Nearly 168 locations from around the world were chosen. The modified intention to treat (mITT) and clinical evaluation (CE) rates of ceftaroline were found to be high, 86.6 and 83.3%, respectively while of ceftriaxone were 77.7% in mITT and 78.2% in CE (File et al., 2011). Many new approved and novel drugs are effective against MRSA infections, but further research is required to determine their efficacy at large scale. The next section discusses few of the most recent antimicrobials that are effective against MRSA infections (Thati et al., 2011).

8.1.1. Oxazolidinones

A novel class of antibiotics known as oxazolidinones are effective against a variety of gram-positive bacteria, including vancomycin- and methicillin-resistant staphylococci. Oxazolidinone blocks protein synthesis by binding to the P-site of the 50S ribosome subunit. The development of oxazolidinone resistance with 23S rRNA does not influence oxazolidinone action when compared with the resistance of other protein synthesis blockers. Its application in surgical infections is made possible by its effective penetration in bone and infiltration into lungs, cerebrospinal fluid, and hematoma (Bozdogan and Appelbaum, 2004). Tedizolid, a brand-new drug among the oxazolidinones, has received approval for the standard 6-days treatment course for skin and soft tissue infections. Compared to linezolid, tedizolid is a drug that is more efficient and offers more benefits (Boucher et al., 2014; Flanagan et al., 2015). Tedizolid's efficiency against chloramphenicol and florfenicol resistant (CFR) isolates harboring methyltransferase gene is also found (Flanagan et al., 2015). Novel oxazolidinone agent cadazolid is a potent agent against *Clostridium difficile* (Gerding et al., 2015), while radezolid is efficient against isolates of *S. aureus* that are resistant to linezolid (Lemaire et al., 2010).

8.1.2. Tetracycline

This class of antibiotic inhibit the bacteria growth by inhibiting the protein synthesis. They attach to the 30S ribosomal subunit and prevent aminoacyl-tRNA from joining the translational mRNA complex, which inhibits the beginning of translation. In several researches, the drug tetracycline was found to bind to the rRNAs 16S and 23S (Chukwudi, 2016). The novel synthetic fluorocycline and eravacyline drugs are effective against infections caused by gram-positive and gram-negative bacteria, including MRSA. Eravacycline is four times more efficient than tigecycline for treating gram-positive bacteria (Zhanel et al., 2016). Omadacycline, an aminomethylcycline, is more efficient to treat CAP and acute bacterial skin and skin structural infections (ABSSSI) (Pfaller et al., 2017).

8.1.3. Fluoroquinolones

Quinolones are among the most common antibacterial drugs used worldwide to treat a range of bacterial diseases in both animals and humans. These drugs are structurally known as quinolones because they have a quinoline ring in their structure. Quinolones and fluoroquinolones prevent bacteria from multiplying by inhibiting their DNA replication pathway. These antibiotics basically damage the bacteria chromosome by

targeting the enzymes gyrase and topoisomerase IV (Aldred et al., 2014). The fluoroquinolone antibiotic, delafloxacin is an drug that is effective against both gram-positive and gram-negative bacteria because of its unique electrochemical characteristics such as being anion at physiological pH and uncharged at acidic pH (Lemaire et al., 2011). In 2011, a research trial was carried out in the USA to evaluate delafloxacin effectiveness in comparison to vancomycin and linezolid. These three drugs were found in following order with highest rates of cure, delafloxacin, linezolid, and vancomycin (Kingsley et al., 2015). Zabofloxacin is another fluoroquinolone drug showing high activity against gram-positive microorganisms, especially against respiratory tract infections due to *St. pneumoniae*. MIC_{50} of zabofloxacin against MRSA was noted high as compared to delafloxacin i.e., 2 mg/ml in and 0.125 mg/ml respectively. Except for these characteristics of zabofloxacin, it is less efficient against gram-negative organisms. Thirdly, nemonoxacin was found to be similar to zabofloxacin in its pattern of activity and its effects against CAP have been also investigated. Fourthly, avarofloxacin efficacy against MRSA is also comparable to that of delafloxacin (Van Bambeke, 2014).

8.1.4. Lipoglycopeptides

Fatty acid chains linked to glycopeptides, a family of antibiotics known as lipoglycopeptides, showed dose-dependent bactericidal action. They prevent the synthesis of cell wall and interfere with the bacterial cell membrane's permeability function. Therefore, the terminal acyl-d-alanyl-d is where the glycopeptide core binds. The alanine chain in the cell wall interacts with hydrophobic filling and hydrogen bonds resulting in a high affinity. This inhibits the cell wall precursors from polymerizing and crosslinking (Damodaran and Madhan, 2011). Three novel drugs from the lipoglycopeptide family have been approved and launched in the market. A lipoglycopeptide called dalbavancin was approved by FDA and European Medicine Agency (EMA) for treating the ABSSSI in 2014 and 2015, respectively (Van Bambeke, 2015). Dalbavancin is a semi-synthetic lipoglycopeptide with a long half-life ~10 days and prolonged duration pf action ~7 days against MRSA with a single dosage of 500 mg (Chen et al., 2007). Dalbavancin is specifically used to treat complex infections in outpatients (Juul et al., 2016). A multi-center study was conducted to treat ABSSSI in 2011–2012 to compare efficacy of dalbavancin with vancomycin. Compared to vancomycin, dalbavancin showed great outcomes with fewer side effects (Boucher et al., 2014).

Ritavancin, a second lipoglycopeptide, authorized by FDA and EMA in 2014 and 2015, which is also a prolonged action lipopeptide used to treat ABSSSI infections (Takahashi and Igarashi, 2018). This drug act by supressing the transglycosylase and transpeptidase enzymes. This antibiotic also exhibits bactericidal activity against broad range of gram-positive pathogens such as vancomycin-resistant enterococci (VRE), VISA, and VRSA due to its enhanced capability of penetration through plasma membrane (Van Bambeke, 2014). Telavancin is another lipoglycopeptdie which is quite effective against hospital-acquired pneumonia (HAP) caused by MRSA (Sandrock and Shorr, 2015). The drug is approved by FDA in 2013 for the treatment of MRSA infections including HAP and VAP following approval for SSSS treatment (Wenzler and Rodvold, 2015). When other antibiotics become ineffective, the EMA has restricted its usage for the treatment of nosocomial pneumonia caused by MRSA (Masterton et al., 2015). Although the lipoglycopeptides are approved for a narrow range of treatment of infections, in the future they will play important role in the treatment of osteomyelitis, bacteremia, and infective endocarditis (Brade et al., 2016).

Other antimicrobial drugs may include doxycycline, clindamycin, and trimethoprim/sulphamethoxazole which are also found effective irrespective of the severity of the disease (Liu et al., 2011).

8.2. Futuristic approaches to treat MRSA infections

As stated by World Health Organization (WHO), among the largest threat to public health, is the rise in antibiotic-resistant microorganisms. Approximately 700, 000 fatalities are caused by antibiotic resistance bacteria each year globally, and by 2050, that number might reach 10 million (Tagliabue and Rappuoli, 2018). A post-antibiotic era, in which ordinary diseases and mild infections might kill, is a very real prospect for the twenty first century, according to a study from WHO (Streicher, 2021). Thus, the development of novel antibiotic-free strategies is urgently required for the management and treatment of antibiotic-resistant bacterial infections.

8.2.1. Herbal medicine

The use of antibiotic stimulators in association with antibiotics is one of the best methods for reducing antibiotic resistance and extending the life of current antibiotics. The most effective combination against MRSA was found β-lactam antibiotic and potassium clavulanate (De Araújo et al., 2013). As listed in **Table 3**, many studies shown that phytochemicals alone or in combination with antibiotic have a great organic potential to exhibit antibacterial activity as well as act as modulators of antibiotic resistance. The majority of these curative effects are attributed to the active secondary metabolites produced by plants (Lakshmi et al., 2013). Phytochemicals act as antibacterial agent by inhibiting efflux pumps, modification of active sites, increasing the permeability of plasma membrane, and alteration of bacterial enzymes as depicted in **Figure 5** (Coutinho et al., 2009). Plant extract along with gentamicin and kanamycin have synergistic effects for example ethanol

TABLE 3 Antibacterial activity of different herbal plants against different strains of methicillin-resistant *Staphylococcus aureus* (MRSA).

Sr. No.	Plant name	Plant part	Extraction method	MRSA strains	ZOI or MIC (μg/ml)	References
1.	*P. nigrum* *H. cordata* *S. baicalensis* *C. sinensis*	Dried Fruit Stem + Leaf Roots Leaves	Sterile water	LA-MRSA	5,000 1,250 1,250 625	Guo et al., 2022
2.	*Z. album*	Arial parts	Ethanol extract	HA-MRSA	312.5–1,250	Sharaf et al., 2021
3.	*C. macrocarpa*	Leaves	Methanol, Ethanol, n-butanol	HA-MRSA	2.0–8.0 256–2,048	Attallah et al., 2021
4.	*C. longa*	Root	Hydroxypropyl methylcellulose	LA-MRSA	ZOI 10–18 mm	Sarwar et al., 2021
5.	*A. pavarii*	Leaf and Stem bark	Methanol	CA-MRSA	1.25–2.50	Buzgaia et al., 2020
6.	*O. lamiifolium* *R. officinalis* *C. roseus* *A. indica* *M. stenopetala*	Leaves	Diethyl ether, ethyl acetate, methanol, ethanol	HA-MRSA	Larger ZOI than antibiotics	Manilal et al., 2020
7.	*A. catechu* *G. mangostana* *I. balsamina* *U. gambir*	Wood Fruit Shell Leaf Leaf + stem	Ethanol	HA-MRSA	1.6–3.2 0.05–0.4 6.3 0.4–0.8	Okwu et al., 2019
8.	*C. sativa* *T. orientalis* *P. guajava*	Leaves	Ethanolic extract	HA-MRSA and CA-MRSA	Larger ZOI than antibiotics	Chakraborty et al., 2018
9.	*C. cyminum* *A. subulatum* *C. verum* *S. aromaticum*	Seeds Seeds Bark Buds	Hydro-distillation	MDR *S. aureus*	29.7 ± 1.7 9.4 ± 1.86 4.8 ± 0.96 5.4 ± 1.08	Naveed et al., 2013
10.	*S. exigua* *E. koreensis*	Root	Tetra-hydroxy Flavanones	HA-MRSA	3.13–6.25	Tsuchiya et al., 1996
11.	*G. glabra* *G. inflata* *G. uralensis*	Root	Flavonoids	HA-MRSA	3.13–12.50	Gupta et al., 2008
12.	*D. capitata* *E. rugulosa* *E. blanda* *G. strictipes* *P. multiflorum*	Apex Wall Apex Root Root	Ethanol extract Ethanol extract Ethanol extract Ethanol extract Ethanol extract	HA-MRSA	1.25 1.43 1.32 1.34 1.34	Zuo et al., 2008
13.	*C. impressicostatum*	Sb	Water extract	HA-MRSA	19.50	Khan et al., 2009
14.	*P. betle*	Leaves	Ethanol extract	HA-MRSA	156–78	Valle et al., 2016
15.	*C. sinensis*	Leaves	Polyphenols	HA-MRSA	50–180	Choi et al., 2015
16.	*C. procera*	Leaves	Aqueous extract	HA-MRSA	12.5	Salem et al., 2014
17.	*E. globulus*	Leaves	Eucalyptus oil	HA-MRSA	4.0	Mulyaningsih et al., 2011
18.	*C. longa*	Root	Ethanol extract	HA-MRSA	217	Gunes et al., 2016

MIC, minimum inhibitory concentration.

extracted of *Turnera ulmifolia* leaves may enhance antibacterial efficacy of antibiotic against MRSA strains. As a possible active efflux regulator, grapefruit oil was found to be beneficial against MRSA (Abulrob et al., 2004). In an interesting study, the traditional Korean medicine known as Sami Hyanglyum-Hwan, *Aucklandiae radix*, *Coptidis rhizome*, *Rhei rhizome*, and *Arecae semen*) restored the anti-microbial activity of ciprofloxacin when evaluated against multiple MRSA strains (Choi et al., 2015).

8.2.2. Synergistic effect of antibiotics with NSAIDs

Several studies have shown that NSAIDs exhibited antimicrobial properties, nevertheless, the exact mode of action

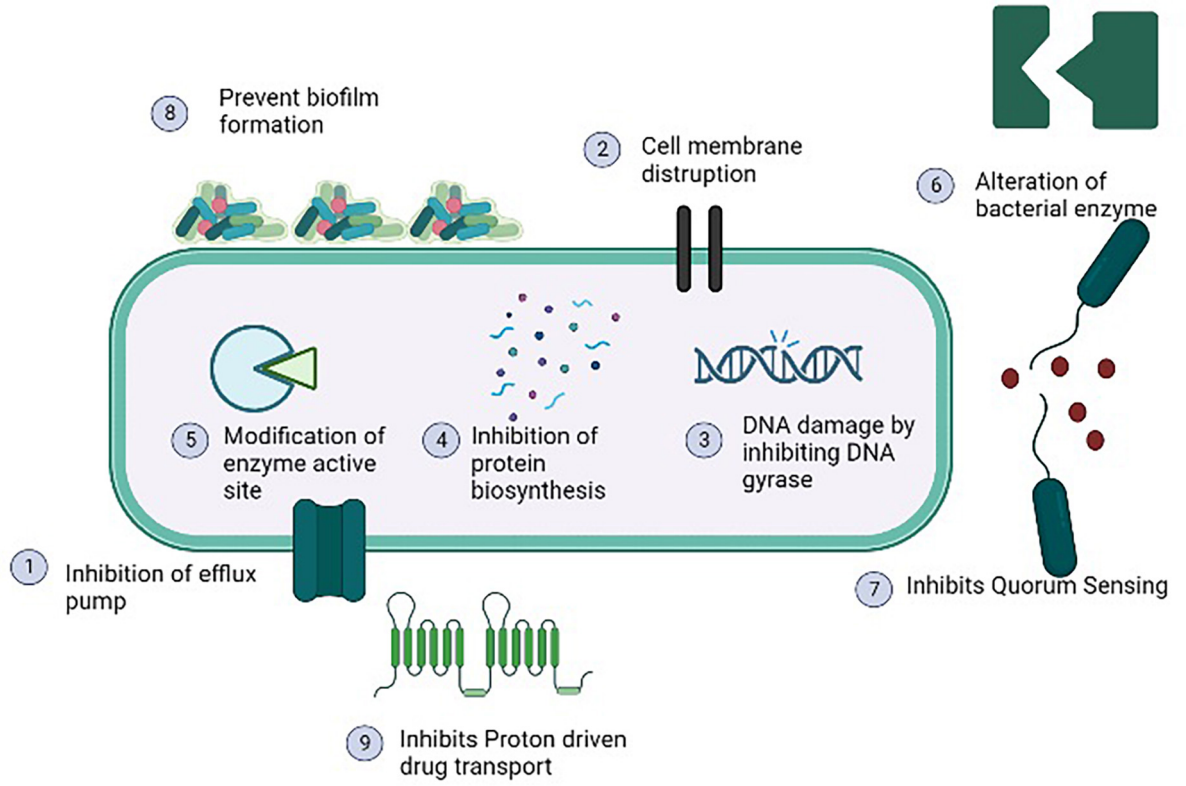

FIGURE 5
Antibacterial mechanisms of various phytochemicals against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

is unclear. Except for mefenamic acid, it has been observed that diclofenac, aspirin, and ibuprofen have antibacterial effects at 5 mg/ml against certain gram-positive bacteria. Due to the presence of lipopolysaccharide in the cell wall of gram-negative bacteria, which is hydrophilic and inhibits most drugs metabolism, the only NSAID that is helpful against gram-negative bacteria is aspirin. The absence of lipopolysaccharide in cell wall of gram positive bacteria, makes it simple for antimicrobial drugs to enter the cells easily (Khalaf et al., 2015). In comparison to the typical therapeutic dosage in use for inflammatory, pain, or fever, NSAIDs have antimicrobial action at significantly lower concentrations (Ong et al., 2007). Opposite to diclofenac, aspirin and ibuprofen demonstrated bacteriostatic and bactericidal action against MRSA strains and may thus be used as antibiotic adjuvants to treat infections (Chan et al., 2017). The treatment of CA-MRSA infections involves the use of NSAIDs and antibiotics. Ineffective outcomes were seen when cefuroxime and chloramphenicol were taken alone to cure MRSA. Although aspirin and ibuprofen have bacteriostatic and bactericidal effects on MRSA strains, however their combination along with cefuroxime and chloramphenicol were examined. It was shown that the combination of ibuprofen/aspirin, chloramphenicol, and cefuroxime have increased antibacterial showing either synergistic or additive effect (Yin et al., 2014). MDR bacterial infections can be treated with NSAID and antibiotic combination (Chan et al., 2017). Another study conducted by Aqib et al. (2021) investigated the effect of antibiotics alone, in combination with NSAIDs, nanoparticles and plant extracts against MDR strains of *S. aureus* including MRSA. It was noted antibiotic in combination with NSAIDs such as meloxicam, diclofenac, aspirin and ibuprofen increases the ZOIs in *in vitro* studies against MRSA and MDR strains of *S. aureus*. The study concluded synergistic correlation between NSAIDs, antibiotics, nanoparticles and plant extracts by calculating fractional inhibitory concentration indices (FICIs).

8.2.3. Nanoparticles as therapeutic agents

Due to the special characteristics of metal nanoparticles (NPs), they are more ubiquitous and inexpensive manufacturing material that are getting much applications in today's world. In 2016, it was noted that the nanometals market based on metal oxides reached USD 4.2 billion. A more rise in NPs manufacturing demand is found to be anticipated by 2025, which is due to increased use of metal based nanomaterials in biomedical research (Gudkov et al., 2022). Research studies going on to explore the potential of nanoparticles as biosensors (Singh et al., 2019), detection and treatment of oncological

disorders (Vimala et al., 2019), and drug delivery have received a lot of interest (Spirescu et al., 2021). It is very interesting to employ metal oxide nanoparticle-based on nanomaterials to treat antibiotic resistant bacterial infections. Now a day, most commonly used metal oxide nanomaterials are silver nitrate, zinc oxide, platinum, aluminum oxide, titanium dioxide, gold, magnesium oxide, iron oxide and sodium alginate (Akhtar et al., 2019; Spirescu et al., 2021; Gudkov et al., 2022; Mendes et al., 2022).

According to the statement from WHO, antibiotic-resistant microorganisms are among the most remarkable barriers to public health and progress. The new paradigm to treat the diseases caused by resistant bacterial strains including MRSA is the use of metal oxide nanomaterials [95]. The NPs act on bacterial cell through various type specific mechanisms such as production of reactive oxygen species (ROS) to induce stress on cell, release of heavy metal ions, alter membrane permeability, DNA damage, protein damage, disrupt the function of efflux pump, act of cell wall and plasma membrane to cause damage and release cellular components outside of cell (Fernando et al., 2018) as illustrated in **Figure 6**. (Shameli et al., 2010) conducted study to evaluate the antibacterial activity of green silver nitrate nanoparticles against MRSA isolates and found that green silver nitrate nanoparticles exhibited the strong antibacterial activity by inhibiting the growth of MRSA in *in vitro* model. Another study was conducted by Lodhi et al. (2021) who evaluated the antibacterial activity of zinc oxide NPs alone and in combination with antibiotic and found increased antibacterial activity when NPs were used in combination with antibiotic. Another study conducted by Wichai et al. (2019) in Thailand who use a complex combination of nanomaterials with other materials (bacterial cellulose + sodium alginate NPs + chitosan + copper sulfate) and found that NPs exhibited a strong antibacterial activity against MRSA strains. A number of studies has been conducted by various researchers from different countries to check the antibacterial activity of various forms of nanoparticles against different strains of *S. aureus* including MRSA are listed in **Table 4**.

8.2.4. Bacteriophages as an alternate to antibiotics

Bacteriophage therapy also known as phage therapy is a type of therapy that uses viruses to kill bacterial pathogens. Due to increasing resistance to antibiotics, phage therapy is considered as cost effective, highly specific and efficient in their mechanism of action against multiple MDR bacteria. Bacteriophages only cause damage to bacteria cells without causing any damage to human and animal cells (Tkhilaishvili et al., 2020). Phage therapy has been found as an effective treatment against multiple bacterial pathogens such as *S. aureus*, *E. coli*, *P. aerogenosa*, *A. baumannii*, *S. pyogenes*, *S. suis*, and *B. cereus* (Yang et al., 2017). This therapy is mostly used in Georgia and Russia for the treatment of those bacterial infections which don't response to antibiotics. A study was conducted by Nandhini et al. (2022) who evaluated the lytic activity of kayvirus phages against MDR *S. aurus*. (Lubowska et al., 2019) also study the genomic, morphology, and lytic properties of three phages which are name as vB_SauM-A, vB_SauM-C, and vB_SauM-D and noted rapid adsorption with bacterial cell, short latent period, and increase lytic activity. Further genome study showed higher G + C content in these phages similar to phage K (Nandhini et al., 2022). As, bacteriophage act so specifically consist of following steps to destroy bacterial cell; (1) Adsorption which is attachment of virus with specific bacteria cell, (2) Penetration of phage DNA or RNA into the bacteria cell, (3) after penetration, phage uses host cell machinery to synthesis early viral proteins, (4) Replication of virus through using the early viral proteins, (5) Synthesis of late viral proteins to assemble again in new complete phage particles, (6) Lastly, new phage particles cause lysis of the bacterial cell and causes its death through release of cellular contents outside the cell and new phage particle starts infecting the bacterial cells (Sausset et al., 2020) as illustrated in **Figure 7**. Staphylococcal bacteriophage (Sb) and PYO bacteriophage are prepared by Eliava Institute, Georgia and available commercially as phage preparations for usage as replacement to antibiotic by humans (Fish et al., 2018). Bacteriophage Sb is a mono phage product and is effective against only one specific bacteria while PYO phage covers multiple bacterial species such as *E. coli*, *S. aureus*, *Streptococcus* spp., *P. aeruginosa*, and *Proteus* spp (Villarroel et al., 2017). The number of isolated phages against MRSA has significantly increased over the past 15 years, and several investigations have found that phages have effective and all-encompassing antibacterial effects as listed in **Table 5**.

8.2.5. Probiotics as therapeutic agents

Utilizing probiotics is a prospective antibiotic substitute. Probiotics are live microorganism known to aid during infection or after antibiotic therapy which upsets the normal gut microbiota. Additionally, they could help to relieve certain other conditions such as irritable bowel syndrome (IBS) symptoms and antibiotic associated diarrhea (McFarland et al., 2021). Probiotic can improve the health of many other body tissues by replenishing their respective microbiota and releasing anti-pathogenic chemicals. So, the word probiotic is not only restricted to gut microbiota but they also provide various other health benefits such as enhances the host immunity by upregulating the immune cells, depression and anxiety disorders, tumor suppression, therapeutic role in COVID-19, overweight and obese patients (Herman, 2019; Shi et al., 2019; Shin et al., 2019; Ceccarelli et al., 2020; Daniali et al., 2020). Firstly, specific strains of Lactobacilli were used and evaluated for antimicrobial properties. Nowadays, many non-pathogenic species of bacterial as well as fungal genera are used as potential source of probiotic such as Streptococcus, Bifidobacterium, Bacillus, Escherichia, Enterococcus, and

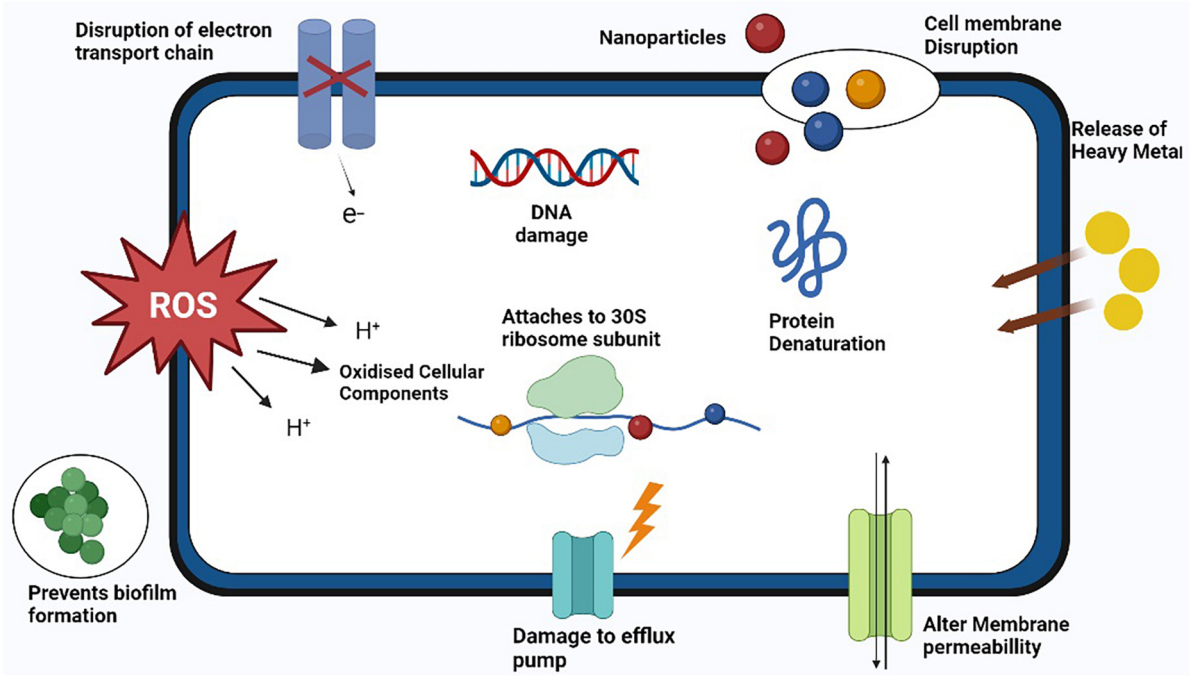

FIGURE 6
Antibacterial mechanisms of action of various nanoparticles against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

TABLE 4 *In vitro* studies on antibacterial activity of nanoparticles against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

Sr. No.	Composition of NPs	MRSA strain	ZOI or MIC	Country	References
1.	Green silver nitrate	HA-MRSA	8.4–8.8 mm	Malaysia	Shameli et al., 2010
2.	Polyacrylonitrile copper oxide	CA-MRSA	7.0 ± 0.05 mm	Taiwan	Wang and Clapper, 2022
3.	Green platinum NPs	MRSA	1.0 μg/ml	Egypt	Eltaweil et al., 2022
4.	Chitosan-gold NPs-Plant extract	MRSA	15.6 μg/ml	Egypt	Hussein et al., 2021
5.	Zinc oxide Zinc oxide + antibiotic	LA-MRSA	125 μg/ml 10.42 μg/ml	Pakistan	Lodhi et al., 2021
6.	Nickle oxide	HA-MRSA	265 μg/mL	Egypt	Rheima et al., 2021
7.	Iron Oxide (IO) NPs IO NPs + Vancomycin IO NPs + Ceftriaxone IO NPs + Gentamicin	HA-MRSA	12 ± 0.21 mm 25 ± 0.3 mm 37 ± 0.21 mm 34 ± 0.2 mm	Malaysia	Majeed et al., 2021
8.	Biosynthesized silver nitrate NP + vancomycin	MRSA	0.39 ± 0.16 mm	Egypt	Awad et al., 2021
9.	Titanium dioxide + erythromycin	HA-MRSA	2–16 mg/L	Pakistan	Ullah et al., 2020
10.	Endolysins + sodium alginate + chitosan NPs	MRSA	22.5 ± 3.1 mm	India	Kaur et al., 2020
11.	Bacterial cellulose + sodium alginate NPs + chitosan + copper sulfate	MRSA	5.0 mm at 0.9 conc.	Thailand	Wichai et al., 2019
12.	Zinc oxide	HA-MRSA	312.5–1,250 μg/ml	India	Umamageswari et al., 2018
13.	Silica silver nitrate	HA-MRSA	2.5–5.0 μg/ml	Taiwan	Chien et al., 2018
14.	Magnesium oxide	MRSA	1.0 μg/ml	USA	Nguyen et al., 2018
15.	Aluminum oxide	MDR-MRSA	1,700–3,400 μg/ml	India	Ansari et al., 2013

NPs, nanoparticles; MIC, minimum inhibitory concentration; ZOI, zone of inhibitions.

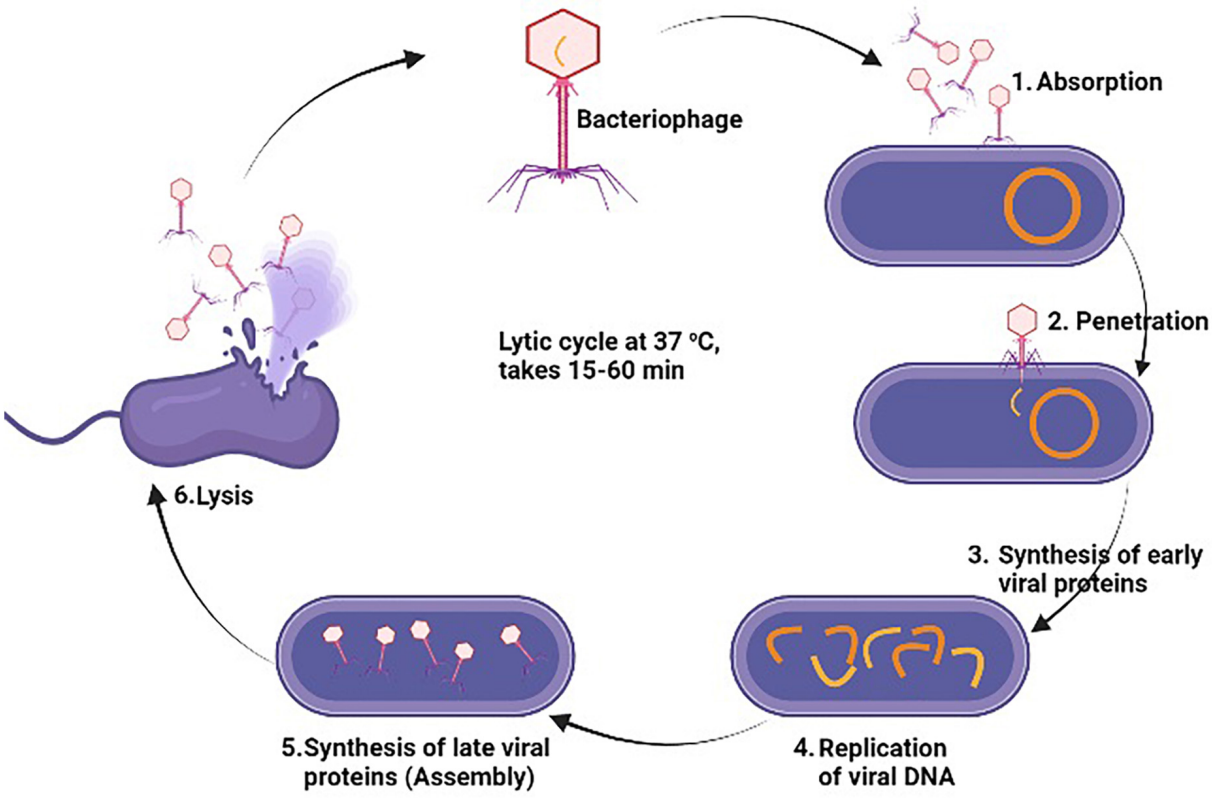

FIGURE 7
General antibacterial mechanism of bacteriophages against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

Saccharomyces from fungi. Various strains of Lactobacillus (*L. rhamnosus, L. plantarum, L. animalis, L. reuteri, L. lactis, L. gasseri, L. curvattus, and L. lacis*), Bacillus (*B. subtilis, B. amyloliquefaciens*), Bifidobacterium (*B. lactis, B. bifidum, B. breve, B. dentium, B. longum, B. infantis, B. catenulatum, B. pseudo catenulatum*), and Saccharomyces (*S. cerevisiae*) are all summarized in **Table 6**. All of the probiotic act by adopting one or more than one mechanism from the following; enhancement of epithelial barrier, increased adhesion to intestinal mucosa, synthesis of antimicrobial molecules, inhibition of pathogen adhesion to intestinal cells, competitive exclusion of pathogenic microorganisms, reducing the pH of gut lumen, and enhancement of immune response (Plaza-Diaz et al., 2019) as illustrated in **Figure 8**.

9. Futuristic approach to prevent MRSA infections

Currently, many MRSA prevention and control interventions are carried out such as judicial use of antimicrobials, hand hygiene, controlling the interaction with *S. aureus* natural reservoirs, preventing transmission from infected patient, decolonization, isolation, disinfection of hospital environment, active surveillance, and many other (Lee et al., 2018). Now there is a need to develop most effective way to control MRSA at animal and human cadre i.e., vaccine production. The problem of antibiotic resistance including MRSA pointing to a notable concern and is under study by Center for Disease Control and Prevention and World Health Organization (Klevens et al., 2007). The development of novel ways to combat antibiotic resistance and production of vaccine very important in this era. MRSA is not well-known to be resistant to any known antibodies (Anderson et al., 2012). However, attempts to develop a potent vaccine against MRSA are under trial by various companies (Adhikari et al., 2012). To develop a potent vaccine against multiple MRSA strains, there is need to use multiple antigens to develop effective immunity against different strains (Aqib et al., 2018a). Additionally, an appropriate adjuvant needs to be added to the vaccine to improve the vaccine function as well as delivery into the host (Adamczyk-Poplawska et al., 2011). To create an effective multi-epitope component immunization against staphylococcal infections, three antigenic determinants are known to be much important which are clustered factor A (ClfA), alpha-enolase (Eno1), and iron regulated surface

TABLE 5 *In vitro* studies on antibacterial activity of bacteriophages against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

Sr. No.	Bacteriophage	Phage isolation source	Phage efficacy assessment assay	Host range	Antibacterial activity method and result	Country	References
	Phage Rih21	Hospital Wastewater	Double layer agar method	MRSA	showed lytic activity	Iraq	Ghayyib et al., 2022
	Phage cocktail APTC-C-SA01	Nasal swabs, soil and sheep feces samples	Plaque assay	Biofilm MRSA and MSSA	> 98% lytic activity	Australia	Liu et al., 2022
	Phage Henu2 + Antibiotics	Sewage sample	Time kill assay	MRSA, MSSA	Growth decreased faster	China	Li et al., 2021
	Phage Sb1 + antibiotics	Commercial purchased	Plague assay and time kill assay	MRSA	Decrease in CFUs after treatment	USA	Kebriaei et al., 2020
	Recombinant chimeric bacteriophage endolysin HY-133	Hypharm GmbH	Broth micro dilution method	MRSA, MSSA, mupirocin-resistant strains	MIC = 0.12–0.5 mg/L, Time kill curve assay showed Bactericidal effect	Germany	Knaack et al., 2019
	Staphylococcal Sb and PYO bacteriophage	Eliava Biopreparations	Microcalori-metry Assay and CFU counting	MRSA	Rapidly inhibited growth of MRSA in both methods	Germany	Tkhilaishvili et al., 2020
	Sb-1 phage	Georgia Eliava Institute	Spot assay	MRSA. MSSA, biofilm	22/28 MRSA sensitive, 16/29 MSSA sensitive, eradicated biofilm	Germany	Tkhilaishvili et al., 2018
	Chimeolysin F (ClyF)		96-well plate method	*S. sureus* multiple strains, MRSA, *S. pyogenes, S. suis, B cereus*	Lytic activity against all *S. aureus* strains and no against other strains	China	Yang et al., 2017
	pq/27 and pq/48	Sewage water	Spot assay	MRSA	Agar method +	Pakistan	Rasool et al., 2016
	Lysostaphin + L. monocytogenes bacteriophage endolysin-ply511	N/A	Peptidoglycan hydrolytic activity	MRSA	Agar method High bactericidal activity	Australia	Turner et al., 2007

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TABLE 6 *In vitro* studies on antibacterial activity of probiotics against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

Sr. No.	Probiotic name/Microbial strain	Evaluation assay	Source of pathogenic organism	Test organisms	Antibacterial effect	Country	References
	Probiotic cellulose	Agar diffusion assay	Colección Española de Cultivos and Urine	*S. aureus*, *P. aeruginosa*, MRSA	Inhibited growth of all	Spain	Sabio et al., 2021
	Chitosan encapsulated strains of *L. lactis* and *L. curvattus*	Agar diffusion assay	Pus, urine, blood	*S. pyogenes, E. coli, K. pneumoniae, S. epidermidis, S. aureus, P. aeruginosa, and S. marcescens*	ZOI shown antibacterial activity against all pathogenic strains	Pakistan	Nasreen et al., 2022
	B. subtilis KATMIRA1933, *B. amyloliquefaciens* B-1895	Agar well diffusion assay	Wound infection	MRSA and MSSA	Growth inhibition seen as ZOI	Iraq	Algburi et al., 2021
	Bifidobacterium strains (*B. lactis, B. bifidum, B. breve, B. dentium, B. longum, B. infantis, B. catenulatum, B. pseudo catenulatum*)	Agar well diffusion and dilution assays, MIC micro-dilution assay	Sahmyook medical center	MDR *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212	ZOI were observed, lower MIC than antibiotics	Korea	Choi and Shin, 2021
	C. accolens	Agar well diffusion assay	Chronic rhinosinusitis patients	*S. aureus* ATCC 25923, MRSA, MSSA	ZOI of inhibitions indicated inhibitory growth	Australia	Menberu et al., 2021
	B. subtilis	Agar radial and spot assays	Animal Health Lab at UOG	Enterotoxic *E. coli* (ETEC), *S. typhimurium*, MRSA	ZOI were observed against all bacterial strains	Canada	Sudan et al., 2021
	L. plantarum CRL 759	Agar slab method, Agar diffusion assay, Optical density method	Human diabetic foot	*P. aeruginosa*, MRSA	Inhibited growth	Argentina	Layus et al., 2020
	Lactic acid bacteria	Agar well diffusion assay	Dairy animals	MRSA	ZOI were observed 16–29 mm	India	Essayas et al., 2020
	L. gasseri YIT 12321	Radial diffusion assay	Respiratory patient	MRSA	ZOI showed growth inhibition	Japan	Ishikawa et al., 2020
	L. animalis 30a-2, *L. reuteri* 4-12E, *L. lactis* 5-12H, *W. cibaria* C34	Agar well diffusion assay	FIRDI, Hsinchu, Taiwan; Chang-Hua Hospital in Taiwan	MRSA, ESBL *E. coli*, *P. aeruginosa*, *B cereus* ATCC 1178, *L. monocytogenes* ATCC 19111, *Y. enterocolitica* BCRC 12986, *S. choleraesuis* ATCC 13312, *S. enteritidis* ATCC 13076, *S. typhimurium* ATCC 13311, *S. fexneri* ATCC 29903, *S. sonnei* ATCC 25931	Represses the growth of all strains	Taiwan	Lin et al., 2020

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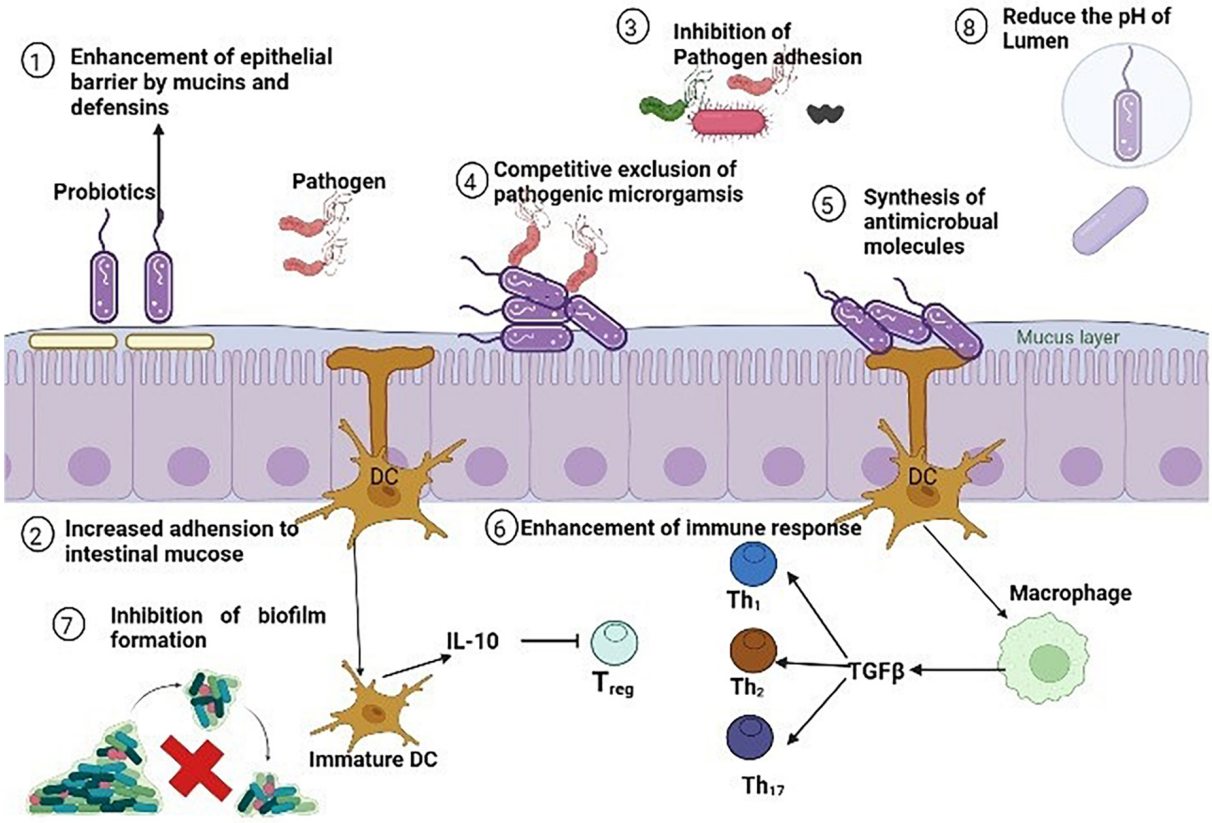

FIGURE 8
Antibacterial mechanisms of various probiotics against methicillin resistant strain of *Staphylococcus aureus* (MRSA).

determinant protein B (IsdB). Eno1 is a polypeptide present in cytoplasm of cell and found in all *S. aureus* strains and has a very well-preserved lineage. Additionally, this protein aids in the mechanism of adhesion and contributes to the spread of infection, so this protein is a potential candidate for vaccine development against multiple *S. aureus* strains (Ghasemi et al., 2016). ClfA, another cell surface protein, aids in the pathogen's adherence to the host. Previous investigations have shown that ClfA plays a key role in the development of staphylococcal illnesses (Garcia-Lara and Foster, 2009). Thus, to initiate a strong, active, and independent immune response to *S. aureus*, it is important to include this surface component as a vaccine agent (Brouillette et al., 2002). Another surface protein that aids in attachment to the cell membrane is IsdB, the third epitope marker (Zapotoczna et al., 2013).

A vaccine is an agent that prevent the infection before its onset and also disrupt the colonization of infection causing organism with host cell and thereby act as long lasting infection prevention agent and extensively reduces the use of antibiotics (Pozzi et al., 2017). A monoclonal antibody is developed by Medimmune, USA based company against α-haemolysin factors of *S. aureus* which may provide protective immunity administered alone or in combination with antibiotics. Another two monovalent trial based vaccines were prepared by USA based company and tested but failed to generate protective levels at the later stages of development. Then two more vaccines StaphVax and V710 were formulated by Nabi Pharmaceuticals, USA containing capsular polysaccharides CP5 plus CP8 and IsdB respectively as an antigenic component. Both of the vaccines provided immunity during animal model but failed in control phase III trials (Shinefield et al., 2002). The failure may be due to certain strains of *S. aureus* such as USA 300 does not contain CPs in their structure, lack of adjuvant in vaccine preparation, *S. aureus* immune evasion virulence factors such as IgG binding protein A have ability to compromise the function of antibodies and make them unable to provide effective immunity (Fowler and Proctor, 2014). However, the trials are underway to make a polyvalent vaccine containing number of antigens such as ClfA, CP5, CP8, secreted toxins (extracellular protein A and B, α-toxin, ESAT-6, *luk*S-PV), MntC, and Fhud2 (Pozzi et al., 2017). Another monoclonal vaccine containing WTA targeted antibodies plus rifampicin

class antibiotic were tested and found protective effects in preclinical trials (Lehar et al., 2015). Moreover, further research is going on and hope soon a better vaccine will be able in market to treat multiple *S. aureus* strains including MRSA.

10. Conclusion

Methicillin resistant strain of *S. aureus* (MRSA) is found to be versatile and unpredictable pathogen with diversity of lineages common between humans and animals indicated its transmission between human and animals. The lineages found common between human and animal were CC398, CC9, CC130, CC97, CC398. Except this, few HA-MRSA and CA-MRSA lineages were identified in animals and LA-MRSA lineages were identified similar to HA-MRSA and CA-MRSA. Therefore, increasing prevalence and genetic adaptation of this pathogen at animal and human cadre exposing it a major threat for public health. This pathogen holds a diversity of host species ranging from humans to food and companion animals and many more. This pathogen has the ability to resist many antibiotics and to escape immune mechanisms through various virulence factors that unable this pathogen to cause mild to life threatening infections. Due to the increasing antibiotics resistance, this article highlighted the importance of alternative ways such as phytochemicals, bacteriophages, nanoparticles, and probiotics alone and in combination to use them as replacement of antibiotic. There is need to work more on these approaches to combat antibiotic resistance and making them available at commercial scale for public. One more approach is to work on successful vaccine production and immunization against MRSA.

Author contributions

MS and AA wrote the whole manuscript. WP structured and funded the project. IM and MK review the manuscript. ZB, AM, MF, and MM drew the illustrations. C-NZ, MK, FS, and NM added the references through endnote. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genetic characterization and virulence determinants of multidrug-resistant NDM-1-producing *Aeromonas caviae*

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The emergence of carbapenemase significantly threatens public health. It is prevalent worldwide but rare in *Aeromonas caviae*. Unlike most bacterial species, *A. caviae* has two distinct flagella systems, which are closely related to biofilm formation. The ability to form biofilms on host tissues or inert surfaces constitutes an important cause of many persistent infections, which causes difficulties in clinical treatment. Here, we report on a multidrug-resistant (MDR) *A. caviae* carrying bla_{NDM-1} with a novel sequence type 1,416. The strong ability of biofilm formation of FAHZZU2447 was verified by a crystal violet assay. The resistome profile and location of the bla_{NDM-1} gene were determined by antimicrobial susceptibility testing, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE), and Southern blot analysis. Moreover, the strain underwent whole-genome sequencing to identify its genomic characteristics. In addition, the bla_{NDM-1} gene was located on a ~243 kb plasmid with genetic context IS*1R*-bla_{NDM-1}-*ble-trpF-dsbD-hp-sul1-qacE*. Phylogenetic analysis indicated the transmission of *A. caviae* in China, Japan, and Thailand. Our study aimed to elucidate the genomic features of bla_{NDM-1}-producing *A. caviae*, thereby clarifying the distribution of *A. caviae* worldwide and emphasizing the harmfulness of biofilm formation to the clinic. Further comprehensive surveillance of this species is needed to control further dissemination.

KEYWORDS

***Aeromonas caviae*, bla_{NDM-1}, whole-genome sequencing, phylogenetic analysis, biofilm formation**

Introduction

Aeromonas spp. is ubiquitous in aquatic environments and has been considered a significant human pathogen since it was isolated from a blood sample in 1954 (Parker and Shaw, 2011). *Aeromonas* spp. is a common contaminant of fish and seafood (Hänninen et al., 1997). It usually causes invasive extraintestinal infections, including biliary tract infections, by ingesting food contaminated with *Aeromonas* spp. (Parker and Shaw, 2011). Until now, a total of 36 species have been described in the genus *Aeromonas*, and all cause widespread infections (Fernández-Bravo and Figueras, 2020). However, according to a recent report, the infections caused by *Aeromonas* spp. in the clinic mainly focused on four species: *Aeromonas caviae*, *Aeromonas dhakensis*, *Aeromonas veronii*, and *Aeromonas hydrophila* (Fernández-Bravo and Figueras, 2020). Among them, *A. caviae* was the most common species but rarely causes biliary tract infection (Janda and Abbott, 2010; Chao et al., 2013).

New Delhi metallo-β-lactamase (NDM) is a metallo-β-lactamase that offers carbapenem antibiotic resistance to hydrolyze to almost all beta-lactam antibiotics, except aztreonam, which significantly threatens public health, worldwide (Nordmann et al., 2011). Since NDM-1 was first detected in 2008, in *Klebsiella pneumoniae* from a patient repatriated from India, it has disseminated worldwide (Yong et al., 2009). However, the current spread of NDM-1 was related to Enterobacterales and limited reports are available of NDM-1-carrying *Aeromonas* spp. (Luo et al., 2022; Xu et al., 2022). To date, only two studies have reported on NDM-1-producing *A. caviae,* which were isolated from sputum and blood, and bla_{NDM-1} genes were located on the plasmid and chromosome, respectively (Luo et al., 2022; Xu et al., 2022). The emergence of NDM-1-harboring *A. caviae* in the clinic is concerning.

Many bacterial species express either single/multiple polar flagella or peritrichous (lateral non-induced) flagella. Few strains possess dual flagellar systems and express two entirely distinct flagella systems: polar flagellum and lateral flagella for swimming and swarming, respectively (Santos et al., 2011). The swimming motility of *A. caviae* in liquid environments is possible by expressing polar unsheathed monotrichous flagellum (*fla*). Furthermore, *A. caviae* produced inducible lateral flagella (*laf*) when cultivated on solid or viscous media. In addition, the phenomenon is associated with the colonization of surfaces, biomass production, and biofilm formation (Gavín et al., 2003). The ability to form biofilms on host tissues or inert surfaces is an important cause of many persistent infections and causes difficulties in clinical treatment that need our attention (Parsek and Singh, 2003).

In previous studies, epidemiological studies have been conducted on carbapenem-resistant *A. caviae* clinical isolates; however, less attention was paid to biofilm formation. In this study, a multidrug-resistant (MDR) *A. caviae* strain carrying NDM-1 with a new sequence type (ST) 1,416 was isolated. The ability of biofilm formation was verified and compared. In addition, the microbiological and molecular mechanisms involved were elucidated. Furthermore, to elucidate the distribution characteristics of *A. caviae*, comprehensive phylogenetic analyses were conducted.

Materials and methods

Isolation and identification of bacteria

Aeromonas caviae strain FAHZZU2447 was isolated from a patient with a biliary tract infection and was hospitalized in a tertiary hospital in Zhengzhou, China. The species was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker, Bremen, and Germany) and genome sequence-based average nucleotide identity (ANI) analysis (Richter and Rosselló-Móra, 2009). The bla_{NDM-1} gene was detected using PCR analysis and Sanger sequencing. The primers used were as follows: bla_{NDM-1}-F, 5′- ATGGAATTGCCCAATATTATGCAC-3′; and bla_{DNM-1}-R, 5′- TCAGCGCAGCTTGTCGGC-3′.

Antimicrobial susceptibility testing

The bacterial resistance was determined using both broth microdilution and agar dilution methods, and the results QQwere interpreted according to the Clinical and Laboratory Standards Institute [CLSI], 2021[1] and European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.[2] *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as quality controls. The antibiotics tested in this study included piperacillin/tazobactam, ceftazidime, ceftriaxone, cefepime, cefotaxime, ciprofloxacin, imipenem, trimethoprim/sulfamethoxazole, amikacin, gentamicin, aztreonam, chloramphenicol, tetracycline, colistin, and tigecycline.

Location of bla_{NDM-1} gene and transferability of plasmids carrying bla_{NDM-1}

The size and number of plasmids of strain FAHZZU2447 were confirmed using S1 nuclease-pulsed field gel electrophoresis (S1-PFGE) (Xu et al., 2018). Using a digoxigenin-labeled bla_{NDM-1} probe, Southern blot analysis was conducted to verify the location of the bla_{NDM-1} gene. Furthermore, a conjugation assay was performed using *E. coli* J53 as the recipient strain to test the transferability of the plasmid carrying NDM-1. Next, transconjugants were selected on agar (OXOID, Hampshire, UK) medium, containing 200 mg/L sodium azide and 2 mg/L meropenem. The verification of transconjugants was carried out by both MALDI-TOF/MS and PCR analysis.

Whole-genome sequencing and bioinformatics analysis

The complete sequence of FAHZZU2447 was obtained using whole-genome sequencing (WGS). Briefly, DNA was extracted using Gentra Puregene Yeast/Bact. Kit (Qiagen, Dusseldorf, Germany) and

1 https://clsi.org
2 https://www.eucast.org/

TABLE 1 Susceptibility of *Aeromonas caviae* FAHZZU2447.

Antibiotics	MIC values (μg/mL)	Antimicrobial susceptibility	Mechanism of resistance
Penicillins			-
Piperacillin/ tazobactam[a]	>128/4	R	
Beta-lactam			bla_{TEM-1B}, bla_{NDM-1}, bla_{MOX-6}, bla_{OXA-18}
Ceftazidime	>128	R	
Ceftriaxone	>128	R	
Cefepime	128	R	
Cefotaxime	>128	R	
Imipenem	8	R	
Aztreonam	32	R	
Fluoroquinolones			-
Ciprofloxacin	16	R	
Aminoglycosides			*aac(3)-IId*, *aph(6)-Id*, *aph(3″)-Ib*
Amikacin	4	S	
Gentamicin	128	R	
Tetracyclines			*tet(A)*
Tigecycline	0.5	S	
Tetracycline	16	R	
Phenicols			*catA1*
Chloramphenicol	8	S	
Polymyxin			–
Colistin	2	I	
Sulfonamide			*dfrA5*, *sul1*, *sul2*
Trimethoprim/ sulfamethoxazole	>8/152	R	
Others			*qacE*, *mph(A)*
Not included in the AST panel	Na	Na	

[a]Tazobactam at a fixed concentration of 4 mg/L. R, resistant; I, intermediary; S, susceptible; NA, not applicable.

TABLE 2 Virulence genes in *Aeromonas caviae* FAHZZU2447.

Functions	Virulence factors	Related genes	Location
Adherence	Lateral flagella	*flgCEIJ*, *fliFGP*, *lafBCEFKSTUX*, *lfgABFGHKLMN*, *lfhAB*, *lfiEHIJMNQR*, *maf-5*	
	Mannose-sensitive hemagglutinin (Msh) pilus, type IV pili	*mshABCDEFG1IJKLMNOP*	
	Polar flagella	*cheABRVWYZ*, *flaBHJ*, *flgABCDEFGHIJKLMN*, *flhABFG*, *fliAEFGHIJKLNOPQR*, *flrABC*, *maf-1*, *motXY*, *nueB*, *pomA2AB2B*	Chromosome
	Tap type IV pili	*TapBCDFMNOPQTUVWY1*, *tppABCDE*	
Secretion system	T2SS	*exeABCDEFGHIJKLMN*	
	T6SS	*atsD*	
Toxin	Hemolysin HlyA	*hlyA*	
Stress adaptation	Catalase-peroxidase	*katG*	

then sequenced on Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) and Oxford Nanopore (Oxford Nanopore Technologies, Oxford, UK) platforms. The hybrid assembly was conducted by Unicycler v0.4.7 (Wick et al., 2017). Multilocus sequence typing (MLST) and virulence genes were identified by pubMLST and VFDB 2022 databases, respectively (Jolley et al., 2018; Liu et al., 2022). Finally, the annotation and bioinformatic analysis were performed using Prokka v1.14.0[3] and an online tool,[4] respectively. The complete genome sequence of *A. caviae* FAHZZU2447 was uploaded to NCBI with the following accession numbers: CP100392-CP100394.

FAHZZU2447 lacks plasmid-mediated quinolone resistance (PMQR) but showed resistance to ciprofloxacin, and the quinolone-resistance-determining region (QRDR) of *gyrA* and *parC* genes was examined for mutations (Yang et al., 2017). Briefly, the QRDR of the *gyrA* and *parC* genes was compared with the sequences of the *A. caviae* complex (GenBank accession numbers: AY027899 and AF435418, respectively) (Arias et al., 2010). Next, OriTFinder was used to predict the complete conjugative modules on a plasmid (Li et al., 2018). Furthermore, the genetic environment surrounding the bla_{NDM-1} gene was characterized using easyfig v2.2.5 (Sullivan et al., 2011). The comparison map of plasmids in this study was generated using Brig v0.95 (Alikhan et al., 2011) and compared with related plasmids in the National Center for Biotechnology Information (NCBI) database.

Motility assays and biofilm formation

The motility of FAHZZU2447 was assessed according to a previous report with minor modifications (Gavín et al., 2002). Briefly, a freshly grown single colony was inoculated into the center of motility agar [0.3% agar in Luria-Bertani broth (OXOID, Hampshire, UK)] and incubated at 37°C for 16–24 h. Next, the motility was assessed by examining the migration of bacteria from the center of the agar toward the periphery of the plate.

Biofilm formation was quantitatively determined through a modified biofilm assay that was based on a previous report (Santos et al., 2011). Briefly, the overnight culture was diluted in LB and dispensed in a 96 well plate (200 μl/well). Wells with only LB broth served as the control. After incubation for 24 h at 37°C without shaking, wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent bacteria. Next, methanol was added to each well to fix the bacteria, and a 0.1% crystal violet solution was used for staining. After washing three times with PBS, absolute ethanol was added to each well and the optical density (OD) was measured at 595 nm. The cutoff OD (ODc) was defined as the mean OD of the control. The following conditions were used to interpret the results (Christensen et al., 1985): non-adherent (OD ≤ ODc),

3 https://github.com/tseemann/prokka

4 www.genomicepidemiology.org

weakly to moderately adherent (ODc < OD ≤ 2 × ODc), and strongly adherent (2 × ODc < OD). Each assay was performed in triplicate. One-way ANOVA was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Phylogenetic analysis

To evaluate the distribution characteristics of *A. caviae*, 150 available genomes were downloaded from the NCBI database. To exclude confounding strains, ANI analysis was performed by pyani v0.2.11[5] (**Supplementary Figure 1**). Phylogenetic analyses were performed using Roary, and a maximum likelihood phylogenetic tree was constructed with MEGA 11 (Page et al., 2015).

Results and discussion

Strain identification and case description

Strain *A. caviae*, designated FAHZZU2447, was isolated from a 67-year-old female patient who was admitted to a tertiary teaching hospital in 2019 in Zhengzhou, China. The patient was admitted for complaints of unexplained nausea and vomiting. The patient underwent cholecystitis resection without postoperative suture removal and biliary T tube drainage. Nausea and vomiting developed after eating dates and cucumbers 7 days earlier, and the vomit contained stomach contents accompanied by abdominal distension. On the day of admission, the patient developed hyperkalemia and underwent dialysis treatment, and piperacillin-tazobactam combined with moxifloxacin was used for anti-infection treatment. On day 7, the patient developed acute kidney failure, and on this day, FAHZZU2447 was isolated from bile. Subsequently, using both MALDI-TOF/MS and ANI analysis, FAHZZU2447 was identified as *A. caviae* (**Supplementary Figure 1**). Unfortunately, the patient was diagnosed with hepatorenal function and electrolyte disturbance on day 28, and the patient's family refused further treatment. On day 31, the dialysis tube was removed, and the patient was discharged home.

Resistome of *A. caviae* FAHZZU2447

Based on the antimicrobial susceptibility testing (AST) results, FAHZZU2447 had a broad drug resistance spectrum and was regarded as an MDR bacterium (**Table 1**). FAHZZU2447 was resistant to most of the tested antibiotics except for amikacin, tigecycline, chloramphenicol, and colistin. According to the ResFinder database results, FAHZZU2447 harbored plenty of antibiotic resistance genes (ARGs) and mediated resistance to multiple agents of antibiotics (**Table 1**). Furthermore, the carbapenem resistance gene bla_{NDM-1} was identified, which is rarely present in *A. caviae*. Currently, two reports related to bla_{NDM-1}-harboring *A. caviae* are available and both were isolated from the clinic in China. However, they were not associated with a biliary tract infection (Luo et al., 2022; Xu et al., 2022). In addition, FAHZZU2447 carries other drug-resistance genes, including beta-lactams (bla_{TEM-1B}, bla_{NDM-1}, bla_{MOX-6}, and bla_{OXA-18}), aminoglycosides [*aac(3)-IId*, *aph(6)-Id*, *aph(3″)-Ib*], tetracyclines [*tet(A)*], phenicols (*catA1*), and sulfonamide (*dfrA5*, *sul1*, *sul2*). Therefore, the resistant phenotype of FAHZZU2447 may mainly be due to the presence of ARGs, except for the resistance to ciprofloxacin, which is associated with mutations in QRDRs. In this study, a total of 83 mutations in *gyrA* codons and 80 mutations in *parC* codons were identified. In addition, substitutions Ser-83-Ile and Ser-87-Ile in *gyrA* and *parC* were found, respectively. As previously described, the mutations at residue 83 of *gyrA* and 87 of *parC* are most frequently encountered and confer a significant increase in the level of quinolone resistance (Goñi-Urriza et al., 2002). However, the substitution in *parC* codon 87 is rare, and this mutation in *A. veronii* strains was only described in one study where it was found to contribute to a higher MIC when co-carrying mutations in *gyrA* codon 83 (Yang et al., 2017).

Toxome and biofilm formation capacity

As shown in **Table 2**, FAHZZU2447 containing virulence genes encoded various functions. In previous studies, it has been indicated that the polar flagellum plays a significant role in bacterial adherence, the initial step that precedes colonization (Rabaan et al., 2001). Migration in the surface mediated by lateral flagella permits fast and local colonization, thereby allowing bacteria to multiply and form microcolonies (Lynch et al., 2002; Santos et al., 2011). Thus, flagella-mediated motility is essential for biofilm formation (Kirov et al., 2004). In addition, biofilm formation is a multifactorial process that involves both pili and flagella (Kirov et al., 2004). Type IV pili (T4P), confirmed to be present in *A. caviae*, can substitute for flagellar roles in biofilm formation (Béchet and Blondeau, 2003). Furthermore, the structure MshA pili also seem to play a significant role in biofilm formation by other species, such as *P. aeruginosa* (Santos et al., 2011). FAHZZU2447 carries multiple virulence genes involved in biofilm formation, including *fla*, *laf*, *fli*, *flg*, *che*, and *Tap* genes. Therefore, we hypothesize that FAHZZU2447 has the ability to form biofilms. The motility of FAHZZU2447 was verified (**Supplementary Figure 2**), and simultaneously, its biofilm formation capacity was assessed using crystal violet. Biofilms were compared with two other

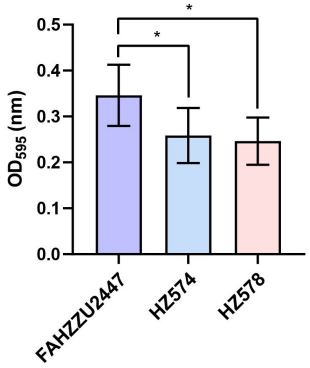

FIGURE 1
Biofilm formation ability of *Aeromonas caviae* FAHZZU2447, HZ574, and HZ578. Bar charts with purple, blue, and pink represent strains FAHZZU2447, HZ574, and HZ578, respectively. The values shown are the mean ± SD of three independent experiments. *$p < 0.05$.

5 https://github.com/widdowquinn/pyani

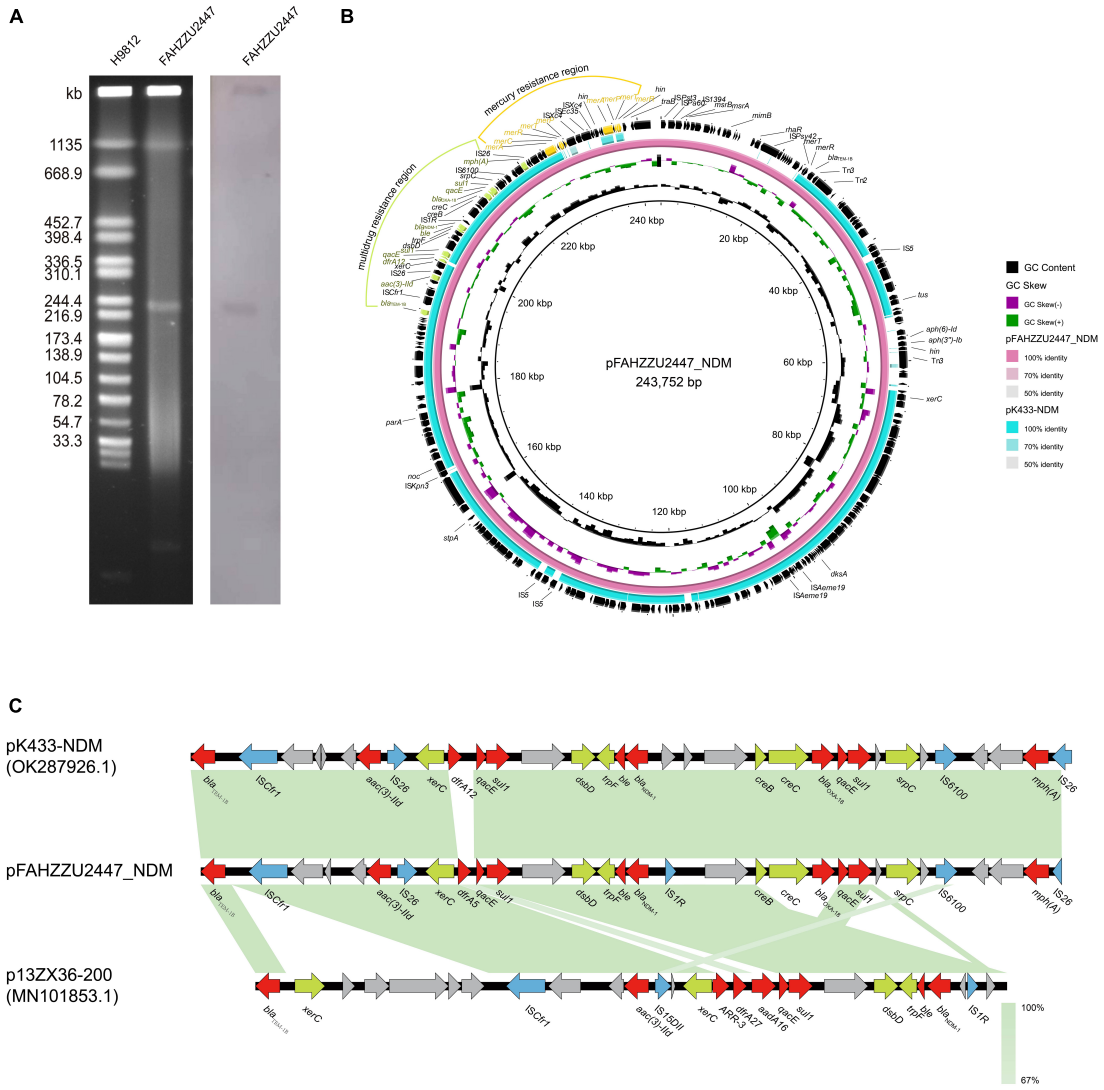

FIGURE 2
The genetic features of the pFAHZZU2447_NDM plasmid. **(A)** Plasmid profiles of *Aeromonas caviae* FAHZZU2447. **(B)** Comparison of the plasmid pFAHZZU2447_NDM with the closely related plasmid pK433-NDM (accession number: OK287926.1). **(C)** Genetic context of bla_{NDM-1} on pFAHZZU2447_NDM, pK433-NDM, and p13ZX36-200 (accession number: MN101853.1). Different color arrows represent different putative functions. Regions with a high degree of homology are indicated in green.

NDM-1-carrying *A. caviae* strains as described in our previous study, which carried fewer virulence genes (Xu et al., 2022).

The mean OD_{595} values of biofilms of the control, FAHZZU2447, HZ574, and HZ578 were 0.11 ± 0.02, 0.35 ± 0.07, 0.26 ± 0.06, and 0.25 ± 0.05, respectively. All three strains were classified as strongly adherent. Notably, the biofilm formation ability of FAHZZU2447 was stronger than that of the other two strains ($p < 0.05$, **Figure 1**). The difference in bioform formation ability may be explained by FAHZZU2447 carrying more biofilm formation-related factors, such as lateral flagella and polar flagella (Xu et al., 2022).

Genomic features of *A. caviae* FAHZZU2447

According to pubMLST, FAHZZU2447 was assigned to a new sequence type ST1416 (*gyrB-groL-gltA-metG-ppsA-recA*: 96-100-370-650-398-173). S1-PFGE and Southern blot analysis confirmed that the bla_{NDM-1} gene was located on the ~243 kb plasmid pFAHZZU2447_NDM (**Figure 2A**). Furthermore, WGS data showed that FAHZZU2447 consists of a 4,540,521 bp chromosome with a GC content of 61.5% and two plasmids of 243,752, and 8,061 bp. *In silico* analysis indicated that plasmid pFAHZZU2447_NDM could not be categorized into known replicon types. In addition, NCBI BLAST analysis revealed that pFAHZZU2447_NDM exhibited 99% nucleotide identity with plasmid pK433-NDM (accession number: OK287926.1), which was from clinical strain *A. caviae* K433 in China (**Figure 2B**). Similar to pFAHZZU2447, pK433-NDM had an unclear plasmid type, and the two plasmids shared a similar backbone. As shown in **Figure 2B**, the bla_{NDM-1} gene is in a multidrug resistance region (MRR) with multiple ARGs and insert sequence (IS) elements (IS*Cfr1*, IS*26*. IS*1R*, and IS*6100*). However, compared with pK433-NDM, pFAHZZU2447_NDM contains an additional mercury resistance region and encodes more ARG and ISs on the plasmid. Genes encoding a small multidrug resistance (SMR) efflux transporter were also found in the MRR, which were deduced to be

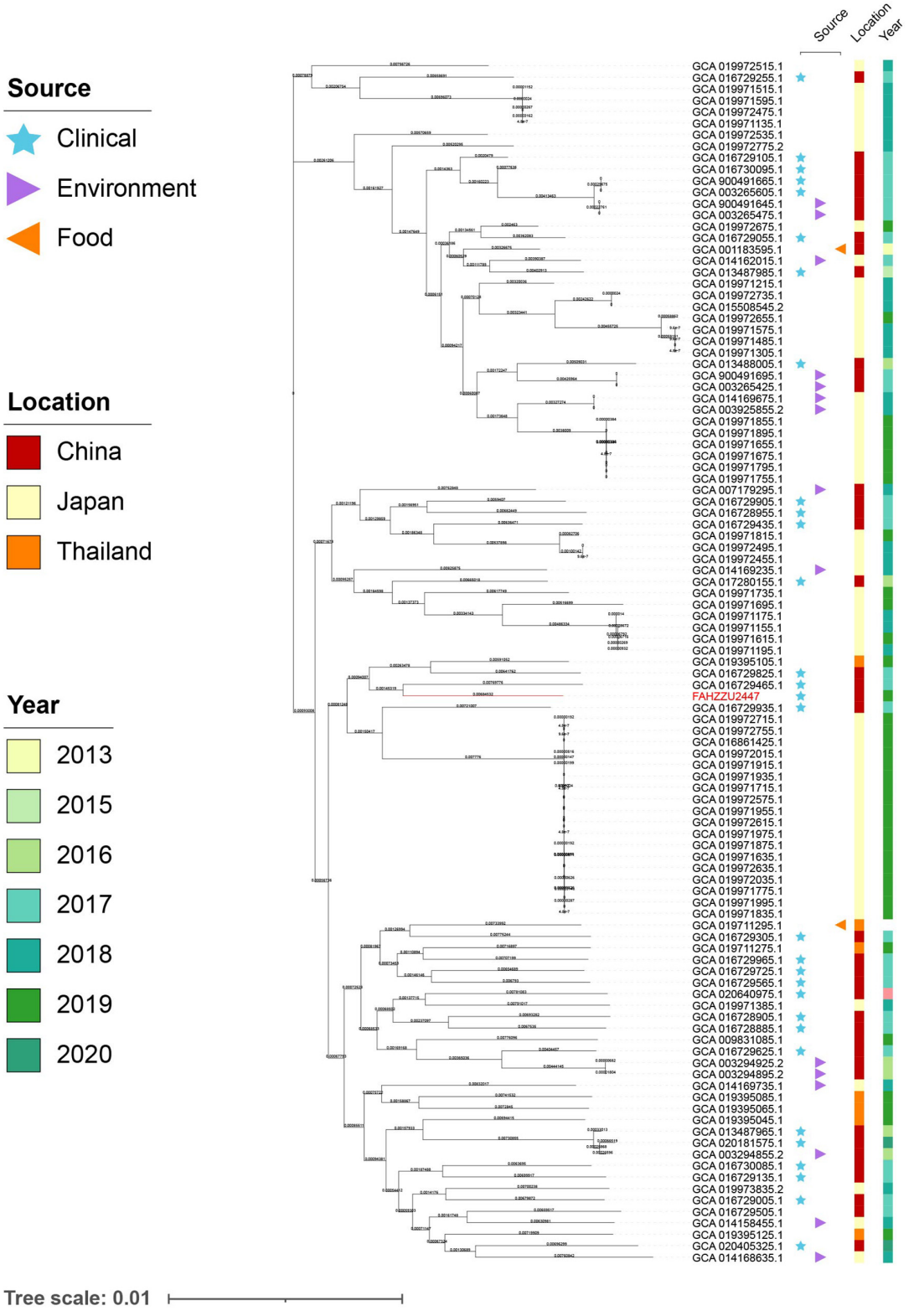

FIGURE 3
The maximum likelihood core-gene phylogenetic tree of *Aeromonas caviae*. The isolation sources, locations, and collection dates of isolates are shown. Blue stars, purple triangles, and orange triangles represent clinical, environmental, and food origins, respectively; the red, orange, and yellow squares represent strains isolated from China, Japan, and Thailand; the light to dark green colors represent different collection times. *A. caviae* FAHZZU2447 is indicated in red.

associated with the efflux system (Kazama et al., 1998; Shen et al., 2021). Furthermore, both pK433-NDM and pFAHZZU2447_NDM lacked a *tra* module, which encodes a primary pilus for conjugation (Chi et al., 2020). In addition, the OriTFinder results indicated that pFAHZZU2447_NDM had incomplete conjugative modules with the absence of a transfer site (*oriT*) and type IV coupling protein

(T4CP) (**Supplementary Table 1**). To verify the transferability of pFAHZZU2447_NDM, conjugation experiments were conducted. However, repeated transformation methods failed, which implied that it was non-conjugative. Moreover, the genetic context of bla_{NDM-1} in pFAHZZU2447_NDM (IS*1R*-bla_{NDM-1}-*ble-trpF-dsbD-hp-sul1-qacE*) was identical to pK433-NDM and p13ZX36-200 (**Figure 2C**, accession number: MN101853.1). Especially, two copies of IS*26* elements surrounding the bla_{NDM-1}-harboring MDR region formed a composite transposon-like structure, which promoted its transfer among various plasmids (Li et al., 2021). The diversity of genetic elements leads to the wide spread of ARGs among bacteria, which needs further attention.

Analysis of phylogenetic relationships

To investigate the distribution of *A. caviae* at the global level, a total of 150 available genomes were downloaded from NCBI (**Supplementary Table 2**). Among these, 139 genomes had a definite source of isolation (including FAHZZU2447), distributed in five continents. Notably, the majority were isolated from Asia (110/139, 79.14%), followed by North America (11/139, 7.91%), South America (9/139, 6.47%), Europe (6/139, 4.32%), and Africa (3/139, 2.16%). Further analysis of the Asian-derived strains showed that transmissions mainly occurred in China, Japan, and Thailand (**Supplementary Figure 3**). Therefore, the maximum likelihood phylogenetic tree of 105 *A. caviae* isolates from China, Japan, and Thailand was constructed. As shown in **Figure 3**, the closest relative of FAHZZU2447 is *A. caviae* Colony274 (GCA 019711295.1) from Thailand. Additionally, strains from China and Thailand (GCA 016729305.1 and GCA 019711295.1), as well as from China and Japan (GCA 016729055.1 and GCA 019972675.1), were closely related, thereby indicating the dissemination of *A. caviae* among countries. *A. caviae* are widespread in aquatic creatures and have been isolated from a variety of seafood. Ingesting seafood has caused *A. caviae* infections (Wu et al., 2019). In a previous survey conducted in Taiwan, it was discovered that *Aeromonas* isolates contaminated 88% of seafood from markets, and 33% of the *A. caviae* produced beta-hemolysin (Wu et al., 2019). Japan and Thailand are the primary aquatic product trading countries of China (Liu and Chen, 2011; Yao et al., 2017). Thus, the trade may have accelerated the spread of *A. caviae*. **Figure 3** shows that most strains were isolated from the clinic, which is a reminder that continuous monitoring is required.

Conclusion

In summary, in this study, a bla_{NDM-1}-carring *A. caviae* FAHZZU2447 from biliary tract infection is reported. FAHZZU2447 harbored various virulence genes and had strong biofilm formation ability, which contributes to persistent infections in the clinic. WGS data indicated that the bla_{NDM-1} gene is located in a multidrug resistance region with various ISs. The phylogenetic analysis shows that the transmission of *A. caviae* mainly occurred in China, Japan, and Thailand. Therefore, continuous monitoring and investigations of *A. caviae* are of utmost importance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in this article/**Supplementary material**.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XH, MC, and RL conceived and designed the experiments. XH, HZ, YL, and JQ collected samples and performed the experiments. XL, HG, JZ, and XM analyzed the data. XH and RL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1055654/full#supplementary-material

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Prevalence of antimicrobial resistance in fecal *Escherichia coli* and *Enterococcus* spp. isolates from beef cow-calf operations in northern California and associations with farm practices

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Antimicrobials are necessary for the treatment of bacterial infections in animals, but increased antimicrobial resistance (AMR) is becoming a concern for veterinarians and livestock producers. This cross-sectional study was conducted on cow-calf operations in northern California to assess prevalence of AMR in *Escherichia coli* and *Enterococcus* spp. shed in feces of beef cattle of different life stages, breeds, and past antimicrobial exposures and to evaluate if any significant factors could be identified that are associated with AMR status of the isolates. A total of 244 *E. coli* and 238 *Enterococcus* isolates were obtained from cow and calf fecal samples, tested for susceptibility to 19 antimicrobials, and classified as resistant or non-susceptible to the antimicrobials for which breakpoints were available. For *E. coli*, percent of resistant isolates by antimicrobial were as follows: ampicillin 100% (244/244), sulfadimethoxine 25.4% (62/244), trimethoprim-sulfamethoxazole 4.9% (12/244), and ceftiofur 0.4% (1/244) while percent of non-susceptible isolates by antimicrobial were: tetracycline 13.1% (32/244), and florfenicol 19.3% (47/244). For *Enterococcus* spp., percent of resistant isolates by antimicrobial were as follows: ampicillin 0.4% (1/238) while percent of non-susceptible isolates by antimicrobial were tetracycline 12.6% (30/238) and penicillin 1.7% (4/238). No animal level or farm level management practices, including antimicrobial exposures, were significantly associated with differences in isolate resistant or non-susceptible status for either *E. coli* or *Enterococcus* isolates. This is contrary to the suggestion that administration of antibiotics is solely responsible for development of AMR in exposed bacteria and demonstrates that there are other factors involved, either not captured in this study or not currently well understood. In addition, the overall use of antimicrobials in this cow-calf study was lower than other sectors of the livestock industry. Limited information is available on cow-calf AMR from fecal bacteria, and the results of this study serve as a reference for future studies to support a better understanding and estimation of drivers and trends for AMR in cow-calf operations.

KEYWORDS

antimicrobial resistance, beef cattle, *Escherichia coli*, *Enterococcus* spp., feces, ceftiofur, tetracycline, cow-calf operations

1. Introduction

Antimicrobial therapy is critical for the treatment of bacterial infections in veterinary medicine; however, resistance to these treatments has been increasing for decades (Thanner et al., 2016) and, as a result, there are concerns about the efficacy of antimicrobials in food-producing animals. It has been well documented that use of antimicrobial drugs is associated with increases in antimicrobial resistant bacteria (Agga et al., 2016; Thanner et al., 2016), but increases in antimicrobial resistance genes (ARG) can also develop in the absence of antimicrobial drug use (Agga et al., 2016). There is consensus that applying pressure on a population of bacteria through antimicrobials will enrich ARGs within that population (Xiong et al., 2018); however, AMR cannot be attributed to exposure to antimicrobials alone (Berge et al., 2010).

Although *Enterococcus* spp. are part of the normal flora in the bovine gastrointestinal tract, there are reports of *Enterococcus* spp. as a causative agent of mastitis in cattle (Devriese et al., 1992; Bradley et al., 2007) and diarrhea in neonatal calves (Ok et al., 2009). *Enterococcus* has, however, been primarily used as a sentinel and potential source of AMR genes for other Gram-positive pathogens. *Enterococcus* spp. are known to be intrinsically resistant to many antibiotics and can develop and confer AMR status to other pathogens (Cameron and McAllister, 2016; Torres et al., 2018). Thus, *Enterococcus* spp. have the potential to cause disease and serve as sentinels for the status of AMR pathogens in an environment. *E. coli* is another component of the normal flora of the bovine enteric system with many strains that have varying pathogenicity. Various *E. coli* strains can cause mastitis or metritis in cows as well as neonatal diarrhea or septicemia in calves (Burvenich et al., 2003; Sheldon et al., 2010; Dubreuil et al., 2016). *E. coli* has also been used as an indicator of AMR prevalence in fecal bacteria and a potential source for transmission of ARGs to other Gram-negative organisms (Cameron and McAllister, 2016), as it acquires resistance easily and can inhabit many types of animals (Tadesse et al., 2012).

California is an important contributor to the U.S. beef industry, with approximately 590,000 beef cows and contributing $3.4 billion in cash receipts from total cattle and calf sales in 2015 (Saitone, 2020). In beef cow-calf operations in the U.S., calves are born and stay at the same location with their dam usually until 6–8 months of age, at which point they are weaned, removed from the dam, and are often placed in a group with animals of approximately the same age and/or size. At this stage of the beef production cycle, there are many sectors that can involve the movement and mixing of animals. Some calves may be moved directly from cow-calf operations to feedlots after weaning until they reach slaughter weight. Alternatively, if there is high forage availability, they may be moved temporarily to a stocker facility before ultimately finishing at a feedlot facility. The time spent in each sector, size of group, and management of the animals are highly variable and depend on many factors including geographic location, producer goals, and access to pasture and/or facilities. Adult cull cows from cow-calf operations often go to slaughter directly at the end of their productive life.

Rates for multidrug resistance (MDR) have previously been shown to be higher in California cattle from various types of beef production systems when compared to nearby states, Washington and Oregon (Berge et al., 2010). Additionally, many AMR studies thus far have focused on feedlot, stocker, and calf ranch operations, where there may be increased selection pressure on bacterial populations through antimicrobial therapy from treatment and/or prevention of disease that develops likely as a result of mixing of animals, transport, and stress (Cameron and McAllister, 2016; Noyes et al., 2016). A 2010 study found that the highest proportion of MDR *E. coli* isolates originated from calf ranches, followed by feedlots, while the least MDR was found in isolates from adult beef cows (Berge et al., 2010). Prophylactic or metaphylactic use of antibiotics and occurrence of disease requiring antibiotic treatment is less common in cow-calf operations than other operation types in the beef production chain (Noyes et al., 2016). There are far fewer studies investigating the levels of AMR that exist in cow-calf operations and not yet one that exclusively investigated the levels of AMR that exist on cow-calf operations in California. Nevertheless, characterization of AMR in cow-calf operations is essential for evaluating and understanding the contribution of the cow-calf sector to AMR in the beef industry, as well as whether specific management and antimicrobial use patterns may be associated with AMR during this production stage before the calves are moved to feedlots where higher selection pressures exist. Previous studies in cow-calf herds have indicated that management and operation-dependent factors can influence the presence of AMR in a group. Specifically, season of collection of samples for testing (spring calves show more AMR than fall calves) (Gow et al., 2008), age of animal (calves show more AMR than feeding cattle or adult cattle) (Yamamoto et al., 2013), and intensity of operation (more intensive operations have more AMR than less intensive operations) (Hille et al., 2017) have all been shown to be associated with increased AMR detection in beef operations.

As part of the ongoing effort on surveillance for AMR, the objective of this cross-sectional study conducted on cow-calf operations in northern California was to assess prevalence of AMR in *E. coli* and *Enterococcus* spp. in beef cattle of different life stages, pasture types, and antimicrobial drug exposure on a herd and individual level. The hypothesis was that the prevalence of AMR in fecal samples varies according to the age of the animal as well as the drugs commonly used in the treatment of sick animals on those

Abbreviations: AMR, Antimicrobial resistance; ARG, Antimicrobial resistance gene; AST, Antimicrobial Susceptibility Test; ASV, Age and source verified; CLSI, Clinical and Laboratory Standards Institute; MDR, Multidrug resistance; MFA, Multiple factor analysis; MIC, Minimum inhibitory concentration; NARMS, National antimicrobial resistance monitoring system; NHTC, Non-hormone treated certified.

operations. The results of this study may serve as reference for future studies on the prevalence of AMR genes in the population of cow-calf operations in California and lead to a better understanding of risk factors for shedding of fecal pathogens carrying ARGs. Further surveillance, risk assessment, and interpretation of these results will help to derive more informed decisions and directions for combatting AMR in the future.

2. Materials and methods

2.1. Farm selection

A convenience sample of beef cow-calf operations in northern California was enrolled in this study either through the network of University of California Cooperative Extension livestock advisors or as clients of the Veterinary Medical Teaching Hospital at the University of California, Davis. Enrollment criteria included farms with a geographic location in northern California and primary production sector as beef cow-calf. No restrictions were placed on the type of operation (organic, conventional, other), herd size, grazing practices, breed of beef cattle, or previous antimicrobial use. All experimental protocols regarding animal use were approved by the Institutional Animal Care and Use Committee (protocol #21174) at the University of California, Davis.

2.2. Animal selection

Fecal samples were collected between June 2019 and August 2020 from cows and/or calves on each farm as a convenience sample, by either the herd veterinarian, extension veterinarian, or cooperative extension livestock advisor. Fecal samples were collected from a combination of calves aged between 1 week to 1 year and adult cows aged between 2 and 10 years either from the rectum or from freshly voided manure (pasture samples) after the animal was observed to defecate. Number and life stage of animal samples was based on number of animals available for sampling and that could be observed defecating within an hour of observation at the time of farm visit with the goal of sampling 5 cows and 5 calves per farm. Individual animal identifier, age, life stage (calf or cow), and breed were recorded when available.

2.3. Fecal sample collection

Fecal samples were collected during a single visit to each farm. Individual disposable gloves were used for collection of each sample and samples were stored in individual 15 mL polypropylene sample tubes. Rectal samples were collected on 8 farms from the recto-anal junction with individual rectal palpation sleeves. Pasture samples were collected as fresh feces (approximately 30–50 g) *via* gloved hand from the top and center of a freshly voided fecal pat, where the individual animal was observed to defecate. Samples were transported on ice to the laboratory at the University of California, Davis where they were kept refrigerated at 4°C if culture could be performed within 48 h or stored at −80°C in tryptic soy broth with 25% glycerol.

2.4. Farm survey and data collection

At time of fecal sample collection, an in-person survey regarding management and production practices was conducted. Information was collected on herd size, breed(s), certification status (organic, natural, or any other specialty certified programs), whether any farm personnel were Beef Quality Assurance (BQA) certified (specialized cattle management and food safety certification program), type of pasture (irrigated vs. dryland), whether the farm incorporated feeding of byproducts, water trough cleaning practices, existence of a current veterinarian-client-patient relationship, and whether the farm had submitted samples in the previous 12 months to a diagnostic laboratory. The survey also included detailed questions regarding antibiotic practices on the farm, specifically use of antibiotics in feed, use of intramammary antibiotics, use of injectable antibiotics, practices related to injectable antibiotics including indication for treatment, method for determining treatment duration and dosage, information recorded regarding treatment, and specific antibiotic(s) used in each method listed above. Treatment with antimicrobials in the past 6 months before sample collection were recorded for all sampled animals based on ranch records, markings on treated animals, or rancher's recollection of treatments.

2.5. Isolation of bacteria

Selective growth media, *E. coli* Chromoselect Agar B and Rapid Enterococci Chromoselect Agar, following manufacturer guidelines (MilliporeSigma, Merck KGaA, Darmstadt, Germany), were used for culture and isolation of the respective bacterial types as previously described (Abdelfattah et al., 2021). Briefly, each fecal sample was streaked on the respective selective media using sterile cotton tipped applicators (Puritan Medical Products Co LLC, Guilford, Maine, USA) and incubated at 44°C (*E. coli*) or 35°C (*Enterococcus* spp.) for 24 h. Both *E. coli* and *Enterococcus* colonies were identified by characteristic blue green colony types on the Chromoselect plates. Two discrete colonies of each bacterial type were selected and purified on 5% sheep blood agar plates (Biological Media Services, University of California, Davis). The pure colonies were stored in tryptic soy broth with 25% glycerol at −80°C until all farm sampling was complete.

2.6. Selecting isolates for antimicrobial susceptibility testing

After initial culture, a total of 698 bacterial isolates (362 *Enterococcus* spp., 336 *E. coli*) were stored. From these, 528 bacterial isolates were selected for antimicrobial susceptibility testing (264 *Enterococcus*, 264 *E. coli*). Exclusion criteria included (1) isolates from a farm where the rectal sleeve was accidentally not changed between samplings, (2) all fecal samples which did not yield at least 2 identifiable isolates for each bacterial type after two culture attempts, and (3) any samples with missing or unknown treatment information. Of the 528 selected, 482 (244 *E. coli* and 238 *Enterococcus* spp.) were selected for antimicrobial susceptibility testing. See Figures 1A,B for flow charts of the isolate selection process for *E. coli* and *Enterococcus* isolates, respectively.

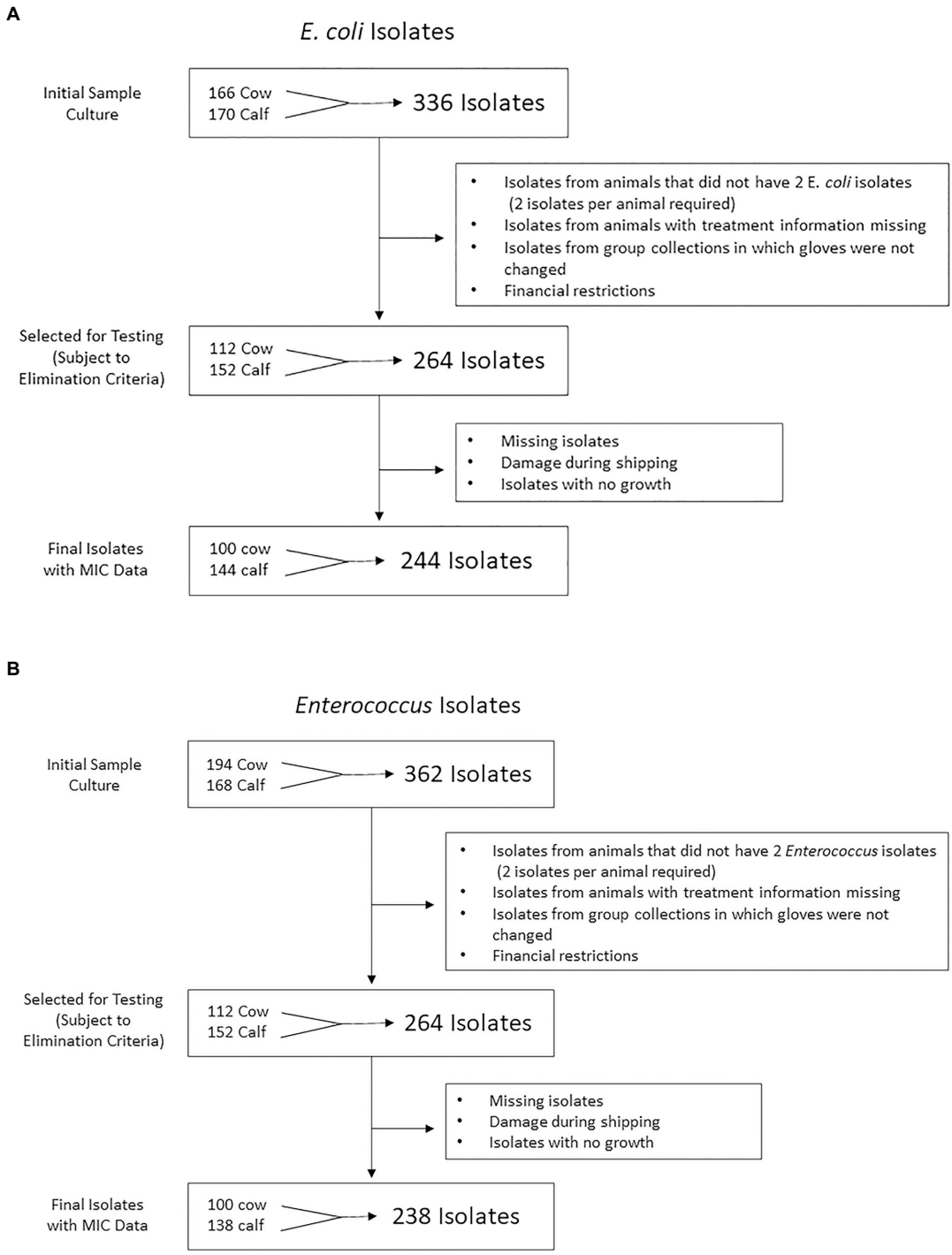

FIGURE 1
Flow chart representation of **(A)** *Escherichia coli* and **(B)** *Enterococcus* isolate numbers at each stage of the study from farm to antimicrobial susceptibility testing. 244 *E. coli* and 238 *Enterococcus* isolates were isolated from cow and calf fecal samples in a study on prevalence of antimicrobial resistance on cow-calf operations in northern California collected between July 2019 and August 2020.

2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was conducted by the broth microdilution method using Sensititre™ system (Thermo Fisher Scientific Inc., Waltham, USA) against a panel of 19 antibiotics on a commercially available BOPO7F Vet Antimicrobial Susceptibility Testing Plate (Table 1). In the AST procedure, 1–5 colonies of *E. coli* or *Enterococcus* were resuspended in 5 mL of demineralized water and the cell suspension adjusted to optical density of 0.5 McFarland using a nephelometer. Next, 10 µl of *E. coli* or *Enterococcus* bacterial suspension was added to 11 mL Mueller-Hinton broth (Thermo Scientific, Remel Inc., KS, USA) and mixed by repeated inversion of

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TABLE 1 Antimicrobials included in the BOPO7F vet antimicrobial susceptibility testing plate, dilution ranges, and breakpoints used for evaluation of 244 *Escherichia coli* and 238 *Enterococcus* isolates (μg/mL). The data was used to interpret the antimicrobial susceptibility test results and estimate the prevalence of antimicrobial resistant *E. coli* and *Enterococcus* in fecal samples from cow-calf operations in northern California collected between July 2019 and August 2022.

Antimicrobial class	Antimicrobial drugs	Dilution range	*E. coli* Breakpoints[a]			*Enterococcus* Breakpoints[a]		
			S	I	R	S	I	R
Penicillins	Ampicillin	0.25–16	≤0.03	0.06–0.12	≥0.25	≤8	.	≥16
Cephalosporins	Ceftiofur	0.25–8	≤2	4	≥8	.	.	.
Lincosamides	Clindamycin	0.25–16	.	.	.	.	.	.
Fluoroquinolones	Danofloxacin	0.12–1	.	.	.	.	.	.
Fluoroquinolones	Enrofloxacin	0.12–2	.	.	.	.	.	.
Amphenicols	Florfenicol	0.25–8	≤4	8	≥16	.	.	.
Macrolides	Gamithromycin	1 to 8	.	.	.	.	.	.
Aminoglycosides	Gentamicin	1 to 16	.	.	.	.	.	.
Aminoglycosides	Neomycin	4 to 32	.	.	.	.	.	.
Penicillins	Penicillin	0.12–8	.	.	.	≤8	.	≥16
Aminocyclitols	Spectinomycin	8 to 64	.	.	.	.	.	.
Sulfonamides	Sulfadimethoxine	256[b]	≤256	-	≥512	.	.	.
Tetracyclines	Tetracycline	0.5–8	≤4	8	≥16	≤4	8	≥16
Pleuromutilins	Tiamulin	0.5–32	.	.	.	.	.	.
Macrolides	Tildipirosin	1–16	.	.	.	.	.	.
Macrolides	Tilmicosin	2–16	.	.	.	.	.	.
Folate pathway antagonist	Trimethoprim-sulfamethoxazole	2/38[b]	≤2/38	-	≥4/76	.	.	.
Macrolides	Tulathromycin	8–64	.	.	.	.	.	.
Macrolides	Tylosin	0.5–32	.	.	.	.	.	.

[a]Breakpoint abbreviations as follows: S, Susceptible; I, Intermediate, and R, Resistant. Adopted from VET01S (5th edition) [Clinical and Laboratory Standards Institute (CLSI), 2020] and M-100 (32nd edition) [Clinical Lab Standards Institute (CLSI), 2022] from Clinical and Laboratory Standards Institute.
[b]Only one dilution available for these antimicrobials.

the tube. Fifty microliters of inoculated Mueller-Hinton broth were dispensed into each well of the 96-well BOPO7F Vet plate using Sensititre™ Autoinoculator and the plates were incubated for 18–24 h at 35°C. The minimum inhibitory concentration (MIC) was read using Sensititre™ Vizion™ Digital MIC Viewing System (Thermo Fisher Scientific Inc., Waltham, USA). The MIC values (lowest concentration of antimicrobial drug that inhibited the growth of bacteria) was interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines [Clinical and Laboratory Standards Institute (CLSI), 2020; Clinical Lab Standards Institute (CLSI), 2022]. Quality control steps included checking for bacterial growth and colony purity by plating 1 μL of the inoculated Mueller-Hinton broth on TSA with 5% sheep blood. Contaminated or no-growth inoculated samples were not read and repeated. In addition, quality control strains (*E. coli* ATCC 35218 and *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212) were run weekly alongside the test samples.

2.8. Breakpoint determination

When available, antimicrobial susceptibility veterinary breakpoints from the Clinical Laboratory Standards Institute (CLSI) were used to interpret MIC results [Clinical and Laboratory Standards Institute (CLSI), 2020], while human CLSI breakpoints were used for bacterial-drug combinations without veterinary breakpoints [Clinical and Laboratory Standards Institute (CLSI), 2020; Clinical Lab Standards Institute (CLSI), 2022]. All breakpoints used in this study were for the bacterium indicated. For antimicrobials in which the BOPO7F Vet AST Plate dilutions included the established breakpoint, "resistant" status was assigned if the isolate grew in or beyond the breakpoint dilution (ampicillin, ceftiofur, sulfadimethoxine, and trimethoprim/sulfamethoxazole for *E. coli* and ampicillin for *Enterococcus*). For antimicrobials in which the testing plate included only dilutions below the established breakpoint, "non-susceptible" status was assigned and included isolates in the intermediate range according to CLSI guidelines or isolates that grew in the highest dilution available. Resistance or non-susceptible status was only assigned to antimicrobials for which breakpoints were available and for which *in-vivo* activity and antimicrobial spectrum were applicable. For antimicrobials that were assigned non-susceptible status (florfenicol and tetracycline for *E. coli* and penicillin and tetracycline for *Enterococcus*), it was not possible to establish resistance because the drug dilutions did not reach the threshold breakpoint; hence growth or no growth at or beyond the breakpoint could not be established. Antimicrobial breakpoints used and dilution ranges for the BOPO7F Vet AST Plate can be found in Table 1.

2.9. Statistical analysis

Data from the ranch survey, individual animal data, and AST results were entered into a spreadsheet (Microsoft Excel, version 16.43, Redmond, WA) and combined using a relational database (Microsoft Access, Version 2010, Redmond, WA). Descriptive statistics for ranch demographics and prevalence of resistance or non-susceptibility for antimicrobials with existing breakpoints were prepared. Univariable generalized linear mixed models with a logit link were prepared for the outcome of resistance or non-susceptibility status of isolates to each antimicrobial with available breakpoint data using the GLIMMIX procedure in SAS (SAS Version 7.15 HF7; SAS Institute, Cary, NC). A random effect was added to account for correlation between isolates from the same animal, since 2 isolates from each fecal sample were required for inclusion in the MIC analysis. A second random effect of farm with animal nested within farm was attempted but led to non-positive G matrices and not explored further. The independent variables were created from the questionnaire data on herd demographics, antimicrobial practices, treatment history, and management practices on the farm. Multivariable generalized linear mixed models were attempted by including all variables from the univariable analysis with $p < 0.2$.

A multiple factor analysis (MFA) was conducted for survey data and antimicrobial susceptibility testing results of the 244 *E. coli* isolates. MFA was conducted to reveal the most important variables that explain the variation in the data set (Pagès, 2002). The dataset consisted of 63 data variables which were organized into 6 groups based on relatedness as follows: (1) herd information: a group of 7 categorical variables specifying farm number, the location of farm, breed distribution, herd size, certification status (e.g., certified organic), type of pasture, and type of production; (2) sampled animals' life stage and treatment history: a group of 7 categorical variables specifying sampled animal life stage, method of fecal sample collection (from rectum or pasture), date of fecal sample collection, whether animal was treated with antimicrobials, and antimicrobial used for treatment (tetracycline, tulathromycin, or florfenicol); (3) antimicrobial resistance group: a group of 8 variables describing AMR for *E. coli* (resistance to ceftiofur, florfenicol, sulfadimethoxine, and trimethoprim/sulfamethoxazole); AMR for *E. coli*; (4) farm antimicrobial use and disease treatment group: a group of 17 categorical variables describing the different injectable and intramammary antimicrobial drugs used in farms and type of treated diseases (respiratory, scours, foot rot, navel infections, wounds, metritis, and mastitis); (5) antimicrobial dosing and record keeping practices: a group of 12 variables describing methods used for determining treatment duration and dosage, and information recorded regarding antimicrobial treatment (e.g., information recorded after antimicrobial treatment such as date, dose, route, withdrawal, and/or product name); and (6) nutrition related factors: a group of 12 categorical variables specifying the provision of byproducts and mineral supplement to calves, and cows (e.g., does the farm feed mineral to preweaned or weaned calves or cows). The groups with loading weights of 0.5 or higher on the first two principal components were retained for interpretation (Hille et al., 2017). The percentage of variability contributed by each group of variables to the principal components and the correlation coefficients for the component variables within each group were estimated (Abdelfattah et al., 2019). Variables within each group with loading weights of ≥0.5 on the first two principal components were also retained for interpretation. The function MFA in FactoMiner package was used to perform the MFA on the dataset. The function *get_mfa_var(res.mfa)* was used to extract the results for the groups and variables. Hierarchical clustering was performed on the MFA principal coordinates using the principal component methods at the animal level (Husson et al., 2010). The identified clusters were described based on the variables that contributed the most to the data variability. Both MFA and hierarchical clustering were performed in R software using FactoMineR for the analysis and factoextra for data visualization (Lê et al., 2008). MFA analysis was not performed for *Enterococcus* data due to the limited number of resistant and non-susceptible isolates.

3. Results

3.1. Survey

A total of 18 cow-calf farms were surveyed and sampled during this study. General descriptive data including the major breed, herd size, pasture type, location, antimicrobial practices, and the number of injectable or oral antibiotics used on farm is shown in Table 2. Other management survey results of interest revealed that most farms (16/18, 89%) had at least one beef quality assurance certified employee, one farm (6%) fed byproducts, 7 farms (39%) had submitted samples to a diagnostic lab in the past year, and 17 (94%) had an established veterinarian-client-patient relationship.

Oxytetracycline was the most common antimicrobial used on farm (used by 14 of 18 farms, or 78%), followed by tulathromycin (10/18, 56%), florfenicol (9/18, 50%), sulfas including sulfadimethoxine and sulfamethoxazole (4/18, 22%), penicillin (3/18, 17%), enrofloxacin (2/18, 11%), and ceftiofur (1/18, 6%). No farm reported using danofloxacin or ampicillin. Regarding the types of diseases that had been treated with antimicrobials in the past 12 months in any cattle on the farm, 13 farms (72%) reported treating infectious bovine keratoconjunctivitis (pinkeye), 13 (72%) reported treating bovine respiratory disease, 10 (56%) reported treating foot rot, 7 (39%) reported treating scours, 6 (33%) reported treating wounds, 5 (28%) reported treating navel infections, 3 (17%) reported treating metritis, and 2 (11%) reported treating mastitis. There were 2 farms (11%) that reported no antimicrobial use because no disease identified as needing treatment was observed during the past year. Only one farm (6%) had routine prophylactic use of antibiotics where all calves received an injection of oxytetracycline between 1 week and 1 month of age, and all farms that used antimicrobials recorded at least one form of information after antimicrobials were administered such as date, dose, route, withdrawal, and/or product name.

3.2. Isolate growth

In total, fecal samples were collected from 187 animals (104 cows, 83 calves) and plated for growth and recovery of *E. coli* and *Enterococcus* isolates. A total of 244 *E. coli* isolates and 238 *Enterococcus* isolates were recovered and tested for antimicrobial susceptibility using broth microdilution method. Of the 104 cow samples plated, 50 samples grew at least 2 isolates of *E. coli* and 50 samples grew at least 2 isolates of *Enterococcus*. Of the 83 calf samples plated, 72 samples

TABLE 2 Descriptive survey data from all farms sampled in a study on prevalence of antimicrobial resistant *E. coli* and *Enterococcus* in fecal samples from cow-calf operations in northern California collected between July 2019 and August 2020.

	# Farms	Farm %
Major breed (>60% of Herd)		
Angus[a]	10	55.56%
Crossbred[b]	7	38.89%
Other	1	5.56%
Herd size		
<100	6	33.33%
100–249	5	27.78%
250–499	3	16.67%
>499	4	22.22%
Pasture type		
>50% Dryland	10	55.56%
≥50% Irrigated Pasture	8	44.44%
Production type		
Conventional	14	77.78%
Other[c]	4	22.22%
Location		
Coastal Range	5	27.78%
Central Valley	8	44.44%
North Central Valley	5	27.78%
Antimicrobial practices		
Antimicrobials in Feed	0	0.00%
Intramammary Antimicrobials	1	5.56%
Number of injectable or oral antimicrobials used		
0	2	11.11%
1 to 2	7	38.89%
3+	9	50.00%

[a]Can indicate either Red or Black Angus.
[b]Cross = Includes Angus cross, Hereford cross, Charolais cross.
[c]Includes Organic, Natural, No Hormone Treated Certified, and/or Age and Source Verified.

grew at least 2 isolates of *E. coli* and 69 samples grew at least 2 isolates of *Enterococcus*. Details regarding the number of samples and resulting isolates can be found in Figure 1.

3.3. *Escherichia coli* antimicrobial susceptibility testing

The distribution of isolates within various drug dilutions tested for each antimicrobial can be found in Table 3. Resistance or non-susceptible data is only shown for those antimicrobials for which established breakpoints by CLSI were available, including ampicillin, ceftiofur, florfenicol, sulfadimethoxine, tetracycline, and trimethoprim-sulfamethoxazole (Table 1). Among the 244 *E. coli* isolates, 88/244 (36.07%) were resistant or non-susceptible to at least one antimicrobial excluding ampicillin, to which all isolates were resistant. Similarly, a large proportion of isolates showed antimicrobial resistance or non-susceptibility to sulfadimethoxine followed by trimethoprim-sulfamethoxazole, while the lowest proportion of isolates showed antimicrobial resistance to ceftiofur. More isolates were classified as non-susceptible to tetracycline than florfenicol. Neither univariable nor multivariable generalized linear mixed models revealed any statistically significant associations between any of the risk factors considered, including record of antimicrobial therapy with the same antimicrobial in the past 6 months, and resistance or non-susceptible isolate status. Although none of the farm-specific variables captured in this study were significantly associated with differences in resistance or non-susceptibility, there were numerical differences between farms in terms of their antimicrobial resistance profile for *E. coli* isolates. Specifically, the highest percentage of resistant or non-susceptible isolates for florfenicol (57%), tetracycline (57%), and trimethoprim-sulfamethoxazole (64%) at the farm level was found on Farm 6, which contributed 14 isolates. Interestingly, Farm 6 did not report the use of any antimicrobials on farm.

3.4. *Enterococcus* antimicrobial susceptibility testing

The distribution of isolates within MICs tested for each antimicrobial can be found in Table 4. Resistance or non-susceptible data is only shown for those antimicrobials for which established CLSI breakpoints were available, including ampicillin, penicillin, and tetracycline (Table 1). Only a small proportion of the total 238 *Enterococcus* isolates, 35/238 (14.7%) were resistant or non-susceptible to at least one antimicrobial. Amongst all isolates tested, antimicrobial non-susceptibility was highest to tetracycline, followed by non-susceptibility to penicillin, and lowest resistance to ampicillin. Similar to the statistical models for the *E. coli* isolates, no significant associations between any of the risk factors and AMR status for *Enterococcus* isolates was found.

3.5. Multiple factor analysis

The first two principal component dimensions of the multiple factor analysis (MFA) explained approximately 8.5% of the variability in the data, i.e., 4.4 and 4.1% of the variance for the first and second principal component dimensions, respectively. The MFA analysis of 63 variables identified four components and 16 variables with a correlation coefficient ≥ 0.5 on both first and second dimensions that accounted for 98.7% of the variability in the data (Figure 2). Herd information (ranch and animals sampled) accounted for 27.7% of the total variability in the data, while antimicrobial dosing and record keeping practices (route, dose, date, withdrawal period, and other tracking information) accounted for approximately 25% of the total variability in the data. Nutrition related factors and farm antimicrobial use and disease treatment accounted for 24.2 and 21.6% of the total variability in the data, respectively (Table 5). The sampled animals' life stage and treatment history as well as antimicrobial resistance data were groups of variables where correlation stayed below the threshold of 0.5.

TABLE 3 Distribution of 244 *E. coli* isolates inhibited by various concentrations of select antimicrobials. Antimicrobial susceptibility was conducted on *E. coli* isolated from fecal samples during a cross-sectional study on prevalence of antimicrobial resistance to *E. coli* and *Enterococcus* from cow-calf operations in northern California between July 2019 and August 2020.

Antimicrobial drugs	% Resistant (Red) or non-susceptible (Blue)	Number of isolates within each MIC[a] (μg/mL)										
		0.12	0.25	0.5	1	2	4	8	16	32	64	GAD**
Ampicillin*	100%		0	0	3	105	125	5	0			5
Ceftiofur*	0.41%		129	110	2	0	0	0				1
Clindamycin	***		0	0	0	0	0	0	0			244
Danofloxacin	***	241	2	0	0							1
Enrofloxacin	***	243	1	0	0	0						0
Florfenicol	19.26%		0	0	0	40	157	27				20
Gamithromycin	***				1	9	64	158				12
Gentamicin	***				244	0	0	0	0			0
Neomycin*	***						241	0	0	0		1
Penicillin	***	0	0	0	0	0	0	4				240
Spectinomycin*	***							23	207	13	0	0
Sulfadimethoxine****	25.41%											62
Tetracycline	13.11%			3	107	101	1	5				27
Tiamulin	***			0	0	0	0	0	9	28		207
Tildipirosin	***				0	14	171	59	0			0
Tilmicosin	***					0	1	0	2			241
Trimethoprim- sulfamethoxazole****	4.92%											12
Tulathromycin	***							158	84	1	0	1
Tylosin	***			0	0	0	0	0	0	0		244

[a]Minimum Inhibitory Concentration.
*Rows do not add up to 244 due to missing data points from plate reader errors.
**Number of isolates that grew in all available dilutions (GAD) of each antimicrobial drug.
***Clinical and Laboratory Standards Institute breakpoints not available for this antimicrobial.
****Antimicrobials for which only one dilution was available for testing.
| (vertical bar) Threshold for breakpoints (any isolates with MICs higher than the vertical bar are considered resistant).
Red highlighted areas: number or percent of isolates classified as resistant to each antimicrobial drug.
Blue highlighted areas: number or percent of isolates classified as non-susceptible to each antimicrobial drug.
Grey areas: specific MIC was not included in the dilution range for the listed antimicrobial drug.

3.6. Hierarchical clustering

Hierarchical clustering was performed on the MFA principal coordinates to aggregate homogeneous clusters. The hierarchical tree suggested clustering into six clusters (Figure 3). The identified clusters were described based on the 16 variables that contributed the greatest to the data variability from the MFA analysis (Table 5). Cluster 5 represented the majority (65.2%) of sampled animals and ranches (12/18). Most animals represented in cluster 5 (85.5%) were on ranches that reported estimation of the dose of antimicrobial drugs based on estimated animal weight, reported recording the date of antimicrobial use (91.2%), reported feeding free choice minerals to calves (94.3%), reported cleaning of water troughs (84.9%), and did not use antimicrobials to treat mastitis (100%). However, 91.8% of animals in cluster 5 were on ranches that also reported that withdrawal periods are not recorded when animals are treated with antimicrobials. Cluster 4 represented two ranches in our study. The farms represented in cluster 4 mentioned that they were not recording the date, route, and withdrawal period of antimicrobial use (100%). One farm represented in cluster 2 mentioned routine use of antimicrobials for prevention of disease and use of antimicrobials for treatment of mastitis. Clusters 1, 3, and 6 represented one herd each. Farms represented in clusters 3 and 6 reported that they were not using antimicrobials for treatment of mastitis and the farm in cluster 6 reported dosing antimicrobials according to veterinarian's orders. The beef operations located in the coastal range were only represented by clusters 5 and 6. The majority of beef ranches in clusters 5 and 6 reported several antimicrobial stewardship or herd health practices including estimation of the dose of antimicrobials based on estimated animal weight, recording of the date of antimicrobial use, feeding free choice mineral to calves, and cleaning of water troughs once a month, and did not use antimicrobials to treat mastitis in comparison to beef ranches included in clusters 1, 2, 3, and 4. A complete description of the six clusters is available in Supplementary Table S1.

4. Discussion

Antimicrobial resistance is a global problem (Thanner et al., 2016), and while much of the attentions is focused on human health implications, the effects of AMR on livestock health may be similar,

TABLE 4 Distribution of 238 *Enterococcus* isolates inhibited by various concentrations of select antimicrobials. Antimicrobial susceptibility was conducted on *Enterococcus* isolated from fecal samples during a cross-sectional study on prevalence of antimicrobial resistance to *E. coli* and *Enterococcus* rom cow-calf operations in northern California between July 2019 and August 2020.

Antimicrobial drugs	% Resistant (Red) or Non-Susceptible (Blue)	Number of isolates within each MIC[a] (ug/mL)										
		0.12	0.25	0.5	1	2	4	8	16	32	64	GAD**
Ampicillin	0.42%		129	73	32	0	2	1	0 |			1
Ceftiofur	***		126	4	4	1	8	23				72
Clindamycin	***		152	5	5	4	15	34	19			4
Danofloxacin	***	2	2	17	67							150
Enrofloxacin	***	3	4	28	100	96						7
Florfenicol	***		0	36	76	58	64	4				0
Gamithromycin	***				177	13	18	16				14
Gentamicin	***				14	60	84	59	21			0
Neomycin	***						52	88	57	36		5
Penicillin*	1.68%	125	3	36	33	23	13	0				4
Spectinomycin*	***							1	19	45	171	0
Sulfadimethoxine****	***											233
Tetracycline	12.61%			161	43	4	0	1				29
Tiamulin	***			103	31	9	5	11	3	0		76
Tildipirosin	***				124	5	1	9	33			66
Tilmicosin	***					128	1	13	54			42
Trimethoprim- sulfamethoxazole****	***											5
Tulathromycin	***							183	21	29	5	0
Tylosin	***			128	1	49	50	7	0	0		3

[a]Minimum inhibitory concentration.
*Rows do not add up to 238 due to missing data points from plate reader errors.
** Number of isolates that grew in all available dilutions (GAD) of each antimicrobial drug.
*** Clinical and Laboratory Standards Institute breakpoints not available for this antimicrobial.
****Antimicrobials for which only one dilution was available for testing.
| (vertical bar) Threshold for breakpoints (any isolates with MICs higher than the vertical bar are considered resistant).
Red highlighted areas: number or percent of isolates classified as resistant to each antimicrobial drug.
Blue highlighted areas: number or percent of isolates classified as non-susceptible to each antimicrobial drug.
Grey areas: specific MIC was not included in the dilution range for the listed antimicrobial drug.

including treatment failures requiring the use of newer and often more expensive antimicrobials (Magnusson et al., 2021).

For our study, the distribution of herd sizes closely represented what has previously been reported for cow-calf operations throughout the state of California (Saitone, 2020). Considering the state's number of beef cow farms, however, our study included a higher proportion of larger herd sizes for the state, since approximately 77% of beef cow farms in California are reported to have fewer than 100 cows, not including hobby farms with less than 10 cows (United States Department of of Agriculture, 2019). Information about the percentage of different breeds, type of production (organic, natural, No Hormone Treated Certified (NHTC), and/or Age and Source Verified (ASV) versus conventional) for beef cow-calf herds or specific antimicrobial practices have not been previously reported. Due to small numbers in each category, organic, natural, NHTC and/or ASV status were combined to represent how management specific to a target consumer may influence AMR patterns overall. Pasture-based forage is common in California, with livestock grazing being California's most extensive land use (Saitone, 2020), but details on dryland versus irrigated pasture for beef cow-calf herds have not been reported.

The types of diseases most treated with antimicrobials reported in our survey, namely pinkeye, respiratory disease, footrot and scours, concur with prior data reported in a large survey on antimicrobial use on California cow-calf operations (California Department of Food and Agriculture, 2020). Use of antimicrobials in feed is an uncommon practice in cow-calf herds and mastitis is not nearly as common in beef as in dairy production systems, so it is not surprising that these practices were not common amongst the farms surveyed. In addition, in California, veterinary oversight is required for the purchase and use of all medically important antimicrobials, which may explain the high percentage of farms that reported having a veterinarian-client-patient relationship (17 of 18 farms surveyed).

Of the *E. coli* isolates, approximately 36% were resistant or non-susceptible to at least one antimicrobial, excluding ampicillin, to which all isolates were resistant. AMR of *E. coli* in cattle or ruminants to various antimicrobials has been observed by other authors to varying degrees, but it is not always clear how resistant status is established. For example, one study from Malaysia found 61.9% of *E. coli* isolated from diseased ruminants to be resistant to trimethoprim sulfamethoxazole compared to 4.9% in our study, 69% resistant to

tetracycline compared to 13.1% non-susceptible in our study, 54.1% resistant to amoxicillin, compared to 100% resistant to ampicillin in our study (Haulisah et al., 2021). Discrepancies may be due to the choice of breakpoint to establish resistant status, meaning that a breakpoint can be chosen based on the species (human versus veterinary) or it can be chosen based on the most similar bacteria for which there is an established breakpoint, both of which are routine practices and depend on the context of the study. Variations in results can also stem from the fact that diseased animals are more likely to have been treated with antimicrobials before isolation of the pathogen or because antimicrobial drug use patterns may vary between countries or regions.

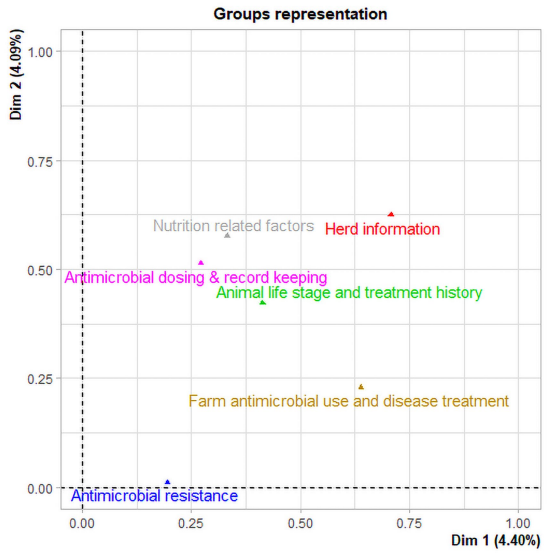

FIGURE 2
Multiple factor analysis of data collected from beef ranches in California in a cross-sectional study evaluating antimicrobial resistance and antimicrobial drug use in 18 cow-calf operations in northern California between July 2019 and August 2020. Two groups (Herd information and Farm antimicrobial use and disease treatment) have the highest coordinates indicating the largest contribution to the first dimension. In the second dimension, the three groups (Nutrition related factors, antimicrobial dosing and record keeping, and sampled Animal life stage and treatment history) have the highest coordinates indicating the largest contribution to the second dimension.

Ampicillin had the highest proportion of resistant isolates of *E. coli* (100%), which was surprising, especially since none of the participating ranches reported any ampicillin use. In this study, a breakpoint of ≥0.25 μg/mL indicating resistance was chosen based on the veterinary literature for ampicillin resistance for treatment of metritis in cattle due to *E. coli* from VET CLSI [Clinical and Laboratory Standards Institute (CLSI), 2020]. By contrast, the human breakpoint is ≥32 μg/mL indicating resistance. The most common MIC for ampicillin in this study was 4 μg/mL (Table 3). Data from this study indicate that the veterinary breakpoint for ampicillin may need to be reevaluated. The lowest prevalence of resistance or non-susceptibility for *E. coli* of all included antimicrobials was for ceftiofur (0.41%), which was also only used by one of the enrolled farms. Restrictions were placed on extra-label cephalosporin use by the Food and Drug Administration in 2012 which aimed to decrease their use in livestock (FDA Federal Register, 2015).

The ampicillin breakpoint used in this study for *Enterococcus* was ≥16 μg/mL indicating resistance. Although dramatically different from the breakpoint for *E. coli*, this human breakpoint was selected due to available data and differences between antibacterial spectrum and bacterium type. As no veterinary breakpoint is available for this bacterium/antimicrobial combination, the human breakpoint was

TABLE 5 Multiple factor analysis (MFA) of 63 data variables collected in a cross-sectional study evaluating antimicrobial resistance status, management factors, and antimicrobial drug use in 18 cow-calf operations in northern California between July 2019 and August 2020. The MFA identified four groups (components) and 16 variables with a correlation coefficient≥0.5 on both first and second dimensions that contribute the most (98.6%) to the variation in the data set.

Identified components	Variation proportion (%)	Component variables	Correlation (R^2)
Herd information	27.7	Sampled ranch	0.99
		Sampled animal	0.99
Nutrition related factors	24.3	Feeding free choice mineral to calves	0.50
		Giving injectable mineral to calves	0.57
		Giving mineral boluses to calves	0.50
		Whether the farm cleans water troughs	0.62
		If they clean water troughs, they use bleach	0.50
Antimicrobial dosing and record keeping practices	25.0	How are antibiotic doses estimated (e.g., estimating weight, standard dose, based on disease, etc.)	0.58
		When an animal is treated, the route is recorded/tracked	0.60
		When an animal is treated, the date is recorded/tracked	0.59
		When an animal is treated, the dose is recorded/tracked	0.57
		When an animal is treated, the withdrawal is recorded/tracked	0.56
		When an animal is treated, other information is recorded/tracked	0.55
		When an animal is treated, nothing is recorded/tracked	0.58
Farm antimicrobial use and disease treatment	21.6	Routine use of antibiotics	0.72
		Use of antibiotics to treat mastitis	0.50

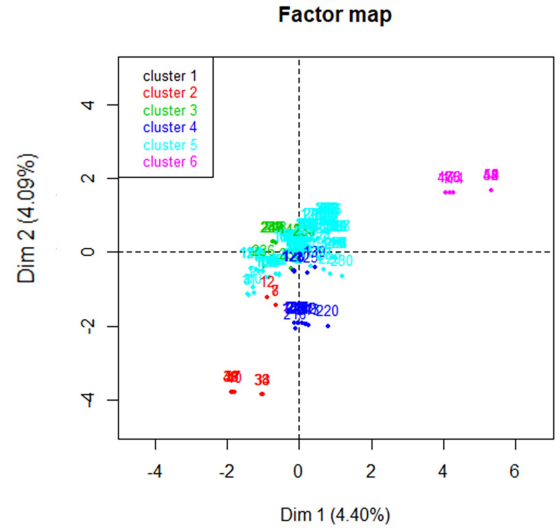

FIGURE 3
Representation of six clusters identified by hierarchical clustering using the results of multiple factor analysis of data collected from beef ranches in California in a cross-sectional study evaluating antimicrobial resistance and antimicrobial drug use in 18 cow-calf operations in northern California between July 2019 and August 2020.

selected as outlined in the methods, resulting in only 1 resistant *Enterococcus* isolate to ampicillin.

Second to ampicillin, the most common drugs for which *E. coli* was resistant or non-susceptible were sulfadimethoxine and trimethoprim-sulfamethoxazole. A 2016 study of AMR in beef cattle found no associations between the prevalence of resistance of *E. coli* isolates to tetracycline, third generation cephalosporin, or trimethoprim-sulfamethoxazole and history of antimicrobial treatment with either ceftiofur or other antimicrobials (Agga et al., 2016). The authors conclude that mixing of treated and non-treated cattle may mask the effect of treatment or that animal-level effects due to treatment are short-lived. However, both ceftiofur and sulfa treatments were uncommon in our study population so that neither hypothesis would explain the prevalence of AMR to this class of antimicrobial observed.

Florfenicol is a relatively new antimicrobial and limited published, peer-reviewed data exist on resistance profiles. It was first approved for use in cattle in 1998 (Food and Drug Administration Health and Human Services, 1996). In this study, 2 animals had a history of being treated with florfenicol and 9 farms indicated that they use it on farm. For *E. coli*, there were 47 (19.26% of isolate pool) non-susceptible isolates to florfenicol, which was the drug with the third highest AMR prevalence, behind ampicillin and sulfadimethoxine. Reports of increasing AMR to florfenicol in *Enterobacteriaceae* exist in the literature. In addition to antibiotic use, mobile genetic elements and horizontal gene transfer are speculated to play a role in the replication of AMR genes resulting in the observed trend of AMR to florfenicol (Fernández-Alarcón et al., 2011; Li et al., 2020). Future research should also investigate genetic elements linked with phenotypic resistance to AMR to florfenicol in enteric bacteria from cow-calf operations to increase understanding of potential factors resulting in higher prevalence of AMR to this drug.

Historically, bacterial resistance to tetracycline has had a high prevalence (Sato et al., 2004; Gow et al., 2008; Yamamoto et al., 2013). In the present study, *E. coli* and *Enterococcus* isolates had a similar, relatively low proportion of isolates resistant to tetracycline, but *Enterococcus* isolates showed the highest proportion of non-susceptibility to this drug.

No biologically relevant survey variables regarding farm description, animal management, or herd level antimicrobial use were significantly associated with AMR in our models while accounting for correlation between isolates from the same animal. Additionally, controlling for correlation between isolates from the same farm led to unstable models with non-positive G matrices indicating a lack of variation in the additional random effect. However, given the high prevalence of AMR at one of the farms, there may be exposures at farm or animal level associated with AMR that were not captured by this survey.

Calves have been shown to carry more AMR bacteria than cows in previous studies (Berge et al., 2010; Yamamoto et al., 2013; Noyes et al., 2016), however calves in two of those studies were less than 4 weeks old. In contrast, calves in our study were up to one year old, and the bacterial AMR profile in neonates may differ from that of older calves. In dairy calves, antimicrobials may be used more often to treat and prevent disease, but in beef calves, the link is less clear. One hypothesis to explain AMR bacteria shed from calves is that AMR is acquired through other routes, such as genetic linkage (Berge et al., 2010) or direct transfer from cows (Yamamoto et al., 2013) and may not be associated with antimicrobial use on farm.

Another study found the frequency of water trough cleaning and size of operation were significantly associated with AMR prevalence (Fernández-Alarcón et al., 2011). Although water trough cleaning was not significantly associated with AMR in our study, MFA analysis showed both water trough cleaning and whether or not farms used bleach to clean water troughs to be two of the variables contributing most to data variability. Other factors that have been found to be statistically significantly linked to AMR in other studies but were not explored in this study include spring versus fall born calves (Gow et al., 2008) and proximity to dairy farms (Berge et al., 2010). None of the farms in the present study were within one mile of a dairy farm and spring versus fall calves was not examined.

Antimicrobial use on farm has been suggested as a contributing factor for the development of AMR, but several studies have indicated that resistance is multifactorial and develops regardless of exposure or use of particular antimicrobials on farm (Berge et al., 2010; Gaze et al., 2013; Agga et al., 2016), findings that may be substantiated by the results of this study. In addition, there is evidence that some AMR genes may be co-selected or have genetic linkages (Berge et al., 2010; Agga et al., 2016), in which resistance to one antimicrobial is genetically linked to resistance to a different antimicrobial and transferred either vertically or horizontally together (Summers, 2006). Alternatively, antimicrobial use in the cow-calf sector may not exert high enough selective pressures on bacterial populations to drive AMR.

Multiple factor analysis showed that herd information (type of ranch and age of animals) and nutrition related factors (cleaning of water troughs, use of bleach by farmers for water trough cleaning, and provision of mineral supplement to calves) accounted for approximately 52% of the total variance in the data. Several studies have shown herd health in farming systems, herd management,

biosecurity, population density, and external pressures to be linked to antimicrobial use (Andrés-Lasheras et al., 2021; Diana et al., 2021). Previous studies reported an association between farm management factors and the prevalence of AMR in *E. coli* isolates (Hille et al., 2017; Markland et al., 2019). Markland et al. (Markland et al., 2019) found that regular cleaning of water troughs and the addition of ionophores to feed were associated with a reduction in prevalence of cefotaxime resistant bacteria in fecal samples of beef cattle on grazing farms in Florida. Beef cattle require several minerals for optimal growth, health, and reproduction. Mineral deficiency may result in anemia, depressed immunity and increased opportunity for bacterial growth and dissemination of resistant bacteria (University of Georgia Extension, 2017). On the other hand, elevated heavy metal supplementation may co-select for antimicrobial resistance of fecal *E. coli* and *Enterococcus* spp. (Jacob et al., 2010). A recent scientific report showed that synthetic smectite clay minerals and Fe-sulfide microspheres have antimicrobial properties and kill antibiotic resistant bacteria including *E. coli* and *Enterococcus* spp. (Morrison et al., 2022) but we did not inquire about the use of these products.

In addition, MFA in this study showed that farm level antimicrobial use, disease treatment, and antimicrobial dosing and record keeping practices accounted for 46% of the total variability in the study data. Similarly, a survey study of antimicrobial use in adult cows on California dairies (Abdelfattah et al., 2019) found that antimicrobial stewardship practices, antimicrobial usage information, and producer perceptions of AMR on dairies accounted for 32.3% of the total variability in the survey data. On the other hand, the sampled animals' life stage and antimicrobial treatment history and in particular the antimicrobial resistance data contributed to a lesser degree to data variability. Given that AMR seemed less variable than other factors describing the animals and farms in the data set, it is not surprising that statistical models were unable to find associations between AMR and animal or farm related factors. Overall, the MFA analysis identified important differences between herds that can be considered in studies that investigate the risk and the associations between farm practices and AMR of fecal bacteria.

Cluster analysis identified some potential regional differences in management practices and antimicrobial use information among cow-calf operations in northern California since the Coastal Range was only represented by two clusters. The cause of the differences could be due to variable access to information or rancher education or due to the influence of veterinarians in the Coastal Range.

One limitation for this study includes the use of a convenience sample of farms that could have introduced bias because the group of farms that are associated with the University of California teaching hospital or extension agents may have similar management tendencies. They could represent farms that have more progressive management, are more attentive to animal health and/or more willing to treat or may be more likely to adhere to legislation regarding antimicrobial use and antimicrobial stewardship. This is a significant factor to consider and, if true, could have biased the study either toward the null because of less antimicrobial use overall (judicious use) or away from the null because these producers may be more likely to watch carefully, identify, and treat any disease conditions that warrant antimicrobials. In addition to selection of farms, selection of animals for sampling was not random, as sampling is logistically challenging in a cow-calf setting. The animals sampled were either being put through the chute for another reason (processing), were physically closest to the chute, or were the easiest animals to collect for sampling (usually more friendly or animals that are visualized more often in this setting).

Some other challenges associated with sampling in this system include limited animal identification, treatment records, and animal restraint. Many of the animal health records were based on the farmer's recollection and therefore are subject to recall bias. In this case, those that were identified as treated were very likely actually treated; however, if a treatment was forgotten, that animal did not have any treatment to associate with AMR isolate status. In addition to logistical challenges, none of the farms put antibiotics in the feed which may have biased this study toward the null; however, it should be noted that this practice is not common in cow-calf operations in California. Finally, the use of human breakpoints for the determination of AMR status when no veterinary breakpoints were available is another limitation, underlining the need for further research into AMR in livestock species. A metagenomic analysis of isolates would have provided further information but was not possible at this time due to financial constraints.

5. Conclusion

AMR is an evolving, multifactorial topic critical to the health of both animals and humans worldwide. Our study generated novel data for cow-calf AMR, an area with knowledge gaps that limit understanding of factors that could be affecting prevalence of AMR. No associations between specific farm management practices including use of antimicrobials and AMR status of bacterial isolates from the same animals were found and antimicrobial resistance as a variable contributed little to the overall variability in the data; therefore, there are likely other factors that are not well understood and/or not captured in this study that are contributing to development of AMR. In addition, this study presents data to show that antimicrobial use in cow-calf operations in northern California is low, which supports other data in the cow-calf sector. The results of this study serve as a reference for future studies on AMR in the population of cow-calf operations in California and beyond and lead to a better understanding of risk factors for shedding of fecal pathogens carrying ARGs. Continued surveillance will allow more informed decisions and directions for combatting AMR in the future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author upon request.

Ethics statement

The animal study was reviewed and approved by UC Davis Institutional Animal Care and Use Committee. Written informed consent for participation was not obtained from the owners because a handout describing the study was provided to owners as well as verbally explained. Interventions on animals were brief and very low risk.

Author contributions

GM and EO designed the study. DW and GM performed field work and collected data. CM, DW, and EO performed laboratory experiments. CM, RP, EA, and GM analyzed the data. CM and EA prepared the manuscript. EO, EA, RP, and GM revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1086203/full#supplementary-material

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Clonal relatedness of coagulase-positive staphylococci among healthy dogs and dog-owners in Spain. Detection of multidrug-resistant-MSSA-CC398 and novel linezolid-resistant-MRSA-CC5

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Introduction: Nasal carriage of coagulase-positive staphylococci (CoPS) in healthy dogs could indicate increased risks of colonization for in-contact people or vice versa. This study determined the nasal carriage rate of CoPS among healthy dogs and in-contact people, their genotypic characteristics and phylogenetic relatedness.

Methods: Nasal samples were collected from 27 households (34 dogs and 41 humans) in Spain. Staphylococci were identified by MALDI-TOF-MS, their antimicrobial resistance (AMR) genes and *spa*-types were tested by PCR/sequencing. The relatedness of CoPS from the same households was assessed by core genome single nucleotide polymorphisms (SNPs) analyses.

Results: *Staphylococcus aureus* carriage was found in 34.1% of humans (including one methicillin-resistant *S. aureus* MRSA-CC5-t2220-SCC*mec* type-IV2B) and 5.9% of dogs; *Staphylococcus pseudintermedius* in 2.4% of humans and 32.4% of dogs; while *Staphylococcus coagulans* was only detected in dogs (5.4%). Remarkably, one human co-carried *S. aureus*/*S. pseudintermedius*, while a dog co-carried the three CoPS species. Household density was significantly associated with *S. pseudintermedius* carriage in households with > than 1 dog and >than 1 human (OR=18.10, 95% CI: 1.24–260.93, p=0.034). Closely related (<15 SNPs) *S. aureus* or *S. pseudintermedius* were found in humans or dogs in three households. About 56.3% *S. aureus* carriers (dog or human) harboured diverse within-host *spa*-types or AMR genotypes. Ten clonal complexes (CCs) were detected among the *S. aureus*, of which methicillin-susceptible *S. aureus*-CC398-IEC-type C (t1451 and t571) was the most frequent, but exclusive to humans. *S. aureus* and *S. pseudintermedius* isolates harboured resistance genes or mutations associated to 9 classes of antimicrobials including linezolid (G2261A & T1584A point mutations in 23S rDNA). The *S. coagulans* isolates were susceptible to all antimicrobials. Most of the *S. pseudintermedius* carried *lukS/F-I, siet,* and *sient* genes, and all *S. aureus* were negative for *lukS/F*-PV, *tst-1, eta* and *etb* genes.

Discussion: Clonally related human-to-human MSSA and dog-to-human MSSP were found. The detection of the MSSA-CC398 clade highlights the need for its continuous surveillance from One Health perspective.

KEYWORDS

Staphylococcus, zoonosis, pets, MSSA-CC398, *Staphylococcus pseudintermedius* carriage, *Staphylococcus aureus* carriage, linezolid resistance

Introduction

Resident bacteria of the mucosal surfaces (such as nasal or oral) and skin of dogs could easily be transmitted to in-contact persons or owners by direct contact or from the household environment (Trinh et al., 2018). Moreover, transmission from humans to pets could also be possible (Orsini et al., 2022). These events (zoonosis and anthroponosis) represent a growing public health problem (Rahman et al., 2020; Orsini et al., 2022).

Two main groups of staphylococci are defined based on rabbit's plasma coagulase activity, *viz*, coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS) (Carroll et al., 2021). Of the two groups, CoPS are more pathogenic (Carroll et al., 2021). *Staphylococcus aureus* is the archetype of the CoPS and relevant animal and human pathogen (Tong et al., 2015); however, other CoPS related to animals have been described. One of the emerging CoPS of clinical importance is *S. pseudintermedius*, a member of the *Staphylococcus intermedius* group (SIG) that comprises four other species, *viz.*; *Staphylococcus intermedius, Staphylococcus delphini,* the recently described *Staphylococcus cornubiensis*, and *S. ursi* (Murray et al., 2018; Perreten et al., 2020; Carroll et al., 2021). In addition to these CoPS, *Staphylococcus coagulans* is another species that was originally described in 1990 as *Staphylococcus schleiferi* subsp. *coagulans* before being classified, based on genomic studies, into a separate species in 2020 (Madhaiyan et al., 2020).

Staphylococcus aureus is an opportunist pathogen frequently found in humans, which colonizes the anterior nose and nasal vestibule of 25–50% of healthy people (Liu et al., 2015; Sakr et al., 2018). *S. aureus* nasal colonization in humans and animals could place individuals at risk of opportunistic infections, and the carriage of specific virulence genes by the isolates can increase their pathogenic potential. In this respect, the following virulence genes are especially relevent in *S. aureus*: *tst* (encodes the toxic shock syndrome toxin, associated with sepsis), *eta* and *etb* (encode the exfoliatin A and B, associated with the staphylococcal scalded skin syndrome), and the *luk-S/F-PV* (encodes Panton Valentine Leucocidin, associated with abscesses and community-acquired pneumonia) (Tam and Torres, 2019; Hu et al., 2021).

The population genetics of *S. aureus* have shown the presence of several clonal complexes (CCs). Remarkably, some of these CCs have somewhat animal host specificity, while others are less host-specific (Chaguza et al., 2022). Methicillin-Resistant *S. aureus* (MRSA) is a globally epidemic bacterium in nosocomial settings and is referred to as healthcare-associated MRSA (HA-MRSA) (Cuny et al., 2010; Cuny et al., 2022). MRSA has also emerged in the community without any relation to the healthcare facilities [community associated (CA)-MRSA] and in the last decade, some CCs (mainly CC398) have been associated with livestock animals (LA-MRSA) (Matuszewska et al., 2022).

Staphylococcus aureus frequently colonizes the skin and nasal cavity of healthy humans, while *S. pseudintermedius* appears to predominantly colonize pets (especially dogs) but could occasionally be found in humans (Cuny et al., 2022). Although infrequent, *S. pseudintermedius* has been also isolated from human skin lesions (Lozano et al., 2017), due en ocassions to dog bites (Börjesson et al., 2015), as well as causing septicemia (Somayaji et al., 2016). Previous reports on nasal colonization in humans by *S. pseudintermedius* in dog-owning households have reported a prevalence of 25–65.9% in dogs and 3–4.5% in humans (Gómez-Sanz et al., 2013c; Han et al., 2016; Rodrigues et al., 2018). Certain isolates of *S. pseudintermedius* have become a major veterinary pathogen of concern due to their frequent multidrug-resistance phenotypes (Priyantha, 2022). Also, severe human infections with methicillin-resistant *S. pseudintermedius* (MRSP) have recently been reported (Priyantha, 2022).

Data about the co-colonization of dogs and in-contact persons with CoPS at the community level are still scarce. Hanselman et al. (2009) reported the presence of *S. pseudintermedius*, *S. aureus*, and S. *schleiferi* subsp. *coagulans* (including methicillin-resistant isolates) in dogs at the household level. In addition, concurrent animal and human colonization by indistinguishable *S. pseudintermedius* and *S. aureus* isolates have been observed (Hanselman et al., 2009). In a previous study performed a decade ago, our research group investigated the household nasal carriage of CoPS in dogs and humans, determining the prevalence of genetic lineages and their recovery rate over a year in animals and owners (Gómez-Sanz et al., 2013c). Nowadays, the abundance of dog ownership in Spain has significantly changed and the number of households with pets has increased. According to available data, there were 20 million registered pets in Spain in 2019 and about 26% of all households own dogs (Asociación Madrileña de Veterinarios de Animales de Compañía, 2019). By implication, sharing and transmission of nasal microorganisms (such as CoPS) between dogs and dog-owning households could be more frequent (Van Balen et al., 2017).

Nasal carriage of CoPS in healthy dogs could be an indicator of increased risk of colonization/infection for people in-contact with these animals, especially if *S. aureus* carries key virulence determinants of special relevance such as *lukS/F*-PV, *tst-1, eta* and *etb* (González-Martín et al., 2020). However, the influence of pet ownership on the diversity of CoPS isolates circulating among dog owners still needs to be fully elucidated (Van Balen et al., 2017). Consequently, this study sought to understand the current epidemiological situation of CoPS nasal carriage in dog-owning households by characterization of the genetic lineages, relatedness between isolates from the same households, antimicrobial resistance (AMR) determinants, virulence factors, and immune evasion cluster (IEC) types of *S. aureus* isolates.

Materials and methods

Samples analyzed and staphylococci recovery

A total of 41 humans and 34 dogs from 27 households were prospectively studied to determine the nasal carriage of CoPS; the sampling was performed in La Rioja region (Northern Spain) between January to March 2022. Household density was classified into four, *viz*: (a) household with a dog and a human (b) household with >1 dog and

a human, (c) household with 1 dog and >than 1 human, and (d) household with > than 1 dog and >than 1 human. The sampled humans and dogs did not have recent hospital stays prior to the study or received antibiotics (at least 3 months before sampling) and the humans had no professional contact with health institutions and did not work in microbiology laboratories. None of the participants had consultations or visits to hospitals in the last 3 months before sample collection. Nasal samples were obtained using sterile swabs with conservation media (Amies BD Life sciences®, New Jersey, USA). All procedures were approved by the ethical committee of the University of La Rioja (Spain) and were carried out following all applicable international, national, and/or institutional guidelines for human samples experiments (as described in the revised Helsinki declaration) and for ethical use of animals (directive 2010/63/EU, Spanish laws 9/2003 and 32/2007, and RD 178/2004 and RD 1201/2005).

Samples were enriched in Brain Heart Infusion broth (BHI; Condalab, Madrid, Spain) supplemented with 6.5% (w/v) NaCl and incubated for 24 h at 37°C. After 24 h of incubation, the broth samples were diluted and carefully dispensed onto four different bacteriological media: blood agar, mannitol salt agar (MSA; Condalab, Madrid, Spain), oxacillin resistance screening agar base (ORSAB with 2 mg/L oxacillin; Oxoid, Hampshire, UK) and ChromAgar LIN (Paris, France) and incubated for 24 h at 37°C. After overnight growth, 3 to 8 different colonies (presenting staphylococci morphology) were randomly selected per sample and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (*MALDI-TOF,* Bruker Daltonics, Bremen, Germany) using the standard extraction protocol. Those isolates identified as CoPS were included in this study.

Antimicrobial susceptibility testing

Susceptibility testing for 13 antimicrobial agents was performed by the disk diffusion method following the recommendations and breakpoints of the European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2022). The antimicrobial agents tested were as follows (μg/disk): penicillin (PEN) (1 or 10 μg, depending on the CoPS species), cefoxitin (FOX) (30 μg to detect methicillin-resistant *S. aureus* (MRSA) isolates), oxacillin (OXA) (1 μg to detect methicillin-resistant *S. pseudintermedius* [MRSP] or *S. coagulans* isolates), erythromycin (ERY) (15 μg), clindamycin (CLI) (2 μg), gentamicin (GEN) (10 μg), tobramycin (TOB) (10 μg), tetracycline (TET) (30 μg), ciprofloxacin (CIP) (5 μg), chloramphenicol (CHL) (30 μg), linezolid (LZD) (10 μg), mupirocin (MUP) (200 μg), and trimethoprim-sulfamethoxazole (SXT) (1.25 μg + 23.75 μg).

Once the antimicrobial resistance phenotype of all CoPS was determined, distinct isolates were selected for further studies (defined as those of different samples or those from the same sample but of different species, and/or different antimicrobial resistance phenotypes).

DNA extraction (for PCR)

For DNA extraction, isolates were seeded on BHI agar and incubated for 24 h at 37°C. An isolated colony was suspended in 45 μL of sterile MiliQ water and added 5 μL of lysostaphin (1 mg/mL) (Sigma). The mixture was vortexed and incubated for 10 min at 37°C. Forty-five μL of sterile MiliQ water, 150 μL of Tris–HCl (0.1 M, pH 8) and 5 μL of proteinase K (2 mg/mL) (Sigma) were added. The final mixture was vortexed and incubated for 10 min at 60°C, then boiled for 5 min at 100°C. To separate and obtain the DNA (supernatant) from debris, the final mixture was centrifuged at 12,000 revolutions per minute for 3 min, and stored at −20°C.

Study of antimicrobial resistance genes

The presence of the following resistance genes was tested by single PCRs, selected according to the antimicrobial resistance phenotype of isolates: beta-lactams (*blaZ, mecA,* and *mecC*), erythromycin and clindamycin (*ermA, ermB, ermC, ermT, mphC, msrA, lnuA,* and *lnuB*), aminoglycosides (*aac6′-aph2″,* and *ant4′*), tetracycline [*tet*(L)*, tet*(M), and *tet*(K)], trimethoprim (*dfrA, dfrD, dfrG* and *dfrK*), and chloramphenicol (*catpC221, catpC223, catpC194, catA, fexA,* and *fexB*). Linezolid or chloramphenicol-resistant isolates were screened for the presence of the linezolid transferable resistance genes (*cfr, cfrB, cfrD, poxtA,* and *optrA*). Mutations in 23S rDNA were also investigated by PCR and amplicon sequencing. The obtained sequences were compared with those of linezolid-susceptible *S. aureus* NCTC 8325 (GenBank accession number CP000253) using the EMBOSS Needle software for nucleotide and amino acid (BLOSUM62 cost matrix) alignments. Primers and conditions of PCRs performed in this study are included in Supplementary Table S1. Isolates were considered multi-drug resistant (MDR) when they were resistant to ≥3 classes of the antimicrobial agents tested (Magiorakos et al., 2012).

Detection of virulence and toxin genes of CoPS

The presence of the genes *tst* (toxin of shock toxic syndrome), *lukS/F*-PV (Panton-Valentine leucocidin), and *eta* and *etb* (exfoliative toxins A and B), was tested by PCR. Immune evasion cluster (IEC) genes (*scn, chp, sak, sea,* and *sep*) were analysed and classified into seven different IEC types (A–G), based on the combination of the positive genes. The *scn* gene (encoding the staphylococcal complement inhibitor) was used as a marker of the IEC system (van Wamel et al., 2006). The presence of the *lukS/F-I, siet,* and *sient* genes was analysed for the *S. pseudintermedius* isolates. Primers and conditions of PCRs performed in this study are included in Supplementary Table S1.

Molecular typing of isolates

All recovered *S. aureus* isolates were characterized by *spa* typing by the PCR/Sanger sequencing. New repeat combinations were submitted to the Ridom *spa* Server.[1] CC398 clone was determined by a specific PCR protocol for the *sau1-hsdS1* variant (Stegger et al., 2011). The clonal complex of the isolates was assigned, when possible, according to the *spa*-types. Moreover, multilocus sequence typing (MLST) was performed in selected *S. aureus* isolates (isolates with *spa*-types repeatedly found and those with new *spa*-types). The seven housekeeping genes of *S. pseudintermedius* (*pta, cpn60, tuf,*

1 https://spa.ridom.de/submission.shtml

ack, purA, sar and *fdh*) were amplified, and the sequence type (ST) was assigned according to the MLST database.[2] The selection of *S. pseudintermedius* isolates for MLST was only on household with dog and human carriers. Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types were determined by multiplex PCRs. Primers and conditions of PCRs performed in this study are included in Supplementary Table S1.

Positive controls (confirmed by sequencing) from the collection of the Universidad de La Rioja were included in all the PCR assays in this study.

Whole genome sequencing

Fourteen selected isolates (11 *S. aureus* and two *S. pseudintermedius*) were whole genome sequenced on the Illumina NextSeq platform. The selection was based on: (a) all MSSA-CC398 isolates, (b) all isolates from households with two or more humans and/or dogs' carriers of either *S. aureus* or *S. pseudintermedius*, (c) the MRSA isolate.

The MagNA Pure 96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA, 4413021) was used to extract genomic DNA according to instructions provided by the manufacturers. The Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) was used for DNA quantification, while Sequencing libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA, FC-131-1096) and sequenced on the NextSeq 500 platform (Illumina, San Diego, CA, USA) using a 300-cycle kit to obtained paired-end 150 bp reads, as previously described (Saidenberg et al., 2022).

Genomic assembly and phylogenetic analysis

All the genomes analysed in this study were *de novo* assembled using SPAdes (v.3.15.5), performing the *in silico* typing with the settings of a minimum of 90% coverage and 80% identity. Core-genome single nucleotide polymorphisms (SNPs) were detected with the NASP pipeline v.1.0.0 (Sahl et al., 2016), mapping against the chromosome reference sequences CA-347 (GenBank accession ID CP006044, *S. aureus*), and SP_11304-3A (GenBank accession ID CP065921.1, *S. pseudintermedius*) for each separate phylogenetic analysis. GATK (v.4.2.2) was used to call SNPs and excluded positions featuring <90% unambiguous variant calls and <10 depth. IQ-TREE (v.2.1.2), was used to construct the phylogenetic trees using ModelFinder with 100 bootstraps. The graphical data was added to the phylogenies with iTOL v.6.6 (Letunic and Bork, 2021).[3]

Genomic typing

The STs were determined with MLST v.2.16 (Jolley et al., 2018),[4] and undefined STs were submitted to the PubMLST database for ST assignment (Larsen et al., 2012). Virulence factors, plasmid replicons, and antimicrobial resistance genes were identified using ABRicate v.0.9.0 and the respective databases VFDB, Plasmidfinder, and Resfinder databases from the Center for Genomic Epidemiology.[5,6] The *spa*Typer v1.0 was used to confirm the *spa* types (Bartels et al., 2014) and mutations associated with AMR were identified using ResFinder v4.1 (Bortolaia et al., 2020) and PointFinder (Zankari et al., 2017).

Genome availability

All the raw genome reads generated from this study have been deposited at European Nucleotide Archive under Study Accession no. PRJEB57210.[7]

Data management and statistics

Data collected were verified and processed and the Statistical Package for Social Sciences (SPSS) Version 26 (IBM, California, U.S.A) was used for analysis. Data were reported as numbers and percentages (for categorical variables) and presented on tables and charts. Data were subjected to univariate logistic regression to compute odd ratio (OR) at a 95% confidence interval (95%CI) between the carriage rate of *S. aureus*/*S. pseudintermedius* and the household densities with significant association ($p < 0.05$).

Results

CoPS nasal carriage in healthy dogs' households

A total of 73 *S. aureus*, 31 *S. pseudintermedius* and two *S. coagulans* isolates were recovered from 75 nasal samples of humans and dogs. After AMR phenotype determination, 52 isolates were selected for further characterization (31 *S. aureus*, 19 *S. pseudintermedius* and 2 *S. coagulans*), corresponding to one isolate per sample or more than one if they presented different species and/or different AMR phenotype (Supplementary Table S2).

Staphylococcus aureus was found in 14 humans (34.1%) (including one individual with MRSA) and two dogs (5.9%) (Figure 1). *S. pseudintermedius* was identified in one human (2.4%) and 11 dogs (32.4%). However, *S. coagulans* was solely identified in two dogs (5.9%) of the same household. Apart from these three species, no other CoPS species were detected in the cultures. Remarkably, one human presented *S. aureus*/*S. pseudintermedius* co-carriage (2.4%) while a dog had co-carriage of all three CoPS species (2.9%) (Figure 1). In total, 14 humans and 12 dogs carried CoPS. Household density was significantly associated with *S. pseudintermedius* carriage in households with > than 1 dog and >than 1 human (OR = 18.10, 95% CI: 1.24–260.93, $p = 0.034$) (Table 1).

2 http://pubmlst.org/
3 https://itol.embl.de/
4 https://github.com/tseemann/mlst
5 https://github.com/tseemann/abricate
6 https://cge.cbs.dtu.dk/services
7 https://www.ebi.ac.uk/ena

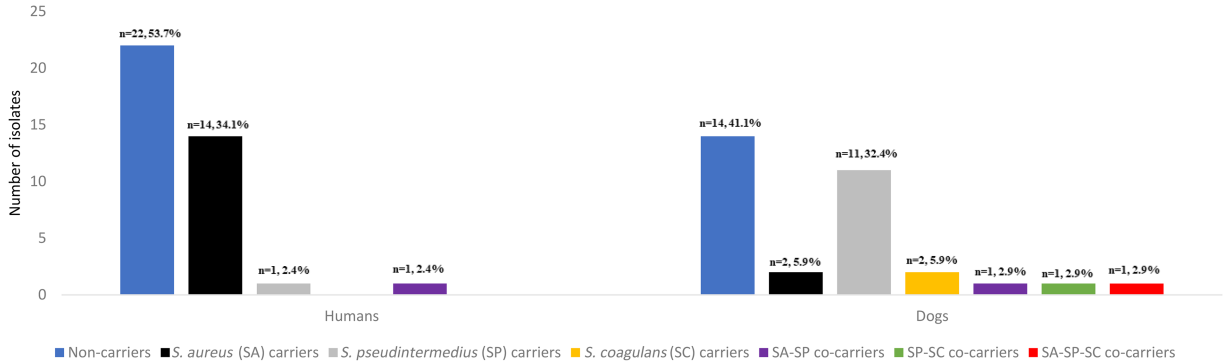

FIGURE 1
Number and prevalence of *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Staphylococcus coagulans* isolates and co-carriage detected in the nasal cavities of healthy humans and dogs.

TABLE 1 Association of household density with nasal *S. aureus* and *S. pseudintermedius* carriage.

Household density	*S. aureus* carriers. Number (%)	*S. aureus* non-carriers. Number (%)	OR (95% CI)	*p* value	*S. pseudintermedius* carriers. Number (%)	*S. pseudintermedius* carriers. Number (%)	OR (95% CI)	*p* value
1 dog and 1 human ($n = 10$)	3 (30)	7 (70)	Referent	Referent	1 (10)	9 (90)	Referent	Referent
>1 dog and 1 human ($n = 3$)	3 (100)	0 (0)	15.0 (0.59–376.7)	0.099	1 (33.3)	2 (66.7)	4.5 (0.19–106.8)	0.352
1 dog and >1 human ($n = 8$)	2 (25)	6 (75)	0.8 (0.09–6.32)	0.814	2 (25)	6 (75)	3.0 (0.22–40.93)	0.410
>1 dog and >1 human ($n = 6$)	5 (83.3)	1 (16.7)	11.7 (0.92–147.57)	0.057	4 (66.7)	2 (33.3)	18.0 (1.24–260.93)	0.034*

*Significant association determined by bivariate regression at 95% confidence interval (CI).

Phenotypic and genetic characteristics of CoPS isolates

The 31 distinct *S. aureus* isolates harboured AMR as follows [percentage of resistant isolates/resistance genotype]: penicillin [77.4/*blaZ*], cefoxitin [9.7/*mecA*], erythromycin-clindamycin-inducible [19.4/*ermT*], erythromycin [9.7/*msrA, mphC*], clindamycin [3.2/*lnuA*], gentamicin-tobramycin [22.6/*aac6′-aph2″*], tetracycline [3.2/*tet*(K)], sulfonamide [3.2/*dfrA*], fluoroquinolones [22.5/amino acid changes in GrlA: S80F, GyrA: S84L], mupirocin (3.2/*mupA*) and linezolid [3.2/G2261A & T1584A point mutations in 23S rDNA] (Figure 2; Table 2). Moreover, the 19 distinct *S. pseudintermedius* isolates harboured AMR as follows [percentage of resistant isolates/resistance genotype]: penicillin [57.9/*blaZ*], erythromycin-clindamycin-constitutive [26.3/*ermB*], tobramycin [15.8/*ant4′*], tetracycline [26.3/*tet*(M)], trimethoprim-sulfamethoxazole [63.2/*dfrA, dfrD, dfrG, dfrK*], and chloramphenicol [5.3/*catA*] (Figure 2; Table 3). No resistance markers were detected in the *S. coagulans* isolates, that were susceptible to all antimicrobial agents tested (Figure 2).

Regarding the genetic lineages of *S. aureus* isolates, the three MRSA isolates from humans (same individual but different AMR phenotypes/genotypes) belonged to the *spa* type t222, associated with CC5. All other isolates were methicillin-susceptible *S. aureus* (MSSA) with 19 different *spa*-types assigned to 10 different CCs. The MSSA-CC398 clone (t1451 and t571) was the most frequently identified (18.8% of *S. aureus* carriers); these isolates were all IEC-type C. Other CCs (*spa*-types) detected were as follows: CC5 (t041), CC7 (t091), CC8 (t121, t126, t1070, t3092), CC15 (t084, t2013), CC30 (t012, t1824), CC45 (t015, t065, t505, t1689), CC97 (t267), CC133 (t4735) and CC152 (t355) (Table 2). For *S. pseudintermedius* isolates, all isolates were methicillin susceptible (MSSP) (including two ST1115) (Table 3).

Clonally related *S. aureus* or *S. pseudintermedius* isolates were found in humans or dogs among 11.1% of households ($n = 3$). Two of the 16 households (household N°11 and 21) positive for nasal *S. aureus* had human carriers with similar clonal complexes (CCs), *spa*-types and IEC types (Table 2). In one of these households (N° 11), MSSA-CC30-*spa*-type t1070 isolates (*scn*-negative) were identified in two humans, however, a different lineage, MSSA-CC8 of the *spa*-type t121 (IEC type-D), was identified in their dog (Table 4). In the second household (N° 21), two humans carried MSSA-CC398 isolates of different *spa*-types (t1451 and t571), although the dog was not *S. aureus* carrier. Moreover, in another household (N° 10), a dog and a human were carriers of the same genetic lineage of *S. pseudintermedius* (MSSP-ST1115); in this household, the human also carried MSSA-CC97-t267 and a dog MSSA-t2013-CC15 (Table 4). All the *S. aureus* isolates were negative for *lukS/F-PV, tst-1, eta* and *etb* genes (Table 2). However, all the *S. pseudintermedius* isolates were positive for *lukS/F-I, siet,* and *sient* virulence genes, but one was only *sient*-positive (Tables 2, 3).

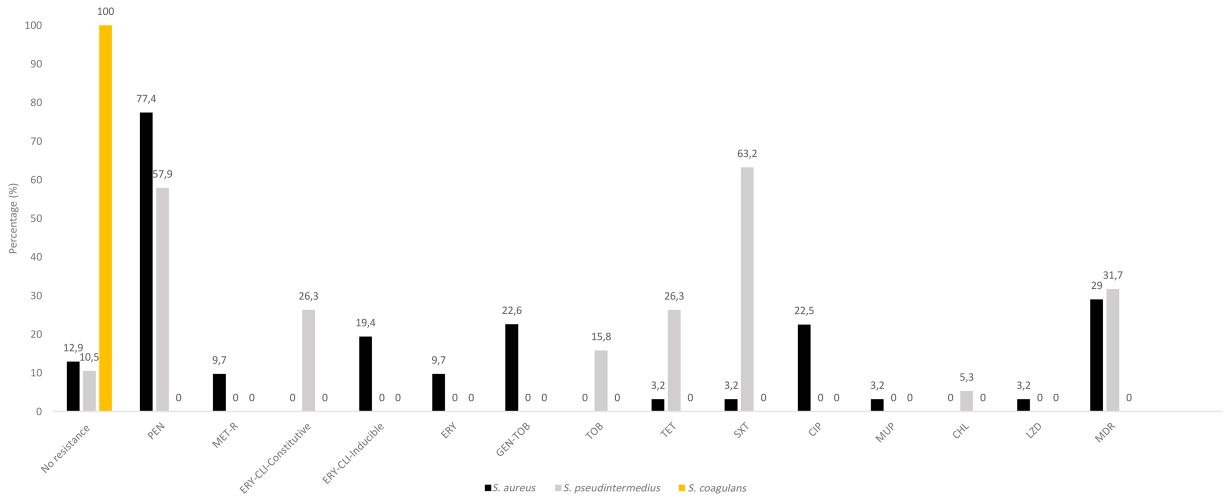

FIGURE 2
Antimicrobial resistance rates in *S. aureus*, *S. pseudintermedius* and *S. coagulans* isolates. Percentages were based on the collection of CoPS (31 *S. aureus*, 19 *S. pseudintermedius* and 2 *S. coagulans*) obtained of different samples or those of the same sample but with different species and/or AMR phenotype. CHL, chloramphenicol; CLI, clindamycin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; MET-R, methicillin-resistant; MDR, multidrug resistance (resistance to three or more classes of antibiotics); PEN, penicillin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline, TOB, tobramycin.

Intra-host variation of genetic lineages or AMR genotypes of CoPS

Nine of the 16 *S. aureus* (56.3%) carriers harboured diverse *spa*-types or AMR genotypes in the same individual (dog or human). Of these, two to four genetically distinct *S. aureus* isolates were detected in these hosts (Table 2). In one human (ID number 3) with both MSSA and MRSA-SCC*mec* type-IV (2B) nasal carriage, three different MRSA-CC5-t2220 isolates with different AMR phenotypes/genes were detected: PEN-FOX-ERY-CLI-CIP-TOB-MUP-LZD/*blaZ, mecA, lnuA, msrA, mphC, mupA*, G2261A point mutation in 23S rDNA; PEN-FOX-ERY-CIP-GEN-TOB/*blaZ, mecA, aac6′-aph2″, msrA, mphC*; and PEN-FOX-ERY-CIP/*blaZ, mecA, msrA, mphC*, respectively; moreover, the MSSA isolate was typed as CC30-t012 and showed resistance only to PEN (*blaZ* positive) (Table 2). Two other humans (ID numbers 57 and 58) from the same household (N° 21) carried *S. aureus* isolates both with similar genetic lineage (CC398) but different *spa* types (t571 and t1451) and similar AMR phenotypes (PEN-ERY-CLI^{ind}-GEN-TOB) (Table 2). In another human *S. aureus* carrier (ID number 60) from a different household (N° 22), isolates with different genetic lineages (CC15 and CC152) were detected (Table 2).

In the *S. pseudintermedius* isolates, 6 of the 12 carriers showed differences and intra-host variations in the AMR phenotypes or AMR genotypes. For instance, one of the dogs (ID number 52) harboured two different MSSP isolates (PEN-SXT/*blaZ, dfrA, dfrG* and PEN-SXT-TOB/*blaZ, dfrG, ant4′*) (Table 3). About 31.7% of the *S. pseudintermedius* had a MDR phenotype (Figure 2). All the three MRSA isolates and some MSSA isolates (20.0%) presented a MDR profile (Tables 2).

Clonal relatedness of CoPS isolates within the same household

Upon WGS, very few SNPs difference (<15) were detected among *S. aureus* isolates from human carriers within the same household (N° 10 and 21) (Figure 3), and these all shared the same repertoire of AMR genes, IEC types and virulence genes and plasmid replicons (Table 4; Figure 3). Concerning the MSSA-CC398 isolates, one isolate from a single person without a co-carrier in their household (N° 17) had >250 SNP differences with those from another household (N° 21) with two human MSSA-CC398 carriers (Supplementary Table S3). The major difference between these MSSA-CC398 isolates was the absence of the *blaZ, aac6′-aph2″*, and *fnbA* genes in the isolate from household number 17 (Figure 3).

Concerning the two *S. pseudintermedius*, each from a dog and human from the same household (N° 10), they were both ST1115 NS identical (zero SNP differences) and shared identical virulence genes (Table 4).

Discussion

Several studies have reported the transmission of CoPS between pets and their owners (Gómez-Sanz et al., 2013c; Han et al., 2016; Sahin-Tóth et al., 2021; Cuny et al., 2022). However, the present study is among the few that studied intra-species and within-host genetic diversities of CoPS in these hosts. Such information can better illustrate the complexity of challenges in the control of AMR in healthy dog-owning households.

The potential influence of dog-ownership on the nasal staphylococcal community (especially *S. aureus* and *S. pseudintermedius* colonization) of dogs and humans needs continuous surveillance. The present *S. aureus* household carriage rate (at least one dog and/or one human) in our study (44.4%) is lower than the 51.2% detected in a previous study performed a decade ago in Spain (Gómez-Sanz et al., 2013c), and the *S. aureus* carriage rate in humans in our study was also lower (34.1% *vs* 41.8%) (Gómez-Sanz et al., 2013c). Concerning studies from other European countries, our *S. aureus* prevalence in humans was higher than those reported in Germany (22%) and Hungary (23.8%) (Holtfreter et al., 2016; Sahin-Tóth et al., 2021).

Data on *S. aureus* nasal carriage rate among healthy dogs in community settings are sparsely available, varying between 2–8%

TABLE 2 Molecular characterization, antimicrobial resistance and virulence profile of the *S. aureus* isolates from humans and dogs.

Host/ID No.	Household ID N°/population in household	N° of isolates	*spa* type	CC	AMR phenotypes	Methicillin susceptibility	MDR phenotype	AMR genes detected	IEC genes/ type	Virulence genes detected (*lukS/F-PV, tst, eta, etb*)
Human/3	2/1 human and 1 dog	3	t2220	CC5	PEN[3]-FOX[3]-ERY[3]-CIP[3]-GEN[2]-TOB[2]-MUP[1]–LZD[1]	MRSA-SCC*mec* type-IV (2B)	Yes	*blaZ*[3], *mecA*[3], *msrA*[3], *mphC*[3] GrlA (S80F)[1], GrlA (S84L)[1], *aac6′-aph2″*[2], *mupA*[1], G2261A & T1584A point mutations in 23SrDNA[1]	Negative	Negative
		1	t012	CC30	PEN	MSSA	No	*blaZ*	Negative	Negative
Human/9	5/1 human and 2 dogs	1	t012	CC30	PEN-CIP-TET	MSSA	Yes	*blaZ*, *tet*(K)	Negative	Negative
		1	t012	CC30	PEN-CIP	MSSA	No	*blaZ*	Negative	Negative
		1	t1824	CC30	PEN	MSSA	No	*blaZ*	*scn, sak, sep*/G	Negative
Human/17	8/2 humans and 2 dogs	1	t4735	CC133	Susceptible	MSSA	No	NT	Negative	Negative
		1	t4735	CC133	CIP-SXT	MSSA	No	*dfrA*	Negative	Negative
Human/20	9/1 human and1 dog	1	t3092	CC8	PEN	MSSA	No	*blaZ*	Negative	Negative
		1	t068	CC8	PEN	MSSA	No	*blaZ*	*scn, sak, sep*/G	Negative
Human/23	10/2 humans and 2 dogs	1	t267	CC97	Susceptible	MSSA	No	NT	*scn, sak*/E	Negative
		1	t267	CC97	Susceptible	MSSA	No	NT	*scn, sak*/E	Negative
Dog/24		1	t2013	CC15	PEN	MSSA	No	*blaZ*	*scn, chp*/C	Negative
Human/26	11/2 humans and 2 dogs	1	t1070	CC30	PEN	MSSA	No	*blaZ*	Negative	Negative
Human/27		1	t1070	CC30	PEN	MSSA	No	*blaZ*	Negative	Negative
Dog/29		1	t121	CC8	PEN-CIP	MSSA	No	*blaZ*, GrlA (S80F)	*scn, sak, sea*/D	Negative
Human/30	12/1 human and 1 dog	1	t041	CC5	Susceptible	MSSA	No	NT	*scn, sak*/E	Negative
Human/38	15/1 human and 2 dogs	1	t505	CC45	PEN	MSSA	No	*blaZ*	*scn, sak*/E	Negative
		1	t065	CC45	PEN	MSSA	No	*blaZ*	*scn, sak*/E	Negative
		1	t015	CC45	PEN	MSSA	No	*blaZ*	*scn, sak, sep*/G	Negative
Human/44	17/1 human and 1 dog	1	t571	CC398	ERY-CLI[ind]	MSSA	No	*ermT*	*scn, chp*/C	Negative
Human/50	19/2 humans and 2 dogs	1	t1689	CC45	PEN	MSSA	No	*blaZ*	*scn, sak*/E	Negative
		1	t091	CC7	PEN	MSSA	No	*blaZ*	*scn, sak, sep*/G	Negative

(Continued)

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TABLE 2 (Continued)

Host/ID No.	Household ID N°/population in household	N° of isolates	*spa* type	CC	AMR phenotypes	Methicillin susceptibility	MDR phenotype	AMR genes detected	IEC genes/ type	Virulence genes detected (*lukS/F-PV, tst, eta, etb*)
Human/57	21/2 humans and 1 dog	1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ, ermT, aac6′-aph2″*	*scn, chp*/C	Negative
		1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*ermT, aac6′-aph2″*	*scn, chp*/C	Negative
		1	t571	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ, ermT, aac6′-aph2″*	*scn, chp*/C	Negative
Human/58		1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ, ermT, aac6′-aph2″*	*scn, chp*/C	Negative
		1	t571	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ, ermT, aac6′-aph2″*	*scn, chp*/C	Negative
Human/60	22/I human and 1 dog	1	t084	CC15	PEN	MSSA	No	*blaZ*	*scn, chp*/C	Negative
		1	t355	CC152	PEN	MSSA	No	*blaZ*	*scn, sak*/E	Negative

ST, Sequence Type. CHL, chloramphenicol; CLI, clindamycin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; PEN, penicillin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; TOB, tobramycin; ERY-CLI^{ind}, erythromycin-clyndamycin inducible.

TABLE 3 Intra-host variation of AMR determinant and virulence factors of *S. pseudintermedius* isolates.

Host/ID	Household ID N°/ population	N° of isolates	AMR phenotypes	MDR phenotype	AMR genes detected	Methicillin Susceptibility/ST	Virulence genes detected
Dog/2	1/1 human and 1 dog	4	PEN[4]-ERY[4]-CLI[4]-TET[4]-SXT[1]-TOB[2]	Yes	*blaZ*[4], *ermB*[4], *tet*(M)[3], *ant4′*[2]	MSSP/NT	*lukS/F-I*[3], *siet*[3], *sient*[4]
Human/23	10/2 humans and 2 dogs	1	Susceptible	No	NT	MSSP/ST1115	*lukS/F-I*[1], *siet*[1], *sient*[1]
Dog/25	10/2 humans and 2 dogs	1	Susceptible	No	NT	MSSP/ST1115	*lukS/F-I*[1], *siet*[1], *sient*[1]
Dog/28	11/2 humans and 2 dogs	1	SXT[1]	No	*dfrK*[1]	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]
Dog/29	11/2 humans and 2 dogs	1	SXT[1]	No	ND	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]
Dog/43	16/2 humans and 1 dog	1	PEN[1]	No	*blaZ*[1]	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]
Dog/39	15/1 human and 2 dogs	1	PEN[1]-SXT[1]	No	*blaZ*[1], *dfrA*[1], *dfrG*[1]	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]
Dog/39	15/1 human and 2 dogs	1	SXT[1]	No	*dfrA*[1], *dfrG*[1]	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]
Dog/49	18/2 humans and 2 dogs	1	SXT[1]	No	*dfrA*[1], *dfrG*[1]	MSSP/NT	*lukS/F-I* [2], *siet*[2], *sient*[2]
Dog/48	18/2 humans and 2 dogs	1	TET[1]	No	*tet*(M)[1]	MSSP/NT	*lukS/F-I* [4], *siet*[4], *sient*[4]
Dog/52	19/2 humans and 2 dogs	3	PEN[3]-SXT[3]-TOB[1]	Yes	*blaZ*[3], *dfrA*[1], *dfrG*[1], *blaZ*[1], *dfrG, ant4′*[1]	MSSP/NT	*lukS/F-I* [3], *siet*[3], *sient*[3]
Dog/53	19/2 humans and 2 dogs	2	PEN[2]-SXT[2]	No	*blaZ*[1], *dfrG*[2], *dfrK*[2]	MSSP/NT	*lukS/F-I* [2], *siet*[2], *sient*[2]
Dog/60	22/1 human and 1dog	1	PEN[1]-ERY[1]-CLI[1]-CHL[1]-SXT[1]	Yes	*ermB*[1], *catA*[1], *dfrD*[1]	MSSP/NT	*lukS/F-I* [1], *siet*[1], *sient*[1]

In superscript is the number of isolates that present the specific phenotype/genotype of AMR. ST, Sequence Type; NT, Not tested; ND, not detected; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; PEN, penicillin; OXA, oxacillin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; TOB, tobramycin.

according to different sources (Fazakerley et al., 2010; Rubin and Chirino-Trejo, 2010; Walther et al., 2012; Sahin-Tóth et al., 2021). Our results further support these numbers: we found a 5.9% carriage rate in dogs. However, in some African and Asian countries, high *S. aureus* nasal carriage is reported in healthy dogs, such as in Nigeria (36.9%), Indonesia (48.0%), Bangladesh (25.0%) and India (35.0%) (Mustapha et al., 2016; Sekhar et al., 2017; Rahman et al., 2018; Decline et al., 2020). The wide variation in nasal *S. aureus* carriage in dogs across the continents could be influenced by the local epidemiology of the *S. aureus*, differences in methodologies, dogs' hygiene, environmental sanitation, antibiotic use in animals, and/or the health status of owners (Collignon and Voss, 2015; Fletcher, 2015; Valiakos et al., 2020).

As it was observed in this study, human *S. aureus* carriers were much more prevalent than dog carriers and indicating that transmission between humans and dogs occurs less frequently when compared to possible intrahousehold human-to-human transmission. Taking into consideration the molecular typing results, the genotypic profiles of *S. aureus* between our studied dog and human isolates were not similar, but those originating from humans in the same households were closely related in several cases, strongly suggesting human-to-human transmission. Moreover, there was a significant association between the household densities and nasal carriage of *S. pseudintermedius* in households with > than 1 dog and >than 1 human, pointing out for the possibility that a relatively higher household population is related to a higher detection rate of *S. pseudintermedius*.

The diverse pattern of the CCs of the MSSA isolates from dogs and in-contact humans corroborated the findings from a previous study in Germany and Spain (Gómez-Sanz et al., 2013c; Holtfreter et al., 2016). Here, a few human *S. aureus* isolates were *scn*-negative which indicates animal-adapted (perhaps dog-adapted) CCs of *S. aureus*. Nevertheless, dogs were carriers of MSSA-*scn*-positive isolates (CC8-IEC-type G and CC15-IEC-type C) suggesting an anthroponotic route of transmission. This is consistent with findings from a previous longitudinal study on *S. aureus* colonization dynamics in humans and dogs in Spain (Gómez-Sanz et al., 2013b). When compared to the current study, this suggests that humans are the major reservoir of *S. aureus* contributing to transmission to dogs. However, it is important to mention that zoonotic transmission from dogs to humans is also possible (Abdullahi et al., 2022).

Concerning *S. pseudintermedius*, 2.4 and 32.4% nasal carriage rates were detected in our studied humans and dogs, respectively. The *S. pseudintermedius* carriage rate among dogs in this study was higher than previously reported for dogs from Spain (22.7%) (Gómez-Sanz et al., 2013c), but lower than those reported in Canada (46%), Germany

TABLE 4 Genetic lineages, virulence and AMR genes of CoPS isolates among households with both dog and human carriers.

Household ID N°/population	*S. aureus*						
	Isolate ID code/ host number	AMR	AMR genes	Virulence genes	IEC type	Plasmid replicons	*spa*-types/ST/ CC
11/2 humans and 2 dogs	X6019/human 26	PEN	*blaZ*	*aur, cap8A-J, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp, seg, sei, sem, sen, seo, seu*	Negative	rep5, rep16, rep19	t1070/ST30/CC30
	X6040/human 27	PEN	*blaZ*	*aur, cap8A-J, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp, seg, sei, sem, sen, seo, seu*	Negative	rep5, rep16, rep19	t1070/ST30/CC30
	X6036/dog 29	PEN-CIP	*blaZ, grlA* (S80F)	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp*	D	rep7, rep20	t121/ST8/CC8
10/2 humans and 2 dogs	X6061/human 23	Susceptible	NT	*aur, splA, splB, splE, hlgA, hlgB, hlgC, lukD-PV, lukE-PV*	E	None	t267/ST97/CC97
	X6065/Dog 24	PEN	*blaZ*	*aur, cap8A-P, clfA, clfB, coa, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp*	C	rep5, rep16	t2013/ST15/CC15
21/2 humans and 1 dogs	X6352/human 57	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6′-aph2″*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	rep13	t1451/ST398/CC398
	X6355/human 57	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6′-aph2″*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	rep13	t571/ST398/CC398
	X6353/human 58	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6′-aph2″*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	rep13	t1451/ST398/CC398
	X6358/human 58	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6′-aph2″*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	rep13	t571/ST398/CC398
Household ID N°/population	*S. pseudintermedius*						
	Isolate number/ host	AMR	AMR genes	Virulence genes	IEC type	Plasmid replicons	ST
10/2 humans and 2 dogs	X6050/dog 25	Susceptible	NT	*lukS/F-I, siet, sient, clpP, hlgB*	Negative	rep7	ST1115
	X6059/human 23	Susceptible	NT	*lukS/F-I, siet, sient, clpP, hlgB*	Negative	rep7	ST1115

CIP, ciprofloxacin; ERY-CLI^{ind}, erythromycin-clindamycin inducible; GEN, gentamicin; PEN, penicillin; TOB, tobramycin; NT, Not tested.

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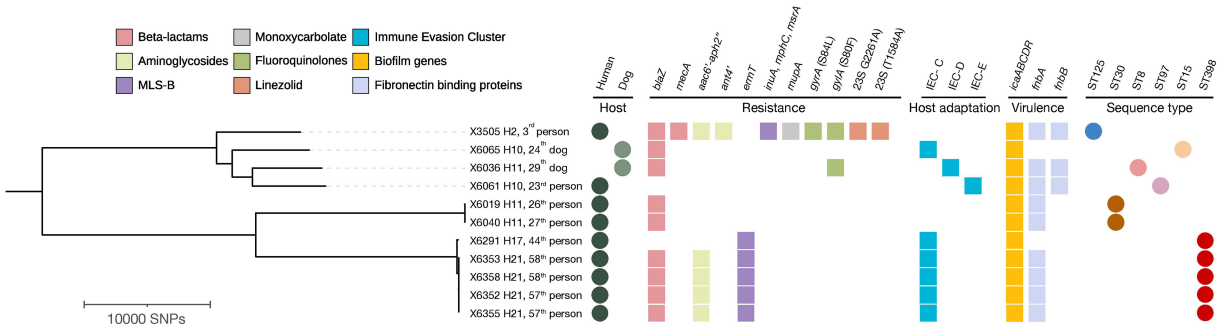

FIGURE 3
Phylogenetic tree based on core genome SNP analysis of 11 MRSA, MSSA-CC398, and other MSSA isolates from households with ≥2 humans and/or dogs' carriers. The presence of antimicrobial resistance genes, immune evasion cluster (IEC), *icaABCDR* operon, fibronectin binding proteins, host type and sequence types (ST) are indicated. The filled circles indicate hosts and sequence types while the filled and squares indicate the confirmed antimicrobial resistance, host adaptation markers and virulence determinants extracted from MLST 2.0, ResFinder, PointFinder and VirulenceFinder. Visuals were obtained using iTOL v6.6. MLS-B, Macrolide-Lincosamide-Streptogramin-B. H, Household.

(37.5%), Australia (85.4%), Korea (65.9%), or Hungary (34.3%) (Hanselman et al., 2009; Bean and Wigmore, 2016; Han et al., 2016; Sahin-Tóth et al., 2021; Cuny et al., 2022). This suggests that the transfer between nasal *S. pseudintermedius* among healthy dogs could depend on the number of dogs in the household. This corroborated the previous reports of diverse MSSP lineages from Germany and France (Haenni et al., 2020; Cuny et al., 2022). Moreover, we did find nasal carriage of *S. pseudintermedius* among a person living with dogs in one of the investigated households. However, this is lower than previously reported in Korea, Spain, and Canada (Hanselman et al., 2009; Gómez-Sanz et al., 2013c; Han et al., 2016). It appears that humans are not natural hosts for *S. pseudintermedius*, but adaptation to humans could occur. A recent large study has shown diversity between the *S. pseudintermedius* isolates of human and dog infections with similar pathogenicity islands and virulence gene-containing prophages (Phumthanakorn et al., 2021). The household in our study with both human and dog *S. pseudintermedius* carriers strongly suggested intrahousehold transmission, as the isolates had no SNP (zero) difference and were confirmed as clones by our genomic analyses. To our knowledge, this lineage (ST1115) has not been reported so far for MSSP from dogs.

In our study, no MRSP was detected among healthy dogs and similar results were obtained in an study in Sweden (Börjesson et al., 2015). However, other studies refer low rates of MRSP nasal carriage in dogs, as 0.9% in Germany, 4.5% in Canada and 2.6% in Norway (Hanselman et al., 2009; Kjellman et al., 2015; Cuny et al., 2022). Moreover, a pooled 4.6% MRSP was reported among healthy dogs in Spain (Gómez-Sanz et al., 2011); of the nine MRSP nasal carriers, one was from a household dog, while the remaining eight were from stray dogs (Gómez-Sanz et al., 2011). The absence of MRSP in our study (healthy animals and household members) and the previously low MRSP in healthy dog studies are remarkably different to the high prevalence in dogs receiving treatment in veterinary clinics in France (16.9%) (Haenni et al., 2014). MRSP seems to be associated with animal-hospital-lineages (Ruiz-Ripa et al., 2021b), whereas isolates that are susceptible or have low AMR levels may represent natural colonizers of dogs. However, among our 19 isolates, three were found to harbour *dfrK*, a gene that is rarely detected in *S. pseudintermedius*, which could be linked to the mobile genetic element *Tn*559 (Ruzauskas et al., 2016; Ruiz-Ripa et al., 2021b). Further characterization of our isolate employing long-read sequencing could help to elucidate this possibility.

The MSSA-CC398 was a predominant lineage in our study, although detected only in humans. Evolutionarily, there are two clades of the CC398 based on the acquisition of SCC*mec* mobile element (carrying *mecA*), *Tn*916 (carrying *tet*(M)), or prophage φsa3 (carrying IEC), *viz:* (a) MRSA-CC398, often considered the predominant LA-MRSA clade (mostly IEC-negative) in Europe, (b) livestock-independent (human-adapted) clade of the MSSA-CC398 (often IEC-positive) (Price et al., 2012; Matuszewska et al., 2022). However, on very rare occasions, MRSA-CC398 carrying the φsa3 (*scn*-positive) and MSSA-CC398-*scn*-negative have been reported among certain *spa* types within the CC398 lineage (Price et al., 2012). Hence, these phenomena make it difficult to categorically classify the CC398 lineages into animal-adapted or human-adapted clades. However, it has been established that the CC398 lineage originated in MSSA from humans, acquired the tetracycline (*tet*(M)) and methicillin (*mecA*) genes and spread to livestock (Price et al., 2012). Thus, the absence of *tet*(M) in all the *S. aureus* and the detection of MSSA-CC398 clade could be inferred as humans-adapted strains. In many cases, MSSA-CC398 clade is associated with the predominant *spa* type t571 and the macrolide resistance gene *ermT* (Mama et al., 2021b). Worryingly, this MSSA-CC398 human clade has been recently considered an emergent lineage in invasive human infections in Spain and other countries (Laumay et al., 2021; Mama et al., 2021b). Concerning MSSA-CC398 in dog-owning households, a previous study by Gómez-Sanz et al. (2013c) also reported MSSA-CC398 of the *spa* type t571. An important similarity between the previous study and the current is that here, MSSA-CC398 isolates were only detected in humans and all were IEC type C of the *spa* type t571 and t1451. Recently, increased detection of penicillin susceptibility phenotype has been detected among invasive MSSA human isolates that have been causing clinical infections (Mama et al., 2021a). This phenotype has been frequently found among CC398 isolates (Mama et al., 2021a), however, most of our isolates are phenotypically resistant to penicillin. In another study by Gómez-Sanz et al. (2013a), about 7.1% of 98 kennel dogs also carried MSSA-CC398-*scn*-negative isolates but of different *spa* types (t034, t5883 and t108), and all were pan-susceptible. Our MSSA CC398 isolates were resistant at least to one antibiotic and worryingly 50% of the isolates were MDR. A major difference between the MSSA-CC398 isolates reported by Gómez-Sanz et al. (2013a) and ours was that here, human isolates were *scn*-positive (IEC type C). Recently in France, the MSSA-CC398 lineage (t571, t1451 and t18379) was also reported in 14.6

and 27.3% of dogs and cats, respectively (Tegegne et al., 2022). Worthy mentioning is the detection of MSSA-CC398-t571-*scn*-negative in a cat (Tegegne et al., 2022). The reason for this variation is not fully understood, however, it could be attributed to the *spa* type associated with the MSSA CC398 isolates or due to the carriage status of the Sa3 prophage (Gómez et al., 2020). The loss of Sa3int prophages in the *scn*-negative isolates is a major determinant for the human-to-animal transmission of MSSA CC398 (Price et al., 2012; Matuszewska et al., 2022). The findings of Gómez-Sanz et al. (2013a,c) and our study suggest the persistence of MSSA CC398 in humans and dogs.

Another finding of special epidemiological relevance is the dual MRSA/MSSA carriage detected in our study as both MSSA-CC30 and MRSA-CC5 were identified in a human household member. In a previous Spanish study, simultaneous carriage of both MRSA and MSSA of the CC398 lineage was reported in a farm worker with occupational exposure (Gómez et al., 2020). In another study among healthcare students in Portugal, concurrent detection of MRSA and MSSA in a single person was also reported (Coelho et al., 2021).

Though all the MRSA isolates had an MDR phenotype, more than 20% of the MSSA and MSSP isolates were also MDR. Generally, the AMR rate was moderate, but the most common AMR in *S. aureus* isolates were to penicillin, aminoglycosides, and erythromycin-clindamycin. Conversely, similarly to previous studies in *S. pseudintermedius*, the predominant AMR phenotype was to sulfamethoxazole-trimethoprim, erythromycin, and tetracycline (Rynhoud et al., 2021). Novel mutations (G2261A & T1584A) in the domain V region of the 23SrDNA of one MRSA isolate was observed, and although the predicted *in silico* resistance did not reveal a currently known AMR phenotype attributable to this mutation, the strain was phenotypically linezolid resistant. The inability to detect the linezolid phenotype from the genome database could be due to that this mutation has not been fully characterized (not previously reported and deposited in the genome database), as opposed to the most frequently detected 23S rDNA point mutation in staphylococci (G2576T) (Gostev et al., 2021; Ruiz-Ripa et al., 2021a). However, recently, novel point mutations in 23S rRNA associated with linezolid resistance in staphylococci have been reported in *S. epidermidis* in Austria (Huber et al., 2021) and in *S. capitis* in China (Han et al., 2022). It could be those novel mutations in domain V of 23S rRNA are silently emerging and mandates the need for close surveillance.

Regarding *S. aureus* virulence factors, all were negative for TSST-1, PVL, ETA and ETB encoding genes. All except one of the nasal *S. pseudintermedius* isolates of dogs and human origins carried the *lukS/F-I, siet,* and *expA* genes. These leucocidins and exfoliatins are responsible for host-specific clinical infections in dogs (Gharsa et al., 2013; Gómez-Sanz et al., 2013c).

Contrary to the findings of Penna et al. (2013), which reported *S. schleiferi* subsp. *schleiferi* carriage among ~32% of healthy dogs in Brazil, only 2 dogs from our study had *S. schleiferi* nasal carriage (5.4%). This finding is similar to the *S. coagulans*-positive dogs (4.9%) from the study of Lee et al. (2019) in Korea, but relatively higher than the 1.0% previously reported in Spain (Gómez-Sanz et al., 2013c). Since its first identification in humans in 1988 (Freney et al., 1988), several *S. coagulans* infections have been reported in humans and pets (May et al., 2005; Abraham et al., 2007; Yarbrough et al., 2017). Furthermore, the recent emergence of *S. coagulans* among healthy and pyodermic dogs has been a relevant global health issue in veterinary medicine due to its high AMR and diverse virulence factors (May et al., 2012; Abraham et al., 2007; Lee et al., 2019). Contrary to this assertion, all *S. coagulans* identified in our study were susceptible to antibiotics tested, likely because the isolates were cultured from healthy dogs.

It is worth mentioning a limitation of this study. As this was a one-point prospective study on the potential transmission of CoPS between dogs and dog-owner, randomly selected households were used. The sample size was relatively small and this could affect the detection of rare AMR phenotypes and virulence genes of CoPS in healthy individuals such as MRSP, *eta* and *tst*.

Conclusion

The nasal carriage of *S. aureus* and *S. pseudintermedius* in healthy dogs' households were moderate. Low rates of MRSA and *S. coagulans*, and no MRSP carriage were detected. Human-to-human MSSA and dog-to-human MSSP transmissions were identified in this study. *S. pseudintermedius* isolates had a homogeneous profile of virulence determinants. The detection of MSSA CC398, an emergent clade that has been implicated in invasive human infections is a relevant health concern and suggests the need for its continuous surveillance of humans, different species of animals and their shared environment from the One Health perspective.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human and animal participants were reviewed and approved by Ethic Committe of the University of La Rioja, Spain. The patients/participants provided their written informed consent to participate in this study.

Author contributions

IA and CT: conceptualization, methodology, and writing — original draft preparation. IA: laboratory experiments and software analysis. CT, IA, MZ, and CL: validation. IA, CT, MS, and AS: formal analysis and data curation. CT, IA, MS, AS, MZ, and CL: writing — review and editing. CT, CL, and MS: supervision. CT: project administration. CT, MZ, and IA: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1121564/full#supplementary-material

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First report of coexistence of bla_{KPC-2}-, bla_{NDM-1}- and *mcr-9*-carrying plasmids in a clinical carbapenem-resistant *Enterobacter hormaechei* isolate

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Introduction: Colistin is regarded as one of the last-resort antibiotics against severe infections caused by carbapenem-resistant Enterobacteriaceae. Strains with cooccurrence of *mcr-9* and carbapenemase genes are of particular concern. This study aimed to investigate the genetic characteristics of a bla_{KPC-2}-carrying plasmid, bla_{NDM-1}-carrying plasmid and *mcr-9*-carrying plasmid coexisting in a carbapenem-resistant *Enterobacter hormaechei* isolate.

Methods: *E. hormaechei* strain E1532 was subjected to whole-genome sequencing, and the complete nucleotide sequences of three resistance plasmids identified in the strain were compared with related plasmid sequences. The resistance phenotypes mediated by these plasmids were analyzed by plasmid transfer, carbapenemase activity and antimicrobial susceptibility testing.

Results: Whole-genome sequencing revealed that strain E1532 carries three different resistance plasmids, pE1532-KPC, pE1532-NDM and pE1532-MCR. pE1532-KPC harboring bla_{KPC-2} and pE1532-NDM harboring bla_{NDM-1} are highly identical to the IncR plasmid pHN84KPC and IncX3 plasmid pNDM-HN380, respectively. The *mcr-9*-carrying plasmid pE1532-MCR possesses a backbone highly similar to that of the IncHI2 plasmids R478 and p505108-MDR, though their accessory modules differ. These three coexisting plasmids carry a large number of resistance genes and contribute to high resistance to almost all antibiotics tested, except for amikacin, trimethoprim/sulfamethoxazole, tigecycline and polymyxin B. Most of the plasmid-mediated resistance genes are located in or flanked by various mobile genetic elements, facilitating horizontal transfer of antibiotic resistance genes.

Discussion: This is the first report of a single *E. hormaechei* isolate with coexistence of three resistance plasmids carrying *mcr-9* and the two most common carbapenemase genes, bla_{KPC-2} and bla_{NDM-1}. The prevalence and genetic features of these coexisting plasmids should be monitored to facilitate the establishment of effective strategies to control their further spread.

KEYWORDS

Enterobacter hormaechei*, multidrug resistance, plasmid, carbapenemase genes, *mcr-9

1. Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) bacteria pose a serious threat to global public health owing to rapid emergence of multidrug resistance (MDR) and limited therapeutic agents available (Ma et al., 2023). Production of carbapenemases, especially

KPC and NDM, is the main mechanism of carbapenem resistance in CRE clinical isolates (Peng et al., 2022). The bla_{KPC} gene is typically located on plasmids of different incompatibility (Inc) groups, such as IncF-, IncI-, IncA/C-, IncX-, and IncR-type plasmids (Chen et al., 2014), and the bla_{NDM} gene is mainly carried by IncX3-type plasmids (Zhang et al., 2017; Yoon et al., 2018). These plasmids, which are often easily transferable, can facilitate the spread of bla_{KPC} and bla_{NDM} resistance genes by horizontal gene transfer among different bacterial populations, complicating clinical therapy and infection control.

Colistin, a cationic polypeptide, is regarded as an antibiotic of last resort for treatment of severe infections caused by CRE (Poirel et al., 2017; Yang et al., 2020). However, the discovery of plasmid-mediated mobile colistin resistance (*mcr*) genes has triggered extensive concern due to the possibility of horizontal transfer of colistin resistance. The *mcr-1* gene was the first *mcr* variant initially reported in China in 2015 in *Escherichia coli* and *Klebsiella pneumoniae* isolates (Liu et al., 2016). To date, various types of *mcr* genes (*mcr-2* to *mcr-10*) have been identified worldwide in Enterobacteriaceae (Ling et al., 2020; Zhang et al., 2022). *mcr-9* is closely related to *mcr-3,* with 65% amino acid identity and 99.5% nucleotide identity (Kieffer et al., 2019). It was first identified in a colistin-susceptible *Salmonella enterica serotype typhimurium* clinical isolate in the United States in 2019 (Carroll et al., 2019) and now has disseminated to various Enterobacteriaceae species, with global distribution in 21 countries across six continents (Li et al., 2020). Surprisingly, reports on coexistence of *mcr-9* and carbapenemase genes (such as bla_{NDM}, bla_{VIM}, bla_{KPC}, bla_{IMP} and bla_{OXA-48}) in Enterobacteriaceae have been increasing worldwide (Chavda et al., 2019; Yuan et al., 2019; Kananizadeh et al., 2020; Liu et al., 2021; Simoni et al., 2021; Yao et al., 2022). These genes may be present in different gene cassettes on a single plasmid or different plasmids from one isolated strain. In this study, we describe cooccurrence of three different MDR plasmids, pE1532-KPC, pE1532-NDM and pE1532-MCR, which carry the bla_{KPC-2}, bla_{NDM-1}, and *mcr-9* genes, respectively, in a single carbapenem-resistant *Enterobacter hormaechei* clinical isolate. To the best of our knowledge, this is the first report of a clinical *E. hormaechei* isolate coharboring *mcr-9* and the two most common carbapenemase genes, bla_{KPC-2} and bla_{NDM-1}.

2. Materials and methods

2.1. Bacterial isolation and identification

Enterobacter hormaechei E1532 was isolated from hydrothorax and ascites samples of a patient in a teaching hospital in Henan, China, in 2015. Bacterial species were identified using the VITEK 2 compact system (bioMérieux, France) as well as *16S rRNA* sequencing. The presence of carbapenemase genes (Chen et al., 2015) and *mcr* genes (*mcr-1* to *mcr-10*) (Rebelo et al., 2018; Borowiak et al., 2020; Kim et al., 2021) was screened by PCR amplification using primers described previously, and the positive products were sequenced using an ABI Sequencer (Life Technologies, CA, United States). The genotype of strain E1532 was analyzed using the multilocus sequence typing (MLST) method to amplify and sequence the seven housekeeping genes (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB* and *rpoB*) (Miyoshi-Akiyama et al., 2013), and sequence type (ST) was defined on the basis of seven allele numbers available on the MLST website.[1]

2.2. Whole-genome sequencing and sequence assembly

Total genomic DNA of *E. hormaechei* E1532 was extracted from cell pellets using a bacterial DNA kit (OMEGA, USA), and the purified DNA was subjected to whole-genome sequencing by a combination of PacBio RS (Pacifc Biosciences, CA, USA) and Illumina NovaSeq (Illumina, CA, USA) sequencing platforms. Paired-end DNA libraries were constructed with an average insert size of 400 bp (ranging from 300 to 500 bp) for Illumina sequencing, and shotgun DNA libraries were generated with a 15 kb insert size (ranging from 10 kb to 20 kb) for PacBio Biosciences sequencing. Clean reads were obtained after filtering the low-quality sequence data and then *de novo* assembled by Unicycler software (Wick et al., 2017). The Illumina-generated short reads were utilized to correct the PacBio-generated long reads using the Pilon tool (Walker et al., 2014).

2.3. Genome annotation and bioinformatics analysis

Prediction and annotation of coding genes and pseudogenes was carried out using the RAST 2.0 algorithm (Brettin et al., 2015). Putative open reading frames (ORFs) were further assessed for functions by BLASTP and BLASTN (Boratyn et al., 2013) against the NCBI RefSeq (O'Leary et al., 2016) and UniProtKB/Swiss-Prot (Boutet et al., 2016) databases. The precise species assignment was further confirmed based on whole-genome sequencing using average nucleotide identity (ANI) analysis with the JSpeciesWS server. PlasmidFinder (Carattoli et al., 2014) was used to examine plasmid replicon type. The presence of antibiotic resistance genes, insertion sequences (ISs), transposons and integrons was analyzed *in silico* using the ResFinder (Bortolaia et al., 2020), CARD (Jia et al., 2017), ISfinder (Siguier et al., 2006), Tn Number Registry (Roberts et al., 2008) and INTEGRALL (Moura et al., 2009) databases. BLASTN and MUSCLE 3.8.31 (Edgar, 2004) were employed for alignment and comparison of the plasmid sequences analyzed in this study with highly homologous plasmid sequences publicly available in NCBI. The circular graph of plasmid sequences and linear comparative graph were constructed by Inkscape 0.48.1 software.[2]

2.4. Plasmid transfer

The filter mating method was used for the plasmid conjugation assay (Ouyang et al., 2018). Briefly, rifampicin-resistant *E. coli* EC600 was used as the recipient. Equal amounts of donor and

1 http://pubmlst.org/ecloacae/
2 https://inkscape.org/en/

recipient strains were mixed together, and mating was performed on the filter membranes of brain heart infusion (BHI) agar plates for 12–18 h at 37°C. The mixtures were spread on doubly selective agar plates containing rifampin together with indicated additional antibiotics for selecting an *E. coli* transconjugant carrying one of the following resistance markers: imipenem for bla_{KPC} (pE1532-KPC) and bla_{NDM} (pE1532-NDM), and azithromycin for *mph*(A) (pE1532-MCR). The presence of the resistance markers carried by transconjugants was confirmed by PCR amplification and sequencing.

For the electrotransformation experiment, streptomycin- and tetracycline-resistant *E. coli* TOP10 was selected as the recipient. Plasmid DNA was extracted using QIAGEN Plasmid Midi Kit (Qiagen, Germany) and transformed into TOP10 competent cells by electroporation. After reviving the bacterial cells for 1 h at 37°C and 200 rpm, positive electroporants carrying the bla_{KPC} or bla_{NDM} or *mph*(A) gene were selected on super optimal broth (SOB) agar plates containing imipenem or azithromycin, and further verified by PCR and sequence analysis.

2.5. Carbapenemase activity assay

The class A/B/D carbapenemase activity of the E1532 strain as well as its transconjugant and electroporant was assessed by the CarbaNP test as described in our previous study (Feng et al., 2022). Overnight bacterial cultures were seeded into MH broth supplemented with 4 μg/ml imipenem and incubated with continuous shaking until the bacterial density reached 1.0–1.4. Cell pellets were collected by centrifugation and subjected to washing with Tris–HCl twice. The sonication process was performed to lyse the bacterial cells. The supernatant from the cell lysis solution was added to substrates I–V at a ratio of 1:1 and then allowed to interact at 37°C for 1–2 h. The phenotypic results of carbapenemase activity were observed by color changes of the mixture.

2.6. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using VITEK 2 System and AST-N334 cards for the following antimicrobial agents: amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefuroxime axetil, cefoxitin, ceftazidime, ceftriaxone, cefoperazone/sulbactam, cefepime, ertapenem, imipenem, amikacin, levofloxacin, tigecycline and trimethoprim/sulfamethoxazole. For polymyxin B, the susceptibility test was performed with the broth microdilution method. The resistance results for tigecycline and colistin were judged according to the breakpoints of European Committee on Antimicrobial Susceptibility Testing (EUCAST)[3]; the breakpoints of other antibiotics were defined following the standard of the Clinical and Laboratory Standards Institute (CLSI). The *E. coli* standard strain ATCC 25922 served as the quality control for susceptibility testing.

3 http://www.eucast.org/

2.7. Nucleotide sequence accession numbers

The complete sequences of chromosomes and plasmids have been submitted to the GenBank database under accession numbers CP114571 (chromosome), CP114573 (pE1532-KPC), CP114574 (pE1532-MCR), CP114575 (pE1532-NDM), and CP114572 (pE1532-4).

3. Results and discussion

3.1. *Enterobacter hormaechei* E1532 coharboring bla_{KPC-2}, bla_{NDM-1} and *mcr-9* genes

E. hormaechei E1532 was found to be resistant to all penicillins, cephalosporins, carbapenems, and fluoroquinolones and showed intermediate resistance to tigecycline; it remained sensitive to amikacin, polymyxin B and trimethoprim/sulfamethoxazole (Table 1). PCR screening and sequencing identification showed that *E. hormaechei* E1532 harbors two carbapenemase genes, bla_{KPC-2} and bla_{NDM-1}, and a newly identified colistin resistance gene, *mcr-9*. MLST analysis revealed that strain E1532 belongs to ST93 (allelic profile: 9–4–14-61-37-4-9), which is a globally disseminated high-risk clone that is frequently reported in China (Peirano et al., 2018; Zhao et al., 2020; Chen et al., 2021).

Whole-genome sequencing revealed that strain E1532 has a single circular chromosome sequence of 4,869,794 bp with an average G + C content of 55.31% and contains a total of 4,717 predicted ORFs. A total of four plasmids, namely, pE1532-KPC, pE1532-NDM, pE1532-MCR and pE1532-4, were present in strain E1532, with circular closed DNA sequences of 39,461 bp, 53,769 bp, 308,217 bp, and 69,180 bp in length with 44, 62, 360, and 90 predicted ORFs, respectively (Supplementary Figure S1; Table 2). Each plasmid consists of backbone regions (responsible for plasmid maintenance, replication and/or conjugal transfer) and one or more accessory modules (acquired DNA regions associated with and bordered by mobile elements) inserted at different sites of the backbone regions (Supplementary Figure S1; Table 2). Antibiotic resistance genes were identified using ResFinder and CARD analysis. The chromosome of E1532 carries three intrinsic resistance genes involved in resistance to β-lactams (bla_{ACT-7}), aminoglycoside [*aph(3′)-Ia*] and fosfomycin (*fosA*). Plasmids pE1532-KPC, pE1532-NDM and pE1532-MCR harbor a total of 19 genes conferring resistance to β-lactams (bla_{KPC-2}, bla_{NDM-1}, bla_{SHV-12}, bla_{DHA-1}, bla_{TEM-1}), aminoglycosides [*aacC2, aphA1, aph(6)-Id, aph(3″)-Ib*], tetracyclines [*tet*(A)], bleomycin (bla_{MBL}), macrolide [*mph*(A)], fluoroquinolones (*qnrB4*), sulfonamide (*sul1*), tunicamycin (*tmrB*), colistin (*mcr-9*), quaternary ammonium (*qacED1*), tellurium (the *ter* locus), and mercuric (the *mer* locus) (Table 3). However, no resistance gene was found on plasmid pE1532-4, which only harbors a Tn*3*-family transposon remnant.

Plasmid pE1532-NDM was successfully transferred to *E. coli* by electrotransformation and conjugation experiments, obtaining the corresponding electroporant pE1532-NDM-TOP10 and transconjugant pE1532-NDM-EC600 (Table 1). In order to investigate the transmissible possibility of conjugative plasmid pE1532-NDM in different hosts, the conserved backbone sequences

TABLE 1 Antimicrobial susceptibility profiles of *Enterobacter hormaechei* E1532 and its transconjugants and electroporants.

Category	Antibiotic	MIC(μg/ml)/antimicrobial susceptibility					
		E1532	pE1532-KPC-TOP10	pE1532-NDM-EC600	pE1532-NDM-TOP10	TOP10	EC600
Penicillins	Amoxicillin/clavulanic acid	≥32/R	≥32/R	≥32/R	≥32/R	4/S	≤4/S
	Piperacillin/tazobactam	≥128/R	≥128/R	≥128/R	≥128/R	≤4/S	≤4/S
Cephalosporins	Cefuroxime	≥64/R	≥64/R	≥64/R	≥64/R	8/S	16I
	Cefuroxime axetil	≥64/R	≥64/R	≥64/R	≥64/R	4/S	16I
	Cefoxitin	≥64/R	≥64/R	≥64/R	≥64/R	≤4/S	≤4/S
	Ceftazidime	≥64/R	32/R	≥64/R	≥64/R	≤1/S	≤1/S
	Ceftriaxone	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S	≤1/S
	Cefoperazone/sulbactam	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S	≤1/S
	Cefepime	≥32/R	16/R	16/R	16/R	≤1/S	≤1/S
Carbapenems	Ertapenem	≥8/R	≥8/R	≥8/R	≥8/R	≤1/S	≤1/S
	Imipenem	≥16/R	8/R	≥16/R	8/R	≤1/S	≤1/S
Fluoroquinolones	Levofloxacin	≥8/R	≤0.25/S	0.5/S	≤0.25/S	≤0.25/S	0.5/S
Aminoglycosides	Amikacin	≤2/S	≤2/S	≤2/S	≤2/S	≤2/S	≤2/S
Glycylcycline	Tigecycline	4/I	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S	≤0.5/S
Sulfanilamides	Trimethoprim/sulfamethoxazole	40/S	≤20/S	≤20/S	≤20/S	≤20/S	≤20/S
Lipopeptide	Polymyxin B	0.25/S	0.25/S	0.25/S	0.25/S	0.25/S	0.25/S

MIC = minimum inhibitory concentration; S = sensitive; R = resistant; I = intermediate.

TABLE 2 Genomic features of the four plasmids carried by strain E1532.

Feature	pE1532-KPC	pE1532-NDM	pE1532-MCR	pE1532-4
Incompatibility group	IncR	IncX3	IncHI2	IncFII
Total length (bp)	39,461	53,769	308,217	69,180
Total number of ORFs	44	62	360	90
Mean G + C content, %	55.67	49.08	47.48	52.97
Length of the backbone (bp)	12,034	34,724	230,863	69,180
Accessory modules	The bla_{KPC-2} region	The bla_{NDM-1} region and IS*Kox3*	The MDR-1 region, the MDR-2 region, IS*Cfr9*-IS*Cfr15*, IS*1B*-IS*Kpn26*, the IS*Kpn21*:ΔTn*6363* region, three separate copies of IS*903B*, IS*Kpn26*, IS*5*, Tn*6362*, Tn*2* and ΔIS*903B*	The Tn3-family transposon remnant

of pE1532-NDM were aligned by BLASTN with the bla_{NDM-1}-harboring IncX3 plasmids available in GenBank. Among the top 100 plasmids with the highest backbone sequence similarity of pE1532-NDM, the plasmids were selected based on the origin of bacterial species or hosts different from pE1532-NDM. A total of 11 plasmids were included and they were mainly isolated from bacterial strains from human. In addition, there were also four plasmids from shrimp, chicken, hospital sewage and unknown source, respectively (Supplementary Table S1). The results of our analysis showed that IncX3 plasmids have an extensive host range. However, only the electroporant pE1532-KPC-TOP10, and not the transconjugant, was obtained by transferring pE1532-KPC into *E. coli*, which may be because pE1532-KPC lacks the conjugal transfer genes in backbone regions (Supplementary Figure S1) and is not self-transmissible (Compain et al., 2014; Jing et al., 2019). Repeated attempts failed to transfer pE1532-MCR into *E. coli* through conjugation and electroporation; this may be attributed to the following facts: (1) pE1532-MCR is a 308 kb megaplasmid which limits the success of conjugation and electroporation. (2) the insertion event occurred within the two conjugal transfer regions

TABLE 3 Drug resistance genes in plasmids analyzed.

Plasmid	Resistance gene	Resistance phenotype	Nucleotide position	Accessory or backbone region located
pE1532-KPC	bla_{KPC-2}	β-lactam resistance	25,914-26,795	The bla_{KPC-2} region
	tet(A)	Tetracycline resistance	15,880-17,079	
pE1532-NDM	bla_{NDM-1}	β-lactam resistance	17,826-18,638	The bla_{NDM-1} region
	bla_{SHV-12}	β-lactam resistance	9,324-10,183	
	bla_{MBL}	Bleomycin resistance	17,457-17,822	
pE1532-MCR	The *ter* locus	Tellurium resistance	64,703-84,651	Backbone region
	aacC2	Aminoglycoside resistance	156,517-157,377	The MDR-1 region
	bla_{DHA-1}	β-lactam resistance	137,136-138,275	
	bla_{SHV-12}	β-lactam resistance	145,679-146,539	
	bla_{TEM-1}	β-lactam resistance	150,685-151,545	
	mph(A)	Macrolide resistance	158,436-159,341	
	qacED1	Quaternary ammonium resistance	139,376-139,858	
	qnrB4	Fluoroquinolone resistance	132,368-133,015	
	sul1	Sulfonamide resistance	139,852-140,691	
	tmrB	Tunicamycin resistance	155,962-156,504	
	strA	Aminoglycoside resistance	263,392-264,228	The MDR-2 region
	strB	Aminoglycoside resistance	264,228-265,031	
	mcr-9	Colistin resistance	252,943-254,562	
	aphA1	Aminoglycoside resistance	224,978-225,793	The IS*Kpn21*:ΔTn*6363* region
	bla_{TEM-1}	β-lactam resistance	115,557-116,417	Tn*2*
	The *mer* locus	Mercuric resistance	105,015-108,991	Tn*6362*

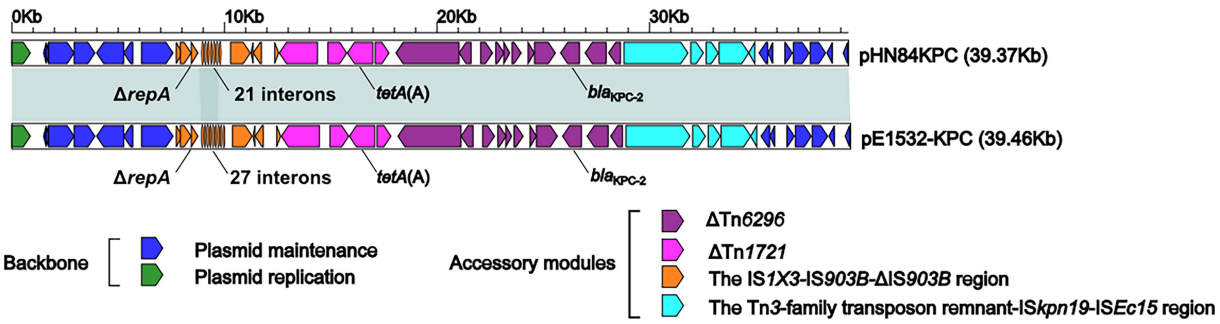

FIGURE 1
Linear comparison of plasmid pE1532-KPC with pHN84KPC (accession number KY296104). Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading denotes shared regions of homology (>95% nucleotide identity).

tra1 and *tra2* might render this plasmid nonconjugative (Supplementary Figure S1). In a carbapenemase activity assay, strain E1532 showed class A + B activity, the pE1532-NDM-harboring electroporant and transconjugant class B activity, and the pE1532-KPC-harboring electroporant A activity (data not shown). Phenotypic susceptibility testing showed these electroporants and transconjugant to be resistant to all β-lactams tested, including carbapenems (Table 1), which was consistent with the presence of carbapenemase genes in these strains.

3.2. Comparative genomics of bla_{KPC-2}-carrying plasmid pE1532-KPC

The entire sequence of pE1532-KPC is highly similar to that of the reference IncR plasmid pHN84KPC (accession number KY296104), with 100% query coverage and > 99% nucleotide identity (Figure 1). pE1532-KPC and pHN84KPC share the most complete IncR backbone gene loci encoding plasmid replication initiation (*repB*) and plasmid maintenance (*parAB*, *umuCD*, *retA*, *vagCD*, *resD*). A single

accessory module, the bla_{KPC-2} region containing two resistance genes, bla_{KPC-2} and *tet*(A), is inserted at a site between *retA* and *vagD* in the backbone of these two plasmids (Supplementary Figure S1). The bla_{KPC-2} region contains the *tet*(A)-carrying ΔTn*1721* cassette (Allmeier et al., 1992) and the bla_{KPC-2}-carrying ΔTn*6296* cassette (Jiang et al., 2010) flanked by the upstream IS*1X3*-IS*903B*-ΔIS*903B* region and the downstream Tn*3*-family transposon remnant-IS*Kpn19*-ΔIS*Ec15* region (Figure 1). The only modular difference between these two plasmids is with regard to the copy number (21 in pHN84KPC, 27 in pE1532-KPC) of the 37-bp tandem repeat within interons, which is located behind the truncated replication initiation gene *ΔrepA* in the bla_{KPC-2} region.

3.3. Comparative genomics of bla_{NDM-1}-carrying plasmid pE1532-NDM

pE1532-NDM displays >99% nucleotide identity (with 100% query coverage) with the reference IncX3 plasmid pNDM-HN380 (Ho et al., 2012) obtained from *K. pneumoniae* isolate CRE380 in China (Figure 2A). pE1532-NDM and pNDM-HN380 have identical backbones that share a set of core genes for plasmid replication (*repB* and *bis*), partition (*parA*), maintenance (*topB* and *stpA*) and conjugal transfer (*pilX* genes) (Supplementary Figure S1). Each of them harbors two accessory modules, IS*Kox3* and the bla_{NDM-1} region, which are inserted at different sites of the plasmid backbone. All resistance genes are located in the bla_{NDM-1} region. This accessory resistance region (18 kb in length) originated sequentially as a truncated IS*26*-bla_{SHV-12}-IS*26* unit, bla_{NDM-1}-carrying ΔTn*125*, IS*3000* and ΔTn*3* (Figure 2B). The truncated IS*26*-bla_{SHV-12}-IS*26* unit was generated by deletion of *yjbJ* (partial)-*yjbK*-*yjbL*-*yjbM* genes and inversion of IS*26* at the 3′ region from the prototype composite transposon-like IS*26*-bla_{SHV-12}-IS*26* unit (Ford and Avison, 2004). ΔTn*125* is a derivative of IS*Aba125*-flanked composite transposon Tn*125* (Poirel et al., 2012), lacking the IS*Aba125* element at the 3′ region and having an interrupted and truncated IS*Aba125* at the 5′ region by insertion of an IS*5* element.

At least four major module differences were identified between the bla_{NDM-1} regions of pE1532-NDM and pNDM-HN380 (Figure 2B). First, the truncated IS*26*-bla_{SHV-12}-IS*26* unit in pE1532-NDM is inverted compared to pNDM-HN380. Second, the bla_{SHV-12} gene cannot form an ORF due to the absence of 1 bp, making it a pseudogene. Third, a 261-bp deletion at the 5′-terminal region of ΔIS*Aba125* was found in pE1532-NDM. Fourth, direct repeats (DRs) have been lost at both ends of the IS*5* element in pE1532-NDM.

3.4. Comparative genomics of the *mcr-9*-carrying plasmid pE1532-MCR

The pE1532-MCR backbone is closely related to the first prototype IncHI2 plasmid R478 (Gilmour et al., 2004) from *Serratia marcescens*, with 95% query coverage and > 99% nucleotide identity, and to another IncHI2 plasmid, p505108-MDR (Shi et al., 2018) from *Cronobacter sakazakii*, with 98% query coverage and > 99% nucleotide identity (Supplementary Figure S2). These three plasmids share the core IncHI2 backbone, including the regions responsible for plasmid replication (*repHIA*, *repHI2*), partition (*parAB*, *parMR*) and conjugal transfer (*tra1* and *tra2* regions) (Supplementary Figure S1).

Linear comparison revealed that the following different regions are present among the backbones of pE1532-MCR, R478 and p505108-MDR (Supplementary Figure S2A). (1) An IS*903B* element in pE1532-MCR but a ΔIS*903D* element in p505108-MDR is inserted between *parR* and *htdA* relative to R478, leading to the interruption of the conjugal transfer region *tra2*. (2) An IS*Kpn26* element is inserted between the backbone genes *orf2034* and *orf222* in pE1532-MCR. (3) An IS*5* element is inserted between *orf1161* and *hha* in pE1532-MCR. (4) Two backbone regions, *hipB-hipA-orf207-orf411* and *orf189-orf426-orf258,* are inserted between *orf1389* and *orf609* in pE1532-MCR and p505108-MDR. (5) *orf159* is interrupted by a Tn*2* element in pE1532-MCR and p505108-MDR. (6) The *klaB* to *orf534* region is truncated by insertion of the MDR-2 region in pE1532-MCR and p505108-MDR relative to R478, leaving only the remnant of the *klaB* and *orf534* genes. (7) An IS*903B* element in pE1532-MCR but a ΔIS*903D* element in p505108-MDR is inserted between *orf2385* and *orf450*, leading to the truncation of both genes. (8) A 377-bp insertion at the 3′-end of *orf444* resulted in the replacement of *orf444* with *orf588* in pE1532-MCR and p505108-MDR. (9) The MDR-1 and MDR-2 regions are inserted into the boundary of *ΔklaB* and *orf819,* respectively, with inversion of the entire backbone region between *ΔklaB* and *orf819* in pE1532-MCR.

To more clearly and intuitively observe the genetic features of the backbone region between *ΔklaB* and *orf819* in these three plasmids, the *ΔklaB* to *orf819* region in pE1532-MCR was reverted compared with that of R478 and p505108-MDR (Supplementary Figure S2B). The genetic differences among them were as follows: (1) An IS*150* element is located between the two backbone genes *orf198* and *ldrB* in R478 but replaced by both the IS*Cfr9*-IS*Cfr15* region and the *orf612-fieF-relB-relE* backbone region in pE1532-MCR and p505108-MDR. (2) IS*186B* is lost between *ldrB* and *orf321* in pE1532-MCR and p505108-MDR relative to R478. (3) Tn*10* is inserted into *orf300* in R478. (4) The *traI* gene in the conjugal transfer region *tra1* is interrupted by a truncated IS*903B* in pE1532-MCR. (5) The IS*1B*-IS*Kpn26* region and IS*Kpn21*-ΔTn*6363* region are inserted into the *mucA* to *orf1404* backbone region in pE1532-MCR, resulting in deletion of backbone genes between *orf318-1* and *mucA* as well as truncation of *mucA* relative to R478; acquisition of the *aphA1a* region in p505108-MDR causes loss of the *orf318-2* to *retA* backbone region as well as truncation of *mucA*. (6) *orf258* is interrupted by an IS*903B* element in pE1532-MCR.

The differences described above involve not only backbone regions but also accessory modules. pE1532-MCR carries a total of 13 accessory modules, and resistance genes are located in the MDR-1 region (Figure 3), the MDR-2 region (Figure 4), the IS*Kpn21*:ΔTn*6363* region (Figure 5), Tn*6362* (Supplementary Figure S3) and Tn*2* (Supplementary Figure S3).

3.5. The MDR-1 region of pE1532-MCR

The MDR-1 region (Figure 3) harbors five antibiotic resistance loci: *qnrB4*-bla_{DHA-1} region (Yim et al., 2013), integron remnant carrying *ΔqacED1* and *sul1*, a truncated IS*26*-bla_{SHV-12}-IS*26* unit (Ford and Avison, 2004), *aacC2-tmrB* region (Partridge et al., 2012), and a truncated IS*26*-*mph*(A)-IS*6100* unit (Liang et al., 2017).

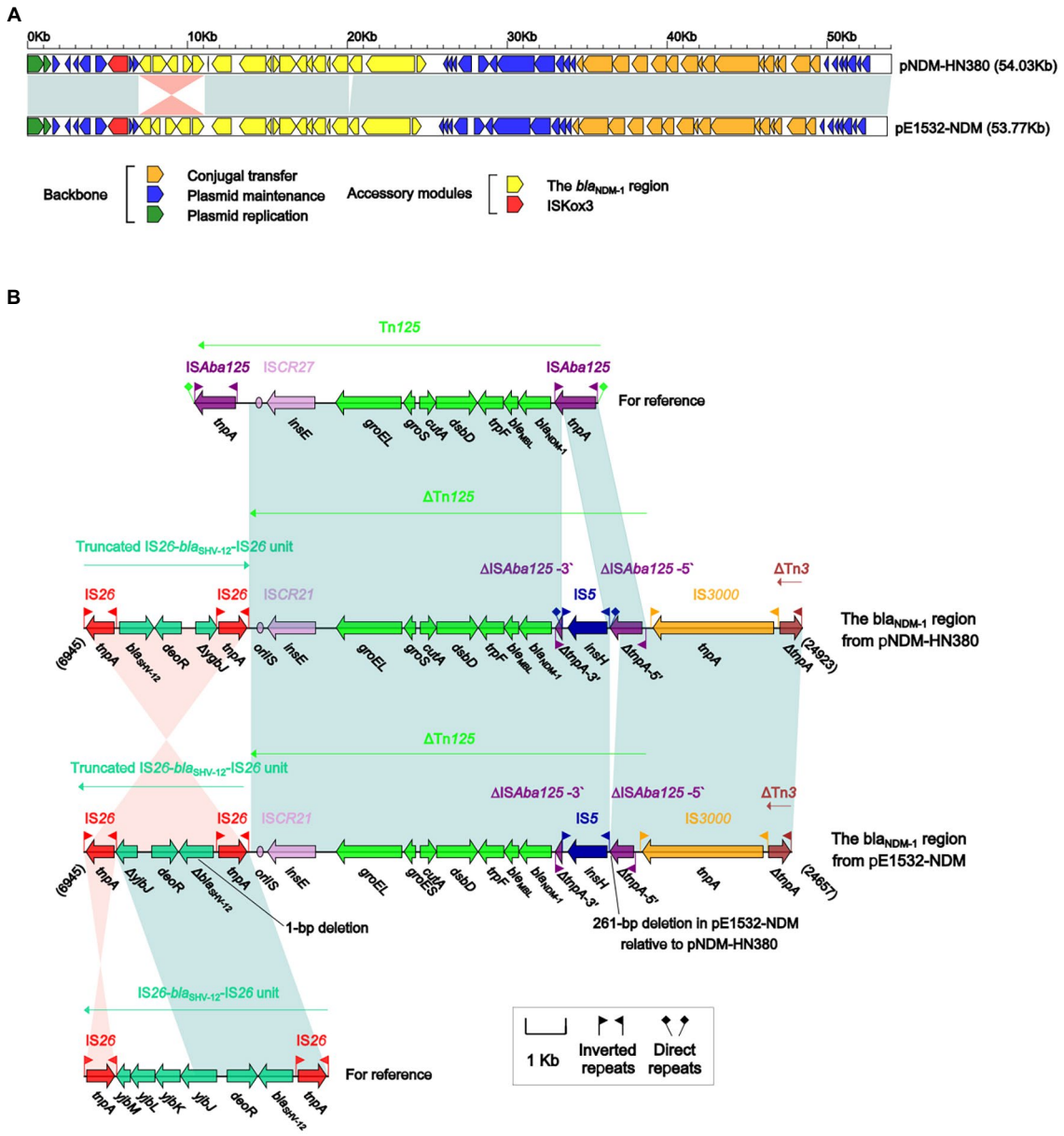

FIGURE 2
Comparison of plasmid pE1532-NDM with pNDM-HN380. **(A)** Linear comparison of the two sequenced plasmids pE1532-NDM and pNDM-HN380 (accession number JX104760). **(B)** The *bla*$_{NDM-1}$ region from pE1532-NDM and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on functional classification. Shading denotes shared regions of homology (≥90% nucleotide identity). Numbers in brackets indicate nucleotide positions within the corresponding plasmids. The accession numbers of the Tn*125* and IS*26*-*bla*$_{SHV-12}$-IS*26* unit for reference are JN872328 and CP003684, respectively.

IS*26*-*bla*$_{SHV-12}$-IS*26* is an IS*26*-flanked extended-spectrum β-lactamase (ESBL) resistance unit lacking direct repeats (DRs) at both ends. A number of IS*26*-based resistance units have been reported with diverse resistance genes (Partridge, 2011). IS*26* can mediate movement of antibiotic resistance genes and formation of composite transposons, which contribute to MDR region assembly (Harmer and Hall, 2016; Harmer et al., 2020). The IS*26*-*bla*$_{SHV-12}$-IS*26* unit in the MDR-1 region has undergone truncation of the upstream IS*26* element and deletion of its 14-bp IRR (inverted repeat right).

Tn*1722* belongs to a Tn*3*-family unit transposon harbored in the tetracycline resistance transposon Tn*1721* (Allmeier et al., 1992) and includes *tnpA*, *tnpR*, *res* and *mcp* genes bounded by 38-bp IRL (inverted repeat left) and IRR. The ΔTn*1722* element in the MDR-1 region is a derivative of Tn*1722* with deletion of *mcp* and IRR, which is also found in pCRE3-KPC (Dong et al., 2020) and plasmid unnamed3 (accession number CP027150). The IS*26*-*mph*(A)-IS*6100* unit contains a macrolide resistance region *mph*(A)-*mrx*-*mphR*(A) (Noguchi et al., 2000) bracketed by IS*26* and IS*6100* elements. These two IS elements, which have almost identical 14-bp IRs, belong to the IS*6* family, and the homologous recombination mediated by them promotes integration of this resistance unit into the MDR region, similar to the IS*26*-bound resistance unit.

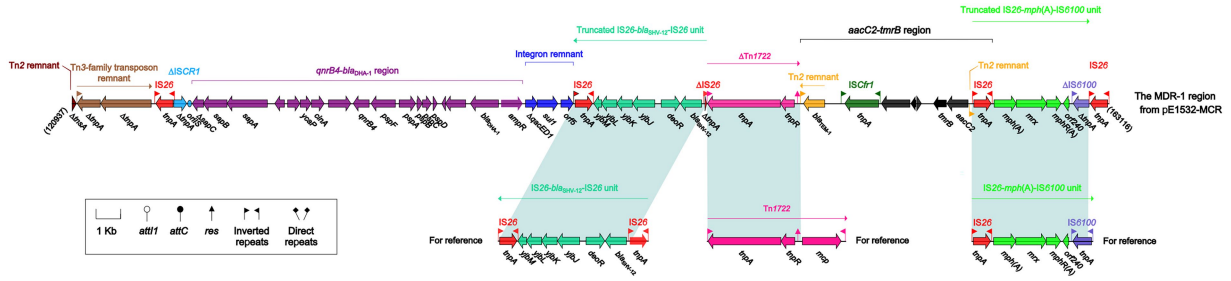

FIGURE 3
The MDR-1 region from pE1532-MCR and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on functional classification. Shading denotes shared regions of homology (≥90% nucleotide identity). Numbers in brackets indicate nucleotide positions within the corresponding plasmids. The accession numbers of the IS*26*-*bla*$_{SHV-12}$-IS*26* unit, IS*26*-*mph*(A)-IS*6100* unit and Tn*1722* for reference are CP003684, KY270852 and X61367, respectively.

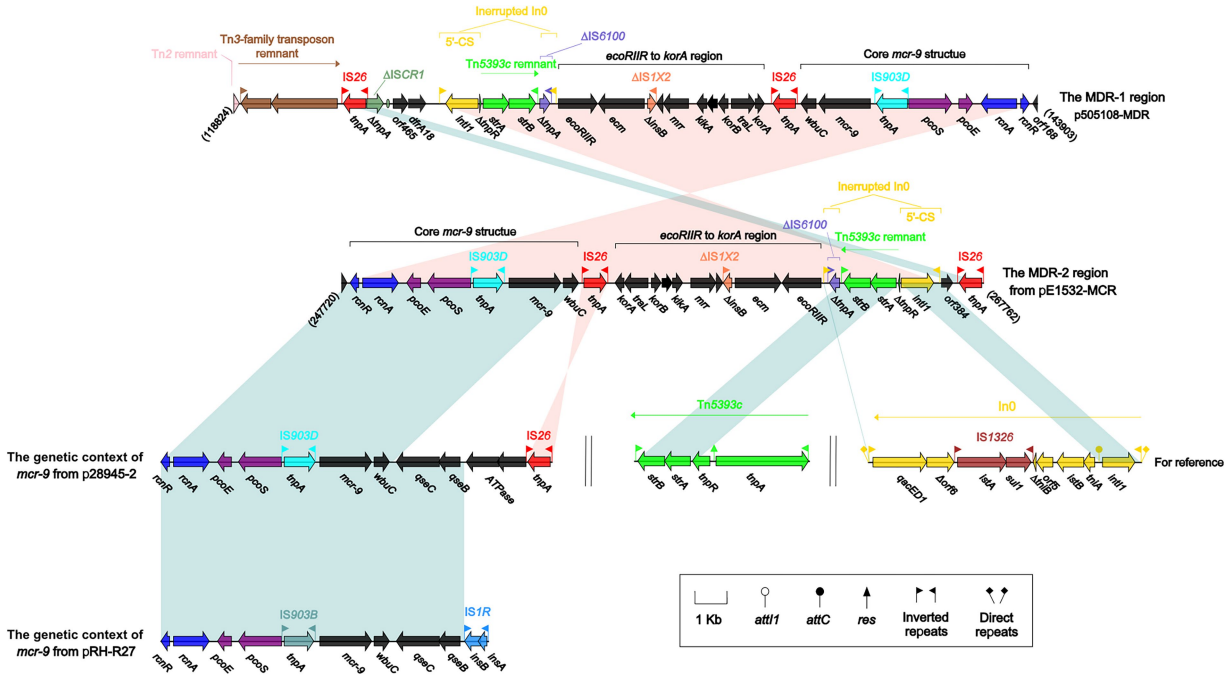

FIGURE 4
The MDR-2 region from pE1532-MCR and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on functional classification. Shading denotes shared regions of homology (≥90% nucleotide identity). Numbers in brackets indicate nucleotide positions within the corresponding plasmids. The accession numbers of Tn*5393c* and In0 for reference are AF262622 and U49101, respectively.

3.6. The MDR-2 region of pE1532-MCR

The MDR-2 region of pE1532-MCR (Figure 4) is highly similar to the MDR-1 region of p505108-MDR (97% query coverage and > 99% nucleotide identity), with a reverse array of the *orf168* to *intI1* region and with the same orientated IS*26* downstream of interrupted In0. Both MDR regions harbor a *mcr-9* gene, and the genetic environments of *mcr-9* are identical to each other. *Mcr-9* is flanked by IS*903D* and IS*26* elements, both belonging to the IS*6* family, which play a vital role in dissemination of resistance genes. The genes upstream of *mcr-9* include *rcnR, rcnA, pcoE* and *pcoS*. However, only the *wbuC* gene is located downstream of *mcr-9*, and a two-component system encoding the genes *qseB* and *qseC*, which are involved in *mcr-9* expression (Kieffer et al., 2019), was not found. The lack of *qseB-qseC* regulatory genes in plasmid pE1532-MCR may explain the phenomenon that strain E1532 carrying *mcr-9* is sensitive to polymyxin B. The gene composition arrayed as *rcnR-rcnA-pcoE-pcoS*-IS*903-mcr-9-wbuC* is the core structure of *mcr-9* resistance cassettes in the *mcr-9*-carrying IncHI2 plasmids from different Enterobacteriaceae (Li et al., 2020). The different encoding genes and IS elements located downstream of *mcr-9* are the main causes leading to the diverse genetic context of *mcr-9* in IncHI2 plasmids.

In addition to *mcr-9*, other resistance genes (*strA*, *strB*) are present in the MDR-2 region. The class 1 integron In0 (accession number U49101) from *Pseudomonas aeruginosa* plasmid pVS1 is an ancestor of more complex integrons with a weak PcW promoter and an unoccupied integration site *attI* in 5′-CS but no gene cassette array

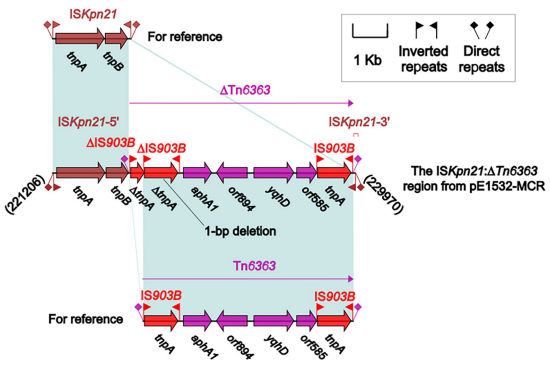

FIGURE 5
The IS*Kpn21*:ΔTn*6363* region from pE1532-MCR and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on functional classification. Shading denotes shared regions of homology (≥90% nucleotide identity). Numbers in brackets indicate nucleotide positions within the corresponding plasmids. The accession numbers of IS*Kpn21* and Tn*6363* for reference are AP012055 and KY978630, respectively.

(Bissonnette and Roy, 1992). ΔIn0 from the MDR region of pE1532-MCR/p505108-MDR contains the 5′-CS composed of the integrase gene *intI1* and promoter gene and is bordered by IRi (inverted repeat at the integrase end) and IRt (inverted repeat at the *tni* end), which resulted from insertion of ΔIS*6100* and Tn*5393c* remnant (Tn*3*-family transposon) carrying the aminoglycoside resistance genes *strA and strB* (L'Abee-Lund and Sorum, 2000).

3.7. The IS*Kpn21*:ΔTn*6363* region of pE1532-MCR

The IS*Kpn21*:ΔTn*6363* region (Figure 5) is composed of IS*Kpn21* and ΔTn*6363* and has undergone two different transposition events: (1) The ISNCY family element IS*Kpn21* with an IRL-*tnpA*-*tnpB*-IRR structure is integrated at a site between the two backbone genes *orf318-2* and *orf1404* in pE1532-MCR and bordered by 5-bp DRs. (2) The transposon ΔTn*6363* is inserted at a site between *tnpB* and the IRR of IS*Kpn21*, splitting IS*Kpn21* into two parts and leaving 9-bp DRs at both ends of ΔTn*6363*. Tn*6363* (accession number KY978628) is an IS*903B*-flanked composite transposon that possesses an IS*903B*-*aphA1*-*orf894*-*yqhD*-*orf585*-IS*903B* structure (Shi et al., 2018). Insertion of an additional element ΔIS*903B* resulted in a 1-bp deletion of IS*903B* upstream of Tn*6363*, thus generating ΔTn*6363* with three IS*903B* elements. ΔTn*6363* is a derivative of Tn*6363* which carries aminoglycoside resistance gene *aphA1*, the embedding of ΔTn*6363* into IS*Kpn21* mediated by IS*903B* facilitates the horizontal transfer and transmission of antibiotic resistance gene in bacterial populations.

4. Conclusion

This is the first report of coexistence of the bla_{KPC-2}-carrying IncR plasmid, bla_{NDM-1}-carrying IncX3 plasmid and *mcr-9*-carrying IncHI2 plasmid recovered from the ST93 multidrug-resistant *E. hormaechei* clinical isolate E1532. These three coexisting MDR plasmids carry a large number of resistance genes, rendering the E1532 isolate resistant to almost all antibiotics tested, including carbapenems. The *mcr-9* gene, which is involved in resistance to the last-resort antibiotic, should be given sufficient attention because it has become widely disseminated worldwide among various species of Enterobacteriaceae. Therefore, epidemiological analysis should be performed to monitor the spread of *mcr-9*-positive strains. Moreover, most of these plasmid-mediated resistance genes are located in or flanked by various mobile genetic elements, such as transposons, insertion sequences and integrons, which facilitate acquisition and horizontal transfer of antibiotic resistance genes across bacterial populations. Not all plasmids can transfer themselves, but non-conjugative plasmids can be mobilized with the help of other conjugative plasmids present in the same donor cell. Therefore, there is the possibility of cotransfer of bla_{KPC-2}-, bla_{NDM-1}-, and *mcr-9*-carrying plasmids. The main limitation in this study is that we did not apply the conjugation assay to evaluate the cotransfer of these three plasmids, which needs to be confirmed by further study. Anyway, the cotransfer and coexistence of *mcr-9* and carbapenemase genes in *E. hormaechei* isolates limit the choice of antibiotics, which will arise a huge risk to clinical treatment and global public health. Further surveillance is necessary to achieve better insight into the prevalence and dissemination mechanism of these coexisting bla_{KPC-2}-, bla_{NDM-1}-, and *mcr-9*-harboring plasmids among clinical isolates.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Genbank- [CP114571 (chromosome), CP114573 (pE1532-KPC), CP114574 (pE1532-MCR), CP114575 (pE1532-NDM), and CP114572 (pE1532-4)].

Ethics statement

The bacterial isolate involved in this study was part of the routine hospital laboratory procedure, and the clinical information of the patient was not involved in this study, so ethical approval and informed consent were not required.

Author contributions

WF and FS conceived and designed the study. LRX and SL carried out the phenotypic characterization. QY and WF carried out the genomic analysis and bioinformatics analysis. WF, QY and FS wrote and revised the manuscript. PX and LLX did the funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1153366/full#supplementary-material

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Genetic characterization of ESBL-producing and ciprofloxacin-resistant *Escherichia coli* from Belgian broilers and pigs

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Background: The increasing number of infections caused by *Escherichia coli* resistant to clinically important antibiotics is a global concern for human and animal health. High overall levels of extended-spectrum beta-lactamase (ESBL)-producing and ciprofloxacin-resistant (ciproR) *Escherichia coli* in livestock are reported in Belgium. This cross-sectional study aimed to genotypically characterize and trace ESBL-and ciproR-*E. coli* of Belgian food-producing animals.

Methods: A total of 798 fecal samples were collected in a stratified-random sampling design from Belgian broilers and sows. Consequently, 77 ESBL-*E. coli* and 84 ciproR-*E. coli* were sequenced using Illumina MiSeq. Minimum inhibitory concentration (MIC) for fluoroquinolones and cephalosporins were determined. Molecular *in silico* typing, resistance and virulence gene determination, and plasmid identification was performed. Scaffolds harboring ESBL or plasmid-mediated quinolone resistance (PMQR) genes were analyzed to detect mobile genetic elements (MGEs) and plasmid origins. Core genome allelic distances were used to determine genetic relationships among isolates.

Results: A variety of *E. coli* sequence types (ST) (n=63), resistance genes and virulence profiles was detected. ST10 was the most frequently encountered ST (8.1%, n=13). The pandemic multidrug-resistant clone ST131 was not detected. Most farms harbored more than one ESBL type, with $bla_{\text{CTX-M-1}}$ (41.6% of ESBL-*E. coli*) being the most prevalent and $bla_{\text{CTX M-15}}$ (n=3) being the least prevalent. PMQR genes (15.5%, n=13) played a limited role in the occurrence of ciproR-*E. coli*. More importantly, sequential acquisition of mutations in quinolone resistance-determining regions (QRDR) of *gyrA* and *parC* led to increasing MICs for fluoroquinolones. GyrA S83L, D87N and ParC S80I mutations were strongly associated with high-level fluoroquinolone resistance. Genetically related isolates identified within the farms or among different farms highlight transmission of resistant *E. coli* or the presence of a common reservoir. IncI1-I(alpha) replicon

type plasmids carried different ESBL genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}32}$ and $bla_{TEM\text{-}52C}$). In addition, the detection of plasmid replicons with associated insertion sequence (IS) elements and ESBL/PMQR genes in different farms and among several STs (e.g., IncI1-I(alpha)/IncX3) underline that plasmid transmission could be another important contributor to transmission of resistance in these farms.

Conclusion: Our findings reveal a multifaceted narrative of transmission pathways. These findings could be relevant in understanding and battling the problem of antibiotic resistance in farms.

KEYWORDS

ESBL, ciprofloxacin, *Escherichia coli*, livestock, WGS

1. Introduction

Escherichia coli remains one of the most important pathogens for humans (Murray et al., 2022), as evidenced by its contribution to mortalities due to drug resistance. Fluoroquinolones and beta-lactam antibiotics are life savers in both human (World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance, 2018) and animal healthcare (World Organization for Animal Health (OIE), 2018): these medications are essential for treating severe illnesses. Resistance to extended-spectrum cephalosporins and fluoroquinolones constitutes a major public health problem because this limits the treatment options for serious bacterial infections (World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance, 2018) and drives the use of the last resort of antibiotic therapy, i.e., carbapenems. The gastrointestinal tract of animals serves as a reservoir of antimicrobial resistance (AMR), which can spread *via* MGEs (Moor et al., 2021). The presence of resistance genes on MGEs enables their dispersion, posing a great hazard to food safety (Partridge et al., 2018). Clinically significant ESBL genes, belonging to the $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} gene families, can successfully disseminate because they are commonly located on plasmids (IncA/C, IncF, IncHI1, IncHI2 IncI, IncK, IncN, IncX plasmids) (Rozwandowicz et al., 2018). In addition, three mechanisms of plasmid-mediated quinolone resistance (PMQR) are known: protection of DNA gyrase and topoisomerase IV from quinolone inhibition by *qnr* genes (ColE plasmids) (Tran et al., 2005), acetylation of quinolones by aminoglycoside acetyltransferase Aac(6′)-Ib-cr (Robicsek et al., 2006) and quinolone accumulation due to quinolone efflux pumps QepAB (Yamane et al., 2007) and OqxAB (ColE plasmids, IncX plasmids) (Hansen et al., 2007; Jacoby et al., 2015; Rozwandowicz et al., 2018). These mechanisms provide low-level resistance (ciprofloxacin MIC range: 0.06–0.25 mg/l); however, they are usually present on multidrug-resistant (MDR) plasmids and facilitate the selection of higher-level resistance making infections with PMQR-carrying pathogens harder to treat (Jacoby et al., 2015). Quinolone resistance in Gram-negative bacteria can also be caused by single amino acid changes in QRDRs in DNA gyrase (*gyrA*) and DNA topoisomerase IV (*parC*) (Karczmarczyk et al., 2011; Gordon and George, 2015). Another mechanism contributing to (fluoro)quinolone resistance is the increased expression of the AcrAB-TolC efflux pump which is regulated by repressor AcrR and other regulators of drug efflux MarAR and SoxRS as well as RNA polymerase RpoB (Amábile-cuevas and Demple, 1991; White et al., 1997; Oethinger et al., 1998; Lindgren et al., 2003; Pietsch et al., 2017) and the AcrB component of the efflux pump itself (White et al., 1997; Blair et al., 2015).

A previous study indicated a high occurrence of ESBL-producing and ciprofloxacin-resistant *E. coli* in fecal samples of broilers and pigs in Belgian farms (De Koster et al., 2021). Possible explanations for these observations include the dissemination of resistant *E. coli* vertically along the production chain from one generation to another (Dierikx et al., 2013; Zurfluh et al., 2014) and resistant *E. coli* residing in the farm environment (Blaak et al., 2015) along with the dissemination of resistant *E. coli* or their resistance genes between farm animals (Hayer et al., 2020). However, the research into the genetic diversity and antibiotic resistance of *E. coli* that colonize livestock in Belgian farms has been limited. Most studies of commensal *E. coli* in livestock, such as the Belgian Antimicrobial Consumption and Resistance in Animals (AMCRA) reports (FAVV-AFSCA, 2021), the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) reports (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021) rely on phenotypic AMR profiles. The lack of whole genome sequencing (WGS) to track MDR and high-risk clones was acknowledged in the latest BELMAP report, which aims to summarize monitoring programs in Belgium and recommends improving monitoring (FOD Volksgezondheid Veiligheid van de Voedselketen en Leefmilieu et al., 2021). An interdisciplinary One Health strategy is essential for tracking AMR's spread between humans, animals and their shared environment. Data on *E. coli* found in food-producing animals should be utilized to identify potential pathways of transmission through which the risk may reach human populations through consumption. To investigate the molecular epidemiology of ESBL-*E. coli* and ciproR-*E. coli,* we used WGS to identify resistance genes, mutations and potential transmission pathways between and among farms.

2. Materials and methods

2.1. Setting, study period and sample/isolate collection

Within the framework of the i-4-1-Health project, a total of 798 fecal samples were collected in a stratified-random sampling design

from conventional broiler ($n = 15$) and multiplier sow farms ($n = 15$) in Flanders, Belgium (September 2017–April 2018). When present, sampling was conducted in different units (broiler houses or rooms with weaned pigs) with a maximum of three units per farm. The farms were included based on the relative level of antibiotic use, meaning that antibiotic use was higher than average compared to the national benchmark value in the respective countries. Farm characteristics and antibiotic use were described previously (Caekebeke et al., 2020).

2.2. ESBL-producing and ciprofloxacin-resistant *Escherichia coli*

Isolation of ESBL- and ciproR-*E. coli* was performed as described by Kluytmans-van den Bergh et al. (2019). A total of 724 ESBL-*E. coli* and 467 ciproR-*E. coli* were isolated from the fecal samples. To investigate the molecular epidemiology, three ESBL-*E. coli* and three ciproR-*E. coli* from each farm were chosen for in-depth analysis including phenotypic characterization and whole genome sequencing. In particular, the first ESBL-*E. coli* and ciproR-*E. coli* isolated from each farm unit were selected.In farms with one sampled unit, three ESBL-*E. coli* and ciproR-*E. coli* with a distinct antibiotic susceptibility profile were selected from that unit. Using these selection criteria, 82 ESBL-*E. coli* [broiler ($n = 45$), pig ($n = 37$)] and 84 ciproR-*E. coli* [broiler ($n = 45$), pig ($n = 39$)] were selected for MIC determination and whole genome sequencing.

2.3. Whole genome sequencing

A single colony was inoculated in 4 ml Mueller Hinton broth and incubated overnight at 35–37°C. The MasterPure Complete DNA & RNA Purification kit (Epicentre, Madison, WI, USA) was used to extract genomic DNA. Libraries were prepared using the Nextera XT sample preparation kit (Illumina, San Diego, CA, USA) and sequenced with 2× 250 bp paired-end sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The sequencing data were submitted to NCBI under BioProject PRJNA905236. Supplementary Table 1 provides an overview of ESBL-*E. coli* and ciproR-*E. coli* sequences and their genetic characteristics used in this study.

2.4. *De novo* assembly, genotyping and phylogenetic analysis

Sequences were trimmed with TrimGalore v.0.4.4[1] and assembled *de novo* using SPAdes v.3.13.0 (Bankevich et al., 2012). Assembly quality was assessed with Quast (Gurevich et al., 2013). The assembled genome was annotated using Prokka v.1.12 (Seemann, 2014). Additional analysis was performed using BacPipe v1.2.6 (Xavier et al., 2020) including the PubMLST database (Achtman scheme) (Jolley et al., 2018), the CARD database (McArthur et al., 2013), ResFinder v4.1 (Bortolaia et al., 2020), VirulenceFinder v2.0.3 (Tetzschner and Lund, 2020) and PlasmidFinder v2.0 (Carattoli and Hasman, 2020). Serotype and pathotype were determined using BioNumerics v7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium). The identification of pathotypes was performed according to the virulence factor database (VFDB) (Chen et al., 2016). *In silico* prediction of fimH type and H and O serotypes was performed using FimTyper 1.0 (Roer et al., 2017) and SeroTypeFinder (Joensen et al., 2015), respectively. Phylogroups were determined using ClermonTyping (Beghain et al., 2018). For core genome multilocus sequence typing (cgMLST), a gene-by-gene approach was employed by generating a study-specific scheme and analyzing allelic loci distances of cgMLST using ChewBBACA (Silva et al., 2018) and visualizing the tree using iTOL v6 (Letunic and Bork, 2021).

2.5. Phenotypic and genotypic antibiotic resistance determination

ESBL production was phenotypically confirmed using the combination disk diffusion method. Ciprofloxacin resistance was confirmed by ciprofloxacin MIC determination using VITEK® MS system (bioMérieux, Marcy l'Etoile, France). In addition, MICs for amoxicillin-clavulanic acid, ampicillin, cefuroxim, cefotaxime, ceftazidime, cefoxitin, fosfomycin, gentamicin, imipenem, meropenem, nitrofurantoin, piperacillin-tazobactam,tobramycin, trimethoprim were determined using VITEK® MS system (bioMérieux, Marcy l'Etoile, France). Furthermore, ciprofloxacin, enrofloxacin, levofloxacin and moxifloxacin were tested for 106 *E. coli* of which 18 were ciprofloxacin-susceptible *E. coli* and 88 were ciprofloxacin non-susceptible *E. coli* using E-tests (bioMérieux, Marcy l'Etoile, France) to identify genome-wide associations between genetic markers and fluoroquinolone resistance levels. Results were interpreted using the EUCAST breakpoint tables v12.0 (The European Committee on Antimicrobial Susceptibility Testing, 2022) and an enrofloxacin breakpoint of MIC≤0.25 mg/l (Hao et al., 2013). After sequencing, known ESBL genes could not be detected in five phenotypic ESBL-*E. coli* (5/82, 6%) (from broiler farms one, four and eight and pig farms three and fifteen); therefore, these isolates were excluded, resulting in 77 ESBL-*E. coli* for further analysis. QRDRs were investigated for mutations conferring resistance within gyrase *gyrA* and *gyrB* and topoisomerases IV *parC* and *parE*. In addition, mutations in *acrB*, *acrR*, *marA*, *marR*, *rpoB*, *soxR*, *soxS* were considered. Mutations and predicted amino acid changes were aligned using clustalw, inbuilt within the CLC genomics workbench v.9.5.3 (CLC bio, Denmark). Prediction of whether amino acid changes affect protein function was performed by Sorting Intolerant From Tolerant (SIFT) (Ng and Henikoff, 2003). Scaffolds containing ESBL or PMQR genes were analyzed using MGEFinder v1.0.3 (Durrant et al., 2020), and ISFinder (Siguier et al., 2006) to detect MGEs and replicon types of plasmids. Scaffolds containing ESBL genes or PMQR represent plasmid sequences were analyzed further on NCBI using blastn search with default settings to the blast database v5. Resistance genes were classified as Rank I (human-associated, mobile ARGs, in ESKAPE pathogens, current threats) or Rank II (human-associated, mobile ARGs emerging from non-pathogens, future threats) (Zhang et al., 2021; Supplementary Table 2).

1 https://github.com/FelixKrueger/TrimGalore

2.6. Statistical tests and visualization

Statistical tests and visualization of the presence of resistance genes, virulence genes and plasmids were performed using R version 4.2.0 (R Core Team, 2020). Differences in the presence of genes were tested using a One-way ANOVA and TukeyHSD test in case of equal variances or a Welch ANOVA and the Games-Howell test in case of unequal variances (mean ± standard deviation and *p*-values are shown). Associations of genetic markers with a phenotype were examined using phi and chi-squared test.

3. Results

3.1. ESBL and PMQR genes in ESBL-producing and ciprofloxacin-resistant *Escherichia coli*

The most abundant ESBL genes detected in *E. coli* isolated from broilers were $bla_{\text{CTX-M-1}}$ (40.5%, $n = 17$) followed by $bla_{\text{SHV-12}}$ (31.0%, $n = 13$). Other ESBL genes detected in broiler isolates were $bla_{\text{CTX-M-32}}$ (2.4%), $bla_{\text{CTX-M-55}}$ (2.4%), $bla_{\text{SHV-2}}$ (2.4%), $bla_{\text{TEM-15}}$ (2.4%), $bla_{\text{TEM-52B}}$ (4.8%) and $bla_{\text{TEM-52C}}$ (7.1%). Three isolates (7.1%) from different broiler farms harbored $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$. $Bla_{\text{CTX-M-1}}$ was also the most common in *E. coli* from pigs (34.3%, $n = 12$), followed by $bla_{\text{CTX-M-32}}$ (22.9%), $bla_{\text{TEM-52C}}$ (11.4%), $bla_{\text{CTX-M-3}}$ (8.6%), $bla_{\text{CTX-M-14}}$ (8.6%), $bla_{\text{CTX-M-15}}$ (5.7%), $bla_{\text{SHV-2}}$ (5.7%), $bla_{\text{TEM-52B}}$ (2.9%) in pig isolates (Figure 1A). Eight of the ciproR-*E. coli* also harbored $bla_{\text{CTX-M-1}}$ ($n = 2$), $bla_{\text{CTX-M-32}}$ ($n = 2$), $bla_{\text{CTX-M-15}}$ ($n = 1$), $bla_{\text{SHV-12}}$ ($n = 2$) and one isolate with both $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$. PMQR genes were found in a relatively low number of ciproR-isolates (14.3%, $n = 12$) (Figure 1B). Of the 84 ciproR-*E. coli*, 12 isolates harbored *qnrS1* (8.9% of the broiler isolates and 15.4% of the pig isolates). Two pig isolates (5.1%) additionally contained the efflux pump OqxAB. A total of 9.5% of the ESBL-*E. coli* from broilers and 8.6% of the ESBL isolates from pigs harbored *qnrS1*. Also, *qnrB19* was detected in 5.7% of porcine ESBL-*E. coli*.

3.2. Other resistance genes, virulence genes and plasmids

In total, 95.8% of the isolates were MDR (i.e., resistant to at least 3 antibiotic classes (Magiorakos et al., 2012)). Genes conferring resistance to aminoglycosides were abundant (overall in 84.5% of the isolates), folate pathway antagonists were present in 90.1% of the isolates, and all isolates harbored multidrug transporter MdfA. Lincosamide resistance was often detected in broiler isolates (ciproR-*E. coli*: 73.3%, ESBL-*E. coli* 83.3%) and beta-lactam resistance was often detected in ciproR-*E. coli* (pig: 64.1%, broiler: 88.9%) (Figure 1C). Plasmid-mediated colistin resistance was found in three pig farms [*mcr-1.1* ($n = 1$), *mcr-2.1* ($n = 2$), *mcr-9* ($n = 1$)] and in one broiler farm [*mcr-9* ($n = 1$)]. Both *mcr-9*- containing isolates did not have the complete *qseC-qseB* two-component system to induce colistin resistance. Highly diverse resistance gene profiles (131 different profiles among 161 isolates) were detected within the same farm and between farms.

The mean number of resistance genes was significantly higher ($p < 0.05$) in ciproR-*E. coli* from pigs (9.44 ± 4.01) compared to

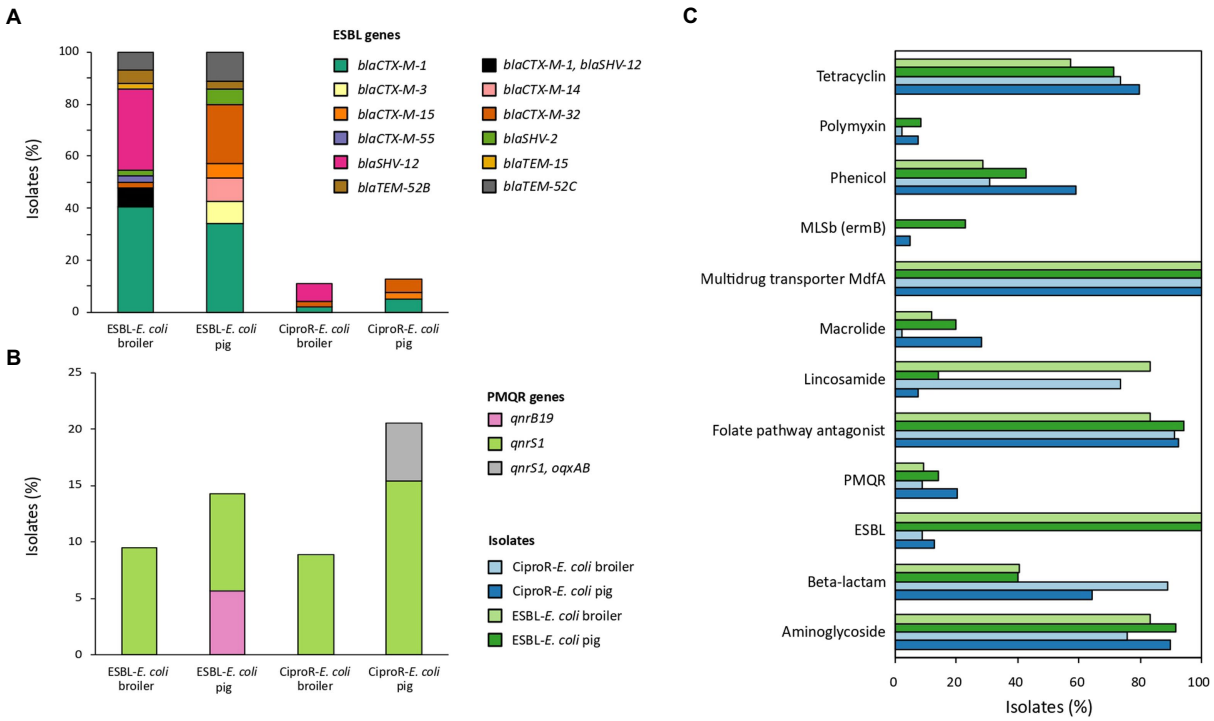

FIGURE 1
The percentage of isolates carrying ESBL genes **(A)**, plasmid-mediated quinolone resistance (PMQR) genes **(B)** and genes conferring resistance to other antibiotic classes **(C)**.

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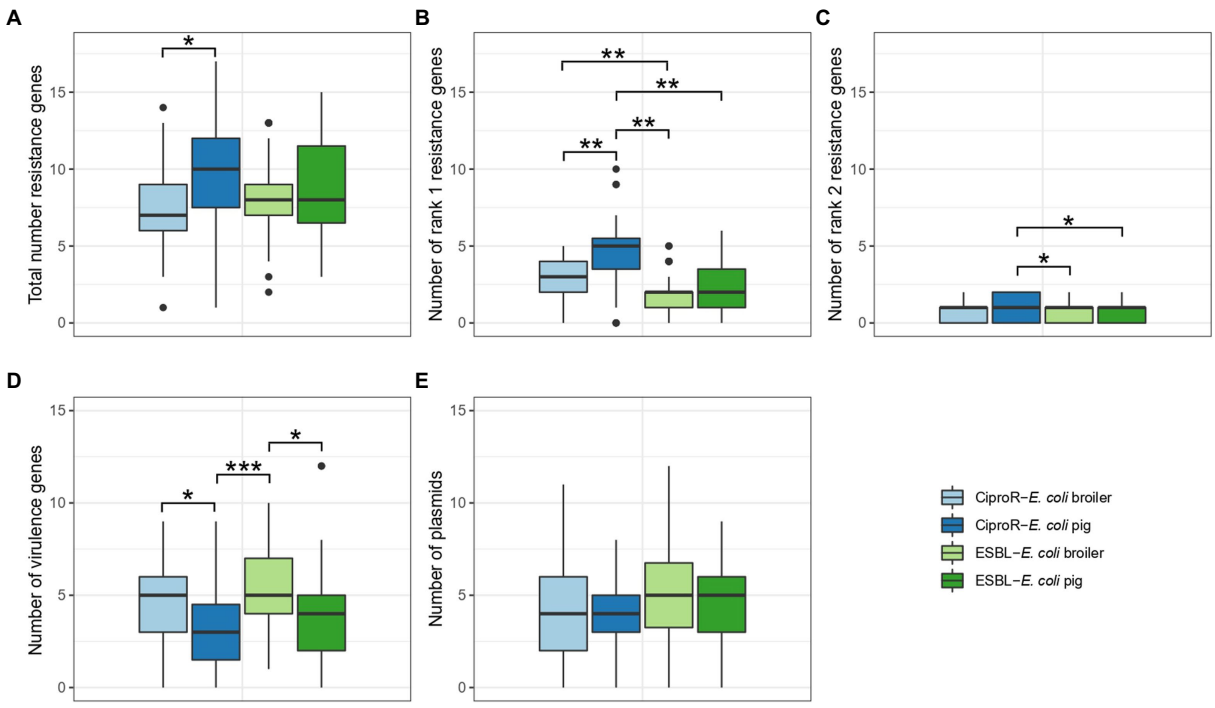

FIGURE 2
Number of resistance genes **(A–C)**, virulence genes **(D)** and plasmids **(E)** in ESBL-*Escherichia coli* and CiproR-*E. coli* isolated from broilers and pigs. Statistically significant differences are indicated according to the level of significance: * (p<0.05), ** (p<0.01), *** (p<0.001) (ANOVA with TukeyHSD or Games-Howell post-hoc tests).

ciproR-*E. coli* from broilers (7.51 ± 2.85) (Figure 2A). Resistance genes that are a current threat to public health, referred to as Rank I resistance genes, were more abundantly present in ciproR-*E. coli* compared to ESBL-*E. coli* and more in pig isolates (4.6 ± 2.4 Rank I resistance genes) compared to broiler isolates (2.8 ± 1.4 Rank I resistance genes) ($p < 0.01$) (Figure 2B). Similar observations can be made for Rank II resistance genes (considered future threats) which were present in higher numbers in porcine ciproR-*E. coli* compared to ESBL-*E. coli* from both broilers and pigs ($p < 0.05$) (Figure 2C). On the other hand, broiler isolates contain a higher number of virulence genes (ciproR-*E. coli*: 4.62 ± 2.23; ESBL-*E. coli*: 5.45 ± 2.60) compared to pig isolates (ciproR-*E. coli*: 3.10 ± 2.25; ESBL-*E. coli*: 3.97 ± 2.81) (Figure 2D). This divergence of resistance and virulence was observed in the higher number of virulence genes (up to 12 genes) and lower number of Rank I resistance genes in ESBL-*E. coli*, while the opposite was seen for most ciproR-*E. coli,* which can carry a higher number of Rank I resistance genes (up to 10 Rank I resistance genes) (Supplementary Figure 1). Fourteen isolates showed a convergence of virulence and resistance (at least 3 Rank I resistance genes and more than six virulence genes) which belonged to ST117, ST189 ($n = 2$), ST648, ST88, ST1011, ST75, ST624, ST115 ($n = 3$), ST48 and ST350 ($n = 2$). Overall, a large diversity was seen in the number of virulence and Rank I resistance genes ranging from lower-risk (one resistance gene and one virulence gene) to high-risk isolates (five Rank I resistance genes and nine virulence genes) (Supplementary Figure 1). On average, four plasmids were detected per isolate and no significant differences in the number of plasmids between the isolates of different origins were detected (Figure 2E). The most common replicon markers (>10% in one or more categories) were IncFIB (52.9%), IncI1-I (gamma) (38.2%), Col (MG828) (30.1%), IncFII (27.7%), IncX1 (25.6%), IncFIC(FII) (23.6%) and p0111 (18.9%). Plasmid replicon IncB/O/K/Z was exclusively detected in broiler isolates (in 23.0% of ciproR-*E. coli* and in 28.9% of ESBL-*E. coli*) (Supplementary Figure 2). Most virulence genes were involved in adherence and invasion (Supplementary Figure 3). The most prevalent virulence genes were *iss* (75%), *gad* (57%), *lpfA* (37%) and *iroN* (37%). A total of 120 different virulence profiles were detected within farms.

3.3. Genotype–phenotype correlations for resistance in ESBL-*Escherichia coli* and ciproR-*Escherichia coli*

More than one type of ESBL gene was detected in most of the sampled farms (73.3%; 22/30 farms). All ESBL genes were associated with very high ampicillin (MIC ≥32 mg/l) and cefotaxime (MIC 8 to ≥64 mg/l) resistance levels ($p < 0.001$), except for two bla_{SHV-2}-harboring porcine isolates which showed cefotaxime MICs below breakpoint (MIC ≤1 mg/l). Strong levels of agreement between ESBL genotype and phenotype were detected for cefuroxime (89.44%, phi coefficient: 0.76), and ceftazidime (86.96%, phi coefficient: 0.77) and an almost perfect level of agreement was detected for cefotaxime (98.14%, phi coefficient: 0.96) (Table 1).

Mutations in QRDR of *gyrA* and *parC* were found in all ciproR-*E. coli*. Sequential acquisition of individual mutations in QRDR of *gyrA* and *parC* led to increasing MICs for all tested fluoroquinolone antibiotics. Predicted amino acid change S83L in GyrA caused low-level resistance to enrofloxacin and moxifloxacin, but not to ciprofloxacin

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TABLE 1 Concordance between ESBL genotypes and cephalosporin phenotypes in *Escherichia coli* isolates from livestock.

Antibiotic	Susceptible phenotype		Non-susceptible phenotype		Agreement (%)	Phi coefficient (95% CI)	*p*-value
	ESBL gene presence	ESBL gene absence	ESBL gene presence	ESBL gene absence			
Cefuroxim	7 (4.3%)	66 (41.0%)	78 (48.5%)	10 (6.2%)	89.44	0.76 (0.69–0.88)	***(<0.001)
Cefotaxime	2 (1.2%)	75 (46.6%)	83 (51.6%)	1 (0.6%)	98.14	0.96 (0.91–1)	***(<0.001)
Ceftazidime	19 (11.8%)	75 (46.6%)	65 (40.4%)	1 (0.6%)	86.96	0.77 (0.67–0.87)	***(<0.001)

CI: confidence interval.

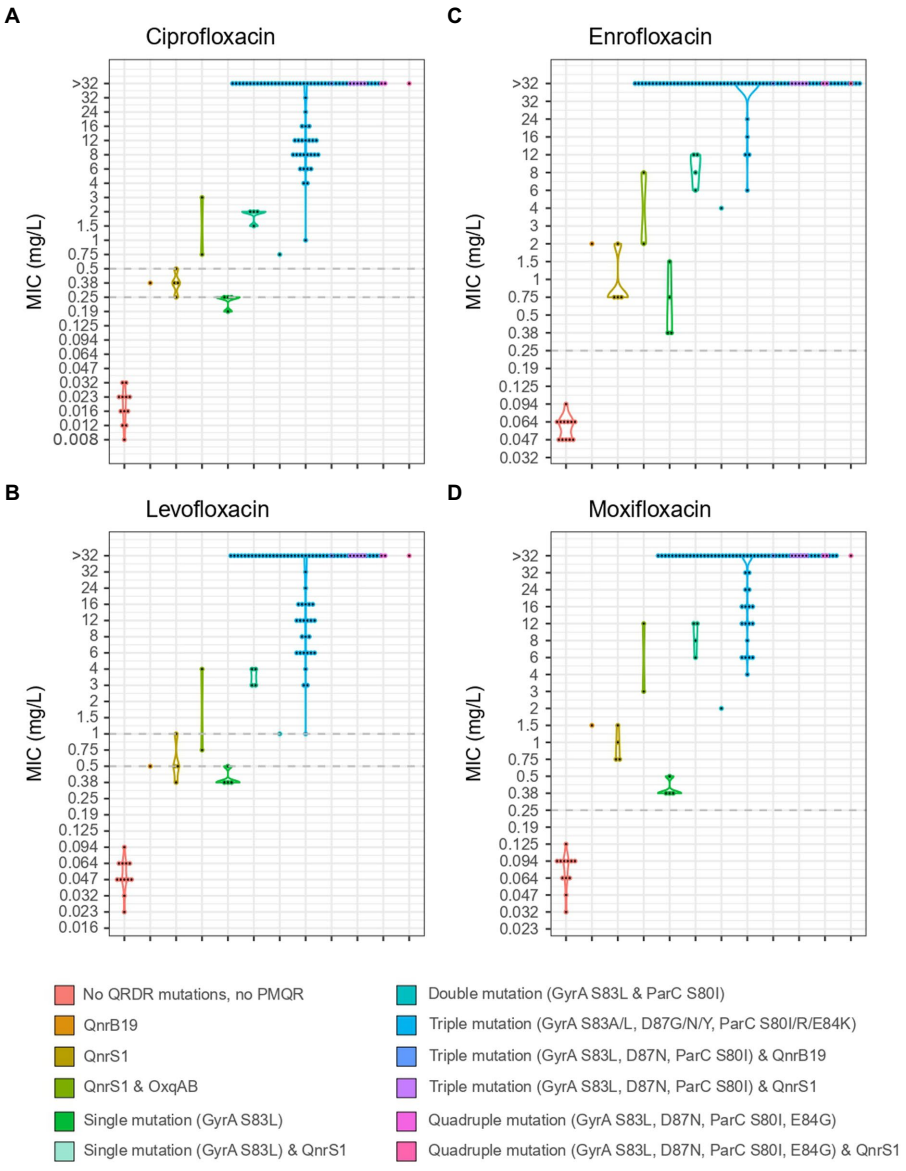

FIGURE 3
MIC values for ciprofloxacin **(A)**, levofloxacin **(B)**, enrofloxacin **(C)** and moxifloxacin **(D)** of 106 isolates from Belgian broilers and pigs in association with the mutations in QRDR regions of GyrA and ParC and the presence of PMQR genes. EUCAST breakpoints are indicated with a horizontal, dotted grey line.

and levofloxacin. Triple or quadruple mutations in QRDR caused high-level fluoroquinolone resistance (MIC>4 mg/l). QnrS1 or QnrB19 alone leads to low-level resistance to enrofloxacin and moxifloxacin and a sensitive/intermediate phenotype for ciprofloxacin and levofloxacin. The presence of both *oqxAB* and *qnrS1* genes lead to a non-susceptible phenotype for all four fluoroquinolones (Figure 3).

GyrA S83L, D87N and ParC S80I were strongly and significantly associated with resistance to fluoroquinolones. Triple mutations in *gyrA*

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(S83L and D87N/Y/G) and *parC* (S80I/R or E84K) were detected in 88% of the ciproR-*E. coli* and confer resistance to all tested fluoroquinolones. Two isolates contained a fourth mutation (GyrA S83L and D87N, ParC S80I and E84G) and one isolate additionally contained the *qnrS1* gene that showed MIC>32 mg/l for all fluoroquinolones. Outside of the QRDR in *gyrA* and *parC*, other mutations were detected in *gyrA*, *parC*, *gyrB*, *parE*, *acrB*, *acrR*, *marR*, *rpoB*, *soxR* and *soxS*, yet, were not positively associated with fluoroquinolone resistance (Figure 4). No mutations were detected in *marA*.

3.4. Genetic context of ESBL genes and PMQR in ESBL-producing and ciprofloxacin-resistant *Escherichia coli*

The specific genetic context of ESBL and PMQR genes (closest MGE and, if possible, identification of plasmid origin or replication) could be identified for 66 isolates (Figure 5). MGEs tended to be present at a fixed distance from the resistance gene. ESBL gene $bla_{\text{CTX-M-1}}$ was commonly found in association with ISEcp*1* upstream

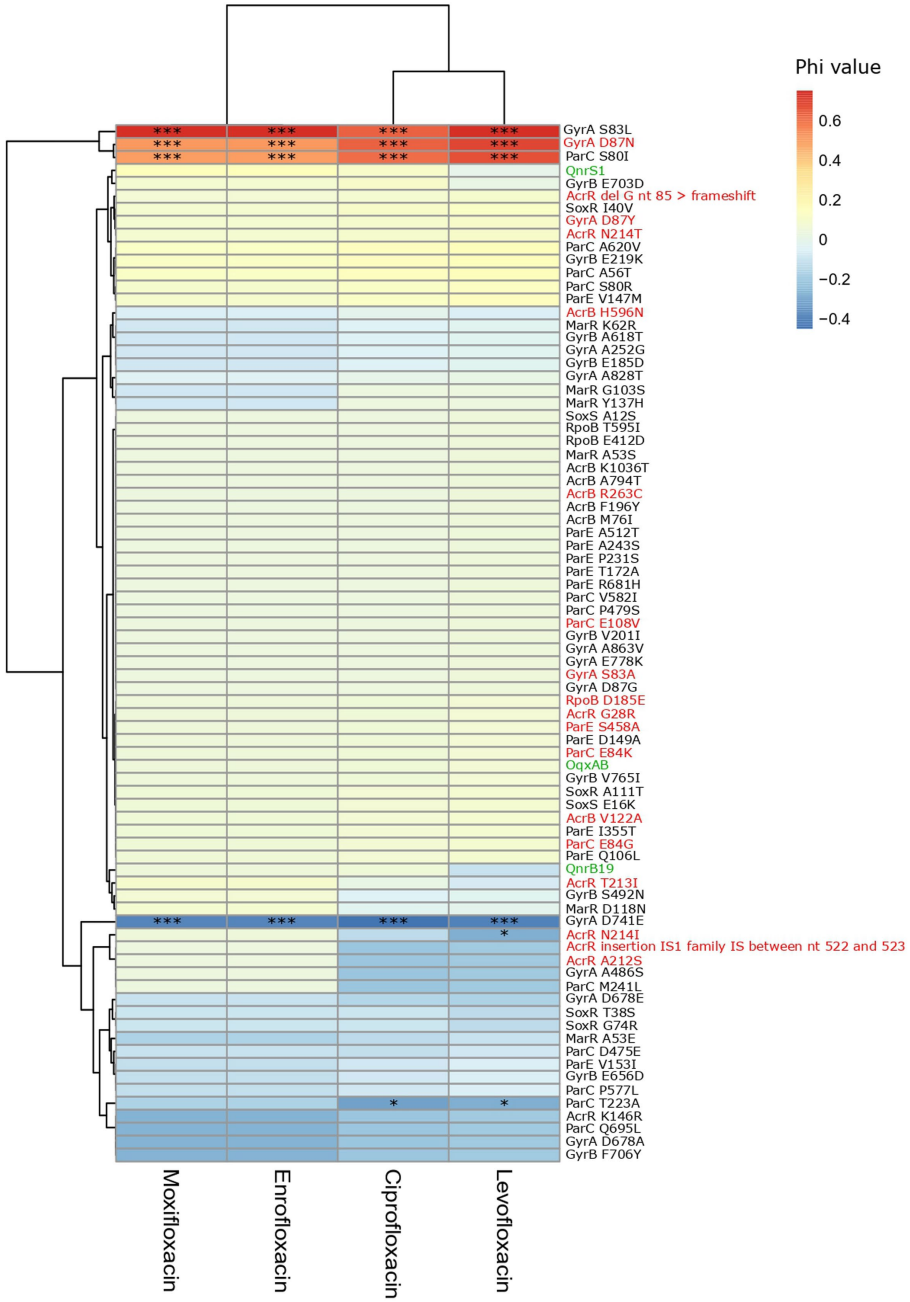

FIGURE 4
Heatmap of the association between the presence of PMQR genes and mutations and fluoroquinolone non-susceptibility. Colors represent the phi values. Negative phi values represent negative associations, positive values represent positive associations between the genes/mutations and the non-susceptibility to the fluoroquinolone antibiotics. PMQR genes are indicated in green, predicted amino acid changes that are likely deleterious for the protein function according to SIFT are indicated in red. *(p<0.05), *** (p<0.001) (Chi-squared test). IS: insertion sequence, nt: nucleotide.

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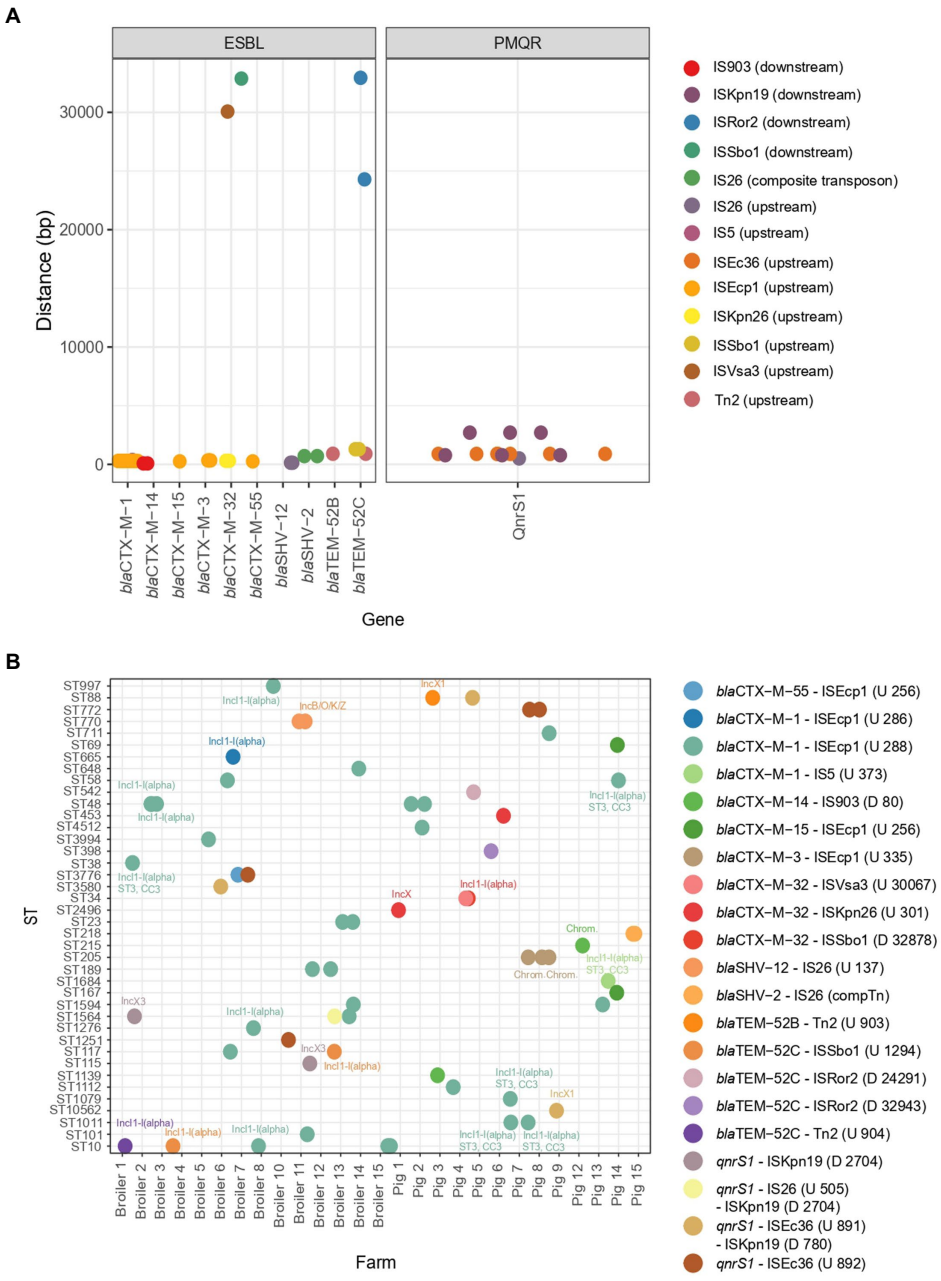

FIGURE 5
Mobile genetic elements and their association to ESBL genes and PMQR genes. **(A)** Distance of mobile genetic elements to the ESBL or PMQR gene. **(B)** The combination of the ESBL/PMQR gene with the closest mobile genetic element for every farm and ST element. The plasmid origins of replication are indicated in the figure. The distance and upstream (U)/downstream (D) location of the mobile genetic element are indicated in the figure legend.

of the gene ($n = 30$) and was always detected on plasmids (Figure 5A). The plasmid IncI1-I(alpha) could be detected in 12 $bla_{\text{CTX-M-1}}$-producing strains and, using pMLST, six of the IncI1-I(alpha) plasmids showed ST3, clonal complex 3. Evidently, this particular MGE circulates in six pig and 13 broiler farms amongst various *E. coli* genotypes, showcasing the remarkable distribution reach of this $bla_{\text{CTX-M-1}}$ harboring plasmid (Figure 5B). Other resistance genes detected on a subset of the $bla_{\text{CTX-M-1}}$-containing sequences are: *aadA5* ($n = 2$), *dfrA17* ($n = 2$), *mdtG* ($n = 1$), *mdtH* ($n = 1$), *mexA* ($n = 1$), *mexB* ($n = 1$), *qnrS1* ($n = 1$), *sul2* ($n = 6$) and *tetA* ($n = 1$), as well as virulence gene *cib* ($n = 10$). One porcine isolate harbored $bla_{\text{CTX-M-1}}$ associated with IS*5* on an IncI1-I(alpha), ST3, CC3 plasmid. The IncI1-I(alpha) plasmid origin of replication could also be detected in association with other ESBL genes, such as $bla_{\text{TEM-52C}}$ ($n = 3$) and $bla_{\text{CTX-M-32}}$ ($n = 1$). The $bla_{\text{SHV-12}}$ gene was detected on an IncN plasmid, without any association of IS elements in four broiler isolates from four different farms or in association with IS26 137 bp upstream of the $bla_{\text{SHV-12}}$ gene on an IncB/O/K/Z plasmid in two isolates from a broiler farm. A composite transposon IS*26* surrounded the $bla_{\text{SHV-2}}$ gene in isolates ($n = 2$) from a pig farm.

Most ESBL genes were located on a plasmid. However, seven ESBL genes [$bla_{CTX\text{-}M\text{-}3}$ ($n = 3$) associated with ISEcp*1*, $bla_{CTX\text{-}M\text{-}14}$ associated with IS903 ($n = 1$), $bla_{CTX\text{-}M\text{-}15}$ ($n = 1$) and $bla_{CTX\text{-}M\text{-}32}$ ($n = 2$)] were predicted to be located on the chromosome. Different IS elements/transposons flanked the $bla_{CTX\text{-}M\text{-}32}$ gene (upstream ISKpn26 ($n = 2$) on an IncX plasmid ($n = 1$) or downstream ISSbo1 on an IncI1-I(alpha) plasmid ($n = 1$) or upstream ISVas3 ($n = 1$)) and the $bla_{TEM\text{-}52C}$ [upstream ISSbo1 ($n = 2$), upstream Tn2 ($n = 1$), downstream ISRor2 ($n = 2$)] in different isolates. The $bla_{TEM\text{-}52B}$ gene was flanked by Tn2 in one porcine isolate and was located on an IncX1 plasmid. Co-localization of QnrS1 with $bla_{CTX\text{-}M\text{-}15}$ ($n = 1$) or $bla_{CTX\text{-}M\text{-}55}$ ($n = 1$) on a predicted plasmid contig was detected (Supplementary Figure 4). In 14 out of 75 isolates (18.7%), co-localization of virulence factor colicin Ib (*cib* gene, polypeptide toxins against *E. coli* and closely related bacteria) with an ESBL gene was detected.

The PMQR gene *qnrS1* was flanked by downstream ISKnp*19* ($n = 6$) and upstream either by ISEc*36* ($n = 7$) or by IS*26* ($n = 1$). For one porcine isolate, the plasmid replicon could be identified as IncX1 harbouring $bla_{TEM\text{-}1B}$. For two broiler isolates from two different farms, QnrS1 could be located on an IncX3 plasmid (Figure 5B). QnrB19 was found to be located on a Col(pHAD) plasmid ($n = 2$); however, no IS elements flanking the gene could be identified. Also, no flanking MGEs could be identified for *oqxAB* genes.

3.5. Typing and possible transmission events of resistant *Escherichia coli* within and between farms

A highly diverse population of *E. coli* was isolated from broiler and pig farms (Figure 6). Overall, 63 different *E. coli* STs were detected with ST10 being the most abundant (13 out of 161 isolates, 8.1%). Phylogroup A was most common in ESBL-*E. coli* from broilers (47.6%) and pigs (57.1%) and in ciproR-*E. coli* from pigs (53.8%), where phylogroup B1 was most common among ciproR-*E. coli* from broilers (31.1%). Phylogroup A was most common among ESBL-*E. coli* from pigs (57.1%) and broilers (47.6%), and ciproR-*E. coli* from pigs (53.8%), while B1 was most common among ciproR-*E. coli* from broilers (31.1%). The number of virulence genes in phylogroups A and B1 was lower compared to phylogroups D and G (Supplementary Figure 5). FimH54 was the most common among ESBL-*E. coli* from broilers (16.7%) and pigs (40.0%) and ciproR-*E. coli* from pigs (41.0%), and fimH32 was most common among ciproR-*E. coli* from broilers (22.2%). With 85 different serotypes among 161 isolates, serotypes were widely diverse.

To determine the genetic relatedness of the isolates, a study specific cgMLST scheme with 3,012 loci was developed. Genetically linked bacterial clusters, with a maximal difference of 10 alleles among them (Schürch et al., 2018; Van Hoek et al., 2020), were identified on several pig ($n = 8$) and broiler farms ($n = 3$) (ST10, ST34, ST205, ST215, ST345, ST453, ST683, ST744, ST1011, ST1140, ST1158). Moreover, the presence of genetically similar resistant bacteria was detected between different broiler farms ($n = 5$) (ST115, ST48, ST155). These results suggest either transmission or a common reservoir between broiler farms. Transmission of *E. coli* ST1594 has likely occurred between a broiler farm and a pig farm as an allelic difference of 3 loci was shown between the two isolates (Figure 6).

3.6. Pathotypes detected in Belgian farm animals: ESBL-producing and ciprofloxacin-resistant ETEC and ESBL-producing EPEC

Most of the *E. coli* isolates were non-pathogenic. However, 12 pathogenic *E. coli* (7.45%) were detected in five pig farms and two broiler farms. ESBL-producing enterotoxigenic *E. coli* (ETEC) were detected in pig farms six ($n = 2$; phylogroup B1, CTX-M-32-producing) and 15 ($n = 2$ from the same pig; phylogroup A; SHV-2-producing) and ciprofloxacin-resistant ETEC were detected in pig farms eight ($n = 2$, ST772, phylogroup A, FimH54) and nine ($n = 1$, ST10, phylogroup A, FimH54). Enterotoxins *sta* and *stb* were present in 4 ETEC strains, *sta* was present in one ETEC strain and *stb* was present in two ETEC strains. The *stb*-containing contigs of the ETEC strains from pig farm 15 also contained the *astA* gene encoding the heat-stable enterotoxin (EAST1) and IS100, an IS21 family insertion element.

ESBL-producing enteropathogenic *E. coli* (EPEC) were detected in pig farm two ($n = 2$) and broiler farms four ($n = 1$) and 12 ($n = 2$). All EPEC strains were atypical because of the lack of bundle-forming pili (BFP). All EPEC strains were fimH54 belonging to phylogroup A; two were ST48 and CTX-M-1-producing strains, one was ST10 and TEM-52C producing strain and two were ST189 and CTX-M-1-producing strain. The latter two contained the IS256 composite transposon to mobilize the cassette of pathogenic virulence genes (*eae*, *espA*, *espB*, *espF*, *astA*, *tir*).

4. Discussion

The study showed that livestock is a reservoir for a large variety of antimicrobial resistance genes, virulence genes and plasmids. More than one type of ESBL gene was detected in most farms and *E. coli* belonging to a variety of STs was found in Belgian broilers and pigs.

The large collection of STs and serotypes of commensal *E. coli* in animals was described before (Ahmed et al., 2017; Duggett et al., 2020; Kaspersen et al., 2020; Massella et al., 2020; Zingali et al., 2020; Leekitcharoenphon et al., 2021). However, the pandemic multidrug-resistant clone ST131 commonly associated with human infections was not detected and $bla_{CTX\ M\text{-}15}$ was rarely found [$n = 3$ from two pig farms (ST4981, ST69, ST167)]. *Escherichia coli* ST131 was also not detected in pig farms in Switzerland during a longitudinal study (Moor et al., 2021). The spread of $bla_{CTX\text{-}M\text{-}15}$ in human-associated *E. coli* is globally linked to IncFII plasmids in ST131 (Rozwandowicz et al., 2018). IncFII plasmids were commonly detected (27.7% of the isolates) in this study but could not be linked to $bla_{CTX\text{-}M\text{-}15}$ or ST131. Instead, CTX-M-1 predominates in *E. coli* from food-producing animals and food in Europe (Ceccarelli et al., 2019; Duggett et al., 2020). We found that the most common ESBL genes were $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$ and ST10 was the most abundant sequence type. This is in line with other reports (Smet et al., 2008; Ahmed et al., 2017; Reid et al., 2017; van Damme et al., 2017; Ceccarelli et al., 2019; Stubberfield et al., 2019; Duggett et al., 2020; Zingali et al., 2020; Leekitcharoenphon et al., 2021; Moor et al., 2021). ST10 has been found in both humans, animals, retail meat and the environment (Oteo et al., 2009; Leverstein-van Hall et al., 2011; Toval et al., 2014; Manges et al., 2015; Reid et al., 2017), is

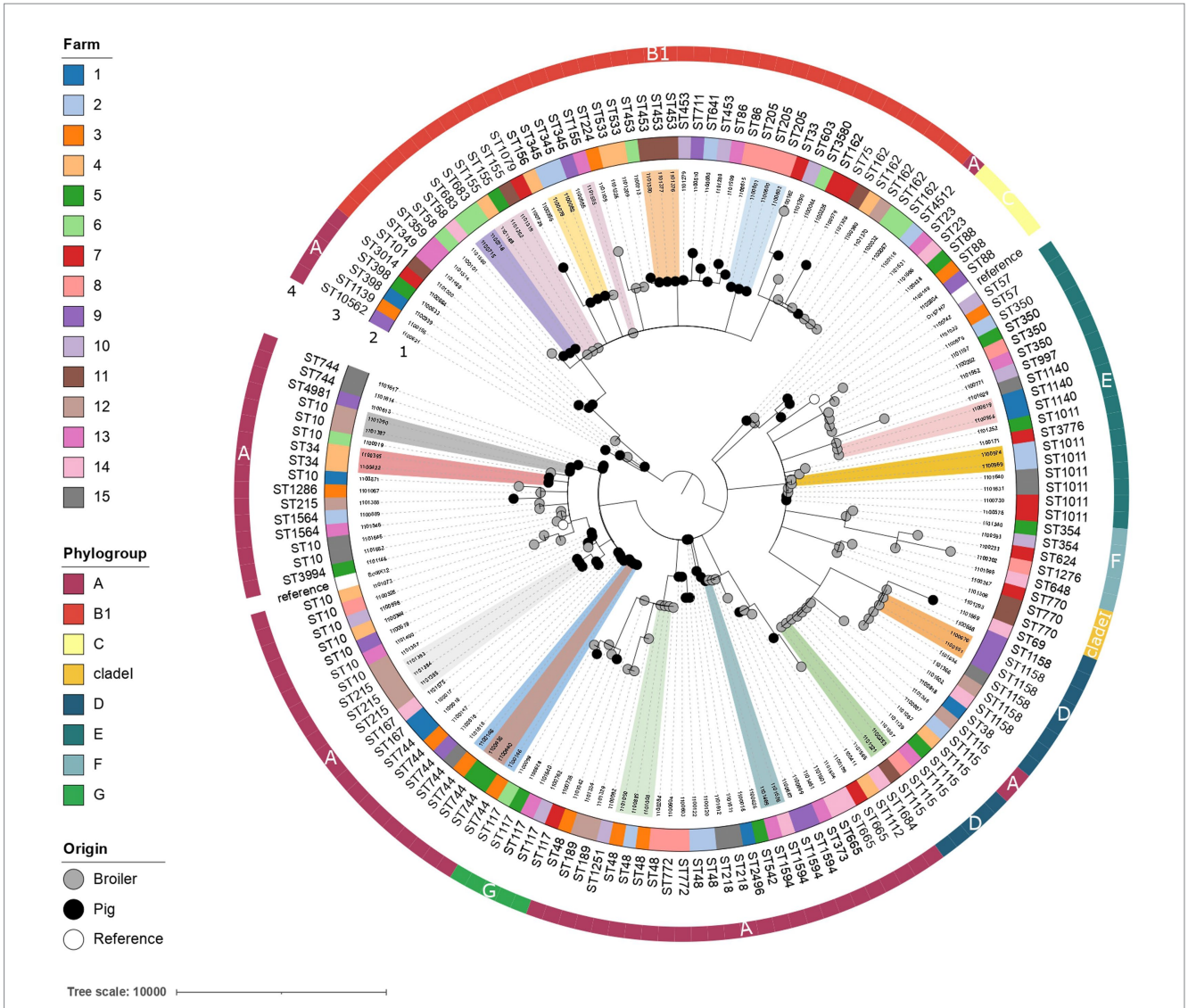

FIGURE 6
Phylogenetic tree of ciprofloxacin-resistant and ESBL-producing *E. coli* from broilers and pigs. The minimum spanning tree is distance-based and was generated by iTOL using cgMLST profile data (3,012 loci). Colored clusters indicate genetically related isolates with ≤10 allelic differences from different broilers/pigs. The isolate IDs are shown in the first ring. The farm is indicated in colored strips in the second ring. Achtmann ST and phylogroups are indicated in rings three and four, respectively. The origin of the isolate is indicated with black (pig), grey (broiler) or white (*E. coli* K12 and *E. coli* O157-H7 reference strains) nodes.

associated with ESBL production (Oteo et al., 2009; Moor et al., 2021), and has been reported as an emerging extra-intestinal pathogen in humans, pigs and broilers (Riley, 2014; Manges and Johnson, 2015; Bojesen et al., 2022). The results from our study combined with published data confirm that ST10 is a potential dominant clonal group of commensal *E. coli* in food-producing animals globally. Other high-risk lineages (ST69, ST117, ST23, ST58, ST648, ST744) of *E. coli* were identified among our isolates. A total of 12 (7.45%) pathogenic *E. coli* strains were detected (ETEC and atypical EPEC), one ST10 TEM-52C-producing strain and two ST189 CTX-M-1-producing strains which contained an IS256 composite transposon to mobilize the cassette of pathogenic virulence genes (*eae*, *espA*, *espB*, *espF*, *astA*, *tir*). These composite transposons can move as a single unit to move these pathogenic virulence genes and disseminate them among bacteria.

The spread of ESBL genes is highly linked to epidemic and highly transmissible plasmids (Rozwandowicz et al., 2018; Kurittu et al., 2021). Most ESBL genes were predicted to be located on plasmids (91%) and were in the proximity of an IS element or transposon that was usually located at a fixed distance from the ESBL gene. The $bla_{\text{CTX-M-1}}$ gene was often associated with ISEcp*1* and IncI1-I(alpha)-ST3 in several broiler and pig farms, as described before (de Been et al., 2014; Zurfluh et al., 2014; Partridge et al., 2018; Rozwandowicz et al., 2018; Ceccarelli et al., 2019; Bernreiter-hofer et al., 2021). ISEcp*1* is known to be associated with ESBL genes. Genes downstream of this IS element can be mobilized through transposition (including chromosomal integration) and are able to enhance ESBL gene expression under its own promotor (Poirel et al., 2003; Ceccarelli et al., 2019; Massella et al., 2020). In our study, the IncI1-I(alpha) plasmid was also found to carry other ESBL genes ($bla_{\text{CTX-M-32}}$ and $bla_{\text{TEM-52C}}$).

These results indicate that the IncI1-I(alpha) plasmid is a major plasmid type contributing to the spread of ESBLs in Belgian farms. Other ESBL-plasmid origin-of-replication combinations were: bla_{SHV-12} on an IncN plasmid or IncB/O/K/Z plasmid, $bla_{CTX-M-32}$ on an IncX plasmid and $bla_{TEM-52B}$ on an IncX1 plasmid. QnrS1 seems to be flanked by different IS elements and was located on IncX1 in a pig farm or IncX3 plasmids in two broiler farms. IncX plasmids were described as widely distributed and to be associated with fluoroquinolone resistance (Dobiasova and Dolejska, 2016). The presence of QnrS1 on IncX1 or IncX3 plasmids was shown before in Germany's pork and beef production chain (Juraschek et al., 2021). QnrB19 could be located on a Col(pHAD) plasmid in two isolates in our study, which was also the case in *Salmonella* spp. from poultry in Nigeria (Jibril et al., 2021).

Co-localization of ESBL genes with virulence factor *cib* was detected in 14/75 isolates (18.7%) and co-localization with other resistance genes (such as *aadA* genes, *dfrA* genes, *aph(3′)-Id*, *aph(6)-Id*, bla_{TEM-1B}, *cmlA1*, *sul* genes, *tetA*, *and qnrS*) was detected. PMQR and ESBL genes localized on the same presumed plasmid contig [*qnrS1* with $bla_{CTX-M-15}$ ($n = 1$) or $bla_{CTX-M-55}$ ($n = 1$)] is concerning. Plasmids co-harboring multiple resistance determinants to critically important antibiotics for human medicine limit treatment options for severe infections and are a threat to public health.

PMQR genes were found in a remarkably low number of isolates and play a limited role in the occurrence of ciproR-*E. coli* in Belgian farms. Ciprofloxacin resistance was caused by mutations in the QRDR region of *gyrA* and *parC* in all ciproR-*E. coli*, of which most showed triple mutations (GyrA S83L and D87N and ParC S80I) significantly associated with high-level fluoroquinolone resistance. In contrast, QnrS1 or QnrB19 alone leads to low-level resistance to enrofloxacin and moxifloxacin and a sensitive/intermediate phenotype for ciprofloxacin and levofloxacin. Despite strong negative correlations between the presence of *qnr* genes and *gyrA* mutations shown previously and the hypothesis that Qnr proteins have a protective effect on quinolone targets (Kaspersen et al., 2020), the presence of QnrS1 combined with GyrA S83L amino acid change was almost always detected in our study. Only two porcine ciproR-*E. coli* isolates did not contain any mutations in the QRDR of *gyrA* and *parC*, instead harbored two PMQR (OqxAB and QnrS1). Although PMQR mechanisms provide low-level resistance (Jacoby et al., 2015), the combination of OqxAB and QnrS1 was sufficient to result in fluoroquinolone resistance above breakpoint.

Pig isolates showed a higher mean number of resistance genes, especially for porcine ciproR-*E. coli*, which could reflect the higher use of antibiotics in pigs compared to broilers (FOD Volksgezondheid Veiligheid van de Voedselketen en Leefmilieu et al., 2021). In contrast, virulence genes were more abundantly present in broiler isolates. Most virulence genes were involved in adherence and invasion (most prevalent virulence genes were *iss, gad, lpfA*), which can contribute to successful colonization and enhanced survival in the gut and the environment (Projahn et al., 2018). Also, the presence of ExPEC-associated virulence factors (such as *astA*, *iss*, *iha*, and *iroN*) is an indication that these commensal *E. coli* in Belgian farms may have pathogenic potential (Mo et al., 2016). Phylogroups A and B1 were the most common and are associated with commensal phenotypes (Johnson and Stell, 2000). In line with this, phylogroups A and B1 carried a lower number of virulence genes compared to phylogroups D and G. However, the pathogenic *E. coli* (ETEC and EPEC) detected in this study belonged to phylogroups A and B1 showing that these phylogroups also have the potential to cause extraintestinal infections.

We identified multiple genetically related clones in different animals of the same farm and of distinct farms. The presence of clonally-related bacteria in different poultry farms suggests a common reservoir or transmission of resistant bacteria. The vertical spread of resistant bacteria from the top to the bottom of the broiler production pyramid (Dierikx et al., 2013; Zurfluh et al., 2014) and resistant *E. coli* residing in the farm environment (Blaak et al., 2015) were previously identified as important transmission routes of resistant bacteria. The diverse profiles of resistance genes, virulence genes and plasmid profiles reflect complex epidemiology. In addition, the detection of plasmid replicons with associated IS elements and ESBL/PMQR genes in different farms and among several STs (such as IncI1-I(alpha) and IncX3) underline that plasmid transmission could be another important contributor to the transmission of resistance.

Our data show the complex epidemiology of ESBL-production and ciprofloxacin resistance in *E. coli* from livestock, suggesting the spread of these resistances involves both dissemination of resistant clones and horizontal transmission of plasmids. This emphasizes how critical it is to curtail the unnecessary use of antibiotics across all levels of the livestock production chain to preserve antibiotic effectiveness. Additionally, further research into plasmid involvement should include sequencing over longer reads to better understand its circulation on farms. The study supports that commensal *E. coli* in livestock should be monitored using WGS. Although not all resistance genes could be associated with MGEs or plasmids and we only sequenced a sub-selection of the resistant strains per farm, we gained valuable information on the genetic characteristics of ESBL-*E. coli* and ciproR-*E. coli* and the transmission of clones and resistance genes in Belgian farms using genomic data.

5. Conclusion

Our research uncovers a multifaceted landscape of ESBL production and ciprofloxacin resistance in Belgian farms. The complex epidemiology with diverse combinations of ESBL genes, ST types, antibiotic resistance and virulence profiles makes it difficult to translate these findings to the impact on human health. Nevertheless, WGS provides detailed information and should be utilized to properly track this complex situation which poses an urgent challenge for preventing the spread of antimicrobial resistance in the broiler and pig production chain.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

Ethical review and approval was not required for the animal study because the procedure to collect fresh fecal droppings is considered to cause no discomfort, and animals were neither handled nor sacrificed

during the study (EC Directive 2010/63). Written informed consent was obtained from the owners for the participation of their animals in this study.

I-4-1-Health Study Group

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Author contributions

SK: original draft writing. MR, BX, CL, DC, KB, KM, JD, MK, JK, and HG: review and editing. SK and MR: data collection. SK, BX, DC, KB, and KM: data analysis. HG and JK: funding acquisition. MB, JK, and HG: project administration. HG, CL, MK, and JK: supervision. SK and MK: data curation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1150470/full#supplementary-material

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Surfaces of gymnastic equipment as reservoirs of microbial pathogens with potential for transmission of bacterial infection and antimicrobial resistance

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Gymnastic equipment surfaces are shared by many people, and could mediate the transfer of bacterial pathogens. To better understand this detrimental potential, investigations on the reservoirs of bacterial pathogens and antimicrobial resistance on the surfaces of gymnastic equipment were performed by analyzing the bacterial community structures, prevalence of viable bacteria, and presence of antimicrobial resistance on both indoor and outdoor gymnastic facilities. The results of high-throughput 16S rDNA amplicon sequencing showed that Gram-positive bacteria on the surfaces of indoor gymnastic equipment significantly enriched, including the opportunistic pathogen *Staphylococcus* strains, while Enterobacteriaceae significantly enriched on surfaces of outdoor gymnastic equipment. The analysis of α-diversities showed a higher richness and diversity for bacterial communities on the surfaces of gymnastic equipment than the environment. Analysis of β-diversities showed that the bacterial communities on the surfaces of gymnastic equipment differ significantly from environmental bacterial communities, while the bacterial communities on indoor and outdoor equipment are also significantly different. Thirty-four bacterial isolates were obtained from the surfaces of gymnastic equipment, including three multidrug *Staphylococcus* and one multidrug resistant *Pantoea*. In particular, *Staphylococcus hemolyticus* 5–6, isolated from the dumbbell surface, is a multidrug resistant, hemolytic, high-risk pathogen. The results of quantitative PCR targeting antibiotic resistance related genes (*intI1*, *sul1* and bla_{TEM}) showed that the abundances of *sul1* and bla_{TEM} genes on the surfaces of gymnastic equipment are higher than the environment, while the abundances of *sul1* gene on indoor equipment are higher than outdoor equipment. These results lead to the conclusion that the surfaces of gymnastic equipment are potential dissemination pathways for highly dangerous pathogens as well as antimicrobial resistance, and the risks of indoor equipment are higher than outdoor equipment.

KEYWORDS

gymnastic equipment, microbiome, bacterial infection, microbial community structure, antimicrobial resistance

Introduction

Transmission of infectious diseases can be accelerated by contamination of pathogens on the surfaces people access. Bacterial transfer rate during routine household activities has been assessed, showing that despite having low transfer efficiency, there are still a sizable number of transmitted bacteria from surfaces to human skin (up to 10^6 cells; Rusin et al., 2002). To provide convenience for people's exercises, a significant amount of public gymnastic equipment has been established in communities, gymnasiums, and universities worldwide. This could potentially increase the risks of bacterial contamination and dissemination. During exercises, one of the most common pathways of bacterial infection transmission is direct skin-to-equipment contact. People sweat as a result of their bodies warming up, which creates a favorable environment for microbial proliferation on the skin. In addition, physical stress damages the body's exterior defenses, opening the door for pathogen invasion (Grosset-Janin et al., 2012).

Antibiotics are the primary treatments for bacterial infections (Laxminarayan et al., 2016). Nevertheless, along with the widespread use of antibiotics in medical and agricultural sectors from the mid-20th century to the early 21st century, antimicrobial resistance (AMR) has emerged and become currently one of the most urgent medical threats (Browne et al., 2021). Antibiotic resistance genes (ARGs) can be disseminated between bacteria *via* horizontal gene transfer mechanisms and spread from environmental bacteria to pathogens, accelerating dissemination of AMR. This dissemination has been evaluated in a number of environmental settings. For instance, hospitals and wastewater treatment plants are ARG reservoirs and hotspot for ARG dissemination, in which the mobile resistome and dissemination dynamics have been extensively studied in recent years (Peter et al., 2020; Zhu et al., 2023). Even in clean environments such as university campus air, multidrug resistant bacterial pathogens in particulate matters were reported (Hu et al., 2023). With these observations, suspicions are raised that public gymnastic equipment that are relatively clean but shared by many people may also become pathways for the transmission of AMR and pathogens. However, despite the high frequency of gymnastic equipment use in university campus, the assessment of the spread of infectious diseases *via* shared gymnastic facilities is rarely reported, and the spread of bacterial infection by these facilities has not been given enough attention.

In order to explore whether gymnastic equipment increases risks for AMR transmission, in this work, we applied microbiomics, bioinformatics, microbiology, and molecular biology methods to explore the potential for transmission of bacteria and resistance *via* gymnastic equipment, providing a comparative analysis of the structures of microbial community, the distribution of pathogenic bacteria, as well as the abundance of ARGs on different gymnastic equipment surfaces. Overall, this investigation emphasized the role and risk of gymnastic equipment as a pathway for the transmission of bacterial infections, which had previously been underappreciated.

Materials and methods

Sample collection and preparation

Samples were collected from the outdoor sports field and the indoor gymnasium at Qingdao University of Science and Technology.

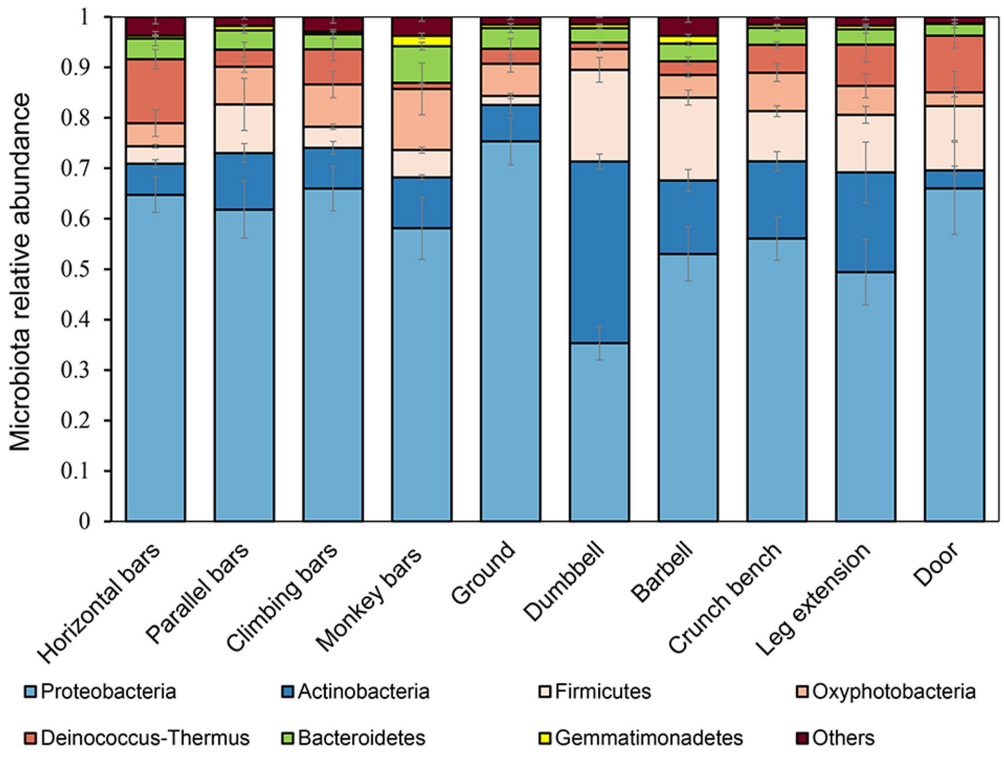

FIGURE 1
Microbial community structures on the surfaces of gymnastic equipment.

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The outdoor equipment includes parallel bars, horizontal bars, climbing bars and monkey bars. The indoor equipment includes dumbbell, barbell, crunch bench and leg extension. Samples collected from the outdoor ground and indoor door were used as the controls for the outdoor and indoor samples, respectively. Sterile cotton swabs soaked in sterile saline were used to repeatedly wipe the gymnastic equipment surfaces that frequently come into contact with the human body. The cotton was then transferred into sterile centrifuge tubes. Three and four replicates were set up for outdoor and indoor control samples, respectively. All samples were stored at 4°C until processing. One milliliter of sterile were added to the centrifuge tubes, which were vortexed for 30 s to prepare the suspension for processing.

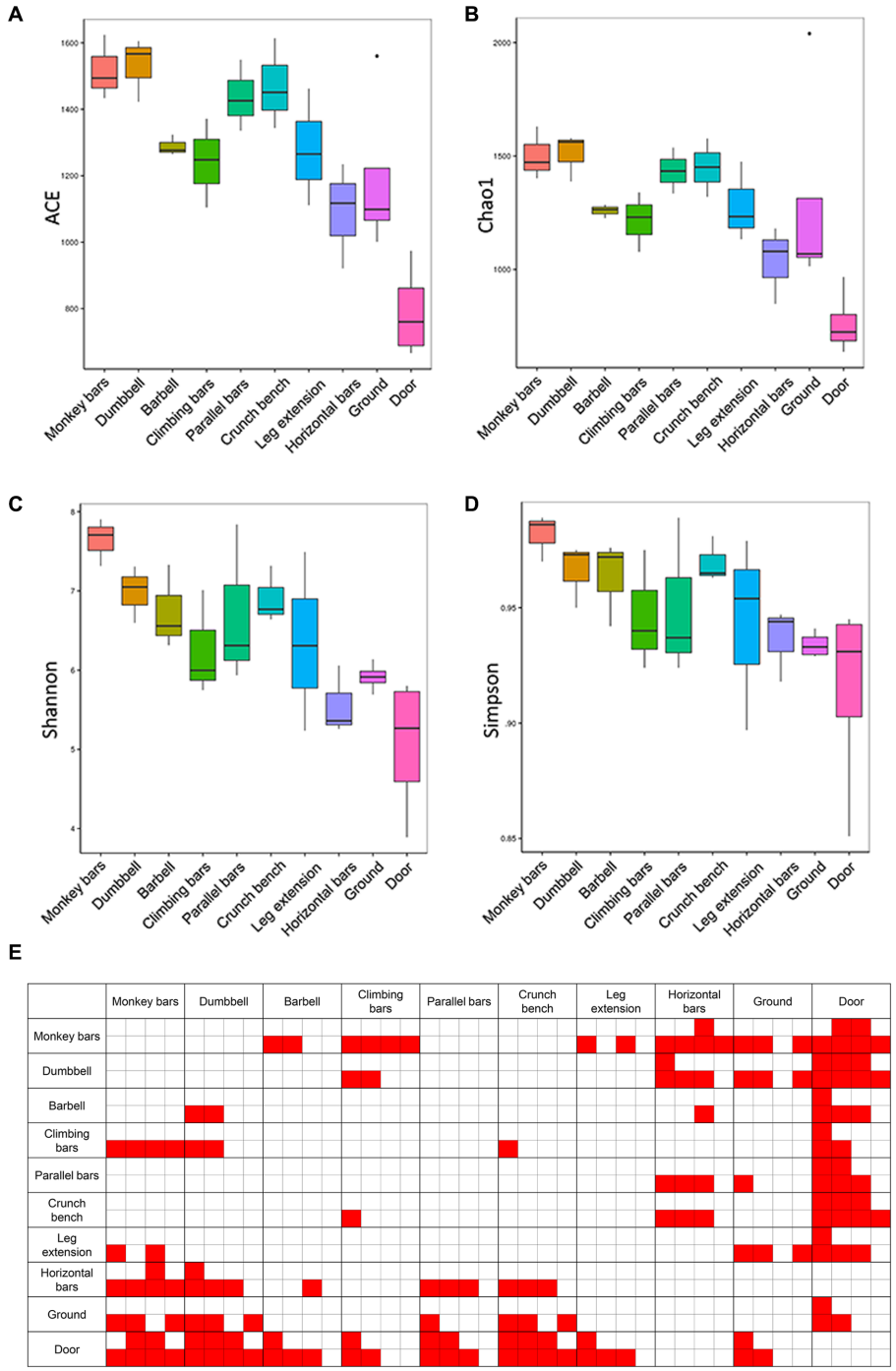

FIGURE 2
Abundances and diversities of microbial communities on the surfaces of gymnastic equipment. **(A)** ACE estimators. **(B)** Chao1 estimators. **(C)** Shannon indices. **(D)** Simpson indices. **(E)** Statistical tests of α-diversity indices. Red, represents *p*-values <0.05. In each comparison box from left to right are comparisons of the ACE estimators, Chao1 estimators, Shannon indices and Simpson indices. The top four small boxes in each comparison box mark the results of the Tukey test and the bottom four small boxes mark the results of the Wilcoxon test.

16S rDNA amplicon sequencing and bioinformatics analysis

Total DNA was extracted from swab samples by Plant Genome DNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing.,China). 16S rDNA amplicon sequencing was performed on the Illumina HiSeq2500 sequencer (Illumina, Inc., San Diego, CA, US). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, Inc., San Diego, CA, United States). Using FLASH v1.2.7 and QIIME v1.7.0, the high-quality clean tags from the raw sequence data were obtained (Caporaso et al., 2010). Chimeras were removed using UCHIME algorithm (Edgar et al., 2011). Using Uparse v7.0.1001 (with default sequence similarity of 97%), Operational Taxonomic Units (OTU) classification was determined (Edgar, 2013). The raw sequencing datasets are available at NCBI SRA repository (accession PRJNA942587).

Bioinformatical analysis and statistics

The analysis of microbial community composition was performed *via* the RDP classifier 2.2 algorithm in Silva database (Version 132) (Wang et al., 2007; Quast et al., 2013). Alpha diversity and beta diversity were calculated by QIIME v1.7.0 and displayed with R software (Version 2.15.3). NMDS, ANOSIM and MRPP analyses were performed with the Vegan package on the R platform. LEfSe (LDA effect size) analysis was performed using LEfSe. The comparison of bacterial community structure and genes abundance of bacteria were analyzed *via* two-tailed *t*-test. Tukey and Wilcoxon tests were used to analyze alpha-diversities.

Bacteria isolation and antimicrobial susceptibility test

Sample suspension was inoculated on blood agar plates and MacConkey plates respectively, followed by incubation at 37°C for 16 h. Genomic DNA of individual colonies was extracted using the Plant Genomic DNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). Subsequently, the universal primer pair 27F (5′ - AGAGTTTGATCCTGGCTCAG - 3′) /1492R (5′ - GGTTACC TTGTTACGACTT - 3′) was used to amplify 16S ribosomal RNA gene by PCR for further sequencing and the taxonomic identification of these isolates. Antimicrobial susceptibility test of the isolates was determined according to the Clinical & Laboratory Standards Institute (CLSI) guidelines using the Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute, 2018).

Quantitative real-time PCR

Genes associated with antimicrobial resistance, including mobile genetic element *intI1* and the three most common ARGs (*intI1*, *sul1*, bla_{TEM}) were determined with qRT-PCR. Primers used in this study are the same as previously reported and shown in Supplementary Table S1 (Barraud et al., 2010; Li et al., 2021; Wei et al., 2023). Two-step Real Time RT-PCR was performed with a StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, United States), according to previously published literature for PCR program (Haenelt et al., 2023). Standard curves for the target genes were constructed using pMD19-T vector. Each sample was analyzed with three biological replicates.

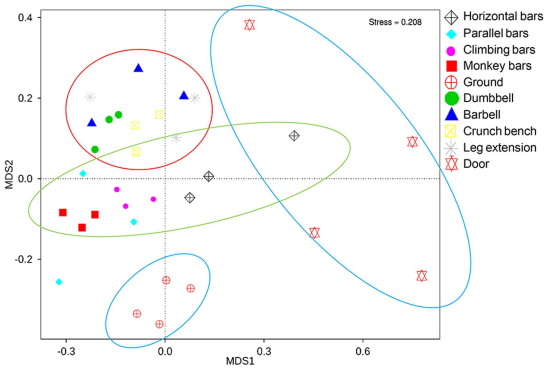

FIGURE 3
NMDS analysis of microbial community on the surfaces of gymnastic equipment. Red circle, represents indoor equipment; green circle, represents outdoor equipment; blue circle, represents controls.

Results

Microbial community structures on gymnastic equipment surfaces

Bacterial microbiomes were determined for each gymnastic equipment sample by high throughput 16S rDNA amplicon sequencing (Figure 1). At phylum level, the main bacterial groups were Proteobacteria, Actinobacteria, Firmicutes, Oxyphotobacteria, Deinococcus-Thermus, Bacteroidetes, Gemmatimondadetes, accounting for more than 96% in the microbial community (Figure 1). Among them, Proteobacteria, the most abundant phylum in the environment also had the highest proportion in our research (Yan et al., 2017). Overall, there were obvious structural differences between the microbial communities on the surfaces of indoor equipment (such as barbells, crunch bench machines, dumbbells, and leg extensions) and those on outdoor equipment (monkey bars, parallel bars, horizontal bars, climbing bars). Compared to outdoor equipment, on the surfaces of indoor equipment, the percentage of Gram-positive bacteria from the phyla Actinobacteria ($p = 7.04 \times 10^{-4}$) and Firmicutes ($p = 1.78 \times 10^{-4}$) increased dramatically, but the percentage of Gram-negative bacteria from the phylum Proteobacteria ($p = 2.00 \times 10^{-3}$) decresed significantly. This may be explained by the proposal that the impact of human is smaller on outdoor equipment because of wind and rain.

At the genus level, observed abundant bacteria on gymnastic equipment include environmentally safe bacteria such as *Methylbacillum*, *Sphingobacterium*, *Hydrophilus*, and *Cynobacterium*, along with opportunistic pathogens like *Acinetobacter*, *Staphylococcus* and *Enterobacter*. Interestingly, *Methylbacillum* and *Sphingobacterium* were prevalent in microbiomes from samples collected from the ground at a high rate of 21.84 ± 3.85% ($n = 4$) and 12.64 ± 6.49% ($n = 4$) respectively,

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FIGURE 4
Bacterial enrichment on the surfaces of different gymnastic equipment. **(A)** Comparison of door and indoor equipment. **(B)** Comparison of ground and outdoor equipment.

significantly higher than other samples ($n = 28$, $p = 1.89 \times 10^{-22}$; $n = 28$, $p = 5.89 \times 10^{-8}$). Meanwhile, the levels of human opportunistic pathogenic *Staphylococcus* found on indoor equipment surfaces were 4.67 ± 3.53% (n = 12), much greater than the 0.47 ± 0.48% in samples collected from the ground (n = 4, $p = 0.036$).

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TABLE 1 Strains isolated from gymnastic equipment surfaces.

Location	Gymnastic equipment	Strains	16S rDNA identification
Indoor	Horizontal bars	1–1	*Bacillus* sp.
		1–2	*Bacillus mycoides*
		1–3	*Bacillus* sp.
		1–4	*Pantoea agglomerans*
	Parallel bars	2–1	*Bacillus altitudinis*
		2–2	*Staphylococcus* sp.
	Climbing bars	3–1	*Bacillus megaterium*
		3–2	*Bacillus* sp.
	Monkey bars	4–1	*Bacillus altitudinis*
		4–2	*Bacillus* sp.
		4–3	*Bacillus* sp.
Outdoor	Dumbbell	5–1	*Bacillus subtilis*
		5–2	*Bacillus* sp.
		5–3	*Bacillus* sp.
		5–4	*Bacillus* sp.
		5–5	*Bacillus oleronius*
		5–6	*Staphylococcus haemolyticus*
		5–7	*Sporosarcina* sp.
		5–8	*Bacillus megaterium*
		5–9	*Bacillus* sp.
		5–10	*Bacillus altitudinis*
		5–11	*Bacillus* sp.
		5–12	*Bacillus subtilis*
		5–13	*Bacillus* sp.
	Barbell	6–1	*Staphylococcus haemolyticus*
		6–2	*Staphylococcus hominis*
		6–3	*Pantoea* sp.
		6–4	*Bacillus* sp.
	Crunch bench	7–1	*Brevibacillus* sp.
		7–2	*Enterobacter hormaechei*
		7–3	*Bacillus* sp.
		7–4	*Bacillus* sp.
	Leg extension	8–1	*Staphylococcus hominis*
Control	Ground	9–1	*Pseudomonas oryzihabitans*

Analysis of microbial community α-diversities on the surfaces of gymnastic equipment

Alpha diversity analysis, including Chao1, ACE, Shannon and Simpson indices, was performed to calculate microbial community richness and diversities (Figure 2). The Shannon and Simpson indices were employed to measure community diversity, and the Chao1 and ACE indices were chosen to measure community richness. As shown in Figure 2, the microbiome in monkey bars had the highest richness and diversity of any piece of equipment, while horizontal bars had the lowest. Besides, the microbial community composition on the surfaces of gymnastic equipment was generally more diverse and rich than those on the surface of the ground and door, assessed with Tukey and Wilcoxon tests, suggesting human activities indeed altered the microbial community.

Similarities between microbial communities

Non metric multidimensional calibration method (NMDS) is a common technique to determine similarities and grouping of high-dimensional data in ecology or microbial ecology (Shepard, 1980). With each point representing a sample, we were able to use NMDS to assess the similarities between microbiomes collected from gymnastic facilities. It can be shown in Figure 3 that a significant difference is present between the surface microbiota of outdoor and indoor equipment, as well as between the surface microbiota of gymnastic equipment and the environmental microbiota (ANOSIM, $p = 0.001$; MRPP $p = 0.001$). This is also a clear support that human activities while using gymnastic equipment has a significant impact on microbiome structures.

Enrichment of microbes on the surfaces of different equipment

In order to identify the microbes enriched on the surface of gymnastic equipment, LEfSe, a technique to analyze enriched bacteria in various microbiomes, was applied (Figure 4). Door and ground sample were used as controls for indoor gymnastic equipment and outdoor gymnastic equipment, respectively. Compared with the control microbiota, human-origin opportunistic pathogens, such as *Staphylococcus*, *Rickettsia*, and *Enterobacter*, showed a significant increase, but ambient bacteria decrease to varying degrees on the surface of gymnastic equipment (Figure 4A). In particular, *Staphylococcus* increased significantly on the surface of indoor equipment, while *Enterobacter* bacteria increased significantly on the surface of outdoor equipment (Figure 4B). This is a strong signal that gymnastic equipment may transmit human pathogens.

Identification of opportunistic pathogens and determination of antimicrobial resistance

With the investigations on microbiomes, we found that on both indoor and outdoor equipment, there is an enrichment of opportunistic pathogens from humans. To further demonstrate that the enriched bacteria were indeed viable and potentially pathogenic, microorganisms from the samples were isolated, and antimicrobial resistance was analyzed. A total of 34 isolates were isolated, including 24 strains of *Bacillus* spp., 5 strains of *Staphylococcus*, 2 strains of *Pantoea*, 1 strain of *Sporosarcina*, 1 strain of *Enterobacter*, and 1 strain of *Pseudomonas* (Table 1). Of the five *Staphylococcus* strains isolated, four was haemolytic, with a clear haemolytic ring (beta-haemolysis) on blood agar plate, demonstrating their strong pathogenicity.

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TABLE 2 Antimicrobial susceptibility test and hemolytic test of *Staphylococcus* spp.

		Parallel bars	Dumbbell	Barbell		Leg extension
	Staphylococcus aureus ATCC25923	*Staphylococcus* sp. 2–2	*Staphylococcus haemolyticus* 5–6	*Staphylococcus haemolyticus* 6–1	*Staphylococcus hominis* 6–2	*Staphylococcus hominis* 8–1
CHL	S	S	R	S	S	S
ERY	I	R	R	R	I	S
KAN	S	S	R	S	R	S
TMP	S	S	S	S	S	S
SXT	S	S	S	S	S	R
CIP	S	S	R	I	S	S
RIP	S	S	S	S	S	S
TET	S	S	S	R	S	S
Hemolytic	No	Yes	Yes	Yes	Yes	No

CHL, Chloramphenicol; ERY, Erythromycin; KAN, Kanamycin, TMP, Trimethoprim; SXT, Sulfamethoxazole; CIP, Ciprofloxacin; RIP, Rifampicin; TET, Tetracycline. R, resistant; S, susceptible; I, intermediate.

Antimicrobial susceptibility was tested for all isolated strains of *Staphylococcus* and *Pantoea* (Tables 2, 3). For *Staphylococcus* strains, antimicrobial resistance was tested against chloramphenicol (CHL), erythromycin (ERY), kanamycin (KAN), trimethoprim (TMP), co-trimoxazole (SXT), ciprofloxacin (CIP), rifamycin (RIF), and tetracycline (TET; Table 2). For *Pantoea* strains, apart from CHL, TMP, CIP, and TET, seven extra antibiotics for Gram-negative strains including streptomycin (STR), nalidixic acid (NAL), ampicillin (AMP), cefepime (FEP), ceftazidime (CAZ), imipenem (IPM), tigecycline (TGC) were tested (Table 3). Four of the five strains of *Staphylococcus* were isolated from indoor equipment and most were multidrug resistance. It's worth nothing that *Staphylococcus haemolyticus* 5–6, in addition to being hemolytic, were also resistant to CHL, ERY, KAN and CIP. For the two *Pantoea* strains, *Pantoea agglomerans* 1–4, isolated from the horizontal bars was sensitive to all antibiotics, while *Pantoea* sp. 6–3, isolated from the barbell, is multidrug resistant bacteria. This finding suggested that the pathogenic bacteria isolated from gymnastic equipment, especially the bacteria carried on indoor equipment with pathogenicity and multidrug resistance, threaten human health.

TABLE 3 Antimicrobial susceptibility test of *Pantoea* spp.

Antibiotics		Horizontal bars	Barbell
	Escherichia coli ATCC25922	*Pantoea. agglomerans* 1–4	*Pantoea* sp. 6–3
CHL	S	S	R
STR	S	S	S
NAL	S	S	I
TMP	S	S	S
AMP	S	S	I
CIP	S	S	S
FEP	S	S	S
CAZ	S	S	I
IPM	S	S	S
TGC	S	S	S
TET	S	S	S

CHL, Chloramphenicol; STR, Streptomycin; NAL, Nalidixic acid; TMP, Trimethoprim; AMP, Ampicillin; CIP, Ciprofloxacin; FEP: Cefepime; CAZ, Ceftazidime; IPM: Imipenem; TGC: Tigecycline, TET, Tetracycline. R, resistant; S, susceptible; I, intermediate.

Abundance of ARGs on the surfaces of gymnastic equipment

To further characterize the potential for transmission of AMR in gymnastic equipment, the abundances of the following genes were quantitatively analyzed: 16S rDNA gene (to represent total bacteria levels), the integron integrase gene *intI1* (to characterize mobile genetic elements capable of transmitting ARGs; Hall, 2012), the sulfonamide resistance gene *sul1* (Razavi et al., 2017), and the β-lactam antimicrobial resistance gene bla_{TEM} (Bonomo, 2017).

As shown in Figure 5, the normalized ARG levels increased on different gymnastic equipment surfaces. The integron integrase gene *intI1* significantly increased on the surfaces of parallel bars, dumbbell, and barbell. The abundances of *sul1* significantly increased on the surfaces of the parallel bars, while the abundances of bla_{TEM} increased significantly on the surfaces of monkey bars, dumbbell, barbell, and crunch bench, indicating that the abundance of ARGs on the surfaces of these sports equipment was higher than that in the environment. When comparing the ARGs abundance of indoor and outdoor equipment, it was shown that the abundance of *sul1* on the surfaces of gymnastic equipment was higher than that of outdoor equipment. Taken together, these data suggest that sports equipment can significantly enrich for ARGs, facilitating their transmission and potentially causing the spread of resistance.

Discussion

In universities, gymnastic facilities are accessible to the entirety of students and faculty, which often include 1,000's of people.

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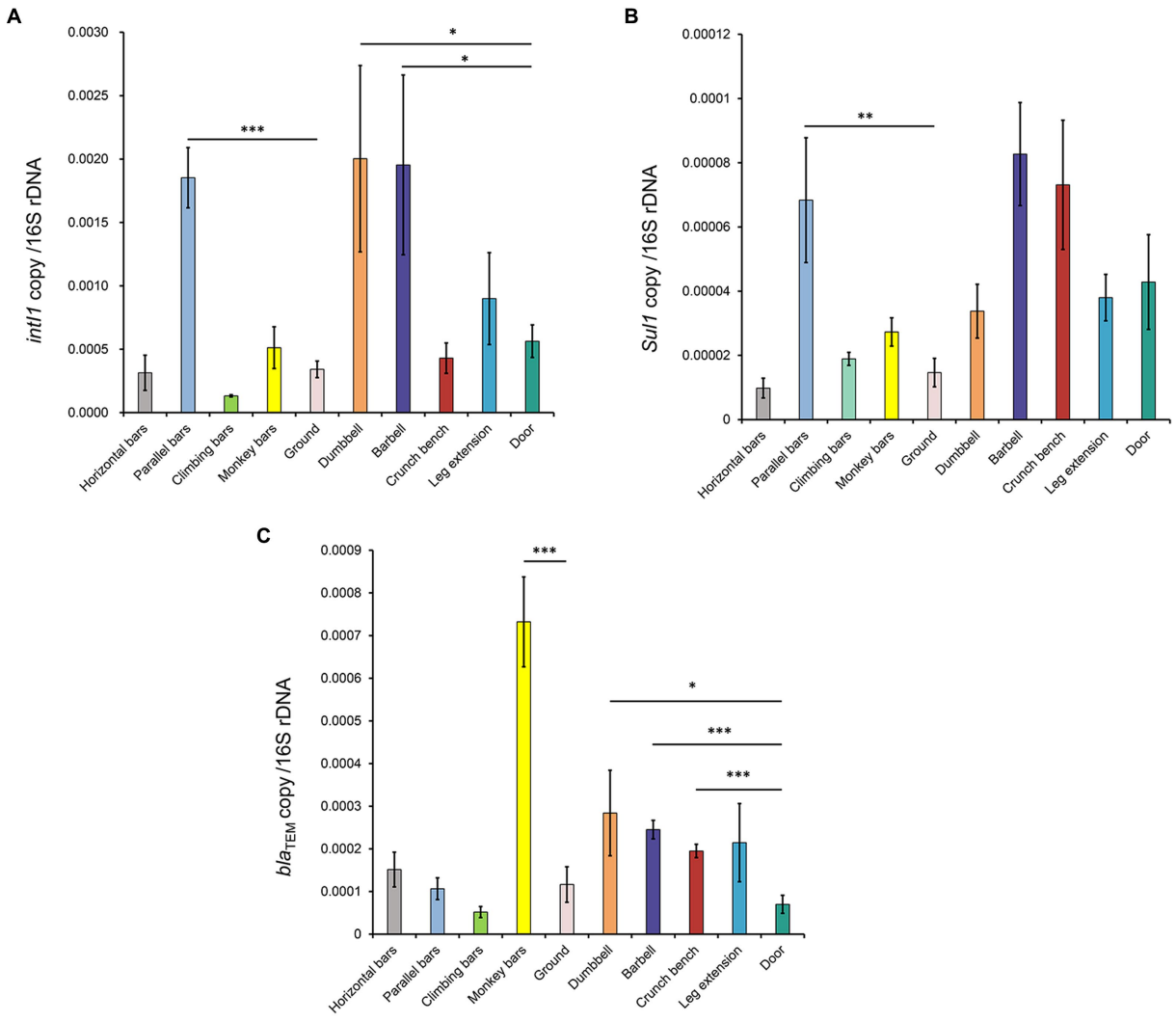

FIGURE 5
Normalized abundance of ARGs on the surfaces of gymnastic equipment. **(A)** *intI1*. **(B)** *sul1*. **(C)** bla_{TEM}. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Considering the frequency of use and direct human contact with equipment, the surfaces of these public gymnastic equipment may act as transmission pathways of pathogenic bacteria, leading to the spread of infection. In this study, the analysis of microbial communities on the surface of gymnastic equipment suggested that the use of gymnastic equipment can indeed significantly change the microbial community structures on the surfaces of equipment, resulting in enrichment of specific pathogenic bacteria on the surface of sports equipment, such as *Staphylococcus* and *Enterobacter*. The rapid increase of bacterial resistance has become one of the urgent problems in the field of medicine and health in the 21st century (Li et al., 2018). In this study, all the 4 strains of hemolytic *Staphylococcus* isolated from indoor equipment were drug-resistant bacteria, among which 3 strains were multidrug-resistant bacteria. In particular, *Staphylococcus hemolyticus* 5–6 isolated from the dumbbell surface is a multidrug-resistant, hemolytic *Staphylococcus* strain, being resistant to 4 of the 8 antibiotics. We summarized the percentage of antibiotic resistance for each isolate and the link between the percentage and presence-absence of resistance genes (see Supplementary materials Table S2, S3; Supplementary Figures S1–S4). By further quantitatively analyzing the abundance of drug resistance genes, we found that not only potential pathogens but also common ARGs (bla_{TEM}, *sul1* and *intI1*) were enriched on the surfaces of gymnastic equipment. This further illustrates the risk of gymnastic equipment in the transmission of bacterial infections.

This study has demonstrated that gymnastic equipment increases risks in the process of bacterial infection transmission. However, it does not mean that we should limit or even avoid using communal gymnastic equipment. On the one hand, the human skin is a powerful barrier against external bacteria. On the other hand, the overall benefits of exercise to improve physical health and immunity outweigh the risk of bacterial infections. However, for certain groups of people, such as those with traumatic skin injuries, those with compromised immune systems and those who are temporarily sick, using public gymnastic equipment for exercise may expose them to the threat of bacterial infections, leading to increased health and economic burdens. To address the above risk of bacterial infection dissemination *via* gymnastic equipment, we recommend that risk assessment and caution is

needed when using public sports equipment for high-risk groups, especially those with surface wounds or lower immunity. Secondly, gymnastic equipment, especially indoor equipment, should be disinfected on a regular basis to prevent the dissemination of bacterial infection and antimicrobial resistance.

Data availability statement

The raw sequencing datasets are available at the Sequence Read Archive (SRA) under BioProject number PRJNA942587.

Author contributions

MZ and YM performed experiments. MZ wrote the manuscript. HX, MW, and LL critically revised the manuscript. LL conceived of the study and oversaw the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1182594/full#supplementary-material

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Molecular epidemiology and comparative genomics of carbapenemase-producing *Escherichia coli* isolates from 19 tertiary hospitals in China from 2019 to 2020

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Background: The clinical use of carbapenems is facing challenges due to increased carbapenemase-producing *Escherichia coli* (CP-EC) infections over the past decade. Meanwhile, whole-genome sequencing (WGS) is an important method for bacterial epidemiological research. We aim to provide more gene-based surveys to explore the genomics and occurrence of CP-EC in China.

Methods: A total of 780 *Escherichia coli* isolates were collected by the China Antimicrobial Resistance Surveillance Trial (CARST) from 2019 to 2020. An antibacterial susceptibility test was performed by using the agar dilution method. CP-EC were detected by the modified carbapenem inactivation method (mCIM), EDTA-modified carbapenem inactivation method (eCIM), and polymerase chain reaction (PCR). Homology analysis was performed by multilocus sequence typing (MLST). A conjugation experiment was performed to verify the transferability of plasmids carrying carbapenemase genes. WGS was conducted to explore the gene-environment of the carbapenemase gene.

Result: Of the 780 *Escherichia coli* isolates, 31 isolates were insensitive to carbapenem with a rate of 4%. Among them, 13 CP-EC isolates had transferability of the bla_{NDM} gene. These isolates belonged to nine distinct sequence types (STs), with some correlation. We found that two (2/13, 15.4%) of the CP-EC isolates that were collected from blood specimens were highly pathogenic and also showed high transferability of the bla_{NDM} gene. In addition, eight (8/13, 61.5%) of the CP-EC isolates were found to be multidrug-resistant.

Conclusion: With the increasing use of carbapenem, CP-EC isolates accounted for nearly half of the total carbapenem-insensitive *Escherichia coli* isolates. Our findings highlight the urgent need to pay attention to CP-EC isolates in bloodstream infections and ESBL-producing CP-EC isolates. Based on the One Health concept, we suggest various measures, including the development of bacterial vaccines, antibiotic management, and establishment of better medical environments, to avoid the outbreak of CP-EC.

KEYWORDS

***Escherichia coli*, carbapenemase, molecular epidemiology, genetic characteristics, bla_{NDM} gene**

1. Introduction

Carbapenems are considered one of the last resort antibiotics against extended-spectrum beta-lactamase-producing gram-negative bacterial infections (Li et al., 2021). However, carbapenem resistance has become a major public health issue, with the World Health Organization listing carbapenem-resistant *Enterobacteriaceae* as the priority bacteria. *Escherichia coli* (*E. coli*), which can cause urinary, respiratory, and bloodstream infections in addition to other pathogens in immunocompromised individuals (Tao et al., 2020), is primarily responsible for community-associated infections, making it difficult to implement traditional preventive measures based on hospital-acquired infections. In China, the isolation rates of carbapenem-resistant *Escherichia coli* (CRECO) have shown an increasing trend in recent years, despite being lower than in previous years. The China Antimicrobial Resistance Surveillance trail (CARST) reported that between 2016 and 2020, the average isolation rates of CRECO were 1.5, 1.5, 1.5, 1.7, and 1.6%, respectively. Although the 2020 isolation rate of CRECO decreased by 0.1% points compared to 2019, it is still higher than the average rate in previous years. Furthermore, the isolation rates of CRECO also varied among regions, with Henan Province showing the highest isolation rates in 2016 and 2018 (3 and 2.9%), and Qinghai Province showing the lowest (0.6 and 0.2%). In 2017, Liaoning Province showed the highest isolation rates (2.8%), while the Tibet Autonomous Region showed the lowest (0.3%). In 2020, Beijing had the highest isolate rates of CRECO (3.2%), while the Tibet Autonomous Region had the lowest proportion (0.2%).

Carbapenem resistance is a growing public health concern due to its widespread dissemination facilitated by carbapenemase, a crucial factor responsible for its transmission. The transfer of genes encoding carbapenemase through conjugative plasmids, transposons, and insertion sequences has led to horizontal transmission (Conlan et al., 2014). In China, carbapenemase-producing *Enterobacterales* (CPE) infections have increased over the past decade, with *E.coli* being the second most common CPE (Deng et al., 2022; Wang et al., 2022). Notably, a majority (76.2%, 16/21) of carbapenemase-producing *Escherichia coli* (CP-EC) cases were detected in a hospital in Shanghai (Zhang et al., 2015). In addition, 35.8% of extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-EC) and 0.9% of CP-EC were isolated from the feces of healthy children's feces in south-central China communities (Liu et al., 2022). Tracing the genetic environment and epidemiology of carbapenemase genes is paramount to public health and aids in designing infection management and prevention strategies.

Polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE) are traditional techniques used in molecular epidemiological research of antimicrobial resistance. However, these techniques have limitations, and whole-genome sequencing (WGS) can be a more effective tool for analyzing antimicrobial resistance. WGS can directly assemble the sequence fragments without designing specific primers to obtain the whole genome of the isolate. The isolate's species, antibiotic resistance genes, virulence factors, and mobile genetic elements can be determined by comparing the whole genome of the isolate with the database. Moreover, genome comparisons of multiple isolates can provide information on the molecular epidemiology of specific resistance genes or mechanisms of antimicrobial resistance. Therefore, WGS and bioinformatics analysis can be more effective in assisting with molecular epidemiological research of antimicrobial resistance. Despite the gene-environment of plasmids has been studied recently, nationwide WGS-based epidemiology of clinical CP-EC has not yet been carried out in China. As a result, we aim to provide more gene-based investigations to explore the genomics and occurrence of CP-EC in China.

2. Materials and methods

2.1. Bacterial strain identification and antimicrobial susceptibility testing

A total of 780 *E. coli* isolates were collected between July 2019 and June 2020 from 19 tertiary hospitals by the China Antimicrobial Resistance Surveillance Trial (CARST) and were preserved by the Institute of Clinical Pharmacology of Peking University. Samples processing, microbial identification, and antibiotic susceptibility testing were performed by the microbiology laboratories of the 19 hospitals, according to a shared protocol. According to CLSI-M100-S31, antibiotic susceptibility testing (AST) was carried out by the reference broth microdilution method. Antimicrobial agents or combinations tested included cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, ertapenem, amikacin, minocycline, tigecycline, ciprofloxacin, and polymyxin E. *E. coli* ATCC25922 was used as the control strain for AST. All tests were performed in duplicate, and three biological replicates per strain were included in each test. PCR was used to amplify the 16SrRNA of the isolates, and the amplified products were sent to Beijing SinoGenoMax Co., Ltd. for sequencing. The isolates were confirmed to be *E. coli* by comparing the sequences in NCBI (https://blast.ncbi.nlm.nih.gov/).

2.2. Detection of carbapenemase phenotypes and genes, and transferability of carbapenemase gene

To detect carbapenemase phenotypes, we employed the modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM), and *Klebsiella pneumoniae* ATCC BAA-1705 and *Klebsiella pneumoniae* ATCC BAA-1706 were utilized as quality controls. We targeted the following carbapenemase genes for PCR detection: bla_{KPC}, bla_{SME}, bla_{IMI}, bla_{GES}, bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{GIM}, bla_{NDM}, and bla_{OXA-48}. To assess the transferability of bla_{NDM}, a conjugation assay was performed on the 13 bla_{NDM}-positive *E. coli* isolates, using sodium azide-resistant *E. coli* J53 as the recipient strain. *E. coli* isolates were analyzed based on genomic sequences using *in silico* MLST and cgMLST analysis on the EnteroBase online platform (http://enterobase.warwick.ac.uk) for *E. coli*. Seven housekeeping gene loci, including *adk, fumC, gyrB, icd, mdh, purA*, and *recA*, were chosen for MLST analysis of *E. coli*. The cluster

analysis is performed by Bionumerics software v.7.0 based on MLST information.

2.3. Whole-genome sequencing and analysis

We performed whole-genome sequencing (WGS) of CP-EC isolates using the Nanopore *De Novo* platform and Circular Library. We prepared the sequencing library using the rapid sequencing kit (Oxford Nanopore Technologies) and subjected the sequencing data to several processing steps. First, we checked the consistency of each base in the original data and assigned a quality value to each base based on the results of the consistency check. Next, we removed residual adapters using strict parameters and filtered out regions with Q20 < 0.8 in the reads to obtain high-quality subreads sequences. These high-quality subreads sequences were then used for subsequent analysis. We assembled the high-quality subreads using the hierarchical genome assembly process (HGAP) 3.0 software, which corrects errors in long reads with short reads. The resulting assembly was further processed using the classic OLC algorithm architecture to obtain the final assembly. To identify transposon sequences, we compared the assembly results with known transposon sequence libraries using RepeatMasker software (using the Repbase database) and RepeatProteinMasker software (using the transposon protein library that comes with RepeatMasker). We also used tandem repeat finder (TRF) software to predict tandem repeats. Contigs were screened for plasmid and resistance gene content using PlasmidFinder and ResFinder tools at the Center for Genomic Epidemiology (CGE) server (http://www.genomicepidemiology.org/services/), respectively. To identify virulence genes related to CP-EC isolates, we utilized VirulenceFinder at CGE and the VFDB database. In addition, we used ISFinder to detect insertion sequences with a threshold of e value1e-5 and generated a gene alignment diagram using Easyfig.

3. Results

3.1. Incidence of carbapenemase gene in carbapenem-resistant *Escherichia coli*

A total of 31 *E.coli* isolates displayed insensitive to meropenem, imipenem, or ertapenem. Among 31 insensitive *E. coli* isolates, the mean minimal inhibitory concentrations (MICs) of meropenem against 50% (MIC_{50}) and 90% (MIC_{90}) were 0.25 and 32 μg/ml, respectively. The MIC_{50} and MIC_{90} values of imipenem were 0.5 and 16 μg/ml, respectively. The MIC_{50} and MIC_{90} values of ertapenem were 4 μg/ml and 128 μg/ml, respectively. Of the 31 isolates, 13 (41.9%), 14 (45.2%), and 30 (96.7%) were insensitive to meropenem, imipenem, and ertapenem, respectively. In addition, 96% (29/31) of isolates exhibited resistance to third/fourth-generation cephalosporins. Clinical information about the 31 *E. coli* isolates is presented in Table 1. Thirteen carbapenemase-producing *E. coli* (CP-EC) isolates were confirmed to produce metallo-β-lactamase using the modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM). Of these 13 CP-EC isolates, only bla_{NDM} was detected, without any other carbapenemase genes. Among these, nine isolates (69.2%) carried bla_{NDM-5}, three isolates (23.1%) carried bla_{NDM-1}, and one isolate (7.7%) carried bla_{NDM-7}. The genome-based phylogenetic analysis revealed the presence of nine CP-EC lineages: ST167 (3 isolates), ST405 (3 isolates), ST744, ST10, ST361, ST1193, ST488, ST410, and ST349 (refer to Figure 1 and Table 2 for details).

The clonal relationship between CP-EC ST448 and ST410 was found to be highly related, with ST410 originating from ST448. In addition, the three NDM-1 producing CP-EC isolates were classified under ST349, ST744, and ST1193, respectively. Notably, NDM-7 producing CP-EC isolate, 19F065, was assigned to ST405. Furthermore, ST167, ST10, and ST744 exhibited close relatedness, with ST167 and ST744 emerging as derivatives of ST10. These findings suggest a complex evolutionary history of CP-EC.

3.2. Plasmid content of carbapenemase-producing *E. coli*

The successful transfer of the carbapenemase gene to *E. coli* J53 recipients was observed in all 13 isolates, with a minimum of a 32-fold increase in the MIC of carbapenems for the transconjugants. Plasmid content was evaluated by PlasmidFinder and is presented in Table 2. IncX3 was the predominant plasmid type observed in CP-EC (8/13, 61.5%), followed by IncFII (3/13, 23.1%). The average length of the plasmids was 50,000 bp, of which p19D107 and p19F412 were longer, with a length of 95,105 bp and 268,028 bp, respectively.

3.3. Genomic analysis of carbapenemase-producing *E. coli*

The plasmids of the 13 CP-EC isolates were found to harbor mobile genetic elements and insertion sequences (ISs), as determined by genomic analysis. IS*Aba125* was frequently located upstream of the genomic region (5/13, 38.5%), while IS*15* was frequently located downstream (9/13, 69.2%). The plasmid carrying bla_{NDM-7}, specifically p19F065, had a similar genetic environment to those carrying bla_{NDM-5}, such as p19F407 and p19R077. Notably, among the nine isolates carrying bla_{NDM-5}, p19R077 and p19F407 exhibited nearly identical genetic environments, with IS*Aba125*, IS*C1041*, and IS*5B* detected upstream of bla_{NDM-5}.

3.3.1. Analysis of carbapenemase gene-environment

The plasmid p19U139, classified as an IncN plasmid, carries the carbapenemase gene bla_{NDM-1}. IS*1X2* and IS*61009* were identified upstream of p19U139, while IS*Ssu9* was found downstream. Meanwhile, the plasmids p19F388 and p19M348, which also carry bla_{NDM-1}, were classified as IncX3 plasmids. Specifically, p19F388 contained two IS*3000* elements, one *Tn2* transposon, and one IS*5* element, while p19M348 had an IS*Aba125* insertion between IS*3000* and IS*5*. These two plasmids shared a similar downstream gene-environment, consisting of an IS*Kox3* element

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TABLE 1 Clinical information of 31 *Escherichia coli* isolates.

Isolates	Gender	Age	Age classification	Year	Specimen classification
19D107	Male	17 days	Children	2019	Secretion
19F065*	Male	60	Adult	2019	Sputum
19R077	Male	79	Elder	2019	Cerebrospinal fluid
19R081	Female	51	Adult	2019	Bloodstream
19R092	Male	36	Adult	2019	Secretion
19U139	Male	53	Adult	2019	Bloodstream
19F053	Male	61	Adult	2019	Drainage
19F054	Male	52	Adult	2019	Bloodstream
19G152	Male	74	Elder	2019	Bloodstream
19M056	Male	35	Adult	2019	Urine
19O014	Female	78	Elder	2019	Bloodstream
19O017	Male	27	Adult	2019	Drainage
19P164	Female	40	Adult	2019	Bloodstream
19U138	Female	77	Elder	2019	Bile
19W025	Female	51	Adult	2019	Secretion
19F384	Female	65	Elder	2020	Bloodstream
19F388	Male	82	Elder	2020	Bile
19F407	Male	12	Children	2020	Bloodstream
19F412	Male	33	Adult	2020	Bloodstream
19G174	Female	75	Elder	2020	Bloodstream
19M348	Male	66	Elder	2020	Drainage
19T247	Male	38	Adult	2020	Bloodstream
19F395*	Female	84	Elder	2020	Bloodstream
19G160	Female	26	Adult	2020	Bloodstream
19G179	Female	66	Elder	2020	Bloodstream
19K198	Female	35	Adult	2020	Drainage
19O191	Female	88	Elder	2020	Bloodstream
19Q278	Female	51	Adult	2020	Urine
19Q287*	Male	54	Adult	2020	Drainage
19R296	Female	83	Elder	2020	Urine
19R360*	Male	75	Elder	2020	Secretion

The age groups in this study were defined as follows: children, defined as patients aged 14 years or younger, and elders, defined as patients aged 65 years or older. ICU sources are indicated by an asterisk (*) in the table.

belonging to the IS*L3* transposase family and multiple IS*26* elements of the IS*6* transposase family, which carried the bla_{SHV-2} gene at a distance. According to the gene alignment diagram, the core structure of the IncX3 plasmid is diverse by carrying different bla_{NDM} subtype genes. For instance, the core structures of IncX3 plasmid carrying the bla_{NDM-1} and bla_{NDM-5} genes are IS*3000*-IS*5*-bla_{NDM-1}-IS*26*-IS*Kox3* and IS*3000*-IS*Aba125*-IS*5*-bla_{NDM-5}-IS*26*-IS*Kox3*, respectively. On the other hand, the IncFII plasmid carrying the bla_{NDM-5} gene has a different core structure: IS*26*-bla_{NDM-5}-IS*Ssu9*-IS*26*-Tn*As1*. For further details, refer to Figure 2A.

3.3.2. Analysis of the bla_{NDM-5} gene-environment in the IncFII plasmid

The gene-environments of the IncFII plasmids, namely p19D107, p19G174, and p19F384, show a high degree of similarity, as all three contain an adjacent reverse IS*26* element located upstream of the bla_{NDM-5} gene. In addition, these plasmids possess IS*26*, ISSsu9, and TnAs1 elements at a distance from the bla_{NDM-5} gene (refer to Figure 2B). Conversely, p19F412 is a longer IncI1-I plasmid that shares a partially similar gene-environment with p19D107.

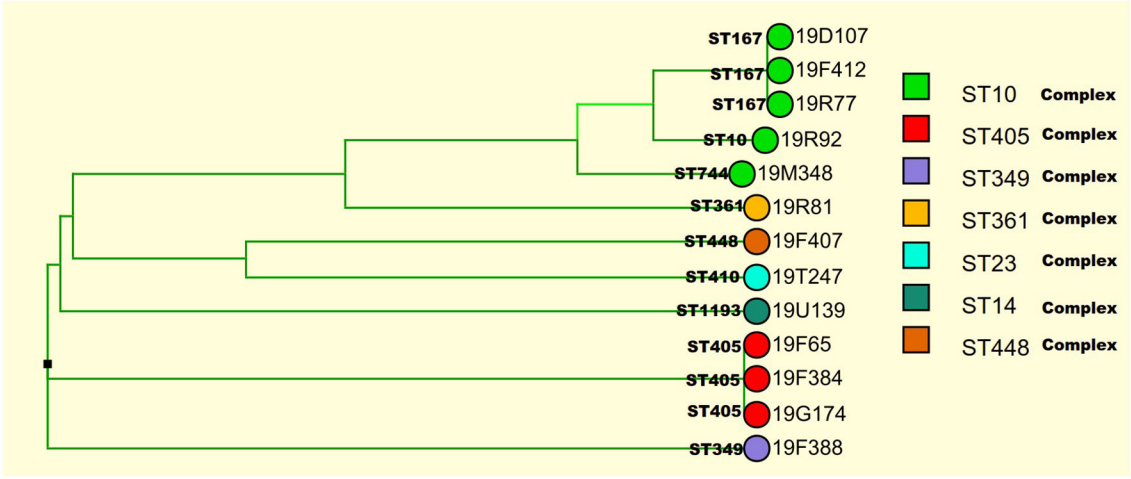

FIGURE 1
Identification of 13 CP-EC lineages using genome-based phylogenetic analysis.

TABLE 2 Phylogenetic analysis and plasmid content of 13 CP-EC isolates.

Isolates	NDM type	Sequence type (ST)	Plasmid type	Plasmid length
19F065	bla_{NDM-7}	ST405	IncX3	46,159 bp
19F388	bla_{NDM-1}	ST349	IncX3	56,904 bp
19F407	bla_{NDM-5}	ST448	IncX3	46,158 bp
19M348	bla_{NDM-1}	ST744	IncX3	54,035 bp
19R077	bla_{NDM-5}	ST167	IncX3	46,163 bp
19R081	bla_{NDM-5}	ST361	IncX3	48,787 bp
19R092	bla_{NDM-5}	ST10	IncX3	45,071 bp
19T247	bla_{NDM-5}	ST410	IncX3	52,884 bp
19U139	bla_{NDM-1}	ST1193	IncN	54,734 bp
19F412	bla_{NDM-5}	ST167	IncI	268,028 bp
19D107	bla_{NDM-5}	ST167	IncFII	95,105 bp
19F384	bla_{NDM-5}	ST405	IncFII	95,073 bp
19G174	bla_{NDM-5}	ST405	IncFII	91,572 bp

3.3.3. Analysis of bla_{NDM-5} and bla_{NDM-7} gene-environment in IncX3 plasmids

p19F065, p19F407, p19R077, and p19T247 are all IncX3 plasmids with the same upstream and downstream gene-environments. These plasmids have a segment of IS3000, IS*Aba125*, and IS*5* upstream and a segment of IS*26*, IS*Kox3*, and Tn*552* downstream. p19R092 has a missing IS*Aba125* upstream, and IS*Kox3* downstream was divided into two segments. The bla_{NDM-5} gene in p19R081 is located at the edge of the genome, so the downstream environment is unknown. Unlike other plasmids, p19R081 has IS*3411* and IS*629*, dividing IS*3000* into two segments (Figure 2C).

3.3.4. Resistome and virulence genes of carbapenemase-producing *E. coli*

The resistome of CP-EC isolates is visualized in Figure 3, revealing the presence of 16 antibiotic resistance genes linked to various antibiotic classes, including β-lactams, aminoglycosides, sulfamide, trimethoprim, quaternary ammonium salt disinfectant, and fosfomycin. Notably, five isolates (5/13, 38.5%) were found to carry extended-spectrum β-lactamase (ESBL) genes, among which bla_{SHV-12} and bla_{CTX-M} were identified in two and three isolates, respectively.

Virulence genes were detected in each isolate with a variable distribution (Figure 4). The majority of isolates (10/13, 76.9%) harbored sequences encoding type IV secretion system (T4SS),

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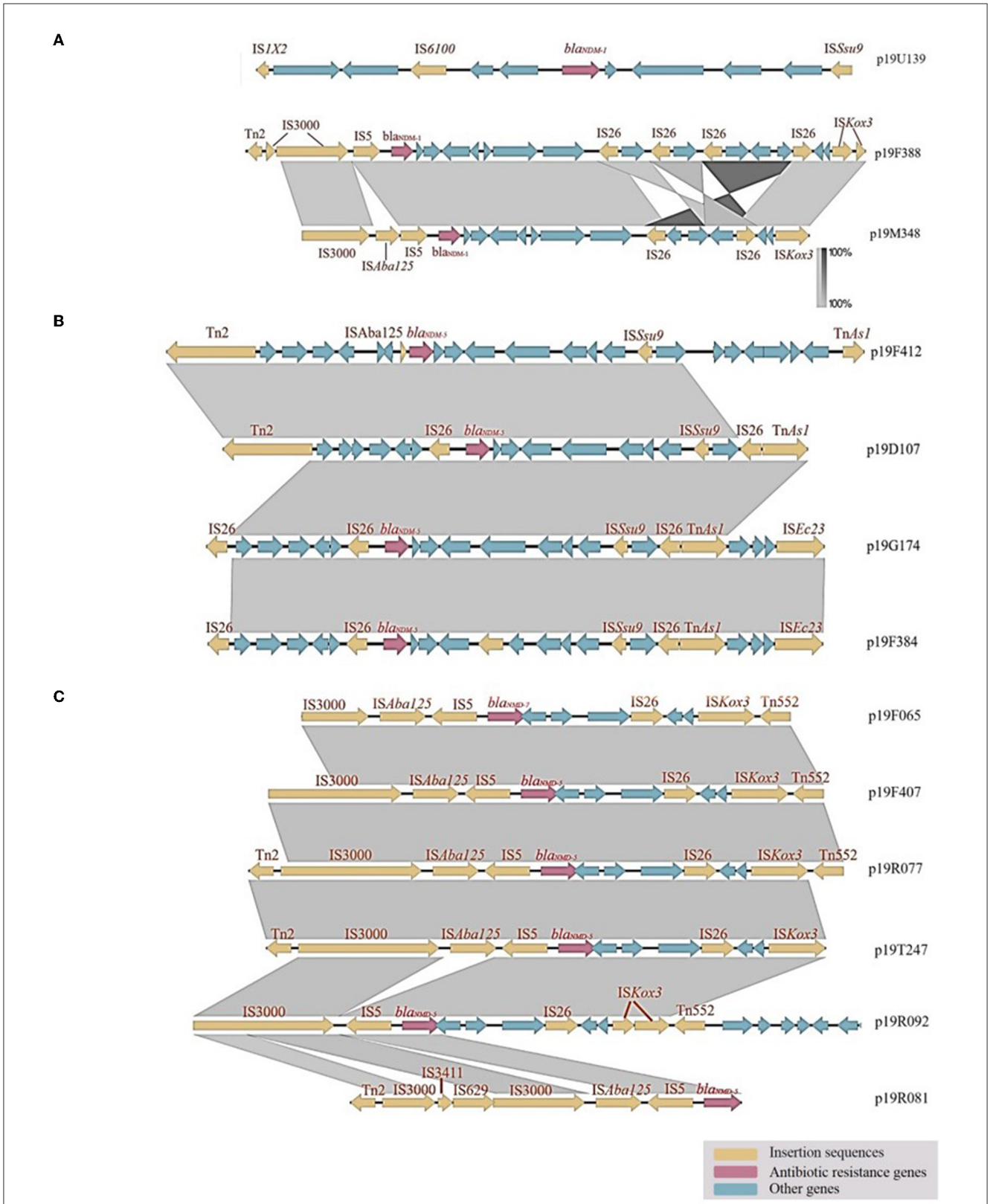

FIGURE 2
Genetic analysis of carbapenemase-producing *E. coli* isolates: Insights from gene alignment and gene-environment analysis of bla_{NDM-1}, bla_{NDM-5}, and bla_{NDM-7} in different plasmids. **(A)** The gene-environment of p19U139 was found to differ from that of other plasmids, with IS*6100* and IS*1X2* belonging to the IS*6* and IS*1* transposase families, respectively. **(B)** In the IncFII plasmid, IS*26* was found to carry both bla_{NDM-5} and *emrE*, a gene related to an efflux protein. **(C)** Analysis of bla_{NDM-5} and bla_{NDM-7} gene-environment in IncX3 plasmids.

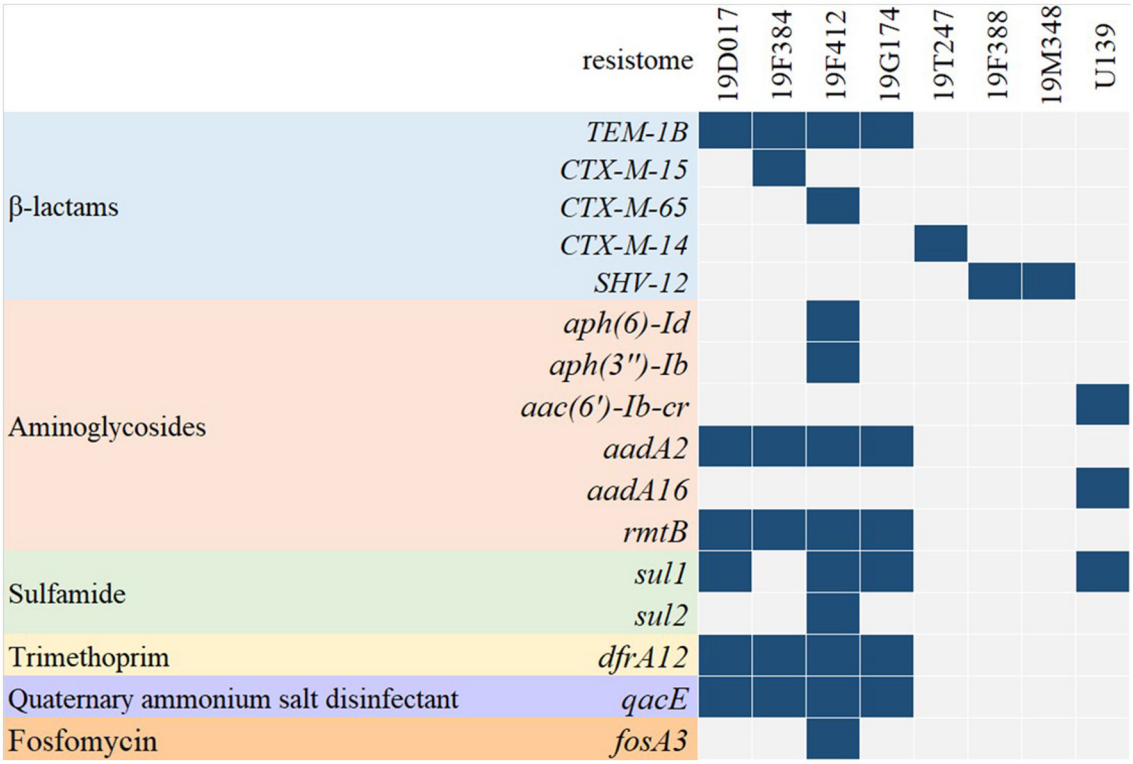

FIGURE 3
Comprehensive analysis of the resistome and prevalence of ESBL genes in CP-EC isolates.

specifically *virB4, virB9, trwD, trwE, trwF, trwG, trwK, ptlH, virD4, icmO/dotL, dotO,* and *dotC* (Christie et al., 2014). In addition, the *traj* gene, related to the plasmid conjugation transfer region, was found in one isolate (Liu G. et al., 2019). In the p19F412 isolate, several sequences encoding adhesion proteins were identified, including *PilU, pilV, pilS, pilR, pilQ, pilO, pilN,* and *pilM*, involved in pili synthesis (Foley et al., 2021); *IutA, iucD, iucC, iucB, iucA, sitD, sitC, sitB,* and *sitA*, encoding iron transport and siderophore proteins (da Cruz Campos et al., 2020); and *icsP/sopA*, associated with outer membrane protease (Tran et al., 2013). Furthermore, the type III secretion system (TTSS) invasion antigen, *IpaH2.5*, was identified in 19F412 and 19R081 (Tran et al., 2013), while the *htp* operons, a molecular chaperone related to heat shock protein Hsp60, were present in four isolates. The colonization factor antigen III-encoding *cofT* genes, facilitating bacterial adherence to the host, were identified in three isolates. Interestingly, IncX3 plasmids carried several genes associated with T4SS, while IncFII plasmids did not possess genes related to TTSS or T4SS. Remarkably, p19F412 and p19R081 isolates contained genes associated with both TTSS and T4SS.

4. Discussion

The prevalence and impact of CPE vary globally. In the United States, CPE is responsible for approximately 9,300 hospital-acquired infections annually, and approximately 50% of patients with bloodstream infections (BSIs) caused by CPE ultimately succumb to the infection (Villegas et al., 2016). Meanwhile, in central China, a surveillance report identified *E. coli* as one of the main pathogens causing bloodstream infections (Tian et al., 2018). In our study, we found that CP-EC from blood specimens accounted for 53.8% (7/13) of the total CP-EC, with bla_{NDM-5}-producing isolates making up the majority, two of which belonged to ST405. These isolates were capable of transmitting carbapenemase genes through IncFII, IncI, IncN, and IncX3, with p19F412 and p19R081 carrying both TTSS and T4SS, making them highly transferable and pathogenic.

The dissemination of CPE isolates is a serious concern as these infections require aggressive and prolonged antibiotic therapy, leading to an increased risk of adverse events and prolonged hospitalization. A recent investigation in Australia found that patients colonized with CP-EC had a significantly longer hospital stay and higher medical costs during an outbreak (Rodriguez-Acevedo et al., 2020). These findings underscore the importance of managing and preventing future outbreaks caused by CP-EC.

The co-occurrence of carbapenemases with other β-lactamases, such as TEM, SHV, or CTX-M, confers resistance in CPE to almost all β-lactam antibiotics (De Angelis et al., 2020). In our study, 61.5% of the 13 isolates belonged to multidrug-resistant CP-EC, with seven isolates producing TEM, CTX-M, and SHV. The co-occurrence of these resistance mechanisms can pose challenges in clinical laboratories since carbapenemases are not inhibited by clavulanic acid, which is used in the standard double-disk test for identifying ESBLs recommended by the Clinical and Laboratory Standards Institute (CLSI). As a result, detecting multiple β-lactamases in CP-EC can be difficult, which may

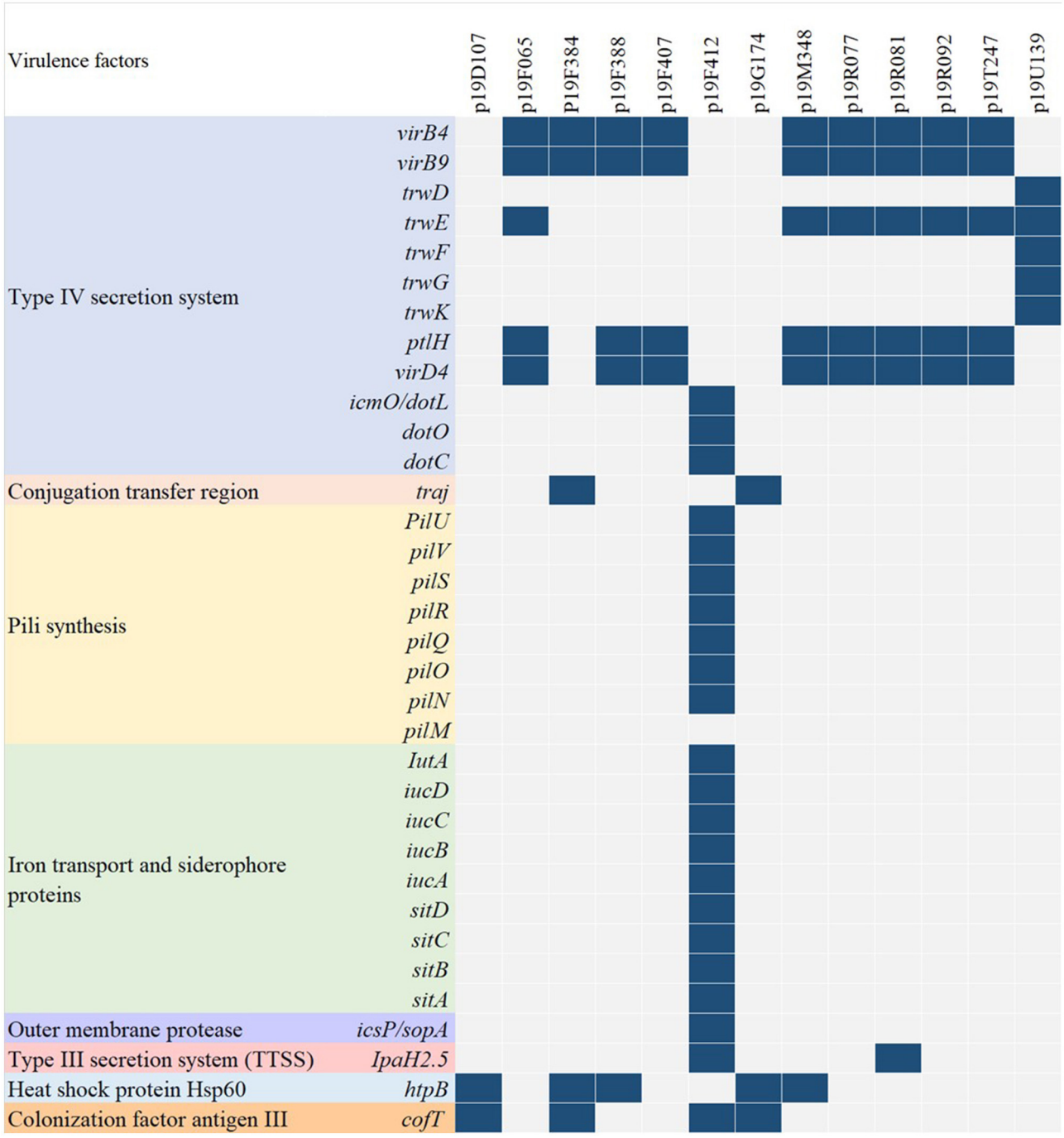

FIGURE 4
Variable distribution and characterization of virulence genes in CP-EC isolates.

lead to heightened transmission and outbreaks in healthcare settings and community-acquired infections. The increased use of carbapenems has led to the gradual detection of CP-EC isolates in the stool samples of healthy individuals in the community (Bezabih et al., 2021). Furthermore, CP-EC isolates are not restricted to human communities or healthcare settings and are increasingly being detected in food-producing animals (Bonardi and Pitino, 2019). CTX-M-producing *E. coli* was the predominant multidrug-resistant *E. coli* isolates in food-producing animals (Michael et al., 2017), with ESBL-producing *E. coli* isolates from pigs in China mainly producing CTX-M (Tian et al., 2009). In addition, CTX-M-14, CTX-M-55, and CTX-M-65 were the most prevalent genotypes among CTX-M-producing *E. coli* isolates from food animals in China (Rao et al., 2014). These isolates may be transmitted to humans through food consumption or contact with animals and can cause serious infections that are difficult to treat. The overuse of antibiotics in food-producing animals has been identified as a major driver of antibiotic resistance, and the detection of CP-EC

isolates in these animals further underscores the urgent need for better antibiotic stewardship practices.

We identified three CTX-M-producing isolates that carried multidrug resistance genes by IncI and IncX3. IncI plasmids have been found to facilitate the transmission of the bla_{CTX-M} between poultry and humans (Kopotsa et al., 2019), highlighting their potential as vectors for expanded carbapenemase gene transmission. Furthermore, IncI plasmids have been shown to promote transmission between mothers and newborns, suggesting that they may increase the transmission of carbapenemase genes in neonatal infections (Hagb et al., 2020).

The clonal relationship analysis revealed that the spread of 13 NDM-producing *E. coli* was polyclonal, with common *E. coli* sequence types such as ST167 and ST405 carrying most bla_{NDM-5}. However, rare *E. coli* sequence types, such as ST744, ST349, and ST1193, were found to carry bla_{NDM-1}. Among the plasmids observed in CP-EC, IncX3 was the predominant plasmid type, accounting for 61.5% (8/13) of isolates. The remarkable stability and efficient horizontal transfer of the IncX3 plasmid can be attributed to its low fitness cost, which can enhance its potential to spread between bacterial populations (Ma et al., 2020). In transconjugants, the presence of the IncX3 plasmid resulted in significantly higher levels of carbapenem resistance and bla_{NDM-5}, compared to transconjugants carrying both plasmids or only the IncFII plasmid (Yang et al., 2020). Notably, studies have demonstrated that the IncX3 plasmid can mediate the transfer of bla_{NDM-5}genes among different *Enterobacter* species over a wide range of temperatures (Liu Z. et al., 2019). The IncX3 plasmid is not only the most prevalent vehicle for bla_{NDM-5} but also a critical platform for the evolution of bla_{NDM-5}, which has led to the emergence of novel NDM variants (Wu et al., 2019). Our findings indicate that the presence of IncX3 plasmid is widespread among CP-EC, which could further limit medication options and exacerbate the burden on the healthcare system.

Our study had several limitations that should be acknowledged. First, while we were able to access the specimens for the CP-EC, we did not have access to information on the patient's diagnosis or antibiotic prescriptions. This lack of information may have impacted our ability to fully understand the epidemiology and clinical significance of CPE infections. Second, although we included hospitals from various regions throughout China, the sample size was limited, which may have restricted our ability to generalize our findings to the entire population of China. Therefore, future studies should focus on collecting more comprehensive clinical data and engage in longer-term surveillance to enable a more in-depth investigation of CPE prevalence and characteristics across China. By doing so, we can gain a more comprehensive understanding of this emerging public health threat and develop effective strategies to control its spread.

5. Conclusion

With the increasing use of carbapenem, CP-EC clinical isolates accounted for nearly half of the total CRECO clinical isolates. We need to pay more awareness to CP-EC in BSI because the isolates were transferable and pathogenicity, which increases the burden on patients and medical settings. Currently, ESBL-producing CP-EC isolates have been found in humans, animals, and the environment, leading to antibiotics' low selectivity. Based on the concept of One Health, we should avoid the outbreak of CP-EC through various methods, including developing bacterial vaccines, originating antibiotic management, and establishing a better medical environment.

Data availability statement

The datasets presented in this article are not readily available because data is precious scientific research and therefore not publicly available. Requests to access the datasets should be directed to michelleko8688@qq.com.

Author contributions

YLi and YLv conceived the study. WK, ST, CC, and TL conducted all experiments. WK and CC analyzed the WGS data and drafted subsequent versions. TL, RL, and YZ interpreted the findings. WK and ST wrote the first draft of the manuscript. All authors acquired the data, critically reviewed this report, and approved the final version. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Spatiotemporal dissemination of ESBL-producing Enterobacterales in municipal sewer systems: a prospective, longitudinal study in the city of Basel, Switzerland

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Background: The contribution of community and hospital sources to the transmission of extended-spectrum β-lactamase producing Enterobacterales (ESBL-PE) remains elusive.

Aim: To investigate the extent of community dissemination and the contribution of hospitals to the spread of ESBL-PE by exploring their spatiotemporal distribution in municipal wastewater of the central European city of Basel.

Methods: Wastewater samples were collected monthly for two consecutive years throughout Basel, Switzerland, including 21 sites across 10 postcode areas of the city collecting either community wastewater (urban sites, n=17) or community and hospital wastewater (mixed sites, n=4). Presumptive ESBL-PE were recovered by selective culture methods. Standard methodologies were applied for species identification, ESBL-confirmation, and quantification.

Results: Ninety-five percent (477/504) of samples were positive for ESBL-PE. Among these isolates, *Escherichia coli* (85%, 1,140/1,334) and *Klebsiella pneumoniae* (11%, 153/1,334) were most common. They were recovered throughout the sampling period from all postcodes, with *E. coli* consistently predominating. The proportion of *K. pneumoniae* isolates was higher in wastewater samples from mixed sites as compared to samples from urban sites, while the proportion of *E. coli* was higher in samples from urban sites (p=0.003). Higher numbers of colony forming units (CFUs) were recovered from mixed as compared to urban sites (median 3.2×10^2 vs. 1.6×10^2 CFU/mL). *E. coli*-counts showed moderate correlation with population size (rho=0.44), while this correlation was weak for other ESBL-PE (rho=0.21).

Conclusion: ESBL-PE are widely spread in municipal wastewater supporting that community sources are important reservoirs entertaining the spread of ESBL-PE. Hospital-influenced abundance of ESBL-PE appears to be species dependent.

KEYWORDS

ESBL-producing Enterobacterales, wastewater, *Escherichia coli*, *Klebsiella pneumoniae*, spatiotemporal distribution

Introduction

Members of the order Enterobacterales are ubiquitous and form a major part of the microbiota of the human and animal intestine (Partridge, 2015). Some of them, such as *Escherichia coli* and the *Klebsiella, Enterobacter, Serratia, and Citrobacter* (KESC) group cause infections, including in the urinary tract, bloodstream, and respiratory tract, as well as intestinal and intra-abdominal infections (Pitout and Laupland, 2008; Qin et al., 2008). Extended-spectrum β-lactamase producing Enterobacterales (ESBL-PE) have been classified as serious and critical threats by public health authorities, such as the Centers for Disease Prevention and Control (CDC) and the World Health Organization (WHO) (Tacconelli et al., 2018; Centers for Disease Control and Prevention, 2019). Despite the implementation of infection prevention and control measures aiming to limit further spread in healthcare settings, the incidence especially of ESBL-producing *E. coli* and *Klebsiella pneumoniae* continues to increase worldwide (Jernigan et al., 2020).

Initially considered mainly hospital-acquired, the current epidemiology of ESBL-PE points to the increasing importance of community-based transmission (Pitout et al., 2005; Chong et al., 2018). Community-acquired ESBL sources include human-to-human transmission within or between households in the open community (Mughini-Gras et al., 2019), foodstuffs (Kluytmans et al., 2013), companion and farm animals (Mughini-Gras et al., 2019), and colonization resulting from global travel, especially to South Asia (Kuenzli et al., 2014). Water bodies worldwide have proven to be vast reservoirs of clinically significant antimicrobial resistant organisms (Aarestrup and Woolhouse, 2020). In line with this, ESBL-PE have been recovered in water samples from Swiss rivers and lakes (Zurfluh et al., 2013), possibly constituting an underappreciated route for further dissemination. Contamination of water bodies by anthropogenic discharges via biological spills, antibiotics and their active metabolites as well as urine and feces, is a significant contributor to the selection and widespread dissemination of antimicrobial resistance (AMR) (Hooban et al., 2021). With these premises, hospital and community sewage may represent relevant sources of ESBL-PE. Yet, the potential variable contribution of different clinically relevant species among Enterobacterales, such as *E. coli* and *K. pneumoniae*, remains to be elucidated.

Although humans are the main source of community-acquired carriage of ESBL-producing species such as *E coli*, transmission within the community alone might not be self-sustaining without mobilization to and from non-human sources (Mughini-Gras et al., 2019). Hence, detailed knowledge on the specific contribution of different sources of ESBL-PE is essential for an efficient allocation of resources and as a basis for tailored infection prevention and control strategies. Most recently, environmental surveillance of municipal wastewater has been shown to be most useful in tracking emerging bacteria and viruses and monitoring their changing epidemiology (Hendriksen et al., 2019; Bagutti et al., 2022).

To obtain a deeper understanding of ESBL-PE circulation in both hospital and community settings, we explored the spatiotemporal distribution of ESBL-PE in urban wastewater collected throughout the sewage system of the city of Basel, Switzerland, representing both community and hospital sources. We further determined the abundance of presumptive ESBL-producing *E. coli* and KESC group to explore bacterial load distribution across the municipal sewage system as well as correlations with socio-economic population determinants and meteorological data.

Materials and methods

Study design

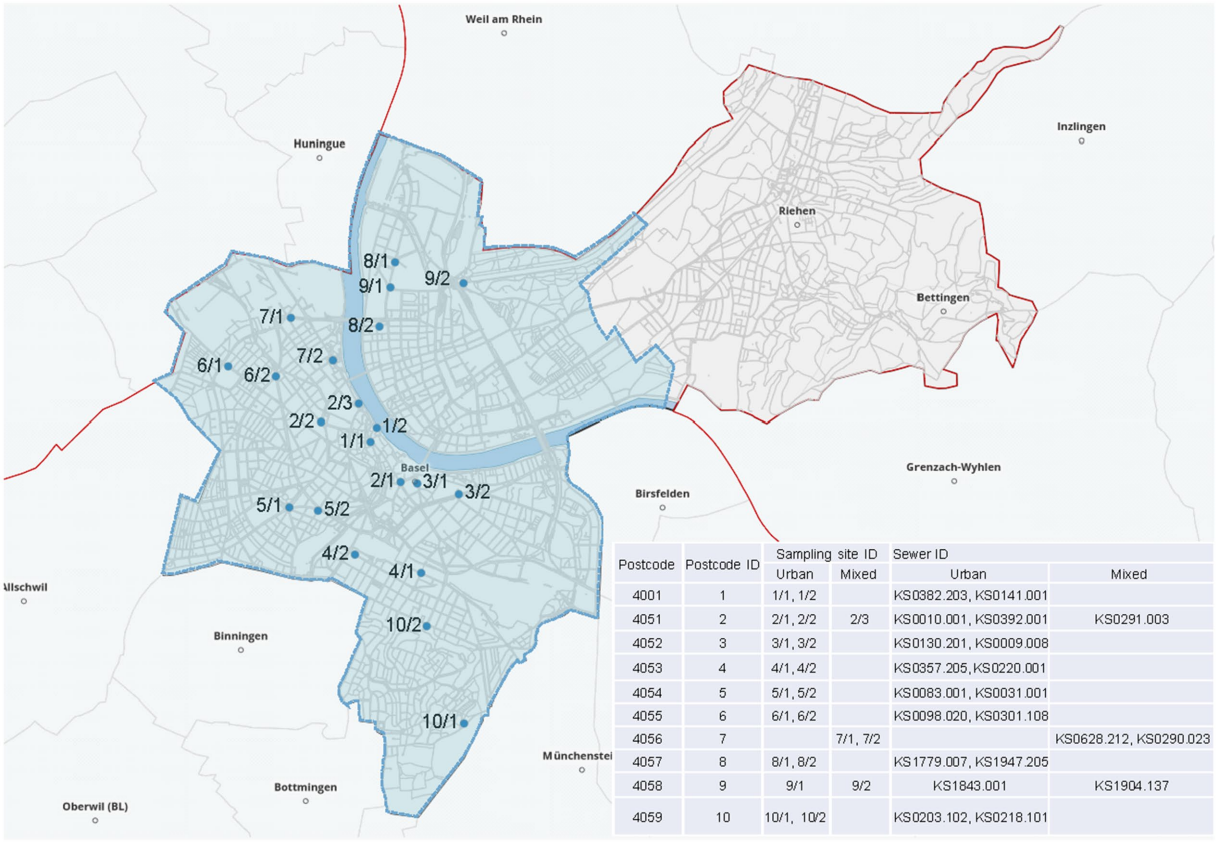
This prospective and cross-sectional longitudinal study (Stadler et al., 2018) (pre-registered on ClinicalTrials.gov; identifier NCT03465683) was performed in the city of Basel, Switzerland, over a 24-month period. Municipal wastewater representing the city's sewage system and covering 44% of the population of Basel was sampled and analyzed for the presence of ESBL-PE. Sampling sites were categorized based on the wastewater sources received, as urban (representing the community without wastewater from healthcare settings) and mixed (representing both community and healthcare settings).

Setting

Basel, a central European city organized in 10 different postcode areas, has a population of around 180,000 inhabitants. Its healthcare system comprises 14 hospitals. Among these, the University Hospital Basel, a >600-bed tertiary care academic center that admits over 35,000 patients per year, constitutes the largest hospital in the city. The sewage system of Basel is an underground sewer network that receives sanitary hospital wastewaters without pre-treatment in accordance with the current national legislation.

Sampling and data sources

Wastewater samples were collected monthly for two consecutive years (June 2017 until June 2019), except for July 2018 during which sampling could not be performed. Therefore, an additional month was included at the end of the study (June 2019). Twenty-one sampling sites (two per postcode area) best reflecting the wastewater of the entire city were chosen. Three sampling sites (4,056/1, 4,056/2, and 4058/2) compile both community and hospital wastewater. One sampling site (4,051/3) was included to capture wastewater from the University Hospital Basel. This sampling point collects community water and 35–40% of the hospital sewage. The remaining 17 sites



Postcode	Postcode ID	Sampling site ID		Sewer ID	
		Urban	Mixed	Urban	Mixed
4001	1	1/1, 1/2		KS0382.203, KS0141.001	
4051	2	2/1, 2/2	2/3	KS0010.001, KS0392.001	KS0291.003
4052	3	3/1, 3/2		KS0130.201, KS0009.008	
4053	4	4/1, 4/2		KS0357.205, KS0220.001	
4054	5	5/1, 5/2		KS0083.001, KS0031.001	
4055	6	6/1, 6/2		KS0098.020, KS0301.108	
4056	7		7/1, 7/2		KS0628.212, KS0290.023
4057	8	8/1, 8/2		KS1779.007, KS1947.205	
4058	9	9/1	9/2	KS1843.001	KS1904.137
4059	10	10/1, 10/2		KS0203.102, KS0218.101	

FIGURE 1
Distribution of the wastewater sampling sites across the city of Basel. The coding 1–10/1–3 refers to postcode area (1–10) and sampling site (1–3) specifying the district. The blue background delimits the city of Basel. Copyright by Google Maps.

receive wastewater from the general population. The specific location of the sampled sewers is displayed in Figure 1. Supplementary Table S1 denotes the sites receiving hospital water, displays population size per postcode area, and defines the catchment area per sampling point. The samples were collected directly from the sewage system by the Civil Engineering Department of the Canton of Basel-Stadt following the specific recommendations of the World Health Organization (WHO).[1]

The population size covered per sampling site was calculated. For this, the Civil Engineering Department drew the sewer system per sampling point on the Geoviewer 5.4 platform and gathered the street addresses covered for each of the sewer catchment areas (Supplementary Table S1). The Statistical Office of the canton of Basel-Stadt provided the number of inhabitants for each address/catchment area. The average population per sampling site was considered as a mean of the sampling years (2017–2019). The overall population covered was calculated based on the average population of Basel along the sampling years (Supplementary Table S1).

Information on socio-economic, population-based data and other key features of the individual districts was provided by the Statistical Office of the canton of Basel Stadt and is publicly available.[2] We obtained meteorological data for Basel overall from the validated weather simulation archive of meteoblue.[3]

Definitions

District refers to the city area covered by two sampling sites that mostly represent a given postcode area. Generated districts are not equivalent to a postcode area because the sewer system did not allow for choosing sampling sites comprising the exact area of the postcode. ESBL-PE refer to phenotypically confirmed ESBL-producing Enterobacterales. Presumptive ESBL-producing isolates refer to those grown in selective media without or prior to an ESBL confirmation test. Meteorological seasons were classified as follows: Winter (December–February), Spring (March–May), Summer (June–August), Autumn (September–November). Calendar months span from January (1) to December (12). Sampling rounds span from 1 (June 2017) to 24 (June 2019, as July 2018 is missing).

1 https://www.who.int/teams/environment-climate-change-and-health/water-sanitation-and-health/sanitation-safety

2 statistik.bs.ch

3 https://docs.meteoblue.com/en/meteo/data-sources/datasets#nems

Wastewater sample collection, processing, and isolation of presumptive ESBL-PE

Samples were collected in sterile 25-mL Falcon tubes (VWR, Dietikon, Switzerland) and transferred to the laboratory within 6 h for processing. Subsequently, 1 mL of wastewater sample was diluted into 9 mL of Enterobacteriaceae Enrichment (EE) Broth (Oxoid, Thermo Fisher Diagnostics, Pratteln, Switzerland) and incubated at 37° C overnight. This enrichment step was pursued to increase sensitivity, hence, to recover as many and phenotypically diverse ESBL-producing Enterobacterales as possible. The enrichments were then diluted 1/100 and 1/1,000 in 1 X Phosphate-Buffered Saline (PBS) medium and 100 µL of both dilutions were spread onto Brilliance™ ESBL plates (Oxoid) and incubated at 37°C for 24 h. This chromogenic medium provides presumptive identification of ESBL-producing *E. coli* (blue/pink) and the KESC group (green). If no colonies grew, 100 µL of the enrichment was streaked onto the plates in undiluted form and incubated at 37° C for 24 h and the initially spread plates were checked after 48 h incubation. One to three colonies per color and/or morphology were chosen and further isolated onto new Brilliance™ ESBL plates.

Species identification, ESBL confirmation, and selection criteria

Species identification of all isolates was performed in duplicate on fresh cultures by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS). The isolates were fixed on the MALDI-TOF MS plate using a CHAC matrix (alpha-cyano-4-hydroxycinnamic acid) (Ziegler et al., 2015) and processed using the MALDI-TOF MS Axima™ Confidence (Shimadzu-Biotech, Reinach, Switzerland) and the SARAMIS™ Database (Spectral Archive and Microbial Identification System, AnagnosTec, Potsdam-Golm, Germany) (Welker and Moore, 2011). Both readouts needed to be consistent for bacterial-species taxonomy assignment. Therefore, no clear identification applied when either both measurements were ambiguous (i.e., below 75% identification) or one of the two duplicates gave no result. If no clear identification was achieved, the spectra were matched against the ribosomal marker-based database PAPMID™ (Mabritec AG, Riehen, Switzerland) (Kassim et al., 2017) applying the same performance criteria. Isolates identified as Enterobacterales and forming colonies of different color and/or morphology were stored in glycerol/Triptic Soy Broth (TSB) (1:1) at −80°C for downstream analysis. Isolates not identified as Enterobacterales, those with unclear or inconsistent identification, and duplicates with the same identification as other selected isolates per sample were discarded and considered as not fulfilling the study inclusion criteria. It should be noted that our MALDI-TOF database at the time of identification could not discriminate some species of the *K. pneumoniae* complex (including *K. quasipneumoniae*, *K. variicola,* and *K. quasivariicola*). Likewise, *E. cloacae* isolates may include other species of the complex, such as *E. ludwigii* and *E. kobei*, as they were not included in the database (Supplementary Table S2; Godmer et al., 2021; Voellmy et al., 2022).

ESBL confirmatory testing was performed and evaluated on all stored isolates according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute CLSI, 2009) and using the Total ESBL Confirm Kit (Rosco Diagnostica, Axon Lab, Baden, Switzerland). The type of cephalosporin tested (cefotaxime and ceftazidime, cefepime) depended on the species and was applied according to the manufacturer's instructions. For *Raoultella* spp., enclosed within the Klebsiella genus till 2001 (Appel et al., 2021), cefotaxime and ceftazidime were used. The plates were incubated at 35°C for 18 ± 2 h. ESBL production was confirmed when the zone of inhibition of the disk with cephalosporine plus clavulanate was ≥5 mm larger than the one around the cephalosporine alone. All isolates fulfilling these criteria were designated ESBL-PE.

Quantification of presumptive ESBL-producing isolates and water temperature measurement

From round 14 (13/08/2018) on, presumptive ESBL-producing isolates were quantified. For this, we included three additional consecutive samplings after the end of the study period (16/07/2019; 19/08/2019; 24/09/2019), resulting in 14 months of quantification. Samples were kept in an insulated box to avoid warming. Samples were plated directly without previous enrichment step. For this, 100 µL of the samples were spread directly and in a 1:10 dilution (TSB) on Brilliance™ ESBL agar and colonies were counted after 24 h incubation at 37°C. Enumeration was performed by counting all colonies according to their color as described before. For the quantification analysis, we distinguished two chromogenic groups according to the manufacturer's manual: *E. coli* as blue/pink and the KESC group as green colonies. We used these categories to assign *E. coli* or KESC group species to the appearing colonies. No further identification was pursued. From round 16 (16/10/2018) on (including the three additional samplings) the wastewater temperature at sampling was recorded for a total of 12 months.

Statistical analyses

Microbiological characteristics were assessed both on an isolate-level and a collapsed wastewater sample-level. Respective differences in distributions were calculated using Chi-squared, Fisher's exact, and Wilcoxon rank sum/Kruskal-Wallis tests—as appropriate. Missing data is indicated throughout. Correlation analyses of sample, socio-economic and meteorological parameters were performed using the Spearman's rank correlation coefficients (rho) ranging from −1 to 1. Meteorological data were standardized across time and merged on a day-level for Basel overall. Socio-economic/population characteristics data were collapsed on a district-year level (medians) by weighting, where appropriate, for population size. Correlation strengths (rho) were defined as no correlation (0), very weak (0.01–0.19), weak (0.20–0.39), moderate (0.40–0.59), strong (0.60–0.79), and very strong (0.80–1.00). To visualize the strength of the relationships between the different variables, the matrix information were plotted using heatmaps (Stata packages "heatplot" (Jann, 2019), "palettes" and "colrspace"). Intracluster correlation was marginal overall and was therefore not considered in the explorative hypothesis tests. All analyses were performed on a multicore system with Stata/MP version 16 (Stata Corp., College Station, Texas, United States). All reported *p*-values are two-sided.

Results

ESBL-PE positive sample confirmation and ESBL-PE species identification

ESBL-PE were recovered from 94.6% (477/504) of all wastewater samples collected during the 2-year sampling period. In total, 1993 isolates were recovered, of which 1,461 met the study inclusion criteria (see "Species identification, ESBL confirmation and selection criteria" section). Supplementary Table S3 details all isolates recovered across samples (space and time), wastewater source, species identified and ESBL profile. Among them, ESBL-production was phenotypically confirmed for 91.3% (1,334/1,461). *Escherichia coli* was by far the most common species identified, accounting for 85.5% of all ESBL-PE (1,140/1,334), followed by *Klebsiella pneumoniae* (11.5%, 153/1,334) (Table 1). Twenty-eight (2%) isolates belonged to other species of the KESC group (10 *Klebsiella* spp., 16 *Citrobacter* spp., and 2 *Enterobacter* spp.). Thirteen (<1%) additional ESBL-producing isolates belonged to *Raoultella ornithinolytica* (11/1,334), *Morganella morganii* (1/1,334) and *Proteus mirabilis* (1/1,334). No additional *Escherichia* species, other than *E. coli* were identified. The proportion of phenotypic ESBL-confirmation positive tests differed across presumptive ESBL-PE (i.e., isolates growing on Brilliance™ ESBL plates), ranging from 97.9% for *E. coli* (1,140/1,164), 96.8% for *K. pneumoniae* (153/158) and 100% for *R. ornithinolytica* isolates (11/11) to less than 1% for *Enterobacter cloacae* (1/52) and *Morganella morganii* (1/27) (Figure 2, for detailed values see Supplementary Table S4).

Spatial distribution

The percentage of wastewater samples harboring ESBL-PE differed across districts ($p = 0.038$), mainly due to two areas (4,057 and 4,053), both with lower proportions of ESBL-PE positive samples (Figure 3A). The proportions of wastewater samples positive for individual species of ESBL-PE differed across districts ($p < 0.001$) (Figure 4A). Of note, we only display species with ≥1 ESBL isolate/s representing ≥0.75% of all isolates tested (≥ 10 isolates of 1,461) in a given postcode area. ESBL-producing *E. coli* and *K. pneumoniae* were recovered from all sampling sites, with *E. coli* consistently predominating across all districts (Figure 4A and Supplementary Table S3). ESBL-producing *R. ornithinolytica*, *E. cloacae*, and *M. morganii* were exclusively recovered from wastewater of unique districts (Figure 4A).

TABLE 1 ESBL-producing species identified across the ESBL-PE isolates (n=1,334), isolates recovered per species and relative abundance.

ESBL species recovered	No. of ESBL positive	%
Citrobacter amalonaticus	2	0.15
Citrobacter freundii	12	0.90
Citrobacter koseri	1	0.07
Citrobacter sp.	1	0.07
Enterobacter cloacae	1	0.07
Enterobacter xiangfangensis	1	0.07
Escherichia coli	1,140	85.46
Klebsiella aerogenes	1	0.07
Klebsiella oxytoca	9	0.67
Klebsiella pneumoniae	153	11.47
Morganella morganii	1	0.07
Proteus mirabilis	1	0.07
Raoultella ornithinolytica	11	0.82

Temporal distribution

The proportion of samples positive for ESBL species did not differ between sampling rounds (1–24) or calendar months (1–12) ($p = 0.501$) (Figure 3B). However, temporal differences in the detection of different ESBL-producing species were observed ($p < 0.001$) (Figure 4B) (criteria for species represented as indicated above). ESBL-producing *E. coli* and *K. pneumoniae* were the only species recovered throughout the entire sampling period (Figure 4B) with ESBL-producing *E. coli* consistently predominating in all sampling rounds (present in >90% of the samples; 475/504), while ESBL-producing *K. pneumoniae* was detected in close to one third of the samples (143/504) (Figure 4B). ESBL-producing *R. ornithinolytica* was consistently recovered along ten sampling rounds in the same district. Instead, ESBL-producing *E. cloacae* and *M. morganii* isolates were only recovered once (Figure 4B).

Comparative analysis of mixed and urban sites

The proportion of wastewater samples harboring ESBL-PE did not differ between mixed (97.9%; 94/96) and urban (93.9%; 383/408) sampling sites ($p = 0.135$) (Supplementary Table S5). The distribution of different ESBL-producing species between mixed and urban sites was similar ($p = 0.076$), except for *K. pneumoniae*, with an increased proportion of detection in mixed sites (50.0% vs. 22.3% in urban sites) (Figure 5A). Among all ESBL isolates recovered during the study period, the proportion of *K. pneumoniae* isolates was higher in wastewater samples from mixed sites (16.9%; 53/313) as compared to samples recovered from urban sites (9.8%; 100/1021) ($p < 0.001$). Contrary, the proportion of ESBL-producing *E. coli* was higher in samples from urban sites (87.0% vs. 80.1%) (Figure 5B) ($p = 0.006$).

Quantification of presumptive ESBL chromogenic groups and correlation analyses

Quantification of bacterial colony counts without pre-enrichment step was performed on 294 samples collected across 14 consecutive months (August 2018 to September 2019). Of these, 253, 227, and 273 samples harbored presumptive ESBL-producing *E. coli* (86.1%), KESC (77.2%) or at least one both groups (92.9%), respectively. Supplementary Table S6 displays all individual concentrations, overall statistics per sample across space and time as well as across urban/mixed sites for both species groups. Overall, a median of 2×10^2 colony forming units per milliliter (CFU/mL) was detected. Presumptive

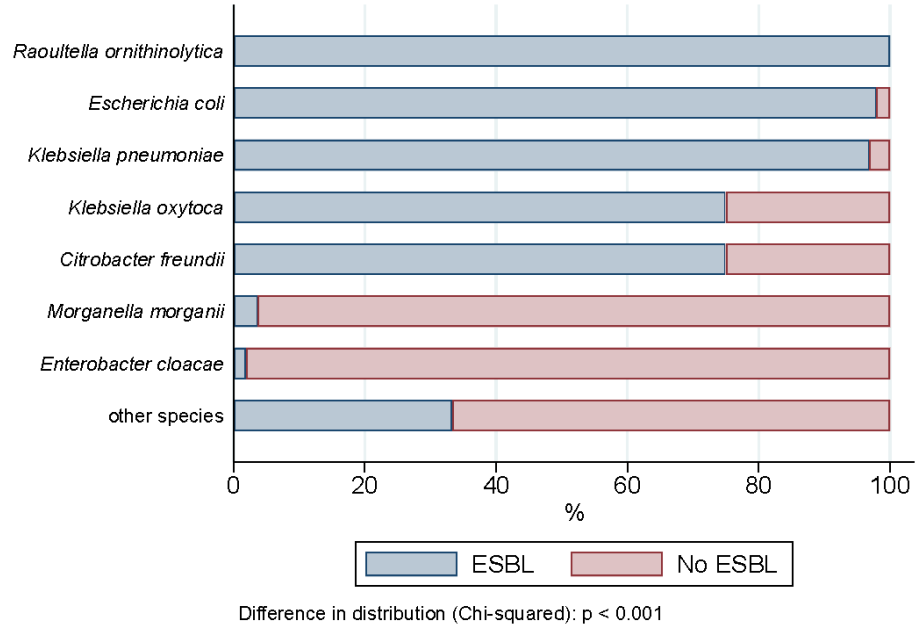


FIGURE 2
Phenotypic ESBL distribution across the enterobacterales recovered (n=1,461).

ESBL-producing *E. coli* and KESC median counts were 9.5×10^1 and 5.5×10^1 CFU/mL, respectively, and differed across sites and sampling months (Supplementary Figure 1). Notably, district 4,056, which collected water from three referral hospitals, enclosed the overall highest CFU/mL numbers of resistant *E. coli* plus KESC (median 505 CFU/mL vs. 200 CFU/mL overall) species group (Figure 6 and Supplementary Table S6). The number of presumptive ESBL-producing *E. coli* and KESC was highest in August, when the median water temperature registered at sampling was also the highest (Supplementary Figures S1D–F). When analyzed by season, the numbers of CFUs were highest in summer for presumptive ESBL-producing *E. coli* and in autumn for KESC and differed across seasons (Figure 6 and Supplementary Table S6).

Correlation analyses were conducted to assess relationships between the quantification of both species groups and different socio-economic and meteorological variables (Figure 7 and Supplementary Table S7). We detected moderate correlations between population size and the number of presumptive ESBL-producing *E. coli*-CFUs (rho = 0.44), while KESC abundance was less related to population size (rho = 0.21) (Figures 8A–D). The median overall counts for mixed sites doubled those from the compiled urban sites (3.3×10^2 vs. 1.6×10^2 CFU/mL) ($p = 0.002$, Figure 8E) with greater differences in KESC counts (1.2×10^2 vs. 3.3×10^1 CFU/mL) as compared to *E. coli* counts (2.0×10^2 vs. 8.5×10^1 CFU/mL).

Discussion

ESBL-producing *E. coli* and *K. pneumoniae* were detected in the vast majority of municipal wastewater samples of the city of Basel, Switzerland, over a two-year study period, indicating widespread dissemination of ESBL-PE in the community and supporting that community sources are important reservoirs entertaining the spread of ESBL-PE. Hospital-influenced abundance of ESBL-PE appears to be species dependent reflected by higher proportions and counts of *K. pneumoniae* but not *E. coli* in wastewater samples from sites receiving both hospital and community wastewater. This finding is further supported by the detection of a moderate correlation between ESBL-*E. coli*-counts in wastewater and population size, yet a weak correlation between counts of other ESBL-PE species and population size.

Our results deviate from the results of a study performed in France on wastewater samples collected in 2011, revealing significantly higher proportions of ESBL-production among *E. coli* isolates recovered from hospital as compared to general urban wastewater (7.5% vs. 0.1%) (Brechet et al., 2014). In line, higher proportions of ESBL-producers were detected among *E. coli* strains isolated from hospital effluents (37%) as compared to municipal sewage (18%) in Poland in a study published in 2013 (Korzeniewska et al., 2013). In addition, this study reported lower proportions of hospital effluents and inflow sewage samples being positive for ESBL-producing *E. coli* as compared to our study (76.5 and 57.1%). Both the French and the Polish studies point to hospitals being a more important reservoir for ESBL-*E. coli*, as compared to the community, over a decade ago. In line with our results and supporting the rising contribution from the community, a recent study on ESBL-producing *E. coli* in urban community wastewater from socio-spatially different communities in Germany also recovered phenotypic ESBL-producing *E. coli* from every wastewater sample across a one-year monthly-based sampling, demonstrating that the general community is an important indirect discharger (Schmiege et al., 2021). Although at a much lower scale, Kutilova and colleagues reported positive ESBL-producing *E. coli* in all samples tested across municipal and hospital wastewaters including different clearing stages from Czech Republic in 2016 (Kutilova et al., 2021). Recent publications from numerous European countries, such as Croatia, Hungary, Germany, Czech Republic, The Netherlands,

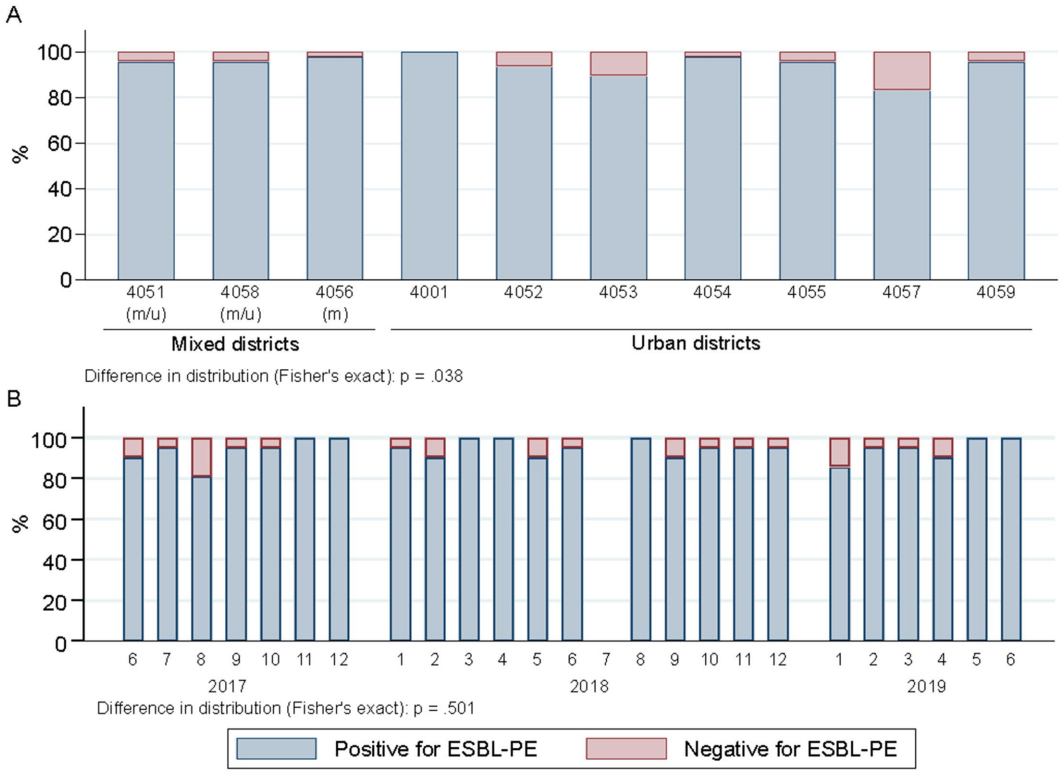


FIGURE 3
Spatiotemporal distribution of ESBL-PE across samples (*n*=504). **(A)** Spatial distribution of ESBL-positive samples across city districts (part of a postcode area). Districts are ordered according to the mixed and urban site distribution (two sites per district, except for 4,051 three). m/u, mixed district comprising one site collecting hospital and urban (m) wastewater and other contributing only with urban wastewater (u); m, district collecting mixed water in both sampled sites. Numerator, number of ESBL samples in given district; denominator, 48 samples per district each except 72 for 4,051. **(B)** Temporal distribution of ESBL-positive samples across sampling months. 1–12 refers to the calendar months January (1) to December (12). Numerator, number of ESBL samples in given sampling point; denominator, 21 samples each. Samples negative for ESBL-PE enclose the following strata: samples with no growth (*n*=14), samples with growth but no Enterobacterales (*n*=10) and samples with Enterobacterales but ESBL negative (*n*=3).

Romania, Slovakia, Norway, and Sweden, evidence that AMR Enterobacterales, including ESBL-producing isolates, are spread throughout the different wastewater systems, even in low-endemic regions (Fagerstrom et al., 2019; Paulshus et al., 2019; Lepesova et al., 2020; Surleac et al., 2020; Hooban et al., 2021; Kutilova et al., 2021; Schmiege et al., 2021; Mutuku et al., 2022; Puljko et al., 2022).

All ESBL-PE recovered in our study belonged to either the Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., and *R. ornithinolytica*) or Morganellaceae (*M. morganii* and *P. mirabilis*) families. ESBL-producing *E. coli* was by far the most common and persistent species, followed by *K. pneumoniae* mirroring the epidemiology of ESBL-PE in clinical samples (Gasser et al., 2019; Antimicrobial Resistance Collaborators, 2022). In line with our results, a recent systematic review showed that the most common producer of ESBL in wastewater is *E. coli* (51/57 studies), followed by *Klebsiella* spp. (29/57 studies) and *Enterobacter* spp. (18/57 studies) (Zaatout et al., 2021).

Median levels of presumptive ESBL-producing *E. coli* (9.5×10^1 CFU/mL wastewater) found in Basel were in line with those recently found among presumptive cefotaxime-resistant *E. coli* in influent wastewater in Croatia (average 1.7×10^1–7.8×10^2 CFU/mL) (Puljko et al., 2022) and between 1 and 10^4 fold lower (10^3–10^6) than in additional European reports on community and hospital effluents (Korzeniewska et al., 2013; Brechet et al., 2014; Kwak et al., 2015; Hocquet et al., 2016; Schmiege et al., 2021; Mutuku et al., 2022). Nevertheless, comparative studies are limited as most available works focused on total coliforms or total *E. coli* (Lepesova et al., 2020). The number of presumptive ESBL-producing *E. coli* and KESC was highest in summer and in autumn, respectively, and differed across seasons. Further, numbers of presumptive ESBL-PE were highest in August, when the median water temperature registered at sampling was also the highest. These observations are supported by previous studies pointing to correlations between increasing outdoor temperatures and antibiotic resistant bacteria (MacFadden et al., 2018; Bock et al., 2022). This study cannot address if the increase observed was related to a selective increase of ESBL-PE or to an overall increase in bacterial counts, as the later was not measured.

Remarkably, ESBL-producing *R. ornithinolytica* was exclusively prevalent in sampling site 4,001/1, representing the city center. *R. ornithinolytica* is widely found in aquatic environments, insects and fishes (Sekowska, 2017). Infections by *R. ornithinolytica* are uncommon in humans but are increasing (Appel et al., 2021).

For some species recovered, i.e., *E. coli*, *K. pneumoniae*, and *R. ornithinolytica*, the selective media Brilliance™ ESBL agar, a validated commercial selective agar used for the identification of presumptive ESBL-producing *E. coli* and KESC group isolates (Blane

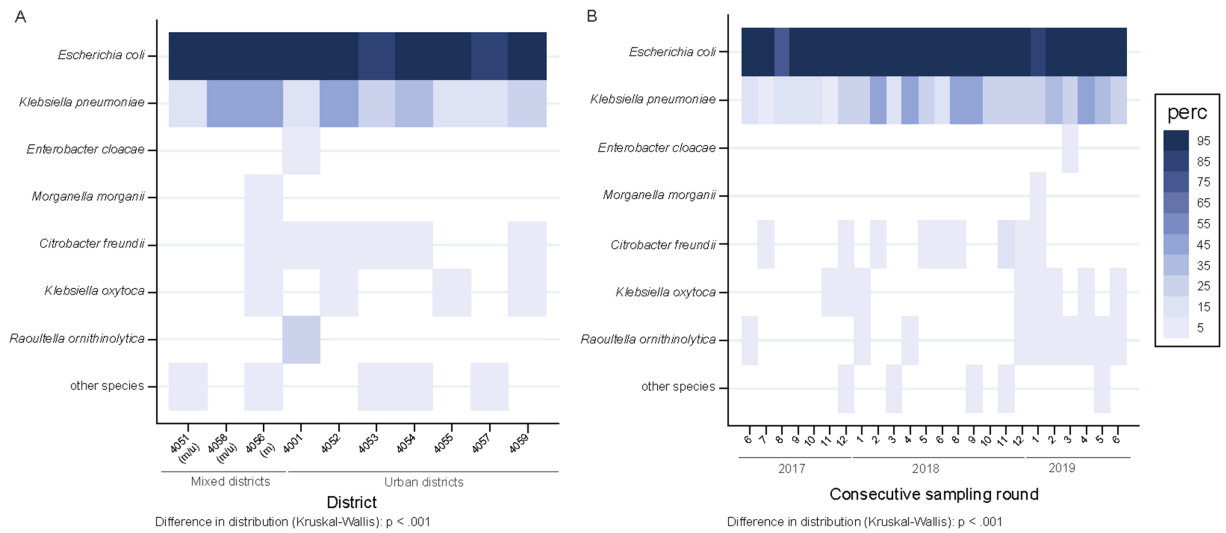


FIGURE 4
Spatiotemporal distribution of ESBL-producing species across samples (*n*=504 overall), represented as percentage of samples positive for ESBL-PE. **(A)** Spatial, sample-level ESBL species distribution across city districts. Districts are ordered according to the mixed and urban site distribution (two sites per district, except for 4,051 three). m/u, mixed district comprising one site collecting hospital and urban (m) wastewater and other contributing only with urban wastewater (u); m, district collecting mixed water in both sampled sites. Numerator, number of samples with ≥1 ESBL isolate/s of a given species with at least 10 isolates (representing 0.75% of all isolates tested; *n*=1,461) in a given district; denominator, 48 samples per district each except 72 for 4,051. Postcodes are ordered according to mixed and urban site distribution. Species are ordered according to the number of recovered isolates per species. **(B)** Temporal, sample-level distribution across consecutive sampling rounds. Numbers 1–12 refer to the calendar months January (1) to December (12). Numerator, number of samples with ≥1 ESBL isolates of a given species in a specific sampling point; denominator, 21 samples each.

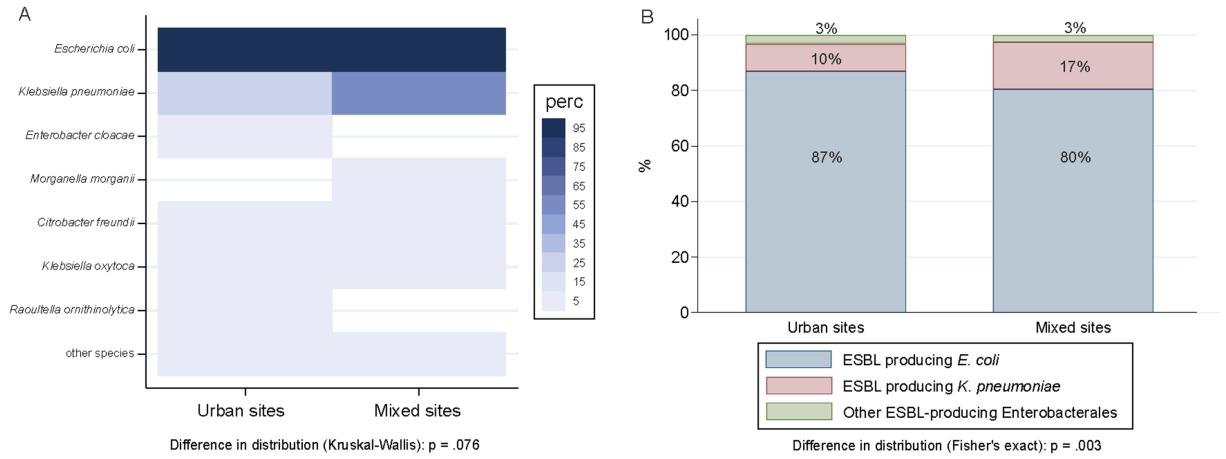


FIGURE 5
Distribution of ESBL-producing species across compiled urban (*n*=96) and mixed sites (*n*=408). **(A)** Sample-level distribution of ESBL-PE as percentage of positive samples. Species are ordered according to the number of isolates per species. Numerator, number of samples with ≥1 ESBL isolate of that given species; denominator, 408 urban samples, 96 mixed samples. Of note, only species with ≥1 ESBL isolate/s representing ≥0.75% of all isolates tested (≥ 10 isolates of 1,461) are displayed. **(B)** Distribution of ESBL-producing isolates (*n*=1,334) clustered per species as *E. coli* (1140), *K. pneumoniae* (153), and others (41). Numerator, number of ESBL-producing isolates of that given species; denominator, 1,021 isolates in urban site, 313 in mixed sites.

et al., 2016; Lee et al., 2021), was an excellent predictor for ESBL-producing isolates (>97%). However, for other species, particularly for *E. cloacae* and *M. morganii*, >96% were ESBL negative. Interpretation of inhibition halos in the phenotypic test for these species suggests expression of inducible (or derepressed) endogenous AmpC β-lactamases, characteristic of these species. Hence, we found that enterobacterial species enclosing 3rd generation cephalosporin resistance mechanisms other than ESBL are consistently recovered. All isolates were recovered in the presence of cefpodoxime, a third generation cephalosporin. Blane, Brodrick (44) assessed the sensitivity and selectivity (suppressed growth of no ESBL producers) of ESBL-PE of Brilliance™ ESBL agar and ChromID ESBL agar, another gold standard media for this purpose that we also tested prior to the study, with and without pre-enrichment to detect ESBL-PE in stool samples.

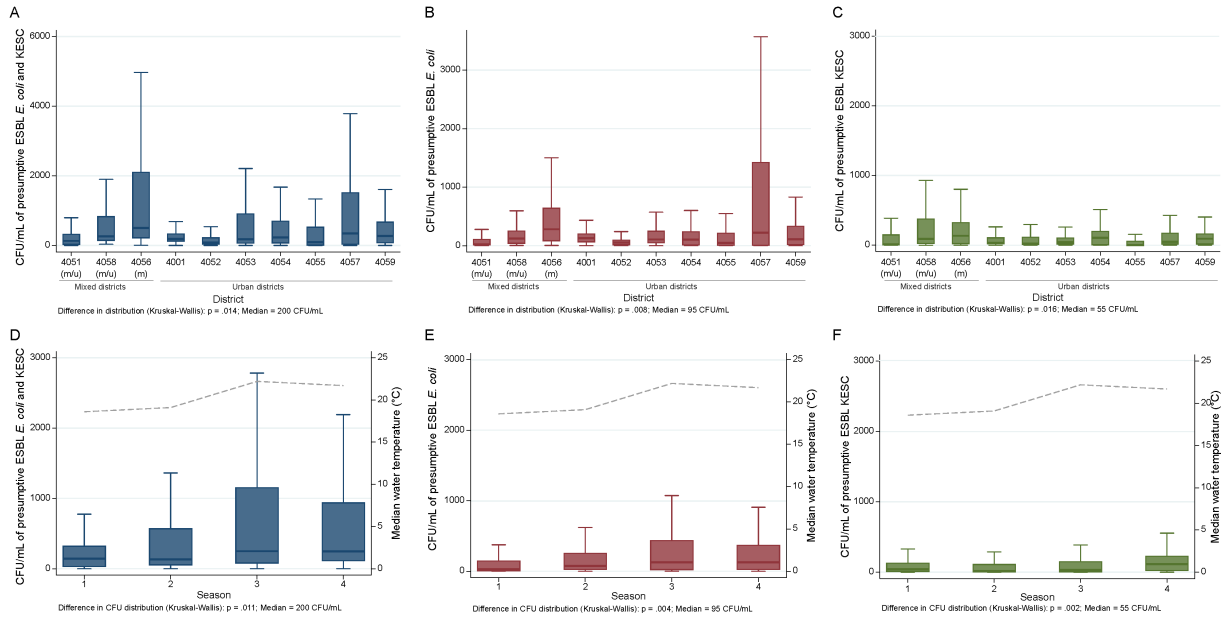


FIGURE 6
Spatiotemporal quantification of presumptive ESBL-producing *E. coli* and KESC groups. **(A–C)** Spatial distribution of presumptive ESBL-producing *E. coli* **(A)**, presumptive ESBL-producing KESC **(B)** and presumptive ESBL-producing *E. coli* plus KESC **(C)** across city districts and median population size. **(D–F)** Temporal distribution of presumptive ESBL-producing *E. coli* **(D)**, presumptive ESBL-producing KESC **(E)** and presumptive ESBL-producing *E. coli* plus KESC **(F)** across sampling seasons and median water temperature (stippled line). (m) mixed site, (u) urban site. 1, Winter; 2, Spring; 3, Summer; 4, Autumn.

Both media performed comparable sensitivity and selectivity for all positive samples recovered. Pre-enrichment with cefpodoxime significantly increased sensitivity (59–98% for Brilliance™ ESBL agar) but reduced selectivity (87–61% Brilliance™ ESBL agar) in both agars, due to increased growth of non-ESBL-PE isolates. These results evidence our pre-enrichment-based isolation method was appropriate in prioritizing sensitivity and species diversity, while increased the range of false positive isolates, which we could be efficiently discriminated in subsequent analyses. On the other hand, the lack of pre-enrichment step for the quantification experiments had the additional effect of maximizing selectivity of ESBL-PE isolates, as no further species discrimination was pursued. We can then expect a higher proportion of ESBL-producing *K. pneumoniae* colonies within the KESC group with respect to the rate of non-ESBL producing *Enterobacter* spp. and *Citrobacter* spp. isolates recovered from the pre-enrichment counterpart, supporting the interpretation of our correlation results using the quantification data.

Our results support that both community and healthcare settings equally contribute to the spread of ESBL-PE. However, it is important to note that the proportion of ESBL-producing *K. pneumoniae* was higher in the mixed sites receiving hospital outlet water from several relevant hospitals in the city, while the proportion of ESBL-producing *E. coli* was higher in urban sites. In fact, district 4,056, which collected water from three referral hospitals, enclosed the overall highest CFU/mL numbers of resistant *E. coli* plus KESC species group. On top, quantification of presumptive ESBL-producing isolates enabled us to proxy resistant *K. pneumoniae* more abundant in mixed sites while having a lower dependency on population size than *E. coli*. The solid positive correlation observed between resistant *E. coli* and population size points to a direct and critical input from the household outlet water. Our results suggest a flow of species from different sources all contributing to the epidemiology of circulating ESBL-PE and reflect the importance of reducing concentrations of environmental pollutants, such as antibiotics and disinfectants, to diminish downstream spread. These findings support tailoring infection prevention and control measures in healthcare settings to the species of ESBL-PE as previously suggested (Tschudin-Sutter et al., 2012, 2017; Tacconelli et al., 2014) as the impact on the further spread of ESBL-producing *E. coli* may be less substantial as compared to the impact on other ESBL-PE-species.

A recent systematic review and meta-analysis on the prevalence of ESBL-PE in wastewater concludes that the prevalence of ESBL-PE in wastewater is increasing over time and that hospital wastewater is the most important repository of ESBL genes (Zaatout et al., 2021). Although antimicrobial resistance *E. coli* and ESBL-*E. coli* are commonly more abundant in untreated hospital wastewater than in community outlets (Hassoun-Kheir et al., 2020), there is consistent scientific evidence that the community represents a relevant source of ESBL-producing *E. coli*, making the effect of hospital effluent on ESBL-producing *E. coli* vague (generally, hospital effluents contribute less than 1% of overall municipal sewage) (Harris et al., 2013; Karkman et al., 2018; Kutilova et al., 2021; Schmiege et al., 2021). As shown in several European studies from Croatia, France, Poland, and Ireland (Galvin et al., 2010; Korzeniewska et al., 2013; Brechet et al., 2014; Puljko et al., 2022), current wastewater treatment processes reduce but do not eliminate all ESBL-PE isolates, and even the proportion of resistant to susceptible isolates released may increase. Thus, identifying sources contributing to the ESBL-PE epidemic is critical to develop targeted interventions.

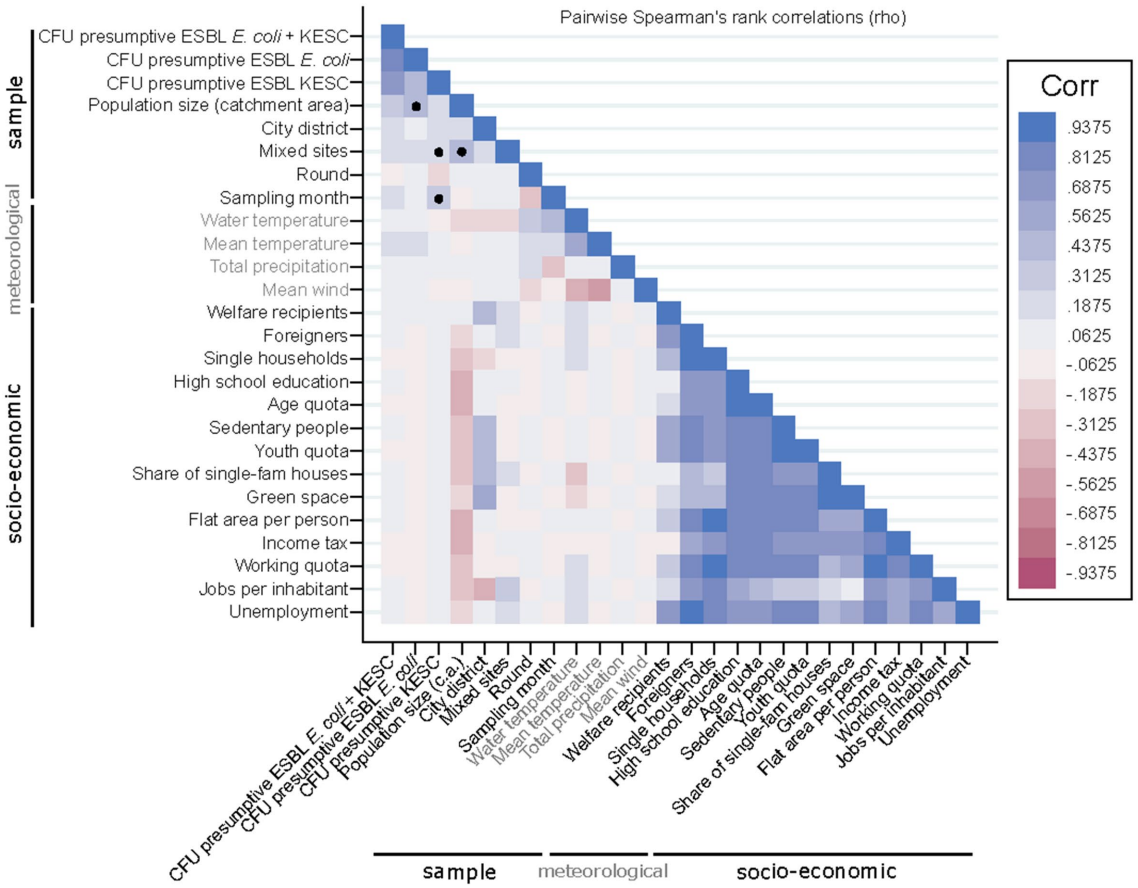


FIGURE 7
Correlation matrix of sample, meteorological and socio-economic parameters. Black dots denote the presumptive ESBL-producing *E. coli* and/or KESC-dependent positive correlations further analyzed and discussed.

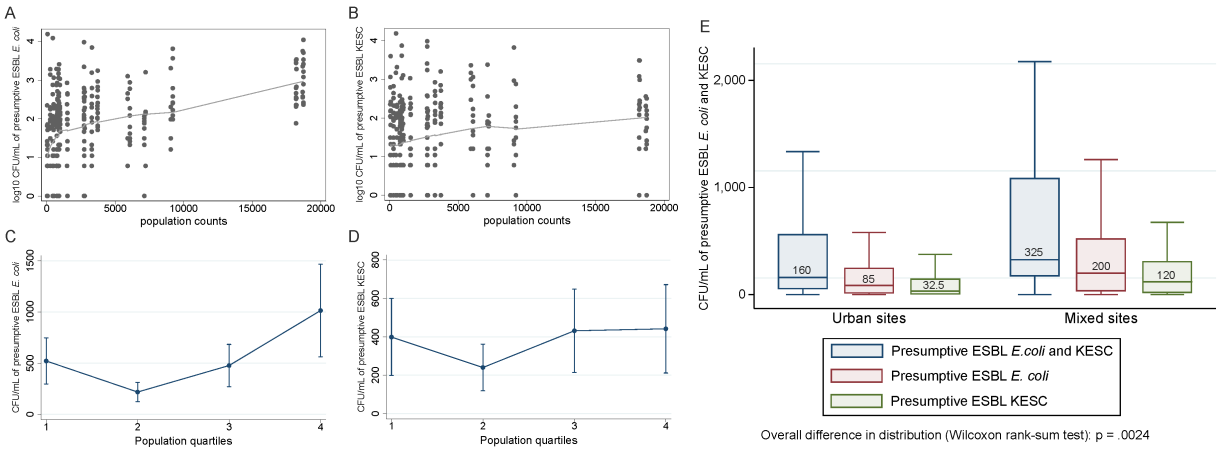


FIGURE 8
Analysis of relevant positive correlations involving presumptive ESBL-producing *E. coli* and/or KESC abundance. **(A,B)** Quantification of presumptive ESBL-producing *E. coli* **(A)** and presumptive ESBL-producing KESC **(B)** colonies across population counts. Trend lines with «Locally Weighted Scatterplot Smoothing» (LOWESS). **(C,D)** Predicted quantification based on negative binomial regression model (unadjusted) of presumptive ESBL-producing *E. coli* **(C)** and presumptive ESBL-producing KESC **(D)** with 95% confidence intervals. Minimum and maximum population size per quartile are as follows (quartile): (1) 39–724, (2) 744–972, (3) 993–3,727, (4) 5911–18,727. **(E)** Quantification of presumptive ESBL-producing *E. coli*, KESC and both groups in urban and mixed sites. Medians are indicated in each boxplot.

Our study has some important limitations. First, results may only be generalizable to countries with a similar prevalence of ESBL-PE. In Switzerland approximately 10% of all clinical *E. coli* strains and *K. pneumoniae* strains are reported as being resistant to third generation cephalosporins, suggesting the presence of ESBLs.[4] Second, our wastewater sampling scheme did not cover the entire population of the city, but only approximately half. However, the sites were chosen to be representative of the entire city, thus selection for areas with different socio-economic characteristics of the population is unlikely. Third, quantification of presumptive ESBL-PE was not carried out during the whole study period and only covered 12 months. Yet, our work covers one of the largest study periods and applies one of the more consistent sampling efforts as compared to former wastewater-based studies (Zaatout et al., 2021). Fourth, our exploratory comparisons and associated hypothesis tests should not be considered as confirmatory. Fifth, we do not present results of ESBL-genotypes. Ongoing investigations on the *resistome*, *mobilome* and *virulome* profile of all recovered isolates together with phylogenomic analyses will further set light on the circulating clones in the sewage pipeline of this European city. Preliminary analyses rule out the presence of carbapenemases in all isolates recovered from wastewater. Likewise, whole-genome comparisons with hospital and foodstuff ESBL-producing isolates recovered across the same study period (2017/2019) will undoubtedly contribute to improve our current understanding on the prevalence and clonal distribution of ESBL-PE in urban settings from a holistic One-Health perspective.

In conclusion, our findings indicate widespread dissemination of ESBL-PE, especially *E. coli*, within the population of a middle European city and support that community reservoirs are similarly important as healthcare-associated reservoirs in entertaining the spread of ESBL-PE. The hospital input of ESBL-producing *K. pneumoniae* seems to contribute to the higher proportions and load recovered from municipal sites collecting hospital wastewater. These results may help develop and implement efficient targeted interventions in the community and hospitals to reduce environmental spread of ESBL-producing clinically relevant species and circulating epidemic clones.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

EG-S analyzed, interpreted the data, and wrote the first draft of the manuscript. CB conceived the study, collected the data, and critically revised the manuscript. JR generated the figures, performed the statistical analyses, and critically revised the manuscript. MA, LM, and RS contributed to data collection. RF and LE collected data and contributed to analysis of data. IS was responsible for the ESBL confirmatory testing of most isolates. AG-M, AE, and PH revised the manuscript. TS provided valuable input on the conceptualization of the study and revised the manuscript. LA-B contributed to data collection and interpretation of results and revised the manuscript. ST-S conceived and supervised the study, analyzed the data, and revised the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1174336/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Spatiotemporal quantification of presumptive ESBL-producing *E. coli* and KESC group colonies. **(A-C)** Spatial distribution across sampling sites among both groups **(A)**, presumptive ESBL-producing *E. coli* **(B)**, and presumptive ESBL-producing KESC **(C)**. **(D-F)** Temporal distribution across sampling month and median water temperature among both groups **(D)**, presumptive ESBL-producing *E. coli* **(E)**, and presumptive ESBL-producing KESC **(F)**. Sampling sites are ordered according to mixed and urban site distribution. Outliers were removed for readability. Boxes, bold lines and whiskers indicate the interquartile ranges, medians, and 1.5 times the interquartile range, respectively.

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Off-label use of antimicrobials among hospitalized children: a retrospective study of 3,406 patients

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Introduction: Off-label drug use is a global problem for which many countries and regions have issued legal provisions or reached an expert consensus. Off-label use is sometimes a necessity, especially since antibacterial drugs have become one of the most widely used drugs in pediatric settings and the issue of causing antimicrobial resistance has increasingly become unavoidable. It also poses additional risks, such as adverse drug reactions.

Methods: Our study analyzed the antimicrobial prescriptions of pediatric inpatients in a large Chinese hospital in the first half of 2021. This retrospective investigation included 6,829 prescriptions, including 2,294 off-label prescriptions. We performed descriptive analyses of prescription antimicrobial agents among pediatric populations and reported the percentages and frequencies.

Results: It was found that off-label use of antibiotics was present in many children (n=1,665, 48.9%) and was most common in newborns (n=328, 82.8%). Among the commonly used antibiotics in pediatric patients, cephalosporins (n=2,778, 40.7%) accounted for a relatively low proportion of offlabel use (n=360, 15.7%), while macrolides (n=628, 27.4%) and penicillins (n=610, 26.6%) accounted for a higher proportion. The off-label type mainly referred to the appropriate population (46.5%) and dosage (dose, 10.0%; frequency of administration, 48.3%).

Discussion: Off-label use was due to imperfect labels, improper medications, or medication errors. Only a few consensuses could apply to pediatric patients. More clinical trials are required to update the consensus, and drug labels must be continuously improved. The prescription behavior of doctors is also needed to be regulated. Rational use of drugs, especially antimicrobials, is the responsibility of all people, including the states, medical institutions, and individuals.

KEYWORDS

off-label, pediatric, antimicrobials, inpatient, evidence-based

1. Introduction

Off-label use of drugs, especially in children, is a common practice worldwide. However, the off-label use of antimicrobials can lead to irrational use and cause many problems, such as bacterial resistance, ultimately leading to fatal consequences in children due to fewer available treatment options. Several countries have issued relevant regulations on this (Zhang et al., 2012; Xie et al., 2020). Before the publication of the new medical practitioners' law on March 1, 2022, off-label use was controversial in China. To provide a reference for clinical applications, various health professional organizations, such as the Chinese Pharmaceutical Association, Chinese

Medical Association, Guangdong Province Pharmaceutical Association, and Shandong Province Pharmaceutical Association, were involved in this endeavor and issued a series of relevant specifications or expert consensuses on the off-label use of drugs. Examples of this include the Consensus on the Use of Antibacterial Drugs (Chen et al., 2015), The Consensus on Management for Off-label Drug Use in Hospitals (2014), The Catalogue of Off-label Usage (The New Usage of 2020) (2020), and the Expert Consensus on Off-Label Drug Use of Shandong Province (2021) (Hou, 2021). Continuous monitoring and improvement are essential for off-label management. We are concerned with how to practice in the clinic and improve evidence-based off-label use.

In this study, we selected the most commonly used antibiotics in pediatric patients in China. The hospital investigated in this study is a large-scale general hospital in China with more than 3,000 beds, including 300 pediatric beds, which is almost the largest in Shandong Province. We retrospectively investigated the use of off-label antibacterial drugs in the pediatric department from January to June 2021. The Expert Consensus on Off-Label Drug Use of Shandong Province (2021) (Hou, 2021) was formed through research and evidence-based evaluation, and it was referred to as the SD Consensus for clinical work. It explored the level of evidence-based clinical off-label drug use and tried to promote rational drug use.

2. Materials and methods

2.1. Data collection and collation

We conducted a retrospective chart review at a Shandong Provincial Hospital. We used the Health Information System (HIS), a non-profit and non-public administrative database, to perform a hospitalization-level drug utilization study and capture clinical and resource utilization data from the entire hospital, especially from pediatric patients.

The demographic data collected from patients in the HIS included age, sex, weight, and race. Other patient-level data included the patient's hospitalization ID, time of the doctor's prescription issuance, admission dates, diagnosis, prescribed drugs, dosage form, dosage strength for each charge (single dose and frequency of administration), and administration route. The major diagnostic category for each patient was assigned using the International Classification of Diseases, 10th edition (ICD-10). The prescription of one drug to one hospitalized patient was regarded as a prescription.

This study included patients less than 18 years of age who had used at least one antimicrobial agent and who were admitted between January 1, 2021, and June 30, 2021. Pediatric patients were divided into the following six age groups: (1) neonatal period (0–28 days); (2) infant period (29 days–1 year); (3) early childhood (>1–3 years); (4) preschool period (>3–6 years); (5) school-age (>6–12 years); and (6) adolescent (>12–18 years).

Off-label use ("off-label") was determined according to the latest version of the drug insert approved by the National Medical Products Administration (2023). Different manufacturers of the same drug were evaluated according to their product instructions. National Medical Products Administration (NMPA) labels, including archived labels for revised labels, were obtained from the publicly available databases NMPA and Yaozh Data (Yaozh Data, 2023). Each prescription was reviewed and classified based on four aspects: indications, appropriate population (no pediatric information and out of the required age range), dosage (dose and frequency of administration), and usage (the administration route). Drug use was considered off-label if the administration route was changed. The drug did not include pediatric information if the patient's age was outside the NMPA-specified age range or if the dosage was over or under the label range. Each type was recorded for multiple off-label uses in the same prescription. If the medication was not adapted to the population or usage, the dosage could not be determined. Sixty-three unique prescription antimicrobials were analyzed, representing 52 drugs based on various formulations and active pharmaceutical ingredients. According to the SD Consensus, off-label uses were marked with the appropriate level of evidence.

2.2. Statistical analysis

We performed descriptive analyses of prescription antimicrobial agents among pediatric populations and reported the percentages and frequencies. Continuous data were reported as the mean ± SD. The antibacterial agents were grouped by drug type according to their common name and pharmacological classification, and the percentages of off-label use were calculated according to the patient's characteristics. All analyses were performed using Microsoft Excel 2010 and SPSS 22.0 software.

3. Results

3.1. General situation

A total of 8,886 prescriptions were registered, but 2,057 were ineligible (Figure 1) because of age (patients either older than 18 years of age could not be determined; $n = 30$), antibacterials used for skin testing ($n = 1{,}965$), or the prescriptions were canceled before being administered ($n = 62$). In the first half of 2021, the total number of pediatric patients discharged from the hospital was 9,320, and 3,406 (36.5%) were treated with antibacterials. Therefore, the analysis was performed on 6,829 antibacterial prescriptions observed in 3,406 inpatients. Their demographic data and medication information are described in Supplementary Table S1. In 1,701 (49.9%) patients with a single drug prescription, 897 (26.3%) with two drugs, and 808 (23.7%) with three or more drugs, we found that the more antibacterial drugs were used, the higher the incidence of off-label use. When a patient was prescribed more than five antibacterial drugs, the incidence of off-label prescriptions exceeded 85% (Table 1). Cephalosporins were the most frequently prescribed medication, issued 2,778 times (40.7%), followed by macrolides (985 times, 14.4%), antibacterial drugs combined with enzyme inhibitors (932 times, 13.6%), carbapenems (708 times, 10.4%), and penicillins (676 times, 9.9%; Table 2). These five types of drugs were prescribed to nearly 90% (89.0%) of all pediatric inpatients treated with antimicrobials. Instead of the most frequently prescribed cephalosporins, macrolides (27.4%) and penicillins (26.6%) were mostly used off-label.

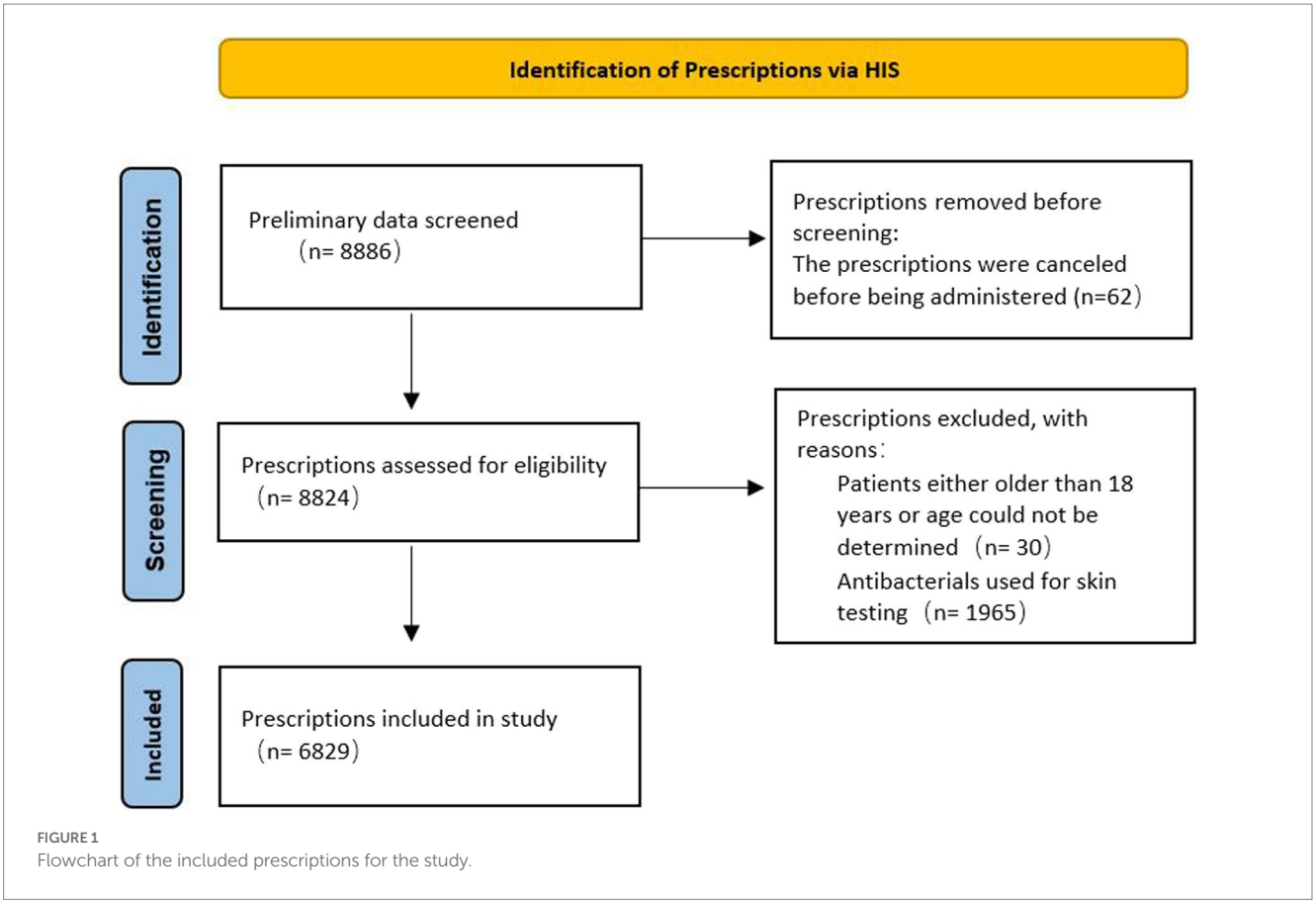

FIGURE 1
Flowchart of the included prescriptions for the study.

TABLE 1 Number of off-label prescription antibacterials per hospitalization case.

Number of antimicrobial prescriptions	Inpatients, *n*	Percentage of all the inpatients (*n*=9,320)	Percentage use across the inpatients with off-label (Patients with off-label/ Patients using antimicrobials, *n*)
1	1,701	18.3%	30.2% (513/1,701)
2	897	9.6%	58.3% (523/897)
3	396	4.2%	73.2% (290/396)
4	211	2.3%	79.6% (168/211)
5 or more	201	2.2%	85.1% (171/201)
Total	3,406	36.5%	48.9% (1,665/3,406)

3.2. Off-label use of antibacterial agents according to pediatric patient age

Off-label antimicrobials in pediatric inpatients were common in all age groups (Table 3). In this study, the off-label use of antibacterial drugs in hospitalized patients was mainly based on age, dosage, and route of administration, which was different from other studies (Carmack et al., 2020; García-López et al., 2020; Haulrig et al., 2021; San Giovanni et al., 2021), so no further analysis of the indications was performed. Among the newborns who used antimicrobials, 82.8% used them off-label. At the same time, a single patient in this age group had the most antimicrobial drug prescriptions (2.32). The frequency of off-label use of antibacterials in this age group was also the highest, at 1.99 ± 1.55. The largest number of hospitalized children were of preschool age (801), followed by 690 school-age children. The most prescribed antibacterial prescriptions were for those in preschool and early childhood (1,513 and 1,307, respectively). Age and frequency were the most frequent off-label factors, and neonatal and preschool children were the top 2. In neonates, off-label age use accounted for up to 46.8%, and off-label use of administration frequency in preschool children accounted for up to 52.7%.

3.3. Off-label use of antibacterial agents according to formulations and drugs

The vast majority of antibiotics were administered intravenously (6,202, 90.8%), and a small percentage were administered orally (627, 9.2%; Table 2). Among these, the incidence of off-label use of

TABLE 2 Antibacterial agents used in hospitalized children.

		Formulation	No. of off-label prescriptions/ Total no. prescriptions, *n*			
		Parenteral	2,164/6,202 (34.9%)			
		Oral	130/627 (20.7%)			
Drug classification	Generic name of drug		Percentage cross all the antimicrobials prescriptions ($n = 6,829$)	No. of prescriptions according to generic name	Percentage cross all the off-label prescriptions ($n = 2,294$)	Off-label type (n)[#]
Cephalosporins			2,778 (40.7%)		360 (15.7%)	
	Cefuroxime	Parenteral		231	183(8.0%)	Age (19) and freq& (164)
	Cefotiam	Parenteral		50	42(1.8%)	Age (42)
	Cefaclor	Oral		208	37(1.6%)	Age (18), dose (17), and freq (2)
	Ceftizoxime	Parenteral		87	35(1.5%)	Age (33) and dose (2)
	Cefazolin	Parenteral		215	19(0.8%)	Freq (19)
	Cefaclor	Oral		16	16(0.7%)	Age (16)
	Ceftriaxone	Parenteral		1,124	12(0.5%)	Dose (7) and freq (5)
Macrolides			985 (14.4%)		628 (27.4%)	
	Azithromycin	Parenteral		568	567(24.7%)	Age (566) and dose (1)
	Azithromycin	Oral		43	15(0.7%)	Dose (13) and freq (2)
	Erythromycin	Parenteral		219	25(1.1%)	Dose (4) and freq (21)
Antibacterial drugs combined with enzyme inhibitors			932 (13.6%)		317 (13.8%)	
	Piperacillin and tazobactam	Parenteral		291	144(6.3%)	Age (140) and dose (4)
	Amoxicillin and clavulanate	Parenteral		133	123(5.4%)	Dose (4) and freq (123)
	Cefoperazone and sulbactam	Parenteral		492	45(2.0%)	Freq (45)
Carbapenems			708 (10.4%)		153 (6.7%)	
	Meropenem	Parenteral		555	148(6.5%)	Age (145), dose (2), and freq (1)
Penicillins			676 (9.9%)		610 (26.6%)	
	Flucloxacillin	Parenteral		666	608(26.5%)	Dose (113) and freq (607)
Oxazolidinones			184 (2.7%)		8 (0.3%)	
	Linezolid	Parenteral		176	6(0.3%)	Dose (3) and freq (3)
Antifungal drugs			169 (2.5%)		115 (5.0%)	
	Fluconazole	Parenteral		119	85(3.7%)	Dose (1) and freq (85)
	Micafungin	Parenteral		18	18(0.8%)	Age (18)
Glycopeptides			136 (2.0%)		5 (0.2%)	
	Vancomycin	Parenteral		115	5	Dose (5) and freq (3)
Nitroimidazoles			133 (1.9%)		16 (0.7%)	
	Ornidazole	Parenteral		75	8	Age (5), dose (2), and freq (1)

(Continued)

TABLE 2 (Continued)

		Formulation	No. of off-label prescriptions/ Total no. prescriptions, *n*			
	metronidazole	Parenteral		44	6	Dose (5) and route (1)
Others			51(0.7%)	27		
	Polymyxin B Sulfate	Parenteral		12	12	Age (12)
	Gentamicin	Parenteral		10	10	Route (10)
Sulfonamides			42 (0.6%)		24 (1.0%)	
	Compound sulfamethoxazole	Oral		42	24(1.0%)	Dose (24)

[#]One prescription may have different types of off-label effects; freq&, frequency of administration.

TABLE 3 Off-label antimicrobial agents used according to patient age.

	Newborn	Infant	Early childhood	Preschool	School	Adolescent	Total
Total *n*. of inpatients	396	538	675	801	690	306	3,406
Total *n*. off-label inpatients	328	242	297	370	302	126	1,665
Percentage of off-label patients	82.8%	45.0%	44.0%	46.2%	43.8%	41.2%	48.9%
Total *n*. of prescriptions	920	1,249	1,307	1,513	1,282	558	6,829
Total *n*. off-label prescriptions	652	319	346	440	371	166	2,294
Percentage of off-label prescriptions	70.9%	25.5%	26.5%	29.1%	28.9%	29.7%	33.6%
Mean no. off-label prescriptions per patient ± SD	1.99 ± 1.55	1.32 ± 0.64	1.16 ± 0.43	1.19 ± 0.46	1.23 ± 0.51	1.32 ± 0.67	1.38 ± 0.89
Analysis based on the type of off-label, *n* (%)							
Age	334 (46.8%)	141 (38.8%)	162 (45.9%)	198 (44.4%)	181 (48.5%)	50 (29.9%)	1,066 (44.1%)
Dose	101 (14.2%)	36 (9.9%)	17 (4.8%)	8 (1.8%)	26 (7.0%)	41 (24.6%)	229 (9.5%)
Administration frequency	278 (39.0%)	185 (51.0%)	174 (49.3%)	235 (52.7%)	162 (43.4%)	75 (44.9%)	1,109 (45.9%)
Administration route	0 (0.0%)	1 (0.3%)	0 (0.0%)	5 (1.1%)	4 (1.1%)	1 (0.6%)	11 (0.5%)

intravenously administered drugs was higher (34.9%). Cephalosporins were used most frequently in pediatric patients in our hospital; consistent with the results of other studies (Zhang et al., 2018), off-label use was relatively rare. Flucloxacillin, azithromycin, and antibacterial drugs combined with enzyme inhibitors are also widely used, and their off-label uses are more common.

Azithromycin had a high percentage of off-label use because its intravenous preparations were not approved for use in children under 16 years of age. The common disease for this age group was *Mycoplasma pneumonia* infection, for which the conventional treatment drug was azithromycin.

Other drugs that were used off-label due to age included meropenem ($n = 145$), piperacillin and tazobactam ($n = 140$), cefotiam ($n = 42$), and ceftizoxime ($n = 33$). Some drugs had no information on administration for children and were regarded as off-label, such as micafungin ($n = 18$), cefaclor of oral formulation ($n = 16$), and polymyxin B ($n = 12$). The main reason for off-label flucloxacillin use was the frequency (two times a day instead of four times a day), which accounted for nearly 50% of all off-label administration frequencies, followed by cefuroxime ($n = 164$), amoxicillin and clavulanate ($n = 123$), fluconazole ($n = 85$), and cefoperazone and sulbactam ($n = 45$). Penicillin and cephalosporins must be administered multiple times daily; however, this is usually not the case. Off-label fluconazole administration mainly occurred in the neonatal period, which was required to extend the interval between dosing in newborns; this is usually clinically ignored. A single dose of flucloxacillin ($n = 113$) was often not standardized; therefore, sulfamethoxazole-trimethoprim

($n = 24$) and cefaclor ($n = 17$) were used. Only two drugs were used off-label administration routes: gentamicin (10/10) and metronidazole ($n = 1$; Table 2).

Azithromycin and meropenem are recommended by the SD Consensus (Hou, 2021). Zhou et al. (2021) published recommendations on off-label use related to the intravenous administration of azithromycin in children. The off-label use of other drugs has not been recommended by expert consensus or guidelines [Chen et al., 2015; The Catalogue of Off-label Usage (The New Usage of 2020), 2020]. No severe adverse reactions were observed in this study.

4. Discussion

Off-label drug use is a clinically inevitable but potentially risky behavior that is more common in pediatric patients owing to the lack of clinical trials in this patient cohort (Gore et al., 2017; Shanshal and Hussain, 2021). Antibacterial agents are one of the most commonly used drugs in pediatrics, and their off-label use often causes serious social problems, such as the production of drug-resistant bacteria (European Medicines Agency, 2015; Zhang et al., 2018; Castagnola et al., 2021; Romandini et al., 2021; Yusuf and Zakir, 2021), which requires more attention. At the same time, further studies are needed on the mechanism of antimicrobial resistance (AMR), for example, "the plasmid paradox," if it also holds true for clinically relevant AMR-harboring CPs and their bacterial hosts, which will greatly affect the measures of antibiotic resistance management (Shen et al., 2022).

This retrospective investigation included 6,829 prescriptions, including 2,294 off-label prescriptions. The main off-label factors were the appropriate population (no pediatric information and out of the required age range) and dosage (dose and frequency). We found that when more antibacterial drugs were prescribed to the same patient, the greater the possibility of off-label use; newborns had the most off-label prescriptions. Only six of the 15 most commonly used antibacterial drugs in pediatrics were evidence-based. Among them, the highest level of evidence was for meropenem, with an evidence level of one, followed by azithromycin (level 5), then other drugs (level 6). The reasons for off-label use were consistent with the results of other studies (Guo and Wang, 2014; Zhang et al., 2018; Wu and Zhou, 2020; Shanshal and Hussain, 2021). These reasons included the disease having no indications for children, the condition requiring an increase in the drug dosage or frequency of administration, and some irregular uses—insufficient single dose, less frequent administration, or changing the route of administration.

Although there are many relevant consensuses and regulations on off-label use in China, few apply to children. The SD Consensus (2022) (Tang et al., 2022) is the first to list a separate catalog for children. Although the consensus provides a vital reference basis for off-label antibacterial drugs in pediatrics, more clinical studies initiated by manufacturers or clinical investigators are also needed. Consensus and guidelines must be tracked and updated promptly.

In addition, we found many irregular uses of antibacterial drugs, which required us to strictly follow specific procedures to manage off-label use for infections at a time when the resistance rate of antibiotics is increasing significantly worldwide (Dyar et al., 2016). Most hospitals in China are restricted to off-label use when there are no reasonable alternative medicines and treatments that severely affect patients' quality of life or cause life-threatening conditions. Adverse drug reactions, contraindications, and precautions are fully considered, and it is ensured that the usage is the best solution. Moreover, off-label use is permitted when medication is for the benefit of patients only and not for experimental research. Third, the user must be supported by advanced evidence-based medicine. Finally, the user must be reported to the Hospital Pharmaceutical Affairs Management and Pharmacotherapy Committee and the Hospital Medical Ethics Committee for approval. Informed consent should also be obtained from all the patients.

Furthermore, off-label use was not only due to imperfect labeling but also to improper medications or medication errors. The prescription behavior of doctors also needs to be regulated. Many doctors and pharmacists know about off-label drug use, but they are more concerned about the efficacy of such drugs than licensed medicines in children (Shakeel et al., 2020). Rational use of drugs, especially antimicrobials, and minimizing the risk are the responsibility of all people, including the state, medical institutions, and individuals. The state shall issue laws, norms, and management systems to regulate off-label use. The NMPA constantly urges the marketing authorization holders of these drugs to fulfill their main responsibility and requires improvements to the information presented. Associations and drug management departments should actively develop guidelines, expert consensus, and catalogs of off-label drugs. Hospitals should implement hierarchical management of off-label use, such as strict management of off-label drug use with few clinical applications, high prices, and uncertain efficacy. For a widely used off-label drug in the clinic that has a definite curative effect and a high level of evidence, NMPA should be promptly suggested to revise the label. Doctors and pharmacists should be encouraged to actively research and explore the clinical effectiveness and safety of drugs, as well as patient benefit evaluation, to provide the theoretical basis for off-label drug use.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Biomedical Research Ethic Committee of Shandong Provincial Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

NH, LT, and KZ conceived and designed the study. LT and KZ organized the database and performed the statistical analyses. LT wrote the first draft of the manuscript. NH wrote the sections of the

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Isolation of Hv-CRKP with co-production of three carbapenemases (bla_{KPC}, $bla_{OXA-181}$ or $_{OXA-232}$, and bla_{NDM-1}) and a virulence plasmid: a study from a Chinese tertiary hospital

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Background: The worldwide dissemination of *K. pneumoniae* isolates is a significant public health concern, as these organisms possess a unique capacity to acquire genetic elements encoding both resistance and hypervirulence. This study aims to investigate the epidemiological, resistance, and virulence characteristics of *K. pneumoniae* isolates that carry both virulence plasmids and $bla_{OXA-48-like}$ genes in a tertiary hospital in China.

Methods: A total of 217 clinical isolates of carbapenem-resistant *K. pneumoniae* (CRKP) were collected between April 2020 and March 2022. The antimicrobial susceptibility test was conducted to evaluate the drug resistance profile. All isolates were screened for the presence of genes encoding carbapenemases (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA-48-like}$), ESBLs genes (bla_{CTX-M}, bla_{SHV}, bla_{TEM}), and virulence plasmid pLVPK-borne genes (*rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg344*) using polymerase chain reaction (PCR) amplification. Clonal lineages were assigned using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). The plasmid incompatibility groups were identified using PCR-based replicon typing (PBRT). The transferability of carbapenemase-encoding plasmids and pLVPK-like virulence plasmids was assessed via conjugation. The plasmid location of *rmpA2* was determined using S1-Pulsed Field Gel Electrophoresis (S1-PFGE) and southern blotting hybridization. The virulence potential of the isolates was assessed using the string test, capsular serotyping, serum killing assay and a Galleria mellonella larval infection model.

Results: Of the 217 CRKP clinical isolates collected, 23% were identified as carrying $bla_{OXA-48-like}$ genes. All $bla_{OXA-48-like}$ isolates exhibited resistance to commonly used clinical antimicrobial agents, except for ceftazidime/avibactam, colistin, tigecycline, trimethoprim-sulfamethOXAzole, polymyxin B, and nitrofurantoin. The main common OXA-48-like carbapenemase enzymes were found to be $bla_{OXA-181}$ and $bla_{OXA-232}$. MLST and PFGE fingerprinting analysis

revealed clonal transmission and plasmid transmission. OXA-48-like producing CRKP isolates mainly clustered in K64 ST11 and K47 ST15. Results of the string Test, serum killing assay (*in vitro*) and *Galleria mellonella* infection model (*in vivo*) indicated hypervirulence. PBRT showed that the $bla_{OXA-181}$ and $bla_{OXA-232}$ producing hypervirulent carbapenem-resistant *Klebsiella pneumoniae* (Hv-CRKP) were mainly carried on ColE-type, IncF, and IncX3. Eight clinical isolates of hv-CRKP were identified as carrying three carbapenem-resistant genes (bla_{KPC}, $bla_{OXA-181\ or\ OXA-232}$, and bla_{NDM-1}). Moreover, Southern blotting hybridization revealed that all eight isolates had a pLVPK-like virulent plasmid (138.9–216.9kb) with an uneven number and size of plasmid.

Conclusion: In our investigation, we have observed the emergence of hv-CRKP carrying $bla_{OXA-48-like}$ genes, which identified two genetic relationships: clonal transmission and plasmid transmission. PBRT analysis showed that these genes were mainly carried on ColE-type, IncF, and IncX3 plasmids. These isolates have been shown to be hypervirulent *in vitro* and *in vivo*. Additionally, eight clinical isolates of hv-CRKP were identified as carrying three carbapenem-resistant genes (bla_{KPC}, $bla_{OXA-181\ or\ OXA-232}$, and bla_{NDM-1}) and carrying a pLVPK-like virulent plasmid. Hence, our findings highlight the need for further investigation and active surveillance of hypervirulent OXA-48-like producing Hv-CRKP isolates to control their transmission.

KEYWORDS

***Klebsiella pneumoniae*, carbapenem-resistance, mobilized virulence factors, bla_{OXA} gene, antimicrobial-resistant genes**

1. Introduction

The Antimicrobial resistance (AMR) is a serious threat to global health, according to the World Health Organization. Among clinical pathogens, *K. pneumoniae* is particularly concerning due to its propensity to acquire multidrug resistance and hypervirulence-encoding mobile genetic elements (Yang et al., 2021). Carbapenem resistance in *K. pneumoniae* is often mediated by plasmid-encoded carbapenemase enzymes, such as bla_{KPC}, bla_{NDM}, and $bla_{OXA-48-like}$ enzyme (Han et al., 2020).

Hypervirulent *K. pneumoniae*, first identified from cases of liver abscess, has been increasingly reported worldwide (Russo and Marr, 2019). A recent study demonstrated that *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2* were the most accurate molecular markers for defining hvKP, all of which have been shown to be located in the virulence plasmid (Russo et al., 2018). In recent years, more and more *K. pneumoniae* isolates integrating both hypervirulence and carbapenem resistance phenotypes have been identified, creating hypervirulent and carbapenem-resistant *K. pneumoniae* that result in devastating clinical outcomes (Yang et al., 2022).

Surveillance studies have revealed that OXA-48-like β-lactamases are among the 2nd or 3rd most common carbapenemases found in Enterobacterales globally (Pitout et al., 2019). OXA-48-like carbapenemases are mainly found in *K. pneumoniae* isolates submitted from hospital sites and have been increasing toward the end of surveillance periods (de Jonge et al., 2016; Karlowsky et al., 2017). Data from global surveillance programs such as SMART (Karlowsky et al., 2017) and INFORM (de Jonge et al., 2016) show that 27% of carbapenemase-producing Enterobacterales (CPE; $n = 1{,}615$) carry $bla_{OXA-48-like}$ carbapenemases (compared to 55% bla_{KPCs} and 26% bla_{NDMs}). In some regions, such as the Middle East, North Africa, and certain European countries like Belgium and Spain, OXA-48-like enzymes were the most prevalent carbapenemases among Enterobacterales (Pitout et al., 2019).

In recent years, cases of OXA-48-like *K. pneumoniae* isolates have been on the rise in China. For instance, OXA-232-producing CRKP was first isolated from five neonatal patients in China in 2017 (Yin et al., 2017), while the first report of OXA-181-producing *K. pneumoniae* from the fecal specimen of a patient in China was in 2020 (Liu et al., 2020). Subsequent reports have documented an increasing number of $bla_{OXA-48-like}$ *K. pneumoniae* isolates in China (Liu et al., 2020; Shi et al., 2020; Jia et al., 2021). In December 2016, the draft genome sequences of three hypervirulent CRKP isolates from India were reported to harbor bla_{OXA} genes ($bla_{OXA-232}$, $bla_{OXA-181}$, and bla_{OXA-1}) along with the *rmpA2* gene (Shankar et al., 2016). While China reported the emergence of OXA-232 carbapenemase-producing *K. pneumoniae* carrying a pLVPK-like virulence plasmid among elderly patients in February 2019, these isolates were not hypervirulent despite carrying a virulence plasmid (Shu et al., 2019). This study aims to investigate the resistance mechanisms and molecular epidemiology of hypervirulent *Klebsiella pneumoniae* isolates producing OXA-48-like carbapenemases in a Chinese tertiary hospital.

2. Materials and methods

2.1. Bacterial isolates and definitions

Between April 2020 and March 2022, the First Affiliated Hospital of Nanchang University in China collected 217 unique clinical

carbapenem-resistant *K. pneumoniae* isolates, characterized by minimum inhibitory concentrations (MICs) of ertapenem >0.5 μg/mL, imipenem >4 μg/mL or meropenem >8 μg/mL. All isolates were identified using the VITEK 2 automated system (bioMerieux, Marcy l'Etoile, France) and the MALDI-TOF MS system (Bruker Daltonics, Billerica, MA, United States) and stored at −80°C until use. The MIC of tigecycline was determined through the *E*-test (AB Biodisk, Solna, Sweden) on Mueller-Hinton media. Susceptibility to colistin and tigecycline was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines,[1] while susceptibilities to other agents were interpreted using the Clinical and Laboratory Standards Institute (CLSI) breakpoints (document M100-S32).

All isolates were screened for the presence of genes encoding carbapenemases (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA\text{-}48\text{-}like}$), ESBLs genes ($bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}), and virulence plasmid pLVPK-borne genes (*rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg344*) using polymerase chain reaction (PCR) amplification, as previously described (Liu et al., 2019). PCR products were visualized by agarose gel electrophoresis and sequencing, and the sequence analysis of PCR products was conducted by Sangon Biotech (Shanghai, China) and aligned in bla_{ST} searches in the NCBI Genbank. Isolates positive for $bla_{OXA\text{-}48\text{-}like}$ genes and virulence genes were further studied.

2.2. Clinical data collection

The clinical data used in this study were obtained from the Electronic Medical Records of inpatients at the First Affiliated Hospital of Nanchang University. The data included patient demographics, date of isolation, clinical diagnosis, specimens, ward admission, antimicrobial treatment, and hospitalization outcomes. The study and consent procedures were approved by the Ethical Committee of the First Affiliated Hospital of Nanchang University.

2.3. Molecular epidemiology analysis: multilocus sequence typing and pulsed-field gel electrophoresis

MLST and PFGE was used to evaluate the genetic relatedness of isolates positive for $bla_{OXA\text{-}48\text{-}like}$ genes and virulence genes.

MLST was conducted in accordance with the protocol outlined on the Pasteur Institute MLST website, using seven conserved housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, and tonB). The resulting MLST amplicons were purified and sequenced by Sangon Biotech in Shanghai, China, and compared to those in the MLST database to determine the sequence type (ST).

PFGE using XbaI from TaKaRa was performed. DNA fragments were then separated via a CHEF DR III apparatus (Bio-Rad, Richmond, CA, United States), with Salmonella serotype Braenderup isolate H9812 serving as a molecular marker. Subsequently, BioNumerics software version 7.6 was utilized to construct a tree diagram using the unweighted Pair-Group Method with Arithmetic means (UPGMA) and the Dice similarity coefficient (SD) with a 1.5% position tolerance. Isolates were considered genetically similar if their Dice coefficient correlation exceeded 80%, in line with the "possibly related (4–6 bands difference)" criteria developed by Tenover et al. (1995).

2.4. Plasmid analyses

2.4.1. Plasmid conjugation

Conjugation was employed to evaluate the transferability of plasmids carrying carbapenemases (bla_{KPC}, $bla_{OXA\text{-}181\ or\ OXA\text{-}232}$, and $bla_{NDM\text{-}1}$) and pLVPK-like virulence plasmid. Eight clinical isolates of CR-hvKP carrying carbapenem-resistant genes (bla_{KPC}, $bla_{OXA\text{-}181\ or\ OXA\text{-}232}$, and $bla_{NDM\text{-}1}$) were used as donors, while rifampicin-resistant *E. coli* EC600 was used as the recipient. Both donor and recipient isolates were cultured in Luria-Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C with shaking (180 rpm) until they reached their exponential growth phase (OD600 = 0.4–0.6). The overnight cultures were then mixed in a 1:1 ratio and incubated at 37°C for 16–20 h. After incubation, 100 μL of the sample was spread onto MH agar plates containing imipenem (5 μg/mL), potassium tellurite (5 μg/mL), and rifampicin (600 μg/mL).

2.4.2. PCR-based replicon typing (PBRT)

Plasmid incompatibility groups were determined using PBRTas previously described in literature (Carattoli et al., 2005; Carattoli, 2009; Carattoli, 2011). PBRT was used to tract the plasmids conferring drug resistance in epidemiological of transconjugants and isolates positive for blaOXA-48-like genes and virulence genes. The identified plasmid incompatibility groups included HI1, HI1b, HI2, I1-γ, L/M, N, FIA, FIB, FIC, FIIA, F, K, B/O, W, Y, P, A/C, T, X, X1, X2, X3, and X4.

2.4.3. S1-pulsed field gel electrophoresis and southern blotting hybridization

Plasmid characteristics were assessed by S1-PFGE. Southern blotting hybridization was performed to determine the plasmid location of the virulence plasmid with a *rmpA2* gene. In brief, the isolates were embedded in 1% Seakem Gold agarose and digested with S1-nuclease (Takara, Otsu, Japan) at 37°C for 30 min, and plasmids were separated on a CHEF DR III apparatus (Bio-Rad, Richmond, CA, United States) for 18 h at 14°C, using a 0.8% agarose gel and run conditions of 6 V/cm and pulse times ranging from 2.16 s to 63.8 s. Plasmid molecular mass standards covering a range from 20.5 kb to 1,135 kb, isolated from Salmonella serotype Braenderup isolate H9812, were used. The transferred plasmids on the S1-PFGE gel were transferred to Hybond-N+ membranes (Amersham), following a previously described protocol (Liu et al., 2017). The probe labeling for *rmpA2* and hybridization were conducted using the DIG-High Prime DNA Labeling and Detection Starter Kit I, following the manufacturer's instructions (CAT.NO.11745832910, Roche, Mannheim, Germany).

2.5. Virulence assessment of transformant

2.5.1. Hyperviscous phenotype detection (string test)

For isolates that were positive for all the aforementioned virulence genes, hypermucoviscosity was defined as present when the viscous

1 http://www.eucast.org/clinical_breakpoints/

string was longer than 5 mm when colonies were stretched on an agar plate.

2.5.2. Serum killing assay

In addition, we performed a serum killing assay to determine *in vitro* virulence, as described in previous literature (Liu et al., 2017). Briefly, serum was collected from healthy individuals and stored at −80°C. An inoculum of 106 CFU mid-log phase bacteria was incubated with 75% pooled human serum, and viable counts were recorded at 0, 1, 2, and 3 h of incubation at 37°C and 200 rpm. Each isolate was tested at least three times. The reaction to serum killing was classified into six grades and categorized as highly sensitive (grade 1 or 2), intermediately sensitive (grade 3 or 4), or resistant (grade 5 or 6). Grade 1 indicated viable counts <10% of the inoculum after 1 and 2 h, and < 0.1% after 3 h. Grade 2 referred to viable counts between 10 and 100% of the inoculum after 1 h and < 10% after 3 h. Grade 3 indicated viable counts exceeding those of the inoculum after 1 h but <100% after 2 and 3 h. Grade 4 referred to viable counts >100% of the inoculum after both 1 and 2 h but <100% after 3 h. Grade 5 referred to viable counts >100% of the inoculum at 1, 2, and 3 h, which decreased during the third hour. Grade 6 referred to viable counts that exceeded those of the inoculum at 1, 2, and 3 h and increased throughout this period. Isolates K. pneumonia ATCC 700603 and the hvKP isolates NTUH-K2044 were used as negative and positive controls, respectively, with serum killing sensitivity of grade 2 and resistance of grade 5.

2.5.3. *Galleria mellonella* infection model

The larvae of *Galleria mellonella* (Gm) was an infection model for the virulent to evaluate study virulence of gram-negative bacteria isolates (Ennis and Sells, 1968; Asai et al., 2022), so we evaluated *in vivo* virulence using the *Galleria mellonella* infection model to assess hypervirulence. Microbial virulence in the *G. mellonella* infection model is typically assessed within 5 d and the most commonly used end point is the survival rate at different time points (Asai et al., 2022). Specific experimental steps was as previously described (Mclaughlin et al., 2014). In brief, 10 pathogen-free *G. mellonella* larvae weighing between 250 and 350 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) were used for each isolate. A mid-log-phase culture was washed and diluted with PBS, and each larva was inoculated by injecting 1×10^6 CFU in a 10 ul aliquot into the hemocoel via the rear left pro leg. Survival rate was recorded every 24 h for 4 days, and larvae were kept in petri dishes at 37°C in the dark. All experiments were conducted in triplicate. The calculation of the LD50 value has been proposed to define hypervirulence in the *Galleria mellonella* infection model for *K. pneumoniae* isolates (Li et al., 2020). The HvKP isolate NTUH-K2044 and PBS were used as controls for high and low virulence, respectively. Statistical analyses were performed and visualized using GraphPad Prism 8.0.

2.6. Statistical analyses

The statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, United States). Categorical variables were compared using either the chi-square test or Fisher's exact test, and a *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Prevalence of CRKP co-carrying pLVPK-like virulence plasmid and *bla*$_{\text{OXA-48-like}}$ carbapenemases genes in a Chinese tertiary hospital

A total of 217 clinical isolates of CRKP were collected from our hospital between April 2020 and March 2022. Among these isolates, 50 (23%) carried both the pLVPK-like virulence plasmid and *bla*$_{\text{OXA-48-like}}$ carbapenemase genes (Figure 1). These clinical isolates were obtained from various clinical specimens, including blood (Pitout et al., 2019), pus (Li et al., 2020), sputum (Khan et al., 2019), and urine (Asai et al., 2022) (Table 1). The ICU occupancy rate for these patients was 62% (31/50), and the overall mortality rate among inpatients involved in the outbreak was 52% (26/50). The median age of patients was 54.7 ± 12.6 years, and the male-to-female ratio was 2.3 (Table 1).

Upon sequence comparison with GenBank, we found that OXA-181 (62%, 31/50) and OXA-232 (38%, 19/50) were the most common carbapenemases identified among the OXA-48-like carbapenemases. All the isolates were found to be positive for the presence of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes. Additionally, the *bla*$_{\text{SHV}}$ gene was detected in over 80% of the isolates. As shown in Figure 2, eight clinical isolates of CRKP carried three carbapenem-resistant genes, including five isolates producing *bla*$_{\text{KPC+NDM+OXA-181}}$ and three isolates producing *bla*$_{\text{KPC+NDM+OXA-232}}$.

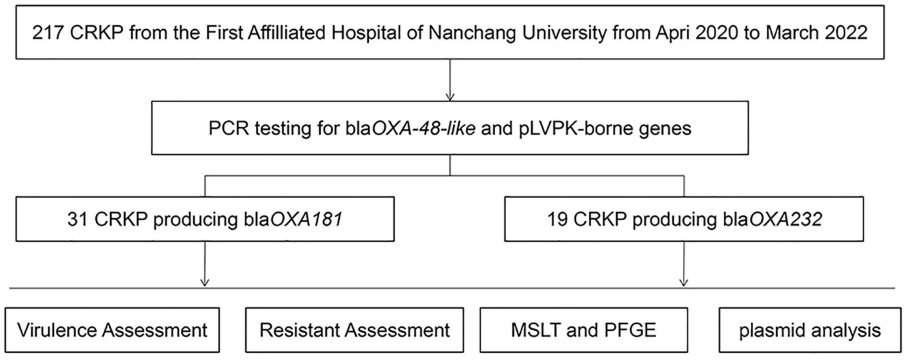

FIGURE 1
Schematic procedure of the study.

TABLE 1 The clinical data of patients infected with *K. pneumoniae* isolates co-carrying pLVPK virulence plasmid and blaOXA-48-like carbapenemases genes.

Patients	Isolates	Age	Gender	Date	Ward	Diagnosis	Specimens	Antimicrobial therapy	Outcome
patient01	oxakp01	58	Male	30-Jun-21	Department of Medical Rehabilitation	Intracerebral hemorrhage	Sputum	Cefoperazone/Sulbactam, Tigecycline	Recovered
patient02	oxakp02	51	Male	30-Sep-20	ICU	Respiratory failure	Sputum	Tigecycline, polymyxin B, Meropenem, Cefoperazone/Sulbactam	Recovered
patient03	oxakp03	54	Female	16-Nov-20	Department of Gastroenterology	Acute respiratory failure	Blood	–	Died
patient04	oxakp04	62	Female	19-Dec-20	Department of Neurosurgery	Hypoxic–ischemic encephalopathy	Blood	–	Recovered
patient05	oxakp05	62	female	19-Dec-20	Department of Neurosurgery	Gallstone with cholecystitis	Blood	Amikacin, Levofloxacin, Cefoperazone/Sulbactam, Imipenem	Recovered
patient06	oxakp06	40	Male	29-Oct-20	Department of Gastroenterology	Brain hernia and ventricular hemorrhage	Blood	Cefoperazone/Sulbactam, Tigecycline, Meropenem, Ceftazidime-avibactam	Died
patient07	oxakp07	57	Male	20-Sep-20	Department of Neurosurgery	Severe pneumonia	Sputum	–	Died
patient08	oxakp08	55	Male	28-Oct-20	Department of Neurosurgery	Septicemia and severe pneumonia	Sputum	Biapenem, Daltomycin, Tigecycline, Imipenem	Died
patient09	oxakp09	65	Male	28-Nov-20	Department of Respiration	Epidural hematoma	Sputum	Meropenem, Tigecycline, polymyxin B, Ceftazidime-avibactam, Amikacin	Recovered
patient10	oxakp10	40	Male	8-Nov-20	Department of Emergency Room	Acute severe pancreatitis, sepsis	Drainage	Tigecycline, Cefoperazone/Sulbactam, polymyxin B, Teicoplanin, Ceftazidime-avibactam	Died
patient11	oxakp11	70	Female	28-Nov-20	Department of Emergency Room	Multiple damages	Sputum	Tigecycline, Linezolid	Recovered
patient12	oxakp12	50	Male	7-Aug-21	ICU	Acute severe pancreatitis	Blood	Biapenem, Daltomycin, Tigecycline	Died
patient13	oxakp13	69	Male	20-Nov-20	Department of Neurosurgery	Acute pancreatitis	Sputum	Imipenem, Linezolid, Ceftazidime-avibactam	Died
patient14	oxakp14	68	Male	4-Dec-20	Department of Infectious Disease	Benign neoplasm of craniopharyngeal duct	Blood	Ceftazidime, Amikacin, Tigecycline, Ceftazidime-avibactam, polymyxin B, Cefoperazone/Sulbactam	Recovered
patient15	oxakp15	68	Male	4-Dec-20	Department of Infectious Disease	Acute severe pancreatitis	Blood	Biapenem, Tigecycline, polymyxin B, Teicoplanin	Died
patient16	oxakp16	24	Female	4-Nov-20	Department of Orthopedics	Lung adenocarcinoma and tracheal stent implantation	Urine	Biapenem	Recovered

(Continued)

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TABLE 1 (Continued)

Patients	Isolates	Age	Gender	Date	Ward	Diagnosis	Specimens	Antimicrobial therapy	Outcome
patient17	oxakp17	87	Female	30-Nov-20	Department of General Practice	Acute severe pancreatitis	Blood	Biapenem, Daltomycin, Tigecycline	Died
patient18	oxakp18	50	Male	14-Aug-21	ICU	Cervical dislocation	Blood	Amikacin, polymyxin B, Cefoperazone/Sulbactam	Recovered
patient19	oxakp19	44	Female	16-Sep-21	ICU	Severe pneumonia	Blood	Imipenem, Tigecycline, Amikacin, polymyxin B, Meropenem, Ceftazidime-avibactam	Died
patient20	oxakp20	43	Male	3-Oct-21	Department of Respiration	Multiple organ failure	Blood	Ceftazidime-avibactam, polymyxin B, Meropenem	Recovered
patient21	oxakp21	43	Male	5-Oct-21	Department of Respiration	Atlas fracture	Blood	Tigecycline, Cefoperazone/Sulbactam, polymyxin B, Teicoplanin, Meropenem	Recovered
patient22	oxakp22	37	Female	24-Nov-22	ICU	Cerebral contusion	Blood	SMZ, Biapenem, Teicoplanin, Tigecycline, Meropenem, Piperacillin-tazobactam	Recovered
patient23	oxakp23	37	Female	24-Nov-21	ICU	Intracerebral hemorrhage	Blood	Linezolid, Meropenem	Died
patient24	oxakp24	50	Female	21-Nov-22	Department of Respiration	Explosive myocarditis	Sputum	Daltomycin, polymyxin B	Died
patient25	oxakp25	37	Female	10-Nov-21	ICU	Spinal injuries	Drainage	polymyxin B	Recovered
patient26	oxakp26	65	Male	1-Nov-21	Department of Respiration	Basal ganglia hemorrhage	Pus	Linezolid, Meropenem, polymyxin B	Recovered
patient27	oxakp27	62	Male	3-Dec-22	Department of Orthopedics	Acute severe pancreatitis and sepsis	Blood	Tigecycline, Cefoperazone/Sulbactam, polymyxin B, Teicoplanin, Ceftazidime-avibactam	Died
patient28	oxakp28	31	Male	9-Dec-21	Department of Medical Rehabilitation	Very severe open craniocerebral injury	Urine	Linezolid, polymyxin B, Teicoplanin	Died
patient29	oxakp29	64	Male	3-Dec-21	Department of General Surgery	Benign neoplasm of pituitary gland	Drainage	Linezolid, Tigecycline, Levofloxacin	Recovered
patient30	oxakp30	46	Male	7-Dec-21	ICU	Benign neoplasm of pituitary gland	Pus	Linezolid, Tigecycline, Levofloxacin	Recovered
patient31	oxakp31	51	Male	2-Jan-22	Department of Emergency Room	Chronic liver failure	Blood	Imipenem	Died
patient32	oxakp32	66	Male	6-Jan-22	Department of Neurosurgery	Brain stem neoplasms	Blood	Ceftazidime, Amikacin, Tigecycline	Died
patient33	oxakp33	46	Male	3-Jan-22	ICU	Chronic liver failure	Pus	Imipenem, Linezolid	Died
patient34	oxakp34	50	Female	3-Jan-22	Department of General Surgery	Esophageal cancer surgery	Drainage	Piperacillin-tazobactam钠, Meropenem	Recovered

(Continued)

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TABLE 1 (Continued)

Patients	Isolates	Age	Gender	Date	Ward	Diagnosis	Specimens	Antimicrobial therapy	Outcome
patient35	oxakp35	71	Male	26-Jan-22	Department of Respiration	Common bile duct stone with cholecystitis	Sputum	Imipenem, Linezolid	Recovered
patient36	oxakp36	68	Male	2-Feb-22	ICU	Cerebral contusion	Sputum	Imipenem, Cefoperazone/Sulbactam, Tigecycline	Recovered
patient37	oxakp37	60	Female	18-Feb-22	ICU	Thalamic hemorrhage	Blood	polymyxin B, Linezolid	Died
patient38	oxakp38	67	Male	18-Feb-22	Department of Infectious Disease	Septic shock	Drainage	Meropenem, polymyxin B	Died
patient39	oxakp39	65	Male	19-Feb-22	Department of General Surgery	Adult Ph acute lymphoblastic leukemia	Drainage	Ceftazidime-avibactam, polymyxin B, Tigecycline, Teicoplanin	Died
patient40	oxakp40	52	Male	25-Feb-22	Department of Neurosurgery	Hepatapostema	Blood	Ceftazidime-avibactam, Tigecycline, Imipenem, Amikacin	Died
patient41	oxakp41	66	Female	27-Feb-22	Department of Emergency Room	Biliary tract infection	Drainage	Cefoperazone/Sulbactam	Recovered
patient42	oxakp42	65	Male	6-Mar-22	Department of General Surgery	Hepatapostema	Wound	–	Died
patient43	oxakp43	55	Male	5-Mar-22	ICU	Hepatapostema	Blood	Biapenem, Tigecycline	Recovered
patient44	oxakp44	57	Male	7-Mar-22	ICU	Hepatapostema	Wound	polymyxin B, Ceftazidime-avibactam	Died
patient45	oxakp45	40	Male	22-Feb-22	Department of Emergency Room	Acute pancreatitis	Sputum	Biapenem, Teicoplanin	Recovered
patient46	oxakp46	57	Male	8-Mar-22	ICU	Paraplegia	Drainage	Amikacin	Recovered
patient47	oxakp47	53	Male	10-Mar-22	Department of Neurosurgery	Biliary tract infection	Drainage	Amikacin, Cefoperazone/Sulbactam	Recovered
patient48	oxakp48	55	Male	11-Mar-22	ICU	Thalamic hemorrhage	Sputum	polymyxin B, Linezolid	Died
patient49	oxakp49	70	Male	14-Mar-22	ICU	Idiopathic thrombocytopenic purpura	Sputum	Meropenem, Teicoplanin	Died
patient50	oxakp50	35	Female	13-Mar-22	ICU	Septic shock	Blood	Meropenem, polymyxin B	Died

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3.2. Molecular characteristics

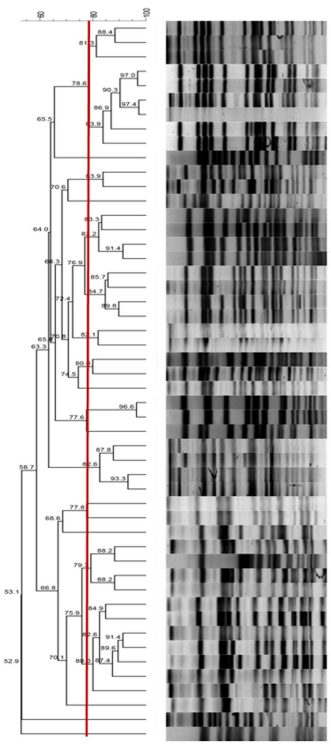
MLST analysis of 50 isolates of OXA-48-like producing CRKP identified nine distinct sequence types (STs), as demonstrated in Figure 2. The most frequently encountered ST was ST11, which accounted for 30 out of 50 isolates, followed by ST15, which was found in eight isolates. No notable differences were observed in the STs of isolates carrying $bla_{\text{OXA-181}}$ versus those carrying $bla_{\text{OXA-232}}$. PFGE analysis demonstrated that CRKP isolates producing both $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$ displayed 19 distinct PFGE patterns, respectively, as depicted in Figure 2. Notably, Cluster A, E, and P exhibited clonal relatedness. Furthermore, both clonal and plasmid transmission was observed based on PFGE analysis. The combined results of PFGE and MLST analysis showed that CRKP isolates co-carrying pLVPK-like virulence plasmid and $bla_{\text{OXA-181 and OXA-232}}$ resistant plasmid mainly clustered in ST11 and ST15 isolates.

3.3. Resistant assessment of *Klebsiella pneumoniae* clinical isolates co-carrying pLVPK-like virulence plasmid and $bla_{\text{OXA-181 and OXA-232}}$ resistant genes

Figure 3 present the antibacterial susceptibility of 50 OXA-48-like producing *K. pneumoniae* isolates. All isolates exhibited resistance to commonly used clinical antimicrobial agents, except for ceftazidime/avibactam, colistin, tigecycline, trimethoprim-sulfamethOXAzole, polymyxin B, and nitrofurantoin. Specifically, the clinical isolates in this study demonstrated complete resistance to Piperacillin-tazobactam, Ticarcillin-clavulanic acid, Cefazolin, Cefepime, Cefoperazone/Sulbactam, Ceftazidime, Ceftriaxone, Aztreonam, and Imipenem (100%). The rates of antibacterial resistance to LevoflOXAcin, CiproflOXAcin, Meropenem, Ertapenem, and Doxycycline were 96, 96, 94, 90, and 90%, respectively, with 48/50, 48/50, 47/50, 45/50, and 45/50 isolates exhibiting resistance to each drug, respectively. Furthermore, these isolates were fully sensitive to Polymixin B and Nitrofurantoin. The tigecycline and colistin MICs were each <1 μg/mL, except for six isolates that had a TGC zone diameter of 4, 4, 8, 8, 8, and 8 mm.

Figure 2 illustrates that all these 50 CRKP carried at least one carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{OXA}) or ESBL genes ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}). As depicted in Figure 2, our study identified eight clinical isolates of Hv-CRKP that carried three carbapenem-resistant genes, namely five of $bla_{\text{KPC+OXA-181+NDM-1}}$ and three of $bla_{\text{KPC+OXA-233+NDM-1}}$. To our knowledge, this is the first report of the co-production of three carbapenemase genes ($bla_{\text{KPC+NDM+OXA181 or OXA232}}$) and the pLVPK-like virulence plasmid in CRKP isolates.

The plasmid-borne resistance to $bla_{\text{OXA-181 and OXA-232}}$ producing CRKP was mainly attributed to ColE-type plasmids (100%, 50/50), IncF plasmids (72%, 36/50), and IncX3 plasmids (26%, 13/50), with IncX3 plasmids always associated with bla_{NDM} (Figure 4). To evaluate the transferability of these resistant plasmids, we selected the



Isoates	Oxa-48-like	Carbapenemase genes	ESBLs genes	Viluerce genes	MLST	Serotype	PFGE	Galleria Mellonella Infection Model
oxakp48	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K47	A	
oxakp09	OXA 232	KPC,OXA232,NDM	TEM,CTX-M,SHV	rmpA2	15	K47	A	<24h
oxakp29	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	IroB	15	K47	A	
oxakp14	OXA 181	OXA181	TEM,CTX-M,SHV	rmpA2,IroB	15	K47	A	
oxakp15	OXA 181	KPC,OXA181,NDM	TEM,CTX-M,SHV	rmpA2	15	K47	A	
oxakp02	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IucA	15	K47	A	
oxakp04	OXA 181	KPC,OXA181	TEM,CTX-M	rmpA	15	K47	A	
oxakp08	OXA 181	OXA181	TEM,CTX-M,SHV	rmpA2	15	K64	A	
oxakp05	OXA 181	KPC,OXA181,NDM	TEM,CTX-M,SHV	rmpA2	15	K64	A	
oxakp39	OXA 181	OXA181,NDM	TEM,CTX-M,SHV	IroB	147	K47	B	24h
oxakp16	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2	11	K64	C	48h
oxakp18	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2,IucA	11	K64	C	
oxakp30	OXA 181	KPC,OXA181	TEM,CTX-M	rmpA,rmpA2,peg344,IroB,IucA	572	K64	D	72h
oxakp03	OXA 181	KPC,OXA181,NDM	TEM,CTX-M,SHV	rmpA2,peg344,IucA	11	K64	E	24h
oxakp33	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA,rmpA2,peg344,IucA	2230	K64	E	
oxakp44	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IucA	11	K64	E	
oxakp49	OXA 181	OXA181	TEM,CTX-M	rmpA	11	K64	E	
oxakp27	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2,IroB,IucA	11	K64	E	
oxakp31	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K64	E	
oxakp13	OXA 181	OXA181,NDM	TEM,CTX-M,SHV	rmpA2	11	K64	E	
oxakp01	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IucA	11	K64	E	
oxakp06	OXA 232	OXA232	TEM,CTX-M,SHV	rmpA	11	K64	F	
oxakp11	OXA 181	OXA181	TEM,CTX-M,SHV	rmpA2,peg344,IucA	11	K64	F	48h
oxakp42	OXA 181	OXA181,NDM	TEM,CTX-M	IroB	147	K64	G	48h
oxakp46	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2	2230	K47	G	
oxakp32	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2,IroB	11	K64	H	<24h
oxakp07	OXA 181	KPC,OXA181,NDM	TEM,CTX-M,SHV	rmpA2	661	K57	I	<24h
oxakp12	OXA 232	OXA232	TEM,CTX-M,SHV	rmpA2	661	K57	I	
oxakp21	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA,IroB	2947	K57	J	<24h
oxakp23	OXA 181	KPC,OXA181,NDM	TEM,CTX-M	rmpA2,IroB	11	K64	K	<24h
oxakp26	OXA 232	OXA232,NDM	TEM,CTX-M	IroB	147	K64	K	
oxakp22	OXA 232	KPC,OXA232	TEM,CTX-M	IroB	11	K64	K	
oxakp25	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	IroB	11	K64	K	
oxakp37	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2,IroB,IucA	11	K64	L	
oxakp43	OXA 232	KPC,OXA232,NDM	TEM,CTX-M,SHV	rmpA2,IucA	11	K64	L	48h
oxakp34	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IroB,IucA	11	K47	M	48h
oxakp47	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K64	N	<24h
oxakp28	OXA 181	OXA181,NDM	TEM,CTX-M	rmpA2,peg-344,IroB,IucA	231	K64	N	
oxakp40	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K64	O	<24h
oxakp45	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K47	O	
oxakp35	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IroB	11	K47	P	<24h
oxakp38	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA,rmpA2,IroB,IucA	11	K57	P	
oxakp24	OXA 232	KPC,OXA232	TEM,CTX-M,SHV	rmpA2,IroB,IucA	11	K47	P	
oxakp36	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA,rmpA2,IucA	11	K64	P	
oxakp41	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IucA	11	K47	P	
oxakp50	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA,rmpA2,peg344,IroB,IucA	11	K64	P	
oxakp10	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2	11	K64	P	
oxakp17	OXA 181	KPC,OXA181	TEM,CTX-M,SHV	rmpA2,IucA	11	K64	Q	<24h
oxakp20	OXA 232	KPC,OXA232,NDM	TEM,CTX-M,SHV	rmpA2,IroB	2947	K54	R	48h
oxakp19	OXA 181	KPC,OXA181	TEM,CTX-M	rmpA2,IroB	1107	K47	S	<24h

FIGURE 2
PFGE typing of 50 clinical OXA-48-like positive CRKP isolates. Genomic DNA from each research strains was digested using Xba I and the digests were subjected to PFGE to generate diagnostic genomic DNA fragmentation fingerprints. The dendrogram of the PFGE profiles was clustered by the UPGAMA on the basis of the Dice similarity by the bionumber software. The red line delineates 80% of the boundary. The strains producing carbapenemases (kpc, OXA, and NDM) are indicated in red font.

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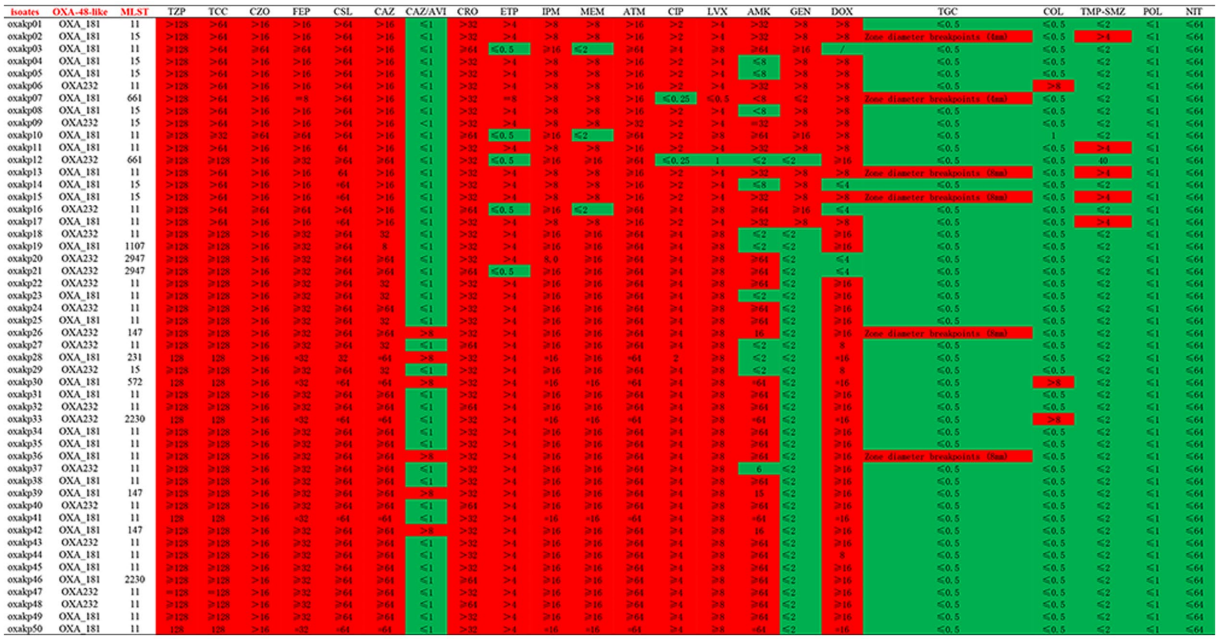


FIGURE 3
In vitro activities of antimicrobial agents and disinfectants against CRKP isolates co-carrying pLVPK virulence plasmid and bla*OXA-48-like* carbapenemases genes.

aforementioned eight isolates that carry three carbapenemase resistance genes and performed the plasmid conjugation experiment. Fortunately, all eight bacterial isolates were successfully conjugated.

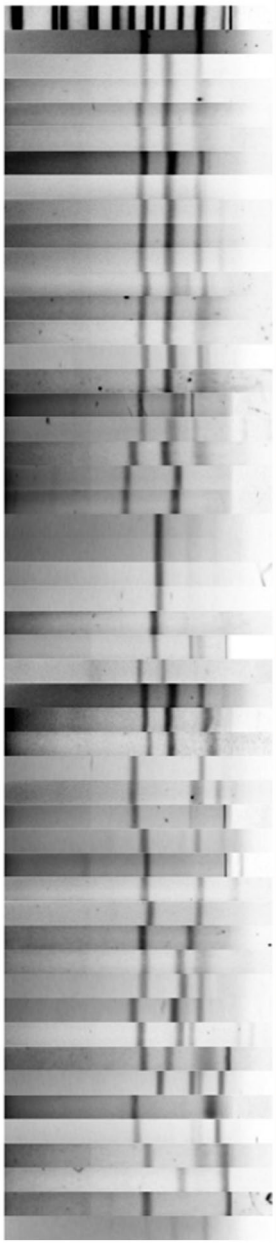
3.4. Virulence assessment of *Klebsiella pneumoniae* clinical isolates co-carrying p-LVPK-like virulence plasmid and $bla_{\text{OXA-181 and OXA-232}}$ resistant genes

As showed in Figure 2, it was observed that all $bla_{\text{OXA-48-like}}$ positive CR-hvKP isolates harbored at least one virulence gene located on a pLVPK-like virulence plasmid (including *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2* genes). The string test was positive for all these isolates. Capsular serotyping showed that 30 isolates were K64, 15 were K47, 4 were K57, and 1 was K54, while K1 and K2 types were not detected. No significant difference was observed between the blaOXA-181 and blaOXA-232 groups. Our findings suggest that *K. pneumoniae* isolates co-carrying p-LVPK-like virulence plasmids and $bla_{\text{OXA-181 and OXA-232}}$ resistant plasmids are mainly clustered in K64 and K47 isolates.

The presence of hypermucoviscosity and plasmid-borne genes resembling pLVPK in the isolated strains suggests hypervirulence. *In vitro* experiments confirmed that the strains exhibited serum resistance, with a survival rate of approximately 78% after 60 min of incubation with serum (Figure 4). To assess virulence *in vivo*, one isolate was randomly selected from each typing cluster based on PFGE. The results, shown in Figure 2, indicate that 19 CRKP isolates were chosen. When a 10^6 CFU suspension of the isolates was used to infect *Galleria mellonella* larvae, 18 isolates had a survival rate of less than 48 h, which was similar to that of a virulent strain of NUTH-K2044 (Figure 2). Eight CRKP isolates, which co-produced three carbapenemases (bla_{KPC}, $bla_{\text{OXA-181}}$ or $_{\text{OXA-232}}$, and $bla_{\text{NDM-1}}$) (Figure 5), were found to harbor a pLVPK-like virulent plasmid (ranging from 138.9 to 216.9 kb) as determined by Southern blotting hybridization of the virulence gene *rmpA2* (Figure 6).

4. Discussion

Bacteria carrying $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-232}}$ are emerging globally, particularly in *K. pneumoniae* and *E. coli*, but their incidence is likely underreported due to laboratory detection challenges (Pitout et al., 2019). The rapid emergence and spread of multidrug-resistant (MDR) and hypervirulent *K. pneumoniae* isolates is a growing concern in clinical settings (Zhang et al., 2020). In recent years, two evolutionary pathways in *K. pneumoniae* have converged, leading to the emergence of carbapenem-resistant and hypervirulent isolates through plasmid recombination and fusion events. Consequently, carbapenem-resistant *K. pneumoniae* (CRKP) and hypervirulent *K. pneumoniae* (hvKP) have merged (Yang et al., 2021). The carriage of a virulence plasmid containing two capsular polysaccharide (CPS) regulator genes (*rmpA* and *rmpA2*) and several siderophore gene clusters (*iucABCD*/*iutA*/*iroBCDN* clusters) is thought to contribute to the hypermucoviscous phenotype of hvKP (Russo et al., 2018; Russo and Marr, 2019). Globally, there has been a growing incidence of MDR and hvKP isolates carrying the $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-232}}$ genes (Pitout et al., 2019), likely resulting from two converging evolutionary pathways of *K. pneumoniae* involving plasmid recombination and fusion events (Zhang et al., 2020; Yang et al., 2021). We report the emergence of hvKP isolates carrying both $bla_{\text{OXA-232}}$ and $bla_{\text{OXA-181}}$ recovered from patients at a teaching hospital in southern China. The high ICU occupancy (62%) and mortality rates (52%) of patients infected with these bacteria highlight the importance of monitoring this isolate. In recent years, an increasing number of *K. pneumoniae*



isoates	String Test	Serum Killing Assay	PBRT
oxakp24	positive	Grade1	IncF,colE
oxakp37	positive	Grade2	IncF,colE
oxakp43	positive	Grade1	IncF,colE,IncX3
oxakp09	positive	Grade4	IncF,colE,IncX3
oxakp27	positive	Grade1	IncF,colE
oxakp38	positive	Grade1	IncF,colE
oxakp45	positive	Grade4	IncF,colE
oxakp32	positive	Grade1	IncF,colE
oxakp47	positive	Grade5	IncF,colE
oxakp50	positive	Grade1	IncF,colE
oxakp30	positive	Grade5	IncF,colE
oxakp31	positive	Grade6	IncF,colE
oxakp07	positive	Grade1	IncF,colE,IncX3
oxakp28	positive	Grade6	colE,IncX3
oxakp23	positive	Grade4	IncF,colE,IncX3
oxakp14	positive	Grade5	colE
oxakp20	positive	Grade5	IncF,colE,IncX3
oxakp10	positive	Grade2	IncF,colE
oxakp35	positive	Grade4	IncF,colE
oxakp26	positive	Grade5	colE,IncX3
oxakp02	positive	Grade6	IncF,colE
oxakp41	positive	Grade2	IncF,colE
oxakp22	positive	Grade5	IncF,colE
oxakp25	positive	Grade6	IncF,colE
oxakp13	positive	Grade5	colE,IncX3
oxakp12	positive	Grade2	colE
oxakp18	positive	Grade4	IncF,colE
oxakp36	positive	Grade5	IncF,colE
oxakp40	positive	Grade4	IncF,colE
oxakp48	positive	Grade5	IncF,colE
oxakp04	positive	Grade4	IncF,colE
oxakp17	positive	Grade1	IncF,colE
oxakp33	positive	Grade5	IncF,colE
oxakp08	positive	Grade2	colE
oxakp44	positive	Grade1	IncF,colE
oxakp05	positive	Grade2	IncF,colE,IncX3
oxakp15	positive	Grade4	IncF,colE,IncX3
oxakp29	positive	Grade6	IncF,colE
oxakp03	positive	Grade5	IncF,colE,IncX3
oxakp06	positive	Grade3	colE
oxakp11	positive	Grade6	colE
oxakp19	positive	Grade6	colE
oxakp01	positive	Grade4	IncF,colE
oxakp42	positive	Grade4	colE,IncX3
oxakp39	positive	Grade1	colE,IncX3
oxakp46	positive	Grade5	colE
oxakp16	positive	Grade5	IncF,colE
oxakp34	positive	Grade1	IncF,colE
oxakp21	positive	Grade6	colE
oxakp49	positive	Grade4	colE

FIGURE 4
S1-PFGE typing of 50 clinical OXA-48-like Positive *K. pneumoniae* isolates. Genomic DNA from each research strains was digested using S1 and the digests were subjected to DNA fragments were separated with a CHEF DR III apparatus.

isolates with integrated hypervirulence and carbapenem resistance phenotypes have been identified, resulting in devastating clinical outcomes (Yang et al., 2022). Our research team found that OXA-resistant isolates with hypervirulence had begun to prevail in China during our active surveillance of CRKP isolates.

In our study, all $bla_{\text{OXA-48-like}}$ positive CR-hvKP isolates exhibited resistance to commonly used clinical antimicrobial agents and harbored at least one virulence gene located on a pLVPK-like virulence plasmid, including *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2* genes. Although OXA-48-like represents weak activity of carbapenemase (Oueslati et al., 2015), all these isolates demonstrated high resistance to carbapenem antimicrobial agents such as imipenem (100%), meropenem (94%), and ertapenem (90%), consistent with the results of another study (Jia et al., 2021). In addition, we detected at least one extended-spectrum β-lactamase (ESBL) gene, such as $bla_{\text{CTX-M}}$, bla_{SHV}, or bla_{TEM}, which might contribute to the observed drug-resistant phenotype.

Horizontal transmission of resistance genes via mobile plasmids is a common dissemination mechanism for carbapenemase-producing Enterobacteriaceae (CPE), resulting in rapid spread of resistance genes across diverse isolates and hosts (Pulss et al., 2018). The high proportion of ST11 (30/50) among the $bla_{\text{OXA-48-like}}$ positive CR-hvKP isolates in our study suggests common clonal origins (Figure 2). Our study further revealed that three clusters of isolates (A, E, and P) were

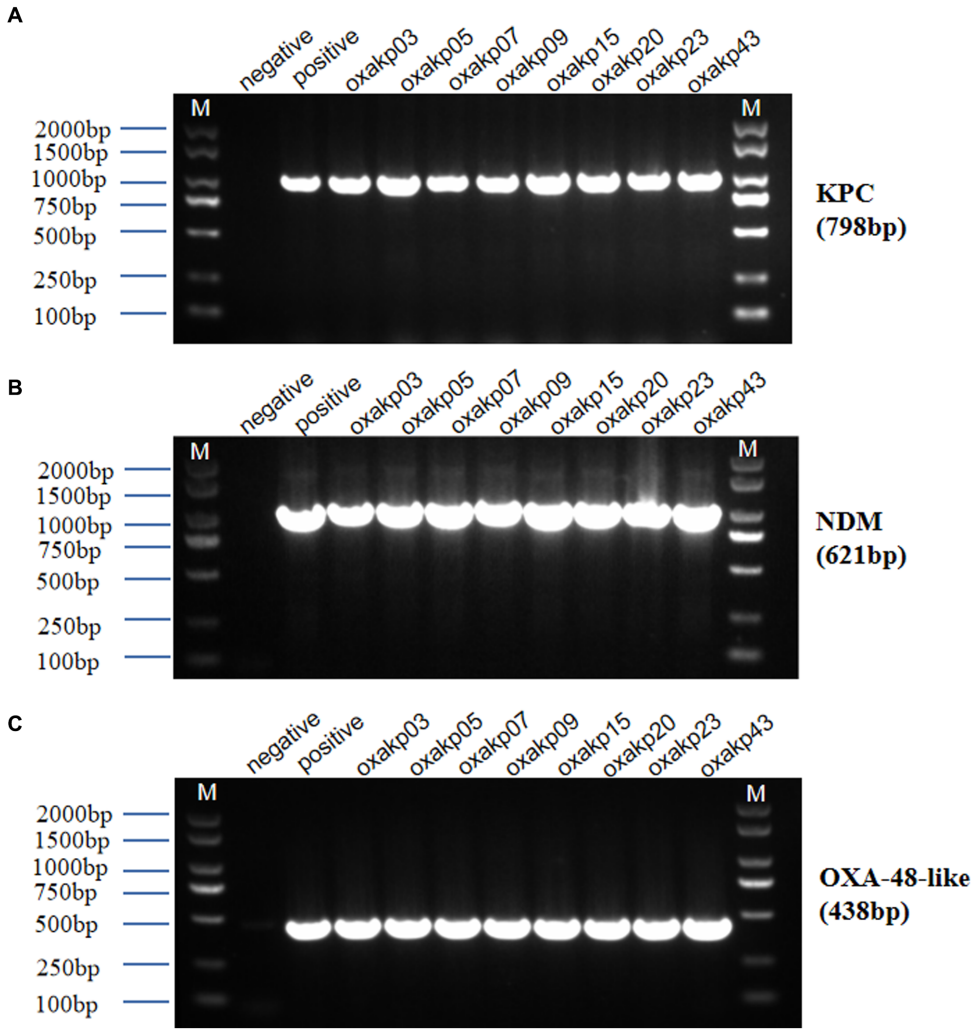


FIGURE 5
Agarose gel electrophoresis was performed for eight clinical isolates of Hv-CRKP carrying three carbapenem-resistant genes **(A–C)**. The gel showed PCR products of expected lengths for the bla_{KPC} gene (approximately 798bp), bla_{NDM} gene (approximately 621bp), and $bla_{OXA-48-like}$ gene (approximately 438bp). M:2000bp size marker, Negative Control did not show amplification.

closely related (Figure 2), with PFGE patterns and MLST demonstrating both clonal and plasmid transmission. These findings suggest that horizontal gene transfer/plasmid transfer plays a crucial role in the dissemination of CR-hvKP strains. Notably, we found a high prevalence of triple positivity for multiple carbapenemases in eight isolates (five producing $bla_{KPC+NDM+OXA-181}$ and three producing $bla_{KPC+NDM+OXA-232}$). Our results are consistent with the continuous global emergence of multidrug-resistant strains (Hu et al., 2014; Khan et al., 2019), which can be sustained by diverse mechanisms, such as R plasmids or transposons (Miller et al., 2014).

In our study, we identified three plasmid replicons (ColE, IncF, and IncX3) with high frequency in our isolates. Previous research has demonstrated that the bla_{NDM} gene can be found in various types of plasmids, including those in the IncF, IncFII, IncN, and IncX3 incompatibility groups (Zhu et al., 2016). The initial acquisition of the OXA-181 gene occurred through the mediation of ISEcp1, which subsequently integrated into Tn2013 and was found on several plasmids such as ColE2, IncX3, IncN1, and IncT. On the other hand, the genetic environment surrounding $bla_{OXA-232}$ is very similar to that of $bla_{OXA-181}$, with the former differing from the latter by only one amino acid substitution (Pitout et al., 2019). Plasmids harboring bla_{KPC} genes, ranging in size from 10 to 300 kb, are commonly found in various incompatibility groups, such as IncF, IncI, IncA/C, IncN, IncX, IncR, IncP, IncU, IncW, IncL/M, and ColE (Chen et al., 2014). In our plasmid conjugation experiment, eight isolates carrying three carbapenemase resistance genes were successfully conjugated. However, Potron A. and colleagues discovered that the plasmid-mediated carbapenem-resistance gene $bla_{OXA-232}$ was located on a small, non-conjugative plasmid called pOXA-232, which carried a ColE-type backbone (Potron et al., 2013; Abdul Momin et al., 2017; Weng et al., 2020). Our research team discovered for the first time the emergence of super resistant bacteria due to the lack of reports on $bla_{KPC+NMD+OXA}$ resistant strains of super carbapenem. Our next challenge is to investigate how these three resistant plasmids can facilitate transfer *in vivo*, as well as the mechanism underlying their coexistence.

pLVPK-like virulent plasmids often have a strong correlation with high hypervirulent phenotypes in *K. pneumoniae*. Several experiments

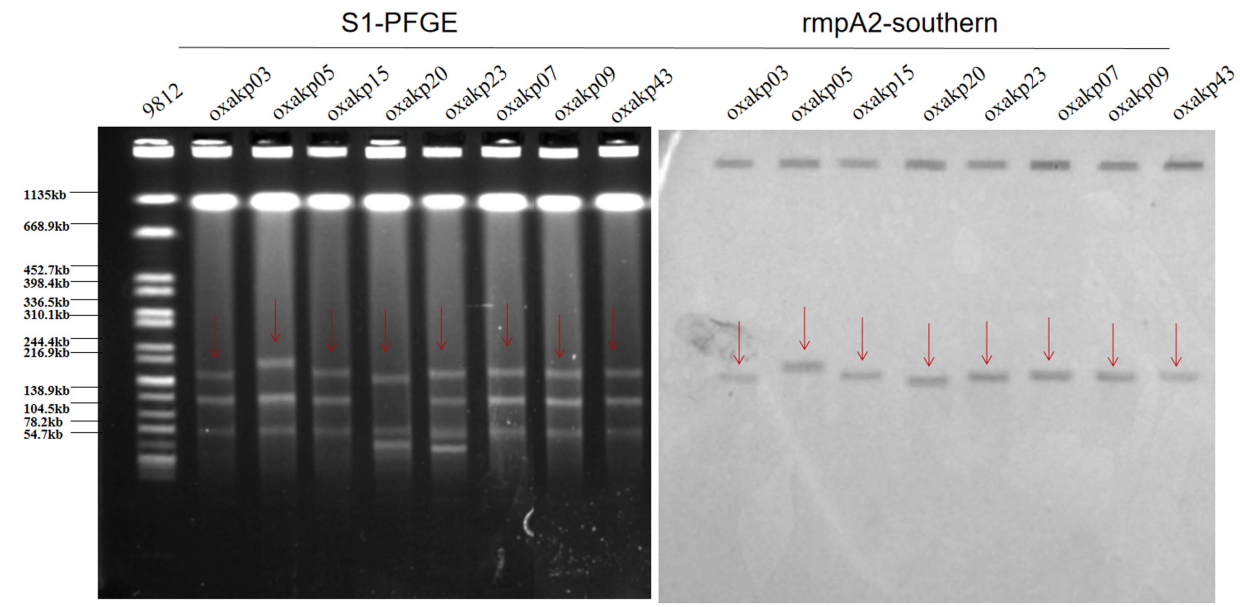


FIGURE 6
S1-PFGE patterns and rmpA2-southern of the eight hv-CRKP strains co-producing three carbapenemases harbored a pLVPK-like virulent plasmid (ranging from 138.9 to 216.9kb).

to confirm the virulent phenotype of these $bla_{\text{OXA-48-like}}$ positive CR-hvKP isolates: hypermucoviscosity (String Test), serum killing assay (*in vitro*) and *Galleria mellonella* infection model (*in vivo*), these isolates have been shown to be hypervirulent. We performed the localization of virulence plasmids for eight strains that carried three resistance plasmids at the same time. Southern blotting hybridization determined that these CRKP carried a pLVPK-like virulent plasmid (138.9–216.9 kb) with uneven numbers and sizes of plasmids. These findings suggest the emergence of hv-CRKP isolates that simultaneously carry three carbapenemases and a virulence plasmid. We speculate that our hv-CRKP isolate may have acquired a virulence plasmid during the evolution of the drug-resistant isolate.

Given these findings, it is essential to carefully monitor and conduct follow-up studies to gain further insights into the epidemiology of multidrug-resistant strains, as well as the possible evolution of successful plasmids and transposition modules that contain three antimicrobial resistance genes ($bla_{\text{OXA-48-like+NDM+KPC}}$) of clinical relevance. Our active surveillance of CRKP isolates led to the discovery of OXA-resistant isolates with hypervirulence that have become prevalent in China. These hypervirulent OXA-resistant isolates carry a pLVPK-like virulence plasmid containing the *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2* genes. Furthermore, we found eight clinical isolates of hv-CRKP carrying three carbapenem-resistant genes: bla_{KPC}, $bla_{\text{OXA-181 or OXA-232}}$, and $bla_{\text{NDM-1}}$. This is the first report of the co-production of three carbapenemase genes ($bla_{\text{KPC+NDM+OXA181}}$ and $bla_{\text{KPC+NDM+OXA232}}$) in CRKP isolates, highlighting the need for active surveillance to control further transmission.

5. Conclusion

This study reports the emergence of hypervirulent OXA-48-like-producing hv-CRKP in our hospital. Based on PFGE and MLST results, two genetic relationships were identified: clonal and plasmid transmission. The most common OXA-like carbapenemases were $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$, which predominantly clustered in K64 ST11 and K47 ST15 isolates. Notably, we identified the co-production of three carbapenemases genes ($bla_{\text{KPC+NDM+OXA181}}$ or $_{\text{OXA232}}$) and a pLVPK-like virulence plasmid in hv-CRKP isolates, which to our knowledge, has not been previously reported. These hv-CRKP isolates carried a pLVPK-like virulence plasmid ranging from 138.9 to 216.9 kb. Therefore, implementation of effective infection control measures is urgently needed to prevent further spread in the region.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

The study was designed by WZ and YL, while T-xP, W-yL, and PL conducted the experiments. SL, Z-yH, and D-DW carried out the analysis. T-XX, PL, and W-JL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genomic epidemiology of nosocomial carbapenemase-producing *Citrobacter freundii* in sewerage systems in the Helsinki metropolitan area, Finland

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Multi-drug resistance is emerging in *Citrobacter freundii*, which is the third most common carbapenemase-producing (CP) *Enterobacteriaceae* in humans in Finland due to recent outbreaks. The objective of this study was to determine if wastewater surveillance (WWS) could detect CP *C. freundii* strains causing infections in humans. Selective culturing was used to isolate CP *C. freundii* from the hospital environment, hospital wastewater, and untreated municipal wastewater in Helsinki, Finland, between 2019 and 2022. Species were identified using MALDI-TOF, and presumptive CP *C. freundii* isolates were subjected to antimicrobial susceptibility testing and further characterized by whole genome sequencing. A genomic comparison was conducted to compare isolates collected from the hospital environment, untreated municipal wastewater, and a selection of isolates from human specimens from two hospitals in the same city. We also examined the persistence of CP *C. freundii* in the hospital environment and the impact of our attempts to eradicate it. Overall, 27 bla_{KPC-2}-carrying *C. freundii* were detected in the hospital environment (ST18; $n = 23$ and ST8; $n = 4$), while 13 bla_{KPC-2}-carrying *C. freundii* (ST8) and five bla_{VIM-1}-carrying (ST421) *C. freundii* were identified in untreated municipal wastewater. CP *C. freundii* was not identified in hospital wastewater. We found three clusters (cluster distance threshold ≤10 allelic difference) after comparing the recovered isolates and a selection of isolates from human specimens. The first cluster consisted of ST18 isolates from the hospital environment ($n = 23$) and human specimens ($n = 4$), the second consisted of ST8 isolates from the hospital environment ($n = 4$), untreated municipal wastewater ($n = 6$), and human specimens ($n = 2$), and the third consisted of ST421 isolates from the untreated municipal wastewater ($n = 5$). Our results support previous studies suggesting that the hospital environment could act as a source of transmission of CP *C. freundii* in clinical settings. Furthermore, the eradication of CP Enterobacteriaceae from the hospital environment is challenging. Our findings also showed that CP *C. freundii* is persistent

throughout the sewerage system and demonstrate the potential of WWS for detecting CP *C. freundii*.

KEYWORDS

wastewater, wastewater surveillance, antimicrobial resistance, *Citrobacter freundii*, carbapenemase-producing *Citrobacter freundii*, carbapenemase-producing *Enterobacteriaceae*

1. Introduction

The World Health Organization has declared antimicrobial resistance (AMR) as one of the top 10 global public health threats, leading to increased mortality and a significant burden for healthcare and economies (WHO, 2021).

Citrobacter spp. are aerobic, Gram-negative, commensal bacteria commonly found within the gastrointestinal tract of humans and animals as well as in environmental sources such as soil and water (Forsythe et al., 2015). *Citrobacter* spp. can cause enteric diseases and extraintestinal infections such as infections of the urinary and respiratory tract. Less frequently, *Citrobacter* spp. also causes serious nosocomial infections in immunocompromised hosts (Forsythe et al., 2015). The occurrence of multi-drug resistance is increasing in *Citrobacters* that can carry extended-spectrum beta-lactamase (ESBL), AmpC beta-lactamases, and carbapenemase-encoding genes such as bla_{KPC-2} (Zhang et al., 2008; Shahid, 2010).

From a hospital infection prevention viewpoint, *Citrobacter freundii* causes concerns due to its exceptional ability to accumulate resistance traits (Yao et al., 2021). The persistence of these strains in the hospital environment and their subsequent spread to patients can cause hard-to-treat infections in compromised hosts. In addition, the accumulated resistance traits could be re-transmitted beyond genus, family, and order boundaries (Majewski et al., 2017) to more virulent organisms such as *Klebsiella pneumoniae*. In the hospital environment, including water fixtures, such as sinks and toilets, affected patients are the suspected initial origin of the carbapenem-resistant organisms (Tofteland et al., 2013; Leitner et al., 2015). Microbes may persist in biofilms in water fixtures and then be dispersed to the surroundings by particles, droplets, or aerosol formation (Kotay et al., 2019). Indeed, it is challenging to verify the transmission of microbes to patients (Kizny Gordon et al., 2017). However, a significant relationship between contaminated sinks and the acquisition of carbapenemase-producing *Enterobacteriaceae* (CPE) in patients indicates the importance of hospital water fixtures and biofilms within them as the environmental reservoir of the CPE epidemics (Smolders et al., 2019). Studies showing hospital rooms as the epidemiological link in CPE outbreaks also support the role of the hospital environment as a source of CPE transmission (van Beek et al., 2019; Räisänen et al., 2021). Although total eradication of CPE in hospital water fixtures with cleaning or disinfection is shown to be difficult (Nurjadi et al., 2021), decreased colonization has been achieved by replacing the sinks and plumbing (Shaw et al., 2018; Smolders et al., 2019).

It has been proposed that wastewater surveillance (WWS) could be used for global surveillance, prediction, and early warning system of AMR (Hendriksen et al., 2019b). However, full integration of human, animal, and environmental AMR monitoring data is still missing. Moreover, there is a need for calibration and standardization of the different methods for reliable data comparison (Pruden et al., 2021). More research studies are needed to clarify the applicability of WWS in observing and predicting the clinically relevant AMR pathogens circulating in the community as well as within asymptomatic carriers. To the best of our knowledge, no previous studies have described the genomic epidemiology of carbapenemase-producing (CP) *C. freundii* in humans, hospitals, and untreated municipal wastewater.

In Finland, *C. freundii* is the third most common CPE in humans and one of the most common CPE-causing clusters (THL, 2022b). During 2012–2018, CP *C. freundii* represented 6.0% of CPE in human specimens (Räisänen et al., 2020), which increased rapidly to 20.0% in 2021 (THL, 2022b). Between 2016–2022, bla_{KPC-2} CP *C. freundii* was detected in 60 human specimens in Finland.

This research was conducted to elaborate on the capacity of WWS to observe CP *C. freundii* strains causing infections. We assessed the similarity between the isolates from the hospital environment, untreated municipal wastewater, and a selection of isolates from human specimens from the national CPE strain collection, which is part of the national infectious diseases register sustained by the National Institute for Health and Welfare (THL). Additionally, we described the occurrence of CP *C. freundii* in the hospital environment and our attempts to eradicate it. The initial motivation for the sampling in the studied hospital arose when an unknown environmental transmission was suspected while one of the CP *C. freundii* clusters described by Räisänen et al. (2021) occurred in the studied hospital.

2. Materials and methods

2.1. Sample collection and bacterial isolation

In this study, samples were collected from a hospital environment, with a specific focus on water fixtures such as sinks and toilets. Wastewater leaving from the same hospital to the municipal sewer was also sampled, as well as untreated municipal wastewater from a local wastewater treatment plant (WWTP) serving the area of the studied hospital (Figure 1). Sampling is described more precisely in the following paragraphs.

2.1.1. Hospital environment

Sampling was conducted six times between December 2019 and July 2021, consisting of up to 27 patient rooms and 228 samples from the unit with two wards in one tertiary care hospital in Finland. The samples were obtained from sink traps (Supplementary Figure 1) and the toilet seat water line (Supplementary Figure 2). Additionally, samples were collected from sink taps (samplings 1–3), bidet showerhead and bidet shower sinks (samplings 1–2), toilet seat and sink plugholes, toilet seat water tank, and the cleaning and disinfection machine (sampling 4). Figure 2 illustrates the sampling timeline.

The samples obtained from the first five samplings (1–5) were collected before or during the cleaning actions (pre-cleaning). The samples were analyzed by THL following the protocol described by van Beek et al. (2019). The samples in sampling 6 (post-cleaning) were collected with sterile swabs (M40 Transystem Amies Agar Gel, Copan Diagnostics, Brescia, Italy) that were initially dampened with buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, United Kingdom). The sampling site was scrubbed with the swab for 15–30 s, and then the swab was moved to the transfer tube. The samples were transferred to the University of Helsinki laboratory in a coolbox and further processed within 12 h from the time of collection.

Each cotton swab was placed into an Eppendorf tube with 1 ml of BPW. The tubes were vortexed, and 100 μl of the suspension was spread on CHROMagar mSuperCARBA (CHROMagar, Paris, France) and incubated aerobically for 18–24 h at 37°C. The remaining 900 μl of the BPW with the cotton swab was also incubated aerobically for 18–24 h at 37°C (enrichment) to detect small quantities of the targeted bacteria. After incubation, the enrichment was streaked with a 10 μl sterile loop on CHROMagar mSuperCARBA and incubated aerobically for 18–24 h at 37°C.

Colony morphology was observed according to the manufacturer's instructions. Overall, 1–5 typical metallic blue colonies were selected from each plate, refreshed with 1 μl sterile loop on CHROMagar mSuperCARBA, and incubated aerobically for 18–24 h at 37°C. Bacterial colonies were subcultured onto individual CHROMagar mSuperCARBA agar plates until a pure culture was achieved. Up to four isolates were subcultured on a bovine blood agar plate (Columbia Blood Agar Base, Oxoid Ltd., Basingstoke, United Kingdom) and incubated aerobically for 18–24 h at 37°C.

2.1.2. Hospital wastewater

Two 1 L grab samples of wastewater from the hospital sewers were collected from two separate locations around the studied hospital. Sampling was performed in July 2021 in concordance with post-cleaning samples of the hospital environment. The samples were transferred to the laboratory in a coolbox and further processed within 12 h from the time of collection. A volume of 100 μl of the wastewater was pipetted and spread on CHROMagar mSuperCARBA and further processed as post-cleaning hospital environment samples without the enrichment step.

2.1.3. Untreated municipal wastewater from local WWTP

Nine 24-h composite samples of untreated municipal wastewater were collected between February 2021 and February 2022 from the WWTP in the city. Wastewater was collected as a part of a consortium project "WastPan" (THL, 2022b). WWTP receives ~296,000 m^3 of wastewater daily, and it serves ~800,000 inhabitants (14.5% of the population of Finland) (Tiwari et al., 2022). WWTP also receives wastewater from the studied hospital. A 1 L sample was delivered to the laboratory in a coolbox, from which 100 μl was pipetted and spread on CHROMagar mSuperCARBA and further processed as the post-cleaning hospital environment samples without the enrichment step.

2.2. Bacterial species identification

Isolates were identified using a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) based Bruker Microflex LT/SH (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) with a score value of >2.0, except for one isolate with a score value of 1.8, and the second-best match for *C. freundii* was identified with a score value of 1.7. Confirmed *C. freundii* isolates were stored at −80°C for further characterization.

2.3. Antimicrobial susceptibility testing

For presumptive CP *C. freundii* isolates, susceptibility to carbapenems was tested with meropenem (10 μg) (Abtek Biologicals Ltd., Liverpool, United Kingdom, or Rosco, Albertslund, Denmark) and ertapenem (10 μg) (Oxoid, Basingstoke, United Kingdom) with a disk diffusion test according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) standard (EUCAST, 2022a). The inhibition zones were measured with a digital caliper. Furthermore, an antimicrobial susceptibility testing was performed with the broth microdilution method using Sensititre EURGNCOL plates (Thermo Fischer Scientific, East Grinstead, United Kingdom) to determine the minimum inhibitory concentration (MIC) of colistin, piperacillin/tazobactam, ceftazidime/avibactam, ceftolozane/tazobactam, and meropenem. The method was performed according to the manufacturer's instructions, except for using 0.9% saline instead of sterile water. *Escherichia coli* ATCC 25922 was included as quality control for each Müeller-Hinton agar patch. The results were interpreted according to EUCAST epidemiological cut-off values (ECOFFs) (EUCAST, 2022b).

2.4. DNA extraction

Isolates were grown in tryptic soy broth (Oxoid, Basingstoke, UK) at 37°C for 16 h. DNA was extracted from cells harvested from 2 ml of culture using the QIAcube Connect instrument (QIAGEN, Hilden, Germany) with a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, United States). The quality of DNA

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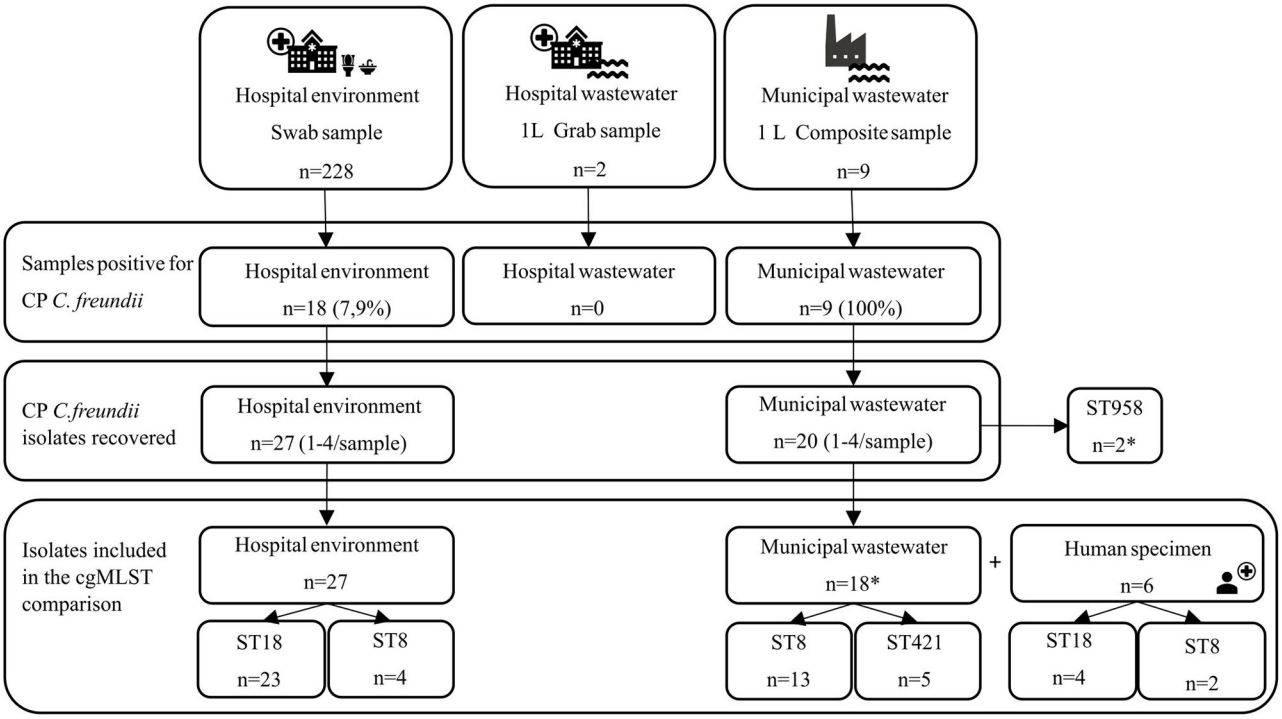

FIGURE 1
Illustration of the collected samples and recovered isolates. ST indicates sequence type. cgMLST indicates core genome multilocus sequence typing. Asterisk indicates two ST958 isolates that were left out of the cgMLST scheme due to poor sequence quality.

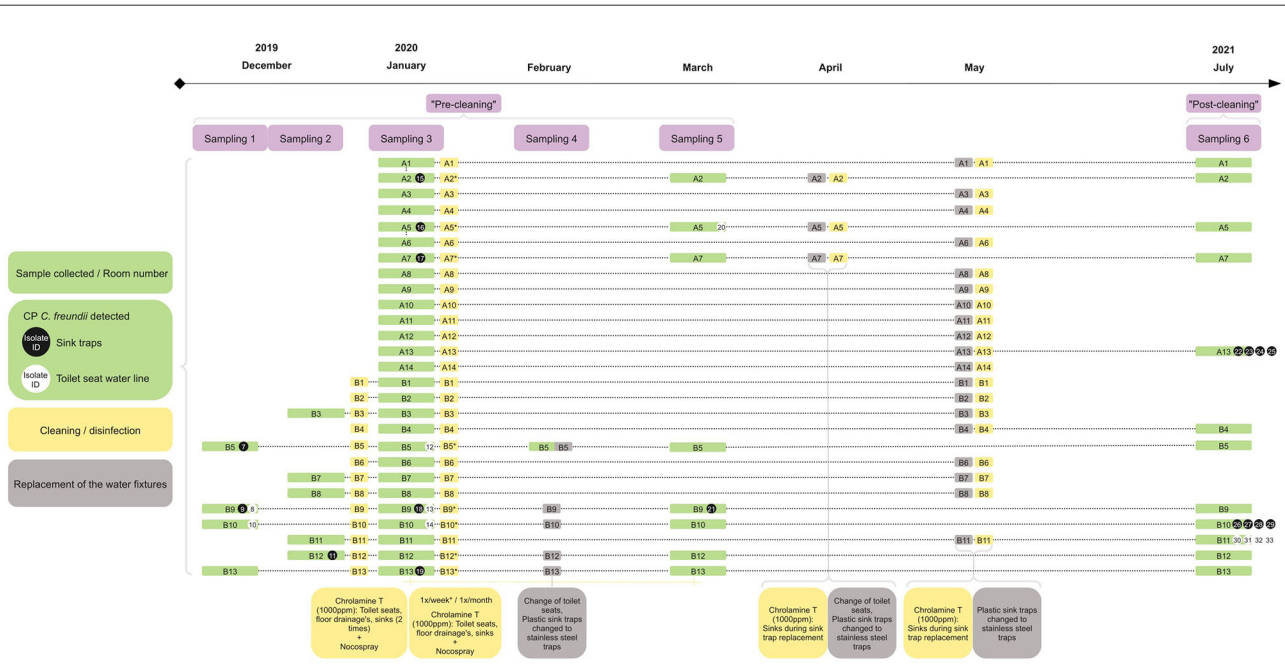

FIGURE 2
Illustration of the hospital environment sampling (purple rectangle) in hospital rooms (green rectangle) and detected carbapenemase-producing (CP) *Citrobacter freundii* isolates in sink traps (black circle) and toilets (white circle). The numbers inside or next to the circles indicate the isolate identification number (ID). Cleaning/disinfection (yellow rectangle) and replacement of the water fixtures (gray rectangle) are shown. Asterisk indicating rooms where cleaning and disinfection was performed a minimum of once per week after Sampling 3.

was assessed by the A260/A280 ratio using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, United States), and DNA quantity was measured using a Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, United States).

2.5. Whole genome sequencing and bioinformatical analyses

The extracted DNA from all 32 presumptive CP *C. freundii* isolates from the post-cleaning hospital environment ($n = 12$) and untreated municipal wastewater samples ($n = 20$) was outsourced for sequencing. Library preparation was performed with a NEBNext Ultra DNA Library Prep Kit for Illumina with 300 bp fragment length. Sequencing was performed with Illumina NovaSeq 6000 (Novogene, Cambridge, United Kingdom) with 2 × 150 bp read length and targeted genomic coverage of 100×.

Sequences were analyzed with Ridom SeqSphere+ software v7.7.5 (Ridom GmbH, Germany) (Jünemann et al., 2013). Quality analysis of the sequences was performed with FastQC v0.1.1.7 (Babraham Institute, 2021), and adapters were removed with Trimmomatic v0.36 (Bolger et al., 2014). Raw reads were assembled with SKESA v2.3.0 using default settings (Souvorov et al., 2018), and quality trimming was performed with an average quality of ≥30 and a window of 20 bases. Remapping and polishing were performed with the BWA-MEM mapping algorithm. Sequencing statistics are presented in Supplementary Table 1. AMR genes were identified from assembled genomes with NCBI AMRFinderPlus 3.2.3 (Feldgarden et al., 2019), using 100% alignment and >90% identity. ResFinder 4.1 (Center for Genomic Epidemiology web server, DTU, Denmark) (Zankari et al., 2017; Clausen et al., 2018; Bortolaia et al., 2020) was used with default settings to specify the allelic variants of beta-lactamase genes. Sequence types (ST) were analyzed using multilocus sequence type (MLST) (Becker et al., 2018) in Ridom SeqSphere+- (Ridom, Munster, Germany). Sequences with novel STs were submitted to PubMLST (Jolley et al., 2018) to assign new STs.

2.6. Genomic comparison of CP *C. freundii* isolates

All isolates, except for two from the untreated municipal wastewater with poor sequence quality, were included in an *ad hoc* core genome multilocus sequence type (cgMLST) scheme with CP *C. freundii* isolates from the pre-cleaning hospital environment samples ($n = 15$). Additionally, six isolates from human specimens from the national CPE strain collection ($n = 6$; ST18 4/48 and ST8 2/11) were included in the cgMLST scheme (Figure 1). The isolates from the human specimens with ST18 were obtained from inpatients treated in the studied unit and have been published previously by Räisänen et al. (2021). The isolates from the human specimens with ST8 were obtained from inpatients treated in another hospital in the same city and within the service area of the studied WWTP. Three isolates were obtained from clinical specimens (ST18, $n = 2$; ST8, $n = 1$) and two from screening specimens (ST18, $n = 1$; ST8, $n = 1$). The specimen type for one isolate was not specified (ST8, $n = 1$).

2.7. Cleaning and other interventions in the patient rooms of the studied hospital

After sampling rounds 1 and 2 in December 2019, the affected rooms were cleaned with 1,000 ppm Chloramine T (KiiltoClean, Finland). Additionally, undiluted Chloramine T was poured into the toilets, floor drainages (4.0 dL each), and sinks (1.5 dL). After a 1-h duration of action, rooms were disinfected with Nocospray (Oxy'Pharm, France). After sampling round 3 in January 2020, the cleaning routine continued as described above, but the patient rooms with CP *C. freundii* findings were cleaned at least once a week, and the other rooms at least once a month. The described routine was followed until the end of March 2020. Starting from February 2020, the toilet seats were replaced, and the plastic sink traps were changed to stainless steel traps (Figure 2).

3. Results

3.1. Hospital environment, hospital wastewater, and untreated municipal wastewater from local WWTP

The pre-cleaning hospital environment samples in December 2019 yielded four CP *C. freundii* isolates from three patient rooms ($n = 3/4$, 75.0%, sampling 1) and one isolate from one patient room ($n = 1/5$, 20.0%, sampling 2). In January 2020, eight CP *C. freundii* isolates were recovered from seven patient rooms ($n = 7/27$, 25.9%, sampling 3). In February 2020, CP *C. freundii* was not recovered from the samples ($n = 0/5$, 0.0%, sampling 4). In March 2020, sampling yielded two CP *C. freundii* isolates from two patient rooms ($n = 2/8$, 25.0%, sampling 5). Post-cleaning samples in July 2021 yielded 12 CP *C. freundii* isolates from three patient rooms ($n = 3/12$, 25.0%, sampling 6). Additional species identified from selective media in post-cleaning samples were *Klebsiella oxytoca* ($n = 4$) and *Klebsiella pneumoniae* ($n = 2$). Two isolates were not identifiable by MALDI-TOF. Figure 2 illustrates the sampling, findings, and interventions.

C. freundii was not recovered from the selective media from hospital wastewater samples ($n = 0/2$, 0%).

In total, 20 CP *C. freundii* isolates were recovered from nine untreated municipal wastewater samples ($n = 9/9$), with up to four CP *C. freundii* isolates in each sample.

3.2. Phenotypic susceptibility

The disk diffusion test and broth microdilution were performed for 32 *C. freundii* isolates recovered from the post-cleaning hospital environment ($n = 12$) and untreated municipal wastewater samples ($n = 20$). All isolates were considered to express phenotypical resistance against meropenem. The phenotypical susceptibility of the isolates is presented in Table 1.

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TABLE 1 Zone of inhibition and MIC of antimicrobials for 32 *Citrobacter freundii* isolated from post-cleaning hospital environment sampling (HE, $n = 12$) and untreated municipal wastewater (WW, $n = 20$).

Sampling site	Time (MM/YY)	Isolate ID	Zone of inhibition (mm)		MIC (mg/L)				
			MRP10	ERT10	COL	P/T4	C/T	CZA	MERO
(ECOFFs)			25	N/A	I/D	8	1	N/A	I/D
HE	07/21	22	**18.3**	17.7	0.5	**>32**	**>8**	1	4
	07/21	23	**18.3**	19	0.5	**>32**	**>8**	1	2
	07/21	24	**19.0**	19.8	0.5	**>32**	**8**	1	2
	07/21	25	**19.7**	18.8	0.5	**>32**	**>8**	1	4
	07/21	26	**22.9**	20.6	0.5	**>32**	**>8**	1	2
	07/21	27	**23.0**	19.9	0.5	**>32**	**>8**	1	2
	07/21	28	**24.2**	21.6	0.5	**>32**	**8**	1	2
	07/21	29	**22.7**	20.2	0.5	**>32**	**>8**	1	2
	07/21	30	**22.0**	19.7	0.5	**>32**	**>8**	1	4
	07/21	31	**24.7**	19.4	0.5	**>32**	**>8**	1	2
	07/21	32	**23.6**	19.9	0.5	**>32**	**>8**	1	4
	07/21	33	**21.6**	18.3	0.5	**>32**	**>8**	1	2
WW	02/21	34	**24.5***	21.1	0.5	**>32**	**1**	<1	0.5
	02/21	35	**18.6***	17.9	0.5	**>32**	**8**	<1	8
	02/21	36	**16.2***	18.6	< 0.25	**>32**	**2**	<1	8
	04/21	37	**16.5***	17.1	< 0.25	**>32**	**8**	<1	8
	05/21	38	**19.0***	20.0	< 0.25	**>32**	**4**	<1	8
	05/21	39	**17.0***	17.0	< 0.25	**>32**	**4**	<1	8
	07/21	40	**14.4**	18.0	0.5	**>32**	**8**	<1	8
	07/21	41	**15.5**	15.9	0.5	**>32**	**>8**	<1	4
	07/21	42	**17.2**	19.0	0.5	**>32**	**4**	<1	4
	08/21	43	**23.8***	17.0	0.5	**>32**	**1**	<1	1
	08/21	44	**15.7***	14.4	0.5	**>32**	**>8**	<1	8
	10/21	45	**21.1***	18.6	0.5	**>32**	**4**	<1	8
	10/21	46	**22.8***	22.2	0.5	**>32**	**8**	<1	8
	11/21	47	**18.4***	24.4	0.5	**>32**	**>8**	<1	4
	11/21	48	**18.9***	23.0	0.5	**>32**	**>8**	<1	4
	01/22	49	**15.7***	21.2	0.5	**>32**	**>8**	<1	8
	02/22	50	**6.0***	7.5	0.5	**>32**	**>8**	8	>16
	02/22	51	**16.1***	23.0	0.5	**>32**	**>8**	<1	4
	02/22	52	**14.0***	18.6	0.5	**>32**	**4**	<1	8
	02/22	53	**13.0***	16.8	0.5	**>32**	**8**	<1	8

Epidemiological cut-off values (ECOFFs) (mm and mg/L) are indicated. N/A indicates that ECOFF is not applicable. I/D indicates insufficient data. Bold lettering indicates Zone of inhibition below and MICs above the ECOFF. MRP10 Meropenem (10 μg). ERT10 Ertapenem (10 μg). Meropenem disk 10 μg by Abtek Biologicals Ltd. (Liverpool, United Kingdom) indicated by * and by Rosco (Albertslund, Denmark) without indicator. COL Colistin, P/T4 Piperacillin/Tazobactam, C/T Ceftolozane/Tazobactam, CZA Ceftazidime/Avibactam, and MERO Meropenem.

3.3. Whole genome sequencing and bioinformatic analyses

In total, four different STs of CP *C. freundii* were identified. In the post-cleaning hospital environment samples, the most prevalent was ST18 ($n = 8/12$, 66.7%), followed by ST8 ($n = 4/12$, 33.3%). In the untreated municipal wastewater, the most prevalent was ST8 ($n = 13/20$, 65.0%), followed by ST421 ($n = 5/20$, 25.0%) and ST958 ($n = 2/20$, 10.0%) (Table 2). All the pre-cleaning hospital environment samples included in the genomic comparison

belonged to ST18 ($n = 15$), and the isolates from human specimens included in the comparison belonged to ST18 ($n = 4$) and ST8 ($n = 2$) (Figure 1).

All isolates ($n = 27$) from the hospital environment carried the bla_{KPC-2} carbapenemase gene. bla_{KPC-2} was also the most prevalent carbapenemase gene in isolates observed in the untreated municipal wastewater ($n = 13/20$, 65.0%), followed by bla_{VIM-1} ($n = 5/20$, 25.0%). Carbapenemase genes could not be identified in two isolates from untreated municipal wastewater ($n = 2/20$, 10.0%). Additionally, 4 to 12 other AMR genes were identified from each isolate. All identified AMR genes in the post-cleaning hospital environment and untreated municipal wastewater isolates are presented in Table 2.

3.4. Genomic comparison of CP *C. freundii* isolates

We identified three clusters [cluster threshold ≤10 allele difference (Jamin et al., 2021)] shown in Figure 3. Cluster 1 comprised isolates belonging to ST18— 23 from the hospital environment and 4 from the human specimens. Cluster 2 comprised isolates belonging to ST8, from which, four, six, and two originated from the hospital environment, untreated municipal wastewater, and human specimens, respectively. Cluster 3 comprised five isolates belonging to ST421 from untreated municipal wastewater. Clusters 1, 2, and 3 were separated by a long distance (up to 1812 allele difference) (Figure 3).

4. Discussion

In this study, we identified closely related CP *C. freundii* strains belonging to ST8 in the hospital environment, untreated municipal wastewater, and human specimens, demonstrating that CP *C. freundii* strains causing infections can be detected in untreated municipal wastewater. However, the absence of ST18 in untreated municipal wastewater raises questions about the sensitivity of culture-based WWS to detect all clinically relevant strains. ST18 has been the dominant ST in human specimens, responsible for an ongoing outbreak since 2016 (Räisänen et al., 2021), whereas ST8 has been rarer.

In this study, we collected the municipal wastewater samples in 2021–2022, whereas the human isolates included in the comparison were from 2019–2020 (ST18) and 2021–2022 (ST8). ST18 was first discovered in a human specimen in 2016 in one hospital located in the same region. The temporal variance between the samples should be noted when interpreting the results. Accordingly, undetected ST18 in municipal wastewater could reflect that it was less frequent in the population during the municipal wastewater sampling, whereas ST8 could have been more common than realized. Hundreds of screening specimens collected from the patients treated in the unit of the studied hospital in 2019 showed a low prevalence of CP *C. freundii* (data not published). However, the true prevalence of asymptomatic carriage in the Finnish population is unknown. Regarding the above, asymptomatic carriage of CP *C. freundii* ST8 may be more common than recognized and, in combination with a slight increase of detected CP *C. freundii* ST8 in clinical specimens in Finland recently, could be reflected in the number of ST8 isolates derived from untreated municipal wastewater. Moreover, a clonal shift in the bacterial population (Shapiro, 2016) could also explain why ST18 could not be recovered from wastewater. *C. freundii* is a commensal bacterium carried within the gastrointestinal tract of both humans and animals (Forsythe et al., 2015). In this study, the animal origin of CP *C. freundii* in untreated municipal wastewater is unlikely, as CPE has never been reported in food producing animals in Finland in routinely performed national surveillance (Finnish Food Authority, 2022); only one case of CP *Escherichia coli* has been reported in companion animals in Finland (Grönthal et al., 2018). However, several studies have reported CPE in wildlife, including wild boars in Algeria (Bachiri et al., 2018) and wild avian species in Spain (Oteo et al., 2018). CPE has not been detected in migratory barnacle geese in Finland (Kurittu et al., 2021), but we cannot comprehensively exclude the possibility of the wildlife origin of CP *C. freundii*, as research data related to CPE in wildlife in Finland is otherwise scarce.

Undetected ST18 in untreated municipal wastewater may indicate its possibly shorter survival time in the wastewater matrix, leading to a quick depletion. In *E. coli*, different strains have shown substantial differences in growth under the same growth conditions (Sekse et al., 2012). CP *C. freundii* belonging to ST8 may have a longer survival time in wastewater, allowing it to survive better in the conditions of the WWTP. ST8 may also be predominant or endemic in the sewerage network or studied WWTP, and isolates may reflect the established microbiome of the sewerage network rather than that of the entire population (LaMartina et al., 2021). The enormous microbial load and a favorable environment in wastewater promote bacterial growth, biofilm formation (Weingarten et al., 2018), and sharing of mobile genetic elements—Even beyond genus, family, and order boundaries (Majewski et al., 2017). All these factors could lead to differences in the AMR bacteria detected in the municipal wastewater compared to those circulating in the population.

CP *C. freundii* was not detected in hospital wastewater, most likely due to the chosen sampling method. A grab sample represents the present situation during the sample collection. Hence, the sample can represent anything from toilet water to gray water, such as showering or cleaning water, leading to substantial differences in the microbiome of that particular sample. A 24-h composite sample would be more representative and less prone to the effects of individual events. Hospital wastewater may also contain greater concentrations of cleaning or disinfection agents that influence the viability of bacteria, and molecular methods could be less sensitive to these effects (Girones et al., 2010).

The genetic relatedness of CP *C. freundii* isolates from the hospital environment, untreated municipal wastewater, and human specimens indicates a possible persistence of the bacteria throughout the sewerage system and a passage of this microbe from patients or the hospital environment to the WWTP. However, we cannot comprehensively prove the directionality, hence further studies are needed to verify this hypothesis. The genetic relatedness identified between the CP *C. freundii* ST18 isolates from the hospital environment and human specimens supports the previous hypothesis of a possible environmental source of transmission of this microbe (Räisänen et al., 2021).

TABLE 2 Genomic characteristics for the 32 *Citrobacter freundii* isolated from post-cleaning hospital environment sampling (HE, $n = 12$) and untreated municipal wastewater (WW, $n = 20$).

Sampling site	Isolate ID	MLST	Resistance genes		
			Carbapenemase genes	Other *bla* genes	Resistance genes other than *bla*
HE	22	ST8	bla_{KPC-2}	bla_{OXA-9}, bla_{CMY-79}, $bla_{CMY-116}$, bla_{TEM-1A}	*aac(6′)-If, aph(6)-Id, aac(3)-IIa, aadA1, dfrA1, sul2, sat2*
	23	ST8	bla_{KPC-2}	bla_{OXA-9}, bla_{CMY-79}, $bla_{CMY-116}$, bla_{TEM-1A}	*aac(6′)-If, aph(6)-Id, aac(3)-IIa, aadA1, dfrA1, sul2, sat2*
	24	ST8	bla_{KPC-2}	bla_{OXA-9}, bla_{CMY-79}, $bla_{CMY-116}$, bla_{TEM-1A}	*aac(6′)-If, aph(6)-Id, aac(3)-IIa, aadA1, dfrA1, sul2, sat2*
	25	ST8	bla_{KPC-2}	bla_{OXA-9}, bla_{CMY-79}, $bla_{CMY-116}$, bla_{TEM-1A}	*aac(6′)-If, aph(6)-Id, aac(3)-IIa, aadA1, dfrA1, sul2, sat2*
	26	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	27	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	28	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	29	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	30	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	31	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa aadA5, dfrA17*
	32	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
	33	ST18	bla_{KPC-2}	bla_{OXA-9}, $bla_{CMY-117}$, bla^{*}_{TEM-XX}	*aph(6)-Id, aac(3)-IIa, aadA5, dfrA17*
WW	34[1]	ST958	NF	bla_{OXA-10}, bla_{CFE}, $bla^{*}_{CMY-100}$	*qnrB, cmlA5*
	35	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	36	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	37	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	38	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	39	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	40	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	41	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	42	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	43[1]	ST958	NF	bla_{OXA-10}, bla_{CFE}, $bla^{*}_{CMY-100}$	*aac(6′)-Ib, aadA2, sul1, qnrB, cmlA5*
	44	ST421	bla_{VIM-1}	bla_{CMY-40}, $bla_{CTX-M-15}$	*aac(6′)-Iic, dfrA16, sul1, qnrS1, qnrB9, mph*(E), *msr*(E)
	45	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-1B}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	46	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla_{TEM-1}	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	47	ST421	bla_{VIM-1}	bla_{CMY-40}, $bla_{CTX-M-15}$	*aac(6′)-IIc, dfrA16, sul1, qnrS1, qnrB9*
	48	ST421	bla_{VIM-1}	bla_{CMY-40}, $bla_{CTX-M-15}$	*aac(6′)-IIc, dfrA16, sul1, qnrS1, qnrB9, mph*(E), *msr*(E)
	49	ST421	bla_{VIM-1}	bla_{CMY-40}, $bla_{CTX-M-15}$	*aac(6′)-IIc, dfrA16, sul1, qnrS1, qnrB9, mph*(E), *msr*(E)
	50	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$, bla^{*}_{TEM-XX}	*aac(6′)-If, aph(3″)-Ib, aph(6)-Id, aadA1, dfrA1, sul2, sat2, qnrS1*
	51	ST421	bla_{VIM-1}	bla_{CMY-40}, $bla_{CTX-M-15}$	*aac(6′)-IIc, dfrA16, sul1, qnrS1, qnrB9, mph*(E), *msr*(E)
	52	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*
	53	ST8	bla_{KPC-2}	bla_{CMY-79}, $bla_{CMY-116}$	*aac(6′)-If, aadA1, dfrA1, sul2, sat2*

MLST, Multi-locus Sequence Type; NF, Not found. AMRfinderPlus v.3.2.3 for antimicrobial resistance genes and ResFinder 4.1. to determine the allelic variants of beta-lactamase genes (*bla*). Beta-lactamase gene detected only by ResFinder 4.1. indicated with *. bla_{TEM-XX} indicates an unidentified TEM-variant due to low alignment. (1) Sequences ($n = 2$) did not exceed the quality requirements for the core genome multi-locus sequence type scheme.

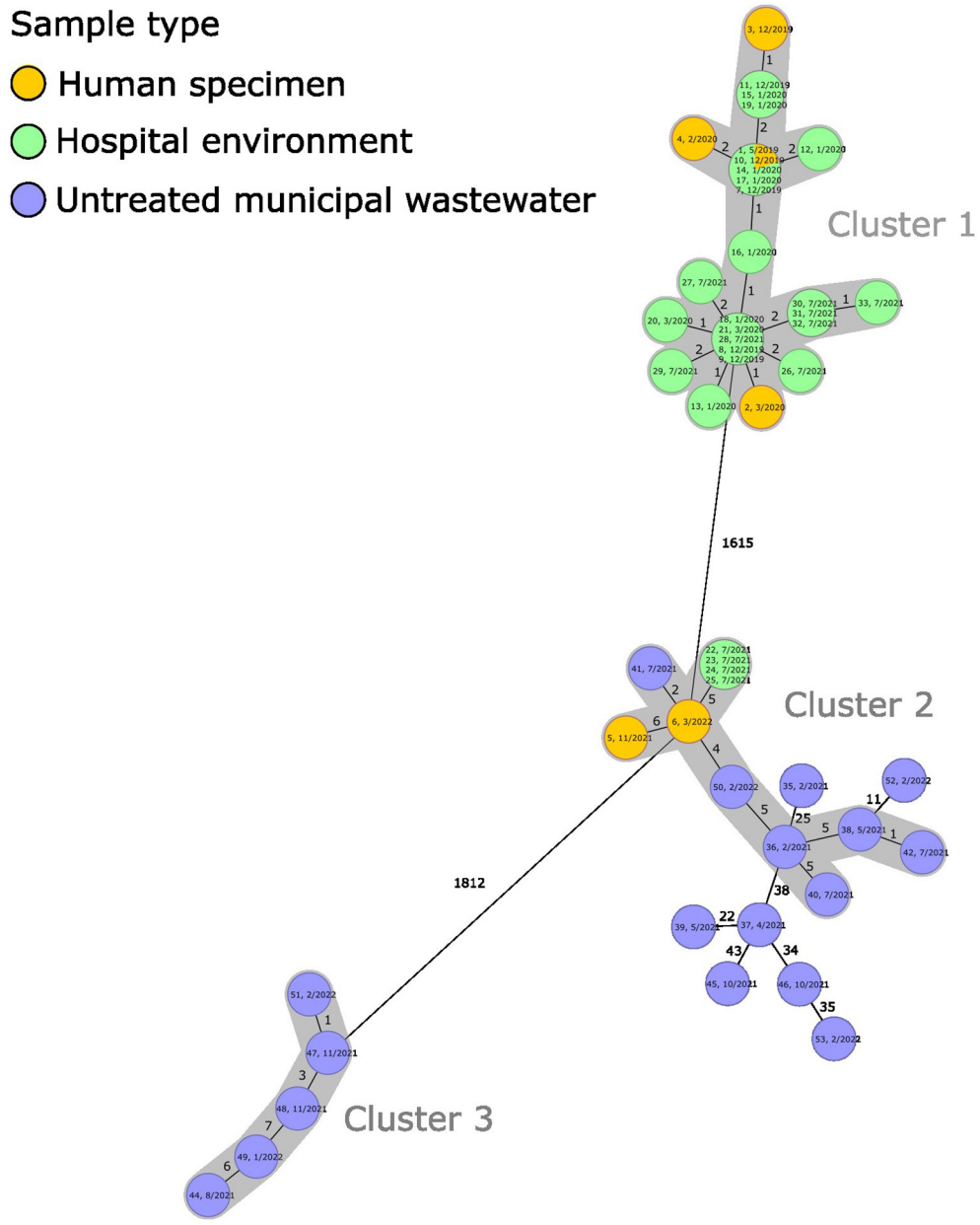

FIGURE 3
A minimum spanning tree of *ad hoc* core genome multilocus sequence typing (cgMLST) of 51 carbapenemase-producing *Citrobacter freundii* isolates, forming clusters 1 (ST18), 2 (ST8), and 3 (ST421). Each circle represents one or multiple identical sequences, and the numbers between the circles indicate the allele differences. Text in the circle indicates the isolate identification number and sample month/year; colors indicate the sample type [human specimen (yellow), hospital environment (green), and untreated municipal wastewater (blue)]. Human specimen isolates (1–6) originated from the national CPE strain collection and were obtained from clinical specimens (isolates 1, 2, and 5), screening specimens (isolates 3 and 4), and an unspecified sample type (isolate 6). Isolates 1–4 have been published previously by Räisänen et al. (2021). The seed genome was NZ_CP007557.1, and the cgMLST was based on 2007 targets, pairwise ignoring missing values.

Sinks and toilets in hospitals may facilitate the storage of human-origin strains (Nurjadi et al., 2021) in biofilms and form a rich ecosystem where plasmids carrying carbapenemase-encoding genes could be transferred through horizontal gene transfer between bacteria (Majewski et al., 2017). Epidemics in hospitals can arise from the patients, but the hospital environment may also facilitate a possible source of transmission of CP *C. freundii* in clinical settings.

CP *C. freundii* was detected both in sink traps and toilets in the studied hospital, but not in the other sampling sites (sink taps, bidet showerhead and bidet shower sinks, toilet seat and sink plugholes, toilet seat water tank, or cleaning and disinfection machine). These findings are in line with the studies that have shown drains to be the most common reservoirs of CPE in the hospital environment (Park et al., 2020; Nurjadi et al., 2021). We observed recurrent findings with clonal isolates in four patient rooms throughout the

study, despite regular treatment with Chloramine T (1,000 ppm) and the replacement of the toilet seats and sink traps (Figure 2). The clonality of the detected isolates reflects the persistence of these strains in the water fixtures rather than the recolonization caused by the patients. Previous studies have shown that cleaning and disinfection reduce the bacterial load, but a total eradication of CPE in hospital water fixtures is rarely achieved (Hopman et al., 2019; Nurjadi et al., 2021) and re-emergence is common despite intensive cleaning or replacement of the water fixtures (Decraene et al., 2018; Shaw et al., 2018; Smolders et al., 2019). Constructional interventions are the only reported successful measures to contain outbreaks (Nurjadi et al., 2021). It is essential to ensure that the surfaces in the hospital environment are easy to keep clean, but more research studies are needed to develop effective eradication methods and technologies that prevent the spread of microbes from sinks and toilets to the surroundings.

A culture-based approach, combined with whole genome sequencing, was chosen to evaluate the convenience of the commercially available culture media in this context. Molecular methods, such as metagenomic sequencing, may provide deeper taxonomic profiles and give a broad overview of the resistance genes. However, metagenomic sequencing may struggle to identify species at the strain level and assign detected resistance genes to particular species (Hendriksen et al., 2019a). Metagenomic sequencing is also expensive to perform and requires expertise that is commonly lacking at the local level (Pruden et al., 2021). Targeted PCR can detect resistance genes in the wastewater (Pärnänen et al., 2019; Probst et al., 2022) but is limited by the target panel and does not provide information about the species carrying the resistance genes (Girones et al., 2010). Pruden et al. (2021) stated that a culture-based approach is attractive since fecal indicator monitoring infrastructure is relatively low cost and already widespread and accessible in many low and low-middle-income countries. However, the culture-based approach has its limitations. For example, not all bacteria are culturable (Stewart, 2012), and the culture media can significantly impact the recovery of the bacteria from the samples (Davis et al., 2005). Nevertheless, culture-based methods can be as sensitive, specific, and accurate as PCR-based methods if the target organism is culturable (Bliem et al., 2018), as is the case with *Enterobacteriaceae*, which are commonly uncomplicated to recover from samples. CHROMagar mSuperCARBA has shown enough sensitivity and specificity to detect CPE in clinical specimens (Soria Segarra et al., 2018). However, wastewater is a complex sample material, and identification of the species of interest by colony morphology on selective media can be difficult. The spectrum of species is much broader than in clinical specimens, and environmental microbes may disturb species identification. In our study, we could only choose a pre-agreed number of colonies from the untreated municipal wastewater samples, and the manual selection of colonies can, by chance, lead to the discarding of some bacterial species or isolates with different STs and enzyme types. The apparent advantage—but also limitation—of culture-based WWS is that the analyses give information about a single bacterial clone. The clones can be described precisely and compared with the clones from various sources. However, studying all relevant clones requires extensive culturing and whole genome sequencing, which is highly laborious and increases costs.

Clinical surveillance is undoubtedly essential in screening and observing AMR pathogens in patients at a higher risk. However, it seems that, despite its limitations, culture-based WWS has the potential to detect AMR pathogens causing infections in the population and possibly also in detecting asymptomatic circulation at the population level. Additionally, WWS is decisive in identifying possible new threats, the persistence or emergence of AMR genes, and following the AMR trends. Research on WWS of AMR still needs to contribute to answering many questions, from standardized methodology to integration of existing AMR data. Longitudinal surveillance, including clinical and wastewater samples, and optimally also data from an extensive screening of the healthy Finnish population, could reveal whether the dominance of ST8 in the wastewater is indicative of its incidence in the population, predicted its elevation in the clinical specimens in Finland, or reflected the microbiome of the WWTP. WWS should not be seen as a competitor or a replacement for clinical surveillance, but as a tool that can provide a broader view of the AMR situation in the population. In the future, the insights provided by WWS would be helpful also in clinical settings and decision-making.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB58690.

Author contributions

VH: conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft, writing—review and editing, and visualization. VJ: conceptualization, methodology, investigation, and writing—review and editing. KR: conceptualization, formal analysis, resources, writing—review and editing, and visualization. V-JA and OL: conceptualization, resources, and writing—review and editing. JJ, IW, and J-ML: resources and writing—review and editing. K-ML, AL, SO, and TP: writing—review and editing and project administration. AH: conceptualization, writing—review and editing, supervision, project administration, and funding acquisition. WastPan study group: resources and project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1165751/full#supplementary-material

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Large-scale analysis of putative plasmids in clinical multidrug-resistant *Escherichia coli* isolates from Vietnamese patients

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Introduction: In the past decades, extended-spectrum beta-lactamase (ESBL)-producing and carbapenem-resistant (CR) *Escherichia coli* isolates have been detected in Vietnamese hospitals. The transfer of antimicrobial resistance (AMR) genes carried on plasmids is mainly responsible for the emergence of multidrug-resistant *E. coli* strains and the spread of AMR genes through horizontal gene transfer. Therefore, it is important to thoroughly study the characteristics of AMR gene-harboring plasmids in clinical multidrug-resistant bacterial isolates.

Methods: The profiles of plasmid assemblies were determined by analyzing previously published whole-genome sequencing data of 751 multidrug-resistant *E. coli* isolates from Vietnamese hospitals in order to identify the risk of AMR gene horizontal transfer and dissemination.

Results: The number of putative plasmids in isolates was independent of the sequencing coverage. These putative plasmids originated from various bacterial species, but mostly from the *Escherichia* genus, particularly *E. coli* species. Many different AMR genes were detected in plasmid contigs of the studied isolates, and their number was higher in CR isolates than in ESBL-producing isolates. Similarly, the bla_{KPC-2}, bla_{NDM-5}, bla_{OXA-1}, bla_{OXA-48}, and $bla_{OXA-181}$ β-lactamase genes, associated with resistance to carbapenems, were more frequent in CR strains. Sequence similarity network and genome annotation analyses revealed high conservation of the β-lactamase gene clusters in plasmid contigs that carried the same AMR genes.

Discussion: Our study provides evidence of horizontal gene transfer in multidrug-resistant *E. coli* isolates via conjugative plasmids, thus rapidly accelerating the emergence of resistant bacteria. Besides reducing antibiotic misuse, prevention of plasmid transmission also is essential to limit antibiotic resistance.

KEYWORDS

***Escherichia coli*, putative plasmid, carbapenem resistance, horizontal gene transfer, whole-genome sequencing**

1. Introduction

The rapid increase of antibiotic-resistant bacteria, which is the consequence of excessive and inappropriate use of antibiotics (Kahlmeter et al., 2003; Costelloe et al., 2010), is a tremendous public health issue because it hinders the proper treatment of infections, thus increasing morbidity and mortality and also healthcare costs (Cosgrove and Carmeli, 2003). It was estimated that in 2019, antimicrobial resistance (AMR) was associated with ~4.95 million deaths worldwide and that AMR bacteria directly caused 1.27 million deaths. *Escherichia coli* is one of the six leading AMR bacteria associated with more than 800,000 deaths (Antimicrobial Resistance Collaborators, 2022). *E. coli*, a Gram-negative bacterial species, is found in the lower intestine of humans and plays a vital role as commensal bacterium, but can also cause urinary tract infections, sepsis and meningitis (McNally et al., 2013), and is the leading cause of community and hospital infections. Many studies have highlighted the AMR threat in healthcare and agricultural settings worldwide, but particularly in low- and middle-income countries (Nhung et al., 2016; Yamasaki et al., 2017; Tran et al., 2019). Even if antibiotic resistance is a global issue, Asia remains the major source of resistance in the world. Almost 35% of the emerging infectious diseases identified in Asia between 1940 and 2004 correspond to the emergence of a new pattern of antimicrobial drug resistance (Jones et al., 2008; Horby et al., 2013). South East Asia including Vietnam is considered as a hot spot for AMR emergence because of the possible access to antimicrobials for humans without prescription despite the regulations and the high antimicrobial usage for livestock (Mao et al., 2015; Carrique-Mas et al., 2020). Despite the National Action Plan to combat AMR implemented since 2013, Vietnam is still heavily affected by the emergence and rapid spread of bacteria, resistant to many antibiotics. A recent large-scale study on 3,153 multidrug-resistant *E. coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* isolates from two intensive care units (ICU) reported a high prevalence of AMR genes and evidence of extensive transmission between ICU patients in Vietnam (Roberts et al., 2022).

The spread of antibiotic resistance is mostly driven by horizontal gene transfer via conjugative plasmids (von Wintersdorff et al., 2016). Majority of carbapenem-resistant *E. coli* strains harbor $bla_{\text{NDM-1}}$ in China (Zhang et al., 2017) and Vietnam (Tran et al., 2015), which emphasizes regionally dissemination of carbapenem-resistant *Enterobacteriaceae* depending on the horizontal transfer of their plasmid-mediated genes. Among the *Enterobacteriaceae, E. coli* can incorporate extracellular plasmids 'naturally' without special treatment (Tsen et al., 2002). According to the NCBI database (accessed on May 31, 2022), up to 17 different types of native plasmids can be found in the *E. coli* genome and they represent up to 14% of *E. coli* genetic material. Unlike chromosomes, plasmids can exist in multiple copies that increase the conjugation frequency and allow the rapid spread of AMR genes within microbial communities (Dimitriu et al., 2021). Plasmid-encoded genes are often carried in complex structures and exist in a variety of plasmid types (Hirabayashi et al., 2021). Given the vital role of plasmids in the acquisition and dissemination of AMR genes, it is important to thoroughly study their characteristics in multidrug-resistant clinical isolates. In the present study, we performed a large-scale analysis of putative plasmids using whole-genome sequencing data retrieved from several published studies on extended-spectrum beta-lactamase (ESBL)-producing and carbapenem-resistant (CR) *E. coli* isolates from Vietnamese patients. The presence of AMR genes on putative plasmids was investigated to assess the risk of transmission particularly of genes associated with resistance to carbapenems.

Abbreviations: AMR, antimicrobial resistance; CR, carbapenem-resistant; MDR, multidrug-resistant; ESBL, extended spectrum beta-lactamase; TE, transposable element.

2. Materials and methods

2.1. Whole-genome sequencing data of multidrug-resistant *Escherichia coli* isolates in Vietnam

In this analysis, we searched all genomic studies investigating multidrug-resistant *E. coli* isolated from clinical samples in Vietnam. We eliminated studies that did not perform whole-genome sequencing using Illumina platforms or did not publish raw sequencing data, leaving 3 studies for inclusion. Raw sequencing data of 751 multidrug-resistant *E. coli* strains previously published by Roberts et al. (2022) (ENA Bioproject: PRJEB29424 and PRJEB28400), Hirabayashi et al. (2021) (provided by the authors), and Tuan-Anh et al. (2020) (ENA Bioproject PRJEB39354) were downloaded from the European Nucleotide Archive (ENA) database. Sixteen samples were from environmental surfaces (environmental isolates) and were collected at the National Hospital for Tropical Diseases, Hanoi, Vietnam. Among the other 735 clinical samples, 720 of the 721 isolates from the study by Roberts et al. (2022) were collected in two ICUs in Hanoi: 275 from the Bach Mai Hospital and 445 from the National Hospital for Tropical Diseases. One duplicate isolate (NHP1391) was excluded from the analysis. The two isolates (MH13 and MH17) from the study by Hirabayashi et al. (2021) were from the Military Hospital 103 in Hanoi, and the 27 isolates from the study by Tuan-Anh et al. (2020) were from the Children's Hospital 1 in Ho Chi Minh city. Two *E. coli* isolates (TN1393 and XP817) were from our laboratory (NCBI SRA BioProject: PRJNA857185) and were from two patients hospitalized in the Saint Paul Hospital and the 108 Military Central Hospital, respectively. Most of the studied samples were collected between 2017 and 2019, except three samples (MH13, TN1393 and XP817) collected in 2012 and 2013. Each isolate is described in Supplementary Table S1.

2.2. Antimicrobial resistance profiling

The *E. coli* isolates from the study by Roberts et al. (2022) were classified into two phenotypes, ESBL-producing isolates and CR isolates, in function of their growth on selective media. The phenotype classification of the other isolates was based (i) on the antimicrobial susceptibility test results extracted from the studies by Hirabayashi et al. (2021) and Tuan-Anh et al. (2020), and (ii) for the two isolates from our laboratory (TN1393 and XP817), on antimicrobial susceptibility testing performed using ampicillin, amoxicillin-clavulanic acid, amikacin, piperacillin, ticarcillin, ticarcillin-clavulanic acid, cefalexin, cefoxitin, ceftazidime, colistin, fosfomycin, ofloxacin, imipenem, penicillin, and trimethoprim-sulfamethoxazole and the

Kirby Bauer disk diffusion method, as previously described (EUCAST, 2020). In total, 4/751 strains were classified as non-ESBL-producing strains (see also Supplementary Table S1).

2.3. Genomic DNA extraction and Illumina sequencing

Genomic DNA (gDNA) of the two isolates from our laboratory was extracted with the Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, Ontario, Canada) following the manufacturer's instructions. The gDNA samples were sheared randomly into small fragments with Covaris S/E210 or Bioruptor to prepare paired-end fragment libraries that were sequenced using an Illumina HiSeq 4,000 system at the Beijing Genomics Institute (Shenzhen, China). According to the information in the articles, gDNA was extracted with the QIACube and QIAamp 96 DNA QIACube HT kit (Qiagen, Hilden, Germany) and sequenced on a Illumina HiSeq X10 machine (Roberts et al., 2022); gDNA was extracted with the Wizard Kit (Promega) and sequenced on a Illumina MiSeq platform (Tuan-Anh et al., 2020); gDNA was extracted with the phenol-chloroform method and sequenced on a MiSeq (MH13 isolate) and on a HiSeq 4000 (MH17 isolate) apparatus (Hirabayashi et al., 2021).

2.4. Bioinformatics analysis

Raw whole-genome sequencing data were analyzed using the bioinformatics workflow described in Figure 1.

2.4.1. De novo assembly of whole-genome sequencing data

The paired-end raw reads were trimmed with Trimmomatic v0.39 (Bolger et al., 2014), using a Phred threshold score < 20, and the following parameters: SLIDINGWINDOW:4:20 LEADING:20 TRAILING:20 MINLEN:50. Trimmed reads were assembled into contigs using SPAdes *de novo* assembler v3.14.1 (Bankevich et al., 2012) with default parameters for genome assembly and with the 'plasmid' flag (plasmidSPAdes) for assembling plasmids (Antipov et al., 2016). Contigs <500 bp were discarded.

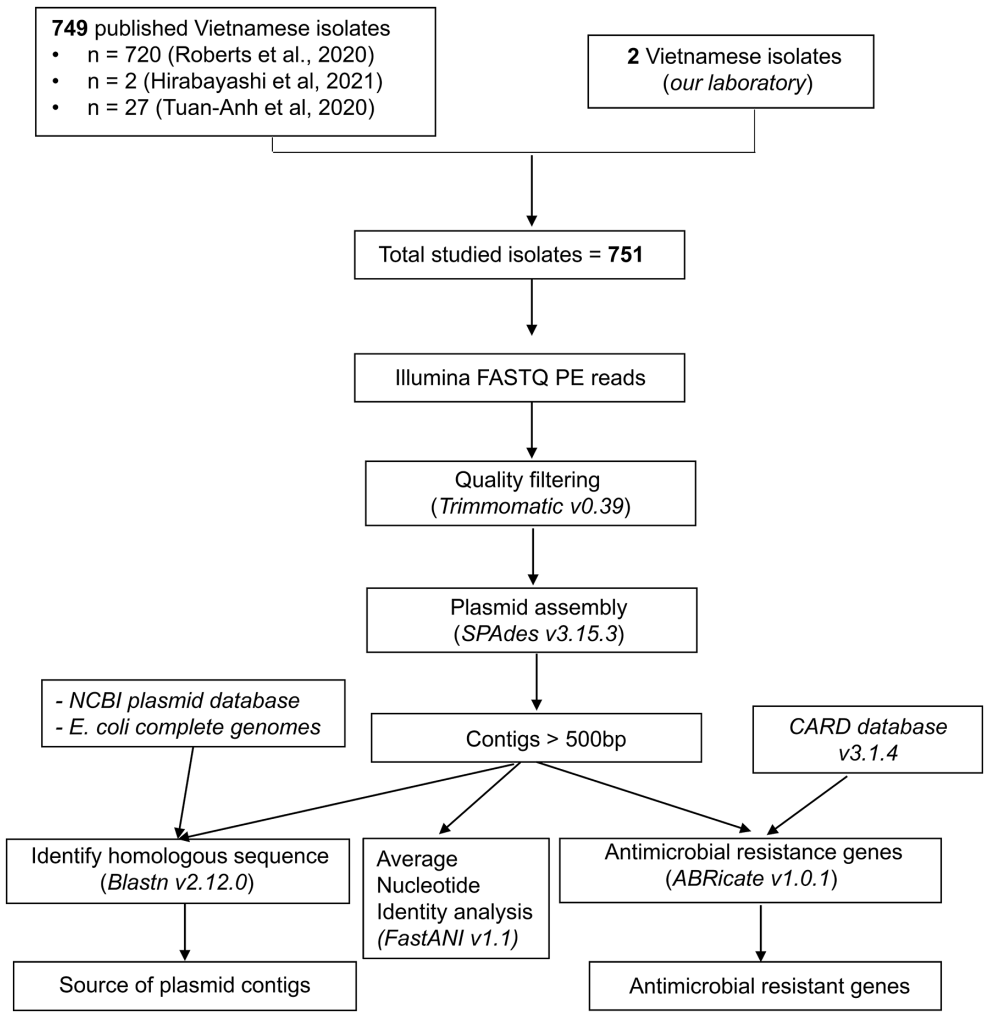

FIGURE 1
Bioinformatics workflow for plasmid analysis using raw sequencing data from multidrug-resistant *E. coli* isolates.

2.4.2. Identification of plasmid contigs and their origin

The contigs assembled with plasmidSPAdes were aligned to the NCBI RefSeq plasmid database (accessed on July 7, 2022) using BLASTn v2.12.0 (Altschul et al., 1990). A contig matched to the nearest neighbor (the highest BLAST bit score) in the plasmid database with a minimal identity of 95% and a minimal query coverage of 50% was defined as "putative plasmid contig." Then, the remaining unassigned contigs were aligned against the 2,174 complete *E. coli* genomes available in the NCBI genome database (accessed on May 31, 2022) with BLASTn v2.12.0, and the previous thresholds were used to identify chromosomal contamination in the plasmidSPAdes assembly.

The taxonomic lineages of the plasmid homologs were obtained using the NCBI Taxonomy database (accessed on July 24, 2022). The plasmid homolog sequences were extracted from the plasmid databases using seqtk v1.3 (Li, 2013). The average nucleotide identity (ANI) between plasmid homologs was estimated using FastANI v1.1 (Jain et al., 2018). All pairs with ANI values <95% were excluded. The plasmid network was then built with Cytoscape v3.9.0 (Shannon et al., 2003).

2.4.3. Identification of chromosome contigs

For each isolate, contigs from the SPAdes assembly were aligned to their putative plasmid contigs from the plasmidSPAdes assembly using BLASTn v2.12.0 (Altschul et al., 1990). All contigs matched to plasmid contigs with a minimum identity of 99% and a minimum query coverage of 99% were filtered out, and the remaining contigs were identified as "chromosome contigs."

2.4.4. Detection of antimicrobial resistance genes

AMR genes carried on contigs were identified using ABRicate v1.0.1 (Seemann, n.d.-a) and the CARD database v3.2.4 (Jia et al., 2017) and the following thresholds: 80% of minimum identity and 50% of minimum coverage. AMR genes were grouped into AMR gene families and drug classes using the CARD database v3.2.4. Contigs were functionally assessed with Rapid Annotations using Subsystem Technology (RAST) version 2.0 (Aziz et al., 2008).

2.4.5. Construction of the sequence similarity network

Pairwise alignments between putative plasmid contigs were carried out using BLASTn in the blastall ("BLAST all against all") program with an e-value threshold of 1×10^{-10} (Camacho et al., 2009). The BLAST best hit for each pairwise alignment was determined. Alignments with identity <99% and minimum coverage <90% and also repetitive alignments were filtered out. The remaining best alignments were used to build the sequence similarity network with Cytoscape v3.9.0 (Shannon et al., 2003).

2.4.6. Multilocus sequence typing analysis

Multilocus sequence typing (MLST) data for the isolates were obtained from the published papers, or determined from the assembled genomes using mlst v2.22.0 (Seemann, n.d.-b) with the *E. coli* species scheme.

2.5. Statistical analyses

Statistical analyses and data visualization were done with R version 4.0.4 (R Core Team, 2016). The numbers of putative plasmid contigs and of AMR genes were compared in resistance phenotype groups with the Kruskal-Wallis test followed by the Dunn's post-hoc tests for multiple pair comparisons using the kruskal.test function and the dunn.test function. The Fisher's exact test was used to compare AMR genes between CR isolates and ESBL-producing isolates through fisher.test function. All *p*-values from multiple testing were corrected with the Benjamini-Hochberg method, and adjusted *p*-values <0.05 were considered significant using the p.adjust function. Correlation plots, box plots and bar plots were generated using the ggplot function from the ggplot2 package version 3.4.0. Binary heatmaps were constructed with the Heatmap function from the ComplexHeatmap package version 2.9.4 (Gu et al., 2016).

3. Results

3.1. Characteristics of putative plasmid contigs

The number of assembled plasmid contigs, using plasmidSPAdes, hugely varied among the 751 isolates, from 0 to 126 (median = 22, mean ± SD = 26 ± 19). In 8/751 isolates, no plasmid contig was identified. As expected, a moderate positive correlation was found between plasmid contig number and total assembly length (Pearson's correlation coefficient, $r = 0.55$) (Figure 2A; Supplementary Table S1). Conversely, no correlation was found between plasmid contig number and sequencing coverage ($r = 0.1$) (Figure 2B; Supplementary Table S1), suggesting that the number of plasmid contigs in an isolate was linked to the presence of plasmids rather than to the sequencing coverage. Moreover, the number of plasmid contigs was higher in CR isolates ($n = 197$) than in ESBL-producing isolates ($n = 550$) ($p = 0.008$) and non-ESBL-producing isolates ($n = 4$) ($p = 0.041$) (Supplementary Figure S1). However, the highest number of plasmid contigs was found in an ESBL-producing strain ($n = 126$).

Among the 19,894 plasmid contigs identified with plasmidSPAdes in the studied isolates, 18,098 (90.97%) were matched in the plasmid database (putative plasmid contigs) and 1,565 (7.87%) were matched to the *E. coli* chromosome with at least 95% sequence identity (Figure 3A). The other 231 contigs (1.16%) could not be matched (i.e., unassigned contigs). However, for 223/231 unassigned contigs, at least one homolog was found in the plasmid database or in the *E. coli* genome database, but with a low BLAST identity (between 70 and 93%). For the other unassigned contigs, a BLASTn homology search against the non-redundant (nr) nucleotide database found that four contigs shared high similarity with the *A. baumannii* chromosome (sequence identity >98%), one contig shared 96% of identity with an *Escherichia* phage, and three contigs could not be aligned to any sequence.

Next, the closest match of the putative plasmid contigs in the plasmid database, which was considered as the plasmid homolog, was investigated. Plasmid homologs were mostly from the *Escherichia* genus (75.79%), followed by the *Klebsiella/Raoultella, Salmonella, Vibrio, Enterobacter, Shigella*, and *Acinetobacter* genera. These genera mostly belong to the *Enterobacteriaceae* family, with the exception of *Vibrio* that belongs to the *Vibrionaceae* family and of *Acinetobacter* that belongs to the *Moraxellaceae* family. Each genus was dominated by a major species (between 66.37 and 99.64% of plasmid homologs for that genus) (Figure 3B). This indicates that plasmids could be shared between genera, but the transmissions of plasmids are more

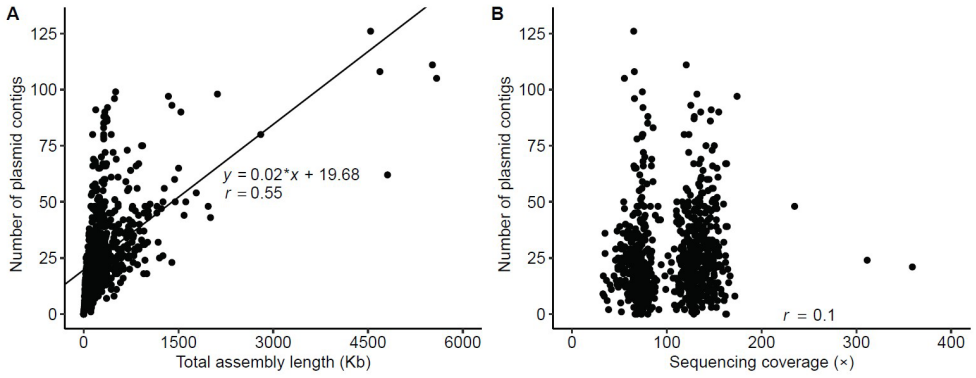

FIGURE 2
Statistics of contigs assembled using the plasmidSPAdes assembler. **(A)** Correlation of the number of plasmid contigs ≥500 bp with the total assembly length. **(B)** Relationship between number of contigs with length ≥500 bp and sequencing coverage. Correlations were assessed using a linear regression model; *r*, Pearson's correlation coefficient.

frequently observed within the same species than those between distinct species. A set of 2,331 plasmid types that represented all plasmid homologs was used for the ANI analysis based on whole plasmid alignments to identify the genetic relatedness among plasmid homologs. This analysis revealed that 1,955/2,331 plasmids from different genera were clustered together with an ANI value >95%, indicating that these plasmid homologs were very closely related. Conversely, 120/2,331 plasmids were in smaller clusters, and 256/2,331 plasmids did not share any similarity with the other plasmids (Figure 3C).

3.2. Antimicrobial resistance genes in the putative plasmid contigs

In total, 5,554 plasmid-carried AMR genes were found in 83.98% (624/743) of isolates with plasmids. Among these genes, 99.06% (5,502/5,554) were detected in contigs that were matched in the plasmid database (Supplementary Table S2) and the other 52 AMR genes were detected in contigs that matched to chromosomes and in unassigned contigs. More than 60% of AMR genes identified in putative plasmid contigs belonged to the major facilitator superfamily antibiotic efflux pump, sulfonamide resistance, trimethoprim resistant dihydrofolate reductase, aminoglycoside acetyltransferase ANT(3″), resistance-nodulation-cell division antibiotic efflux pump, macrolide phosphotransferase, CTX-M β-lactamase, and TEM β-lactamase families (Supplementary Table S2 and Supplementary Table S2). The mean number of AMR genes detected in plasmid contigs for each isolate was nine. Overall, the number of AMR genes in plasmid contigs was higher in CR isolates than ESBL-producing isolates ($p < 0.001$). As expected, non-ESBL-producing isolates, which had a lower resistance phenotype, had fewer AMR genes than CR and ESBL-producing isolates (Figure 4A). Both CR and ESBL-producing isolates harbored many different plasmid-carried AMR genes belonging to >40 different AMR gene families. Conversely, only five AMR gene families [i.e., major facilitator superfamily antibiotic efflux pump, sulfonamide resistance, TEM β-lactamase, aminoglycoside 6-phosphotransferase APH (6), aminoglycoside 3″-phosphotransferase were found in non-ESBL-producing isolates]. Although CR and ESBL-producing isolates showed similar frequencies of AMR gene families across the putative plasmid contigs, the frequency of genes encoding OXA, NDM and KPC β-lactamases, which are implicated in carbapenem resistance, varied between CR and ESBL-producing isolates (Figure 4B and Table 1). The presence/absence of AMR genes classed into AMR gene families was further investigated in each isolate. AMR genes belonging to the major facilitator superfamily, sulfonamide resistance, trimethoprim resistant dihydrofolate reductase, and aminoglycoside acetyltransferase ANT(3″) families were highly represented in the studied isolates. While there was no clear separation between groups on the binary heatmap of AMR gene family across the studied strains, the presence of AMR genes in isolates was not likely associated with their resistance phenotype, sequence type (MLST), location, and study. However, isolates belonging to the sequence type 617 (ST617) could be grouped in clusters on the basis of their AMR gene profile, and these clusters included mainly CR isolates (Figure 4C).

3.3. AMR genes and carbapenem resistance

Then, the analysis focused on 22 AMR genes that are associated with resistance to carbapenems. AMR genes belonging to the efflux pump and β-lactamase families were found in both CR and ESBL-producing isolates. Efflux pump genes (*TolC, adeN, adeI, adeJ* and *adeK*) were found in <1% of the studied strains. *MarA* and its homolog *ramA*, which encode bacterial transcriptional activators involved in regulating the AcrAB/TolC multidrug efflux pump, and porin genes were detected in ~11% of isolates. Carbapenemase-encoding genes ($bla_{\text{CTX-M}}$, bla_{KPC}, bla_{NDM} and bla_{OXA}) were detected in CR isolates and also in ESBL-producing isolates. Specifically, $bla_{\text{CTX-M-27}}$, a β-lactamase-encoding gene that leads to resistance to carbapenems, cephalosporins, and penams, was the most frequently detected CR gene in putative plasmids (9.64% of CR and 15.82% of ESBL-producing isolates). Its presence in plasmids could be responsible for high resistance to antibiotics in CR and ESBL-producing isolates. Moreover, the frequency of $bla_{\text{KPC-2}}$, $bla_{\text{NDM-5}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{OXA-181}}$ (β-lactamase-encoding genes) on putative plasmid contigs was higher in CR than ESBL-producing isolates (Fisher's exact test: adjusted

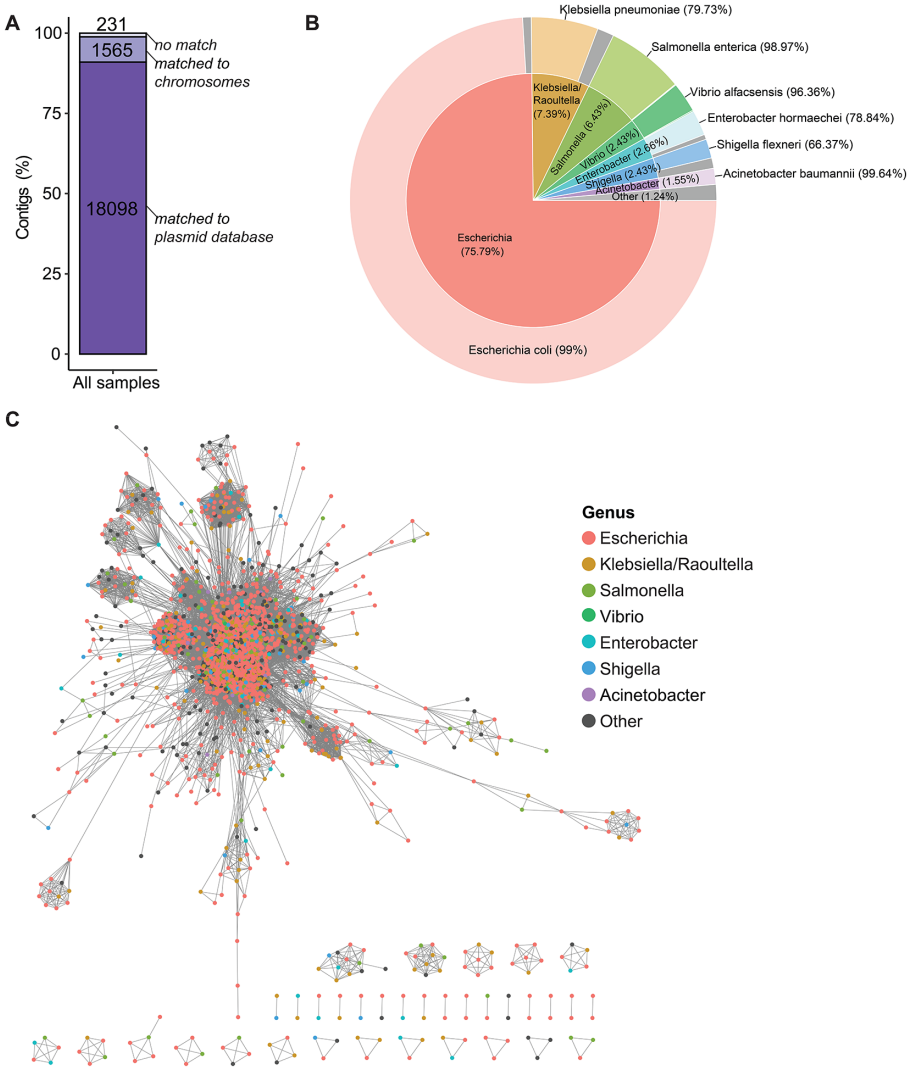

FIGURE 3
Origin of the putative plasmid contigs. **(A)** BLASTn homology search of contigs against the plasmid database and the complete *E. coli* genomes using the following thresholds: 95% of minimum identity and 90% of minimum query coverage. The numbers in the bars indicate the number of contigs. **(B)** Pie chart showing the percentages of plasmid homologs that matched to the putative plasmid contigs grouped by host genus and host species. "Other" (in gray): other species/genera with matching <1%. **(C)** Clustering of plasmid homologs by average nucleotide identity with a cut-off value of 95%. Each node represents a plasmid homolog and is color-coded in function of the host genus.

p-values <0.05). These genes are probably strongly associated with carbapenem resistance (Figure 5; Table 1). Additionally, >90% ($n = 178/197$) of the studied CR isolates carried at least one copy of the bla_{KPC}, bla_{NDM}, or bla_{OXA} gene in plasmid or chromosome contigs. Specifically, 67 of these CR isolates carried these genes only on plasmid contigs, 60 only on chromosome contigs, and 51 on both plasmid and chromosome contigs. Together, these findings indicated that carbapenem hydrolysis by KPC, NDM, and OXA carbapenemases is the major mechanism underlying carbapenem resistance in *E. coli* isolates with a significant contribution from plasmids.

3.4. Highly conserved structural organization of plasmid-encoded β-lactamase genes

Next, a sequence similarity network was built using 304 putative plasmid contigs that harbored bla_{KPC}, bla_{NDM} and bla_{OXA} genes to obtain a global view of the genetic organization of β-lactamase-encoding genes on plasmids. Plasmid contigs carrying the same β-lactamase-encoding gene shared a high degree of genetic similarity (sequence identity >99% and minimum query coverage of 90%) in pairwise alignments. All contigs carrying different bla_{NDM} genes were grouped into one cluster. Contigs carrying different bla_{OXA} genes showed a great diversity of genetic structures and were organized in four clusters. Moreover, plasmid contigs harboring bla_{OXA-48} were classified in two sub-clusters. Although the clusters of the plasmid contigs carrying bla_{OXA-1} and bla_{KPC-2} did not share any sequence similarity, they were connected through a contig that contained both the bla_{OXA-1} and bla_{KPC-2} genes (Figure 6A). The genetic environment surrounding β-lactamase-encoding genes was similar in CR and in ESBL-producing isolates. In agreement with the result of the sequence similarity network, the structural organization of β-lactamase-encoding genes within each cluster was highly conserved across plasmid contigs (Figure 6B). Transposable elements (TEs; including transposons and insertion sequences), which play important roles in the spread of AMR genes,

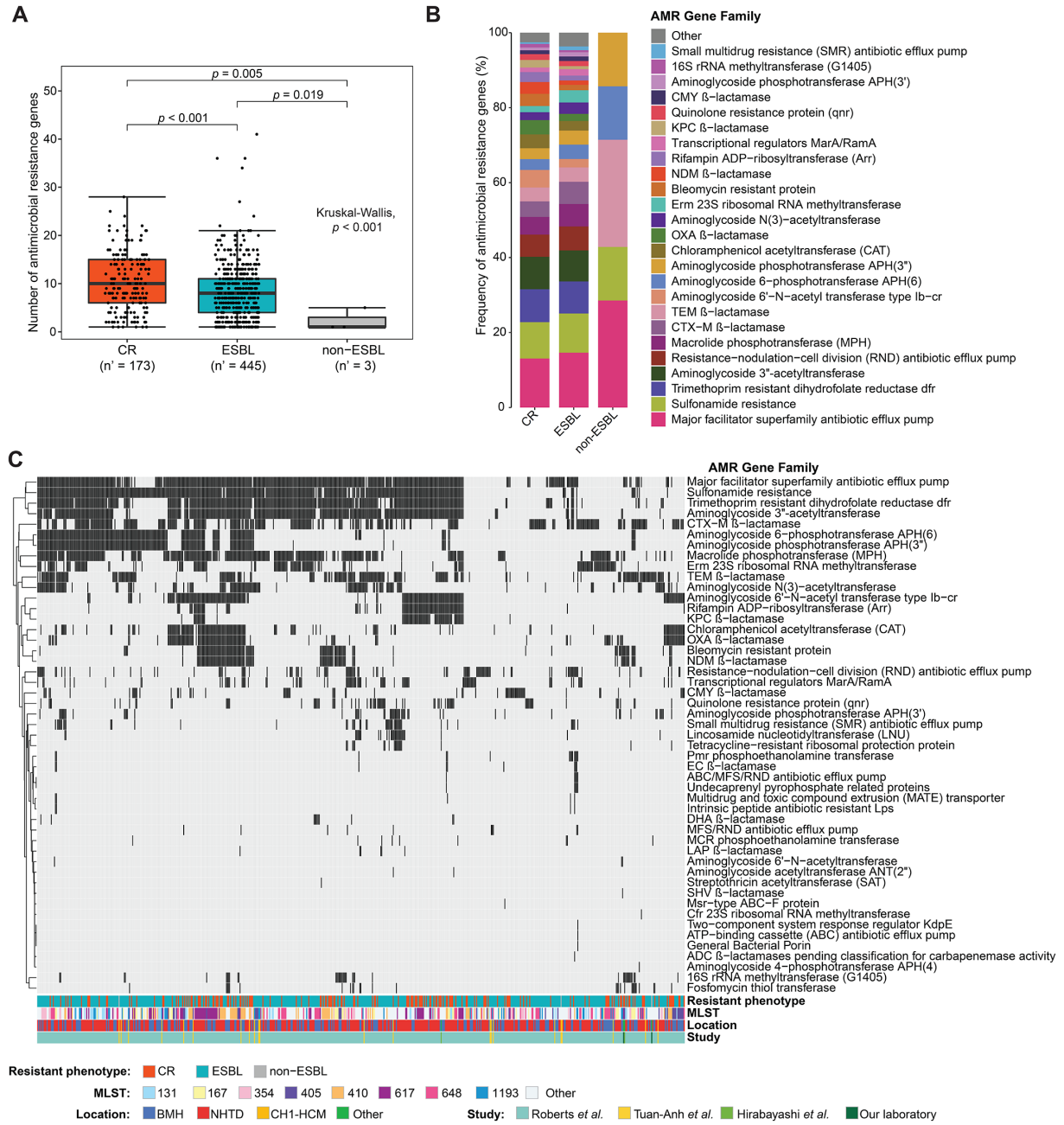

FIGURE 4
Antimicrobial resistance (AMR) genes in putative plasmid contigs. **(A)** Box plot showing the number of AMR genes detected in putative plasmid contigs of isolates grouped in function of their resistance phenotype; n', number of isolates with the putative plasmid contigs carrying AMR genes. *P*-value calculated with the Kruskal-Wallis test followed by the Dunn's *post-hoc* test and corrected for multiple testing with the Benjamini-Hochberg method. **(B)** Frequency of AMR genes, classified into AMR gene families, detected on putative plasmid contigs in carbapenem-resistant (CR) isolates, extended-spectrum beta-lactamase (ESBL)-producing isolates and non-ESBL-producing isolates. AMR genes with frequency<1% were merged in the "Other" group. **(C)** Heatmap showing the presence (black) and absence (gray) of AMR gene families across isolates. Only isolates carrying at least one AMR gene were included. Hierarchical clustering was performed using Euclidean distances. The color bars at the bottom of the heatmap indicate the resistance phenotype, sequence type (MLST), location, and study from which the sequencing data of the isolates were taken. BMH, Bach Mai Hospital; NHTD, National Hospital for Tropical Diseases; CH1-HCM, Children's Hospital 1-Ho Chi Minh city.

particularly bla_{KPC}, bla_{NDM} and bla_{OXA}, in healthcare settings (Partridge et al., 2018), were often located close to the β-lactamase-encoding genes.

4. Discussion

The emergence of ESBL-producing and CR *Enterobacteriaceae* is a public health threat worldwide, including Vietnam, because the latest generation of β-lactam antibiotics would become ineffective against infectious diseases caused by these species. Importantly, AMR *Enterobacteriaceae* are spreading not only in hospital settings, but also in the communities and in agricultural settings (Carrique-Mas et al., 2020). Dissemination of AMR genes by mobile genetic elements, especially plasmids, is considered the main mechanism of the emergence of drug resistance in bacteria (Partridge et al., 2018). In this study, the plasmid assemblies of 751 multidrug-resistant *E. coli* from

TABLE 1 Presence of AMR genes associated with resistance to carbapenems on putative plasmid contigs.

AMR gene	CRE isolates (n=197)		ESBL isolates (n=550)		Fisher's exact test	
	No. isolates carry AMR genes	Occurrence (%)	No. isolates carry AMR genes	Occurrence (%)	Normal P	Adjusted P*
CTX-M-27	19	9.64	87	15.82	0.033	0.103
KPC-2	36	18.27	31	5.64	<0.0001	<0.0001
NDM-5	46	23.35	37	6.73	<0.0001	<0.0001
NDM-1	4	2.03	1	0.18	0.019	0.069
NDM-4	3	1.52	4	0.73	0.388	0.840
NDM-7	3	1.52	2	0.36	0.118	0.324
OXA-1	44	22.34	52	9.45	<0.0001	<0.0001
OXA-9	1	0.51	1	0.18	0.458	0.840
OXA-23	0	0.00	1	0.18	1	1
OXA-339	0	0.00	1	0.18	1	1
OXA-70	1	0.51	0	0.00	0.264	0.645
OXA-48	12	6.09	7	1.27	0.001	0.004
OXA-181	10	5.08	7	1.27	0.004	0.019
OXA-928	0	0.00	1	0.18	1	1
SHV-12	0	0.00	1	0.18	1	1
TolC	1	0.51	4	0.73	1	1
marA	13	6.60	44	8.00	0.639	1
ramA	9	4.57	21	3.82	0.673	1
adeN	1	0.51	1	0.18	0.458	0.840
adeI	1	0.51	3	0.55	1	1
adeJ	1	0.51	3	0.55	1	1
adeK	1	0.51	3	0.55	1	1

*P-values were corrected for multiple testing with the Benjamini-Hochberg method.

hospitals in Vietnam were generated using short-read sequencing data from four studies in Vietnam. Overall, the studied isolates contained many putative plasmids that harbored a great diversity of AMR genes, particularly carbapenemase-encoding genes.

4.1. Genetic similarity of plasmids

The BLASTn homology search of contigs (assembled with plasmidSPAdes) against the NCBI RefSeq plasmid database and *E. coli* complete genomes (to determine the closest relative putative plasmid contigs) showed that apart from *E. coli* origin, putative plasmid contigs were found to be originated from other *Enterobacteriaceae* species or from the *Vibrionaceae* and *Moraxellaceae* families. More importantly, despite their different species/genus origin, theplasmid homologs shared high genomic similarity, which is broadly in agreement with a previous study on the prokaryotic plasmidome (Redondo-Salvo et al., 2020). These findings suggest that genetic exchange events by plasmid conjugation occur frequently within the *Enterobacteriaceae* family, especially in *E. coli* species but also between species. The potential plasmidic horizontal transfers between bacterial species, e.g., *A. baumanni* and *E. coli* have been reported (Chatterjee et al., 2017; Cooper et al., 2017). This aspect is fundamental to explain the ecology of AMR and to understand the emergence of drug resistance in the different bacterial species of public health interest.

4.2. Molecular epidemiology of CTX-M β-lactamases

ESBL-producing and CR isolates harbored many different AMR genes on their plasmids. The most frequently detected genes encoded antibiotic efflux pumps that eliminate antimicrobial agents from host cells (Kumar et al., 2020). Genes encoding CTX-M β-lactamases also were frequently identified on plasmids of ESBL-producing and CR isolates, as already previously observed in clinical ESBL-producing *E. coli* isolates (Ma et al., 2005; Ghenea et al., 2022). Strikingly, each CTX-M variant is widespread over a particular geographic area, thus providing epidemiological evidence for the transmission of β-lactamase-encoding genes within and between communities (Canton and Coque, 2006; Rossolini et al., 2008). In the present study, the $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ genes were widely represented on plasmids of ESBL-producing and CR strains. Previous studies showed that the genes encoding these CTX-M variants are predominant in ESBL-producing *E. coli* isolates from humans and farm animals in Vietnam (Biedenbach et al., 2014; Nguyen et al.,

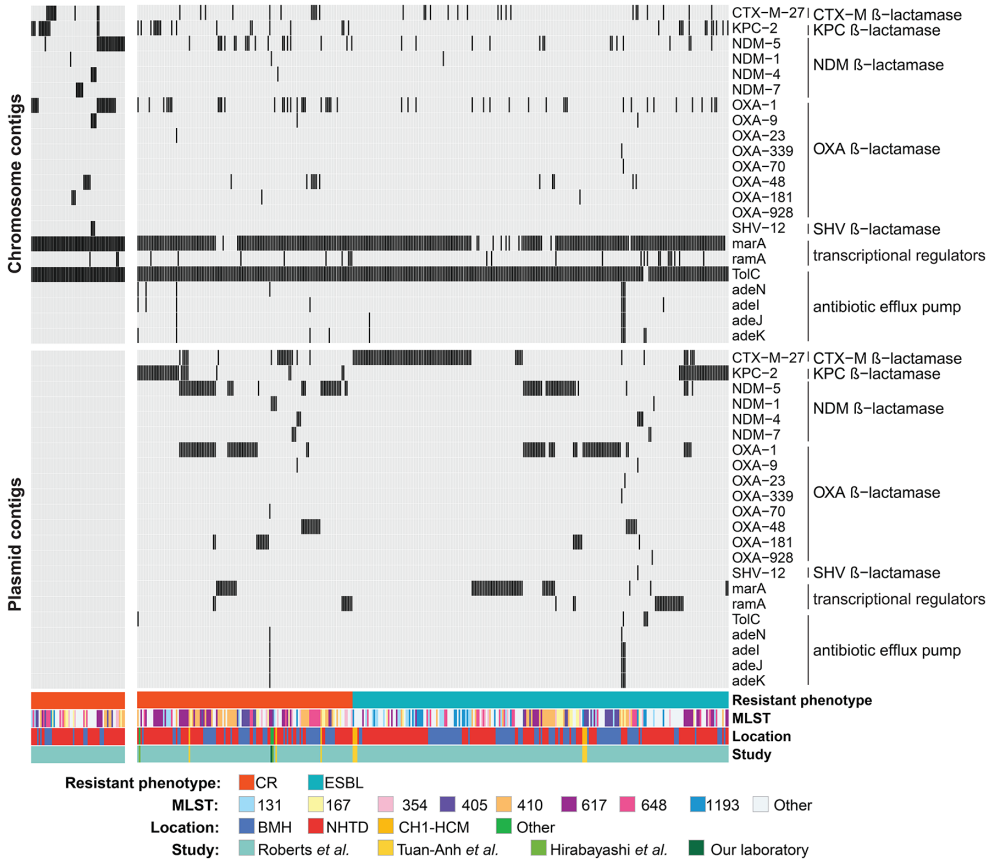

FIGURE 5
Carbapenem resistance (CR) genes on putative plasmid and chromosome contigs across isolates. All isolates carrying at least one carbapenem resistance gene on putative plasmid/chromosome contigs are shown on the right, whereas CR isolates without any carbapenem resistance genes on plasmid/chromosome contigs are on the left heatmap. Black and gray colors indicate presence and absence of AMR genes, respectively. The color bars at the bottom of the heatmaps indicate the resistance phenotype, sequence type (MLST), location and study from which the sequencing data of the isolates were taken. BMH, Bach Mai Hospital; NHTD, National Hospital for Tropical Diseases; CH1-HCM, Children's Hospital 1-Ho Chi Minh city.

2019). CTX-M-15 is the dominant CTX-M variant worldwide. Other CTX-M variants (e.g., CTX-M-55, CTX-M-27) that were initially specific to some world regions have now spread globally due to international travel (Bevan et al., 2017; Nakayama et al., 2020). Thus, monitoring the emergence of plasmid-carried $bla_{\text{CTX-M}}$ genes, particularly genes encoding new variants, could provide important information for developing strategies to reduce the transmission of AMR genes via plasmids in clinical settings.

4.3. Molecular epidemiology of carbapenemases

Plasmid-carried carbapenemase-encoding genes are considered the main cause of the resistance phenotype in CR *E. coli*. In our analysis, genes encoding the KPC-2, NDM-5, OXA-1, OXA-48, and OXA-181 β-lactamases were more frequently detected on plasmids in CR than ESBL-producing isolates. The bla_{KPC} gene was first detected on a plasmid of a carbapenem-resistant *K. pneumoniae* strain in the southern United States in 2001 (Yigit et al., 2001). It has then rapidly spread to other bacterial pathogens, including *E. coli,* worldwide, including in Vietnam (Petrella et al., 2008; Woodford et al., 2008; Richter et al., 2011; Linh et al., 2021). Similarly, plasmid-carried $bla_{\text{NDM-5}}$ was identified in patients in the United Kingdom and the United States (patient hospitalized in India) in 2016–2017 (Hornsey et al., 2011; Rojas et al., 2017), and in multidrug-resistant clinical *E. coli* strains in many countries (Pitart et al., 2015; Soliman et al., 2016; Zou et al., 2020). In Vietnam, plasmid-encoded $bla_{\text{NDM-1}}$ was detected in clinical *Enterobacteriaceae* isolates from a surgical hospital in 2010 (Tran et al., 2015). To our knowledge, Roberts et al. (2022) were the first to describe the presence of $bla_{\text{NDM-5}}$ in clinical isolates in Vietnam. In our study, plasmid-carried $bla_{\text{NDM-1}}$, but not $bla_{\text{NDM-5}}$, was detected in the XP817 and MH-13 isolates collected in 2012–2013, whereas plasmid-carried $bla_{\text{NDM-5}}$ was detected in clinical samples isolated in 2017–2019. This suggests the emergence of plasmid-carried $bla_{\text{NDM-5}}$ in Vietnam approximately in 2017, close to the year of its first report in other countries.

Plasmid-encoded OXA β-lactamases can hydrolyze β-lactam substrates, including carbapenems (Evans and Amyes, 2014). The KPC, NDM and OXA enzymes can mediate carbapenem resistance. Their hydrolytic activity depends on the expression level of the genes encoding these enzymes that is mostly influenced by their genetic environment and copy number (Roth et al., 2011; Evans and Amyes, 2014; Paul et al., 2017). This could explain why some ESBL-producing isolates that harbor these β-lactamase-encoding genes do not show any phenotypic resistance to carbapenems.

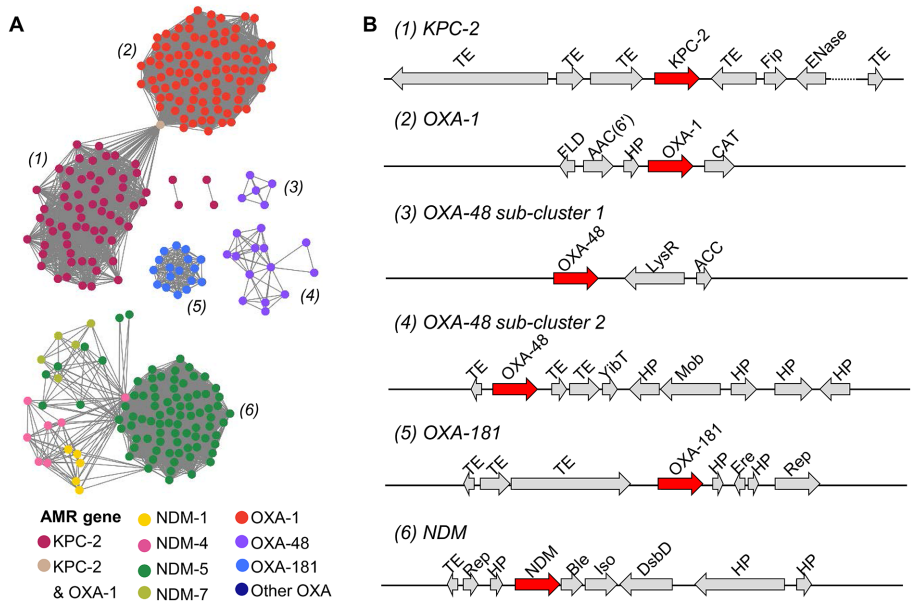

FIGURE 6
Characterization of putative plasmid contigs containing genes encoding the KPC, NDM and OXA β-lactamases in *E. coli* isolates. **(A)** Sequence similarity network of 304 plasmid contigs harboring bla_{KPC}, bla_{NDM}, and bla_{OXA} genes. Pairwise alignments between contigs were done with BLASTn, reciprocal BLAST, and an E-value threshold of $1x10^{-10}$. Each node represents a plasmid contig and is colored in function of the carried gene. Each plasmid contig cluster is labeled with a number. **(B)** The most frequent genetic organization of β-lactamase-encoding genes in plasmid contig clusters. Gene products from the RAST annotation are shown. TE, transposable element; Fip, filamentous phage production; ENase, plasmid conjugative transfer endonuclease; FLD, S-(hydroxymethyl) glutathione dehydrogenase; AAC(6′), aminoglycoside 6′–N–acetyl transferase type Ib–cr; HP, hypothetical protein; CAT, chloramphenicol O-acetyltransferase; LysR, LysR transcriptional regulator; ACC, acetyl-coenzyme A carboxyl transferase, YibT, uncharacterized protein YibT; Mob, mobilization protein; Ere, erythromycin esterase; Rep, DNA replication protein; Ble, bleomycin resistance gene; PRAI, phosphoribosylanthranilate isomerase; DsbD, cytochrome C-type biogenesis protein DsbD.

The sequence similarity network based on pairwise alignments of plasmid contigs harboring the bla_{KPC}, bla_{NDM} and bla_{OXA} genes captured a high degree of similarity between contigs carrying the same AMR gene. This confirmed the results by Roberts et al. (2022) showing that AMR genes are transmitted within and between hospital settings in Vietnam. The genetic environment around the bla_{KPC-2} and bla_{NDM} genes is largely consistent with what reported by previous studies (Dortet et al., 2012; Cuzon et al., 2013; Martins et al., 2020). Specifically, our analysis showed that bla_{KPC-2} is located between two TEs, whereas bla_{NDM} genes are close to the bleomycin resistance gene located downstream of a TE and share the same promoter. Conversely, bla_{OXA} genes were found in several clusters with different environmental structures, as previously reported by Evan & Amyes (Evans and Amyes, 2014). This could be explained by spontaneous DNA rearrangements following acquisition of the bla_{OXA} by horizontal transfer.

4.4. Limitations

Our study, based on short-read whole-genome sequencing data, provided general and predictive information on plasmid profiles in 751 ESBL-producing and CR *E. coli* isolates. This work has certain limitations. We could only assemble partially plasmids from short-read data using the existing assembly tool and therefore part of plasmid information remains unknown. Another limitation of our study was sample isolation bias since most of the studied strains were isolated from patients of two hospitals in northern Vietnam. Nevertheless, the current study serves as a good foundation for further analysis on a larger scale to have a more detailed look into resistance plasmid epidemiology at the national and global level.

5. Conclusion

This study highlights the high potential of horizontal transfer and dissemination of AMR genes encoded by plasmids in multidrug-resistant *E. coli* isolates in Vietnam. These putative plasmids could be transferred among different *Enterobacteriaceae* species and genera. The number of plasmid-carried AMR genes was higher in CR than in ESBL-producing isolates, especially β-lactamase-encoding genes. The genetic environment surrounding carbapenemase-encoding genes in plasmid contigs was highly conserved with the presence of transposable elements that might facilitate their spread in microbial communities.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and

institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

QHN and TTTT: study concept and design. TTHL, STN, K-OTN, and DVQ: data acquisition. QHN, TTHL, STN, and TTTT: data analysis and interpretation. QHN, TTHL, STN, JH, A-LB, and TTTT: drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1094119/full#supplementary-material

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Genome-wide identification and oxacillinase OXA distribution characteristics of *Acinetobacter* spp. based on a global database

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Objective: To use genomic analysis to identify *Acinetobacter* spp. and to explore the distribution characteristics of ß-lactamase oxallicinases (*bla*OXA) among *Acinetobacter* species globally.

Methods: Genomes of global *Acinetobacter* spp. were downloaded from GenBank using Aspera batch. After quality check using CheckM and QUAST software, the genomes were annotated using Prokka software to investigate the distribution of *bla*OXAs across *Acinetobacter* spp.; a phylogenetic tree was constructed to explore the evolutionary relationship among the *bla*OXA genes in *Acinetobacter* spp. Average-nucleotide identification (ANI) was performed to re-type the *Acinetobacter* spp. BLASTN comparison analysis was implemented to determine the sequence type (ST) of *Acinetobacter baumannii* strain.

Results: A total of 7,853 genomes were downloaded, of which only 6,639 were left for further analysis after quality check. Among them, 282 *bla*OXA variants were identified from the genomes of 5,893 *Acinetobacter* spp.; *bla*OXA-23 (n=3,168, 53.8%) and *bla*OXA-66 (2,630, 44.6%) were the most frequent *bla*OXAs, accounting for 52.6% (3,489/6639), and the co-carriage of *bla*OXA-23 and *bla*OXA-66 was seen in 2223 (37.7%) strains. The 282 *bla*OXA variants were divided into 27 clusters according to the phylogenetic tree. The biggest clade was *bla*OXA-51-family carbapenem-hydrolyzing enzymes composed of 108 *bla*OXA variants. Overall, 4,923 *A. baumannii* were identified out of the 6,639 *Acinetobacter* spp. strains and 291 distinct STs were identified among the 4,904 *bla*OXA-carrying *A. baumannii*. The most prevalent ST was ST2 (n=3,023, 61.6%) followed by ST1 (n=228, 4.6%).

Conclusion: OXA-like carbapenemases were the main *bla*OXA-type β-lactamase spread widely across *Acinetobacter* spp. Both *bla*OXA-23 and *bla*OXA-66 were the predominant *bla*OXAs, among all *A. baumannii* strains, with ST2 (belonging to CC2) being the main clone disseminated globally.

KEYWORDS

***Acinetobacter* spp., *Acinetobacter baumannii*, OXA-23, OXA-66, ST2**

1. Introduction

Acinetobacter spp. is one of the most frequent non-fermentative gram-negative coccobacilli that is widely distributed among humans as well as in the external environment. It predominantly colonizes and infects hospitalized patients, with a variety of nosocomial infections implicated (Bergogne-Bérézin and Towner, 1996). High rates of antibiotic resistance in *Acinetobacter* spp. have been documented in numerous reports, with strains possessing OXA-type carbapenem-hydrolyzing β-lactamases (CHβLs) being particularly of concern (Lee et al., 2016). Infections caused by such strains are often extremely difficult to eradicate due to their widespread resistance to the major groups of antimicrobial agents.

Currently, *Acinetobacter* spp. has been assigned into 144 different *Acinetobacter* species, including 68 with species names and 76 unnamed taxa., with *A. baumannii*, *A. nosocomialis*, and *A. pittii* being the most frequent ones in healthcare setting (Lupo et al., 2018; Qin et al., 2021). With the rapid development of matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and whole genome sequencing (WGS) technology, the current classification of *Acinetobacter* spp. urgently needs to be updated, since different species have been reported to cause different infections and show different drug resistance characteristics (Antunes et al., 2014; Zajmi et al., 2022; Longo et al., 2023).

Till date, *A. baumannii* has been found to be of the greatest clinical importance among *Acinetobacter* species, owing to its association with a variety of nosocomial infections, including pneumonia, bacteremia, urinary tract infection, and secondary meningitis (Lee et al., 2017). Specifically, ventilator-associated pneumonia caused by such strains in intensive care units (ICUs) is of great concern (Harding et al., 2018); even more worrying is the extremely and rapidly developed drug-resistance of the strains, with extensive drug-resistance and pan-drug-resistance of *A. baumannii* being a public health threat (Magiorakos et al., 2012). Moreover, such strains possess the capacity of long-term survival, resulting in enhanced opportunities of transmission across patients (Antunes et al., 2014).

Multiple investigations have shown that diverse OXA β-lactamases, especially the carbapenem-hydrolyzing oxacillinase (Tooke et al., 2019), play quite important roles in the resistance of *A. baumannii* (Kengkla et al., 2018). In particular, *bla*OXA-23, *bla*OXA-24, *bla*OXA-51, and *bla*OXA-58 have been found to be primarily associated with carbapenem resistance in *A. baumannii*, with *bla*OXA-23 being the most widespread gene in most countries (Khurshid et al., 2020; Wang et al., 2021), and *bla*OXA-24 and *bla*OXA-58 being the dominant genes in specific regions (Salehi et al., 2019). *bla*OXA-51 is a chromosomally encoded β-lactamase that has been demonstrated to be universally present in all *A. baumannii* strains, and resistance to carbapenems have been reported when the genetic environment around the gene promoted the expression of *bla*OXA-51 gene (Takebayashi et al., 2021). Furthermore, global clone groups 1 (ST1) and 2 (ST2) have been the two major clonal groups of carbapenem resistance spreading globally (Douraghi et al., 2020; Palmieri et al., 2020).

In recent years, the number of *bla*OXA variants have been continuously increasing. Correspondingly, the grouping of *bla*OXA enzymes has also changed based on amino acid sequence similarity (Walther-Rasmussen and Høiby, 2006). Till date, more than multiple *bla*OXA variants have been identified (Boyd et al., 2022; https://www.ncbi.nlm.nih.gov/gene/?term=OXA). However, the prevalence and evolution of *bla*OXAs among *Acinetobacter* spp. have remained unknown as the major β-lactamase, and whether the dissemination of *bla*OXA genes is related to specific clones would require further exploration.

In this study, we aimed to analyze the prevalent distribution of *bla*OXAs among global *Acinetobacter* spp. from all publicly available genome sequences. A phylogenetic tree of *bla*OXA variants was constructed to explore the evolutionary relationship among them. Furthermore, all the *Acinetobacter* spp. analyzed were identified by average nucleotide identity (ANI) comparison, and sequence types (STs) of the *bla*OXA-carrying *A. baumannii* were explored to investigate the relationship between prevalent *bla*OXAs and predominant STs.

2. Materials and methods

2.1. Download of *Acinetobacter* spp. genomes

A total of 7,853 *Acinetobacter* spp. genomes were downloaded from NCBI genome database[1] using Aspera, on December 21, 2021. The genomic quality of the 7,853 strains was filtered by CheckM and QUAST software (Gurevich et al., 2013; Parks et al., 2015). The conditions for being considered a high-quality genome included completeness ≥ 90% and contamination < 5%. The quantity of contig was required to be ≤500 bp and N50 ≥ 40,000 bp; thus, 1,214 genomes that did not meet the above conditions were filtered out.

2.2. Investigation of *bla*OXAs across *Acinetobacter* spp.

Prokka (Seemann, 2014) is a fast prokaryotic genome annotation software that was used to annotate the genomes of all 6,639 strains in our study, in order to avoid the differences in genome gene prediction by different annotation methods. Distributions of blaOXA for each genome were determined by Blast analysis using a self-building blaOXA database retrieved from the National Database of Antibiotic Resistant Organisms.[2] Genomes harboring blaOXA were selected for further analysis.

2.3. Phylogenetic tree of *bla*OXA variants within *Acinetobacter* spp.

MUSCLE version 3.8.31 (Edgar, 2004) was used for nucleotide sequence alignment of 282 *bla*OXA genes. Then, the generated

Abbreviations: CHβL, Carbapenem-hydrolyzing β-lactamase; MALDI-TOF MS, Matrix-associated laser desorption ionization–time of flight mass spectrometry; WGS, Whole genome sequencing; ICU, Intensive care unit; ANI, Average nucleotide identification; ST, Sequence type; SNP, Single nucleotide polymorphism; MLST, Multi-locus sequence typing.

1 https://www.ncbi.nlm.nih.gov/genome/browse/

2 https://www.ncbi.nlm.nih.gov/pathogens/refgene/#blaOXA

multiple sequence alignment file was used to build a maximum likelihood (ML) phylogenetic tree by RAxML version 8.2.11 (Alexandros and Stamatakis, 2014), with Bootstrap being set as 500 and M (model setting) being selected as "GTRCAT." Finally, this tree was visualized with iTOL software (Letunic and Bork, 2019).

2.4. Species identification of *Acinetobacter* spp.

Average-nucleotide identification was performed for the genomes of all 6,639 strains (Jain et al., 2018; Supplementary Table S1), and 95% was set as the cutoff value for species identification. Briefly, the genome sequences of type strains for *Acinetobacter* were obtained based on the NCBI type strain list. FastANI version 1.3 (Jain et al., 2018) was used to calculate the ANI values with all 6,639 genomes were as query, and all genomes of type strains belonging to this genus as reference. When the ANI value between a query genome and the type strain genome was greater than or equal to 95%, this query genome were given the same species name with the type strain. When the ANI value was less than 95%, this query genome was treated as *Acinetobacter* spp.

2.5. Analysis of the sequence types of *Acinetobacter baumannii*

A self-made Perl program was used to extract the nucleotide coding sequence of the gene from each *A. baumannii* genome sequence file (GBK format). Concurrently, seven allele sequences sequences and the MLST (Pasteur) profiles of *A. baumannii* were downloaded.[3] The sequence type (ST) for each genome was determined as follows: (1) all genes for each genome were searched against the housekeeping gene sequences via BLASTN, and the blast results were filtered with stringent criteria (*E*-value = $1e^{-5}$, identity = 100%, and coverage = 100%) to obtain the seven conserved gene-type profiles; (2) this conserved gene-type profiles in each genome were compared with the MLST profiles to determine the ST for each genome.

2.6. Strain meta information acquisition

Strain meta information including isolation country, and date, host, and source, etc. was extracted from the downloaded gbk file in batches using perl script. Species identification, OXA distribution as well as STs were integrated in an excel for the further analysis.

2.7. Statistical analysis

SPSS software was used for statistical analysis, and chi-square test was used to compare the difference in distribution of *bla*OXA between *A. baumannii* and non-*A. baumannii Acinetobacter* spp.; $p < 0.05$ was taken as significant.

3 https://pubmlst.org/

3. Results

3.1. Distribution of *bla*OXA across global *Acinetobacter* spp.

Out of the 6,639 *Acinetobacter* spp., 5,893 (88.8%) strains were found to carry 9,581 *bla*OXAs, which were assigned to 282 *bla*OXA variants (Figure 1), with *bla*OXA-23 ($n = 3,168$, 47.7%) and *bla*OXA-66 ($n = 2,630$, 39.6%) being the most predominant *bla*OXAs. In addition, *bla*OXA-82 ($n = 571$, 8.6%), *bla*OXA-69 ($n = 302$, 4.5%), *bla*OXA-58 ($n = 202$, 3.0%), *bla*OXA-72 ($n = 192$, 2.9%), *bla*OXA-64 ($n = 175$, 2.6%), and *bla*OXA-65 ($n = 169$, 2.5%) were found to be common. Other *bla*OXAs were scattered, as shown in the Figure 1. Globally, the earliest enzyme identified was *bla*OXA-78 in 1943 in the United States. In 1980s, *bla*OXA-64 and *bla*OXA-69 were the main OXA variants, whereas *bla*OXA-214, *bla*OXA-235, *bla*OXA-500, and *bla*OXA-506 emerged successively in 1990s. Each *Acinetobacter* spp. seemed to have contained only one *bla*OXA before 1996, and subsequently co-existence of two *bla*OXA variants appeared. Since the identification of the first combination of *bla*OXA-23 and *bla*OXA-66 in *A. baumannii* in Singapore in 1996, it has been increasingly prevalent each year (Figure 2; Supplementary Table S2). The combinations of *bla*OXA variants detected were diverse, and distributed all over the world; a combination of three distinct *bla*OXA variants appeared in the cerebrospinal fluid of an inpatient in Italy in 2005.

Functionally, the 282 *bla*OXA variants were assigned to three classes, 239 being carbapenem-hydrolyzing-class-D-β-lactamase (Table 1), and nine belonging to oxacillin-hydrolyzing-class-D-β-lactamase (Table 2); whether the remaining 34 *bla*OXA variants possessed carbapenem-hydrolyzing activity would require further analysis (Table 3).

3.2. Phylogenetic tree of *bla*OXA variants across *Acinetobacter* spp.

Phylogenetically, the *bla*OXA variants within *A. baumannii* were divided into 27 distantly related clusters, according to the phylogenetic tree constructed based on SNPs (Figure 1). Among the 27 clades, 11 (including 239 *bla*OXA variants) belonged to carbapenem-hydrolyzing-class-D-β-lactamase (Figure 1; Table 1). Six clades, including nine *bla*OXA variants, belonged to oxacillin-hydrolyzing-class-D-β-lactamase (Figure 1; Table 2), and 10, including 34 *bla*OXA variants, were unknown with regard to antibiotic-hydrolyzing activity (Figure 1; Table 3). It was noteworthy that seven clades consisted of single *bla*OXA gene, namely *bla*OXA-9, *bla*OXA-50, *bla*OXA-232, *bla*OXA-568, *bla*OXA-542, *bla*OXA-308, and *bla*OXA-299. Moreover, two clades were unnamed, one group consisting of *bla*OXA-296 and *bla*OXA-666, and another group consisting of *bla*OXA-294, *bla*OXA-295, *bla*OXA-297, and *bla*OXA-298 (Table 2).

3.3. Species assignment of *Acinetobacter* spp.

Average-nucleotide identification calculations based on BLAST+ (ANIb) analysis (Richter et al., 2016) showed that 6,417 out of 6,639 *Acinetobacter* spp. belonged to 70 species while the remaining 222

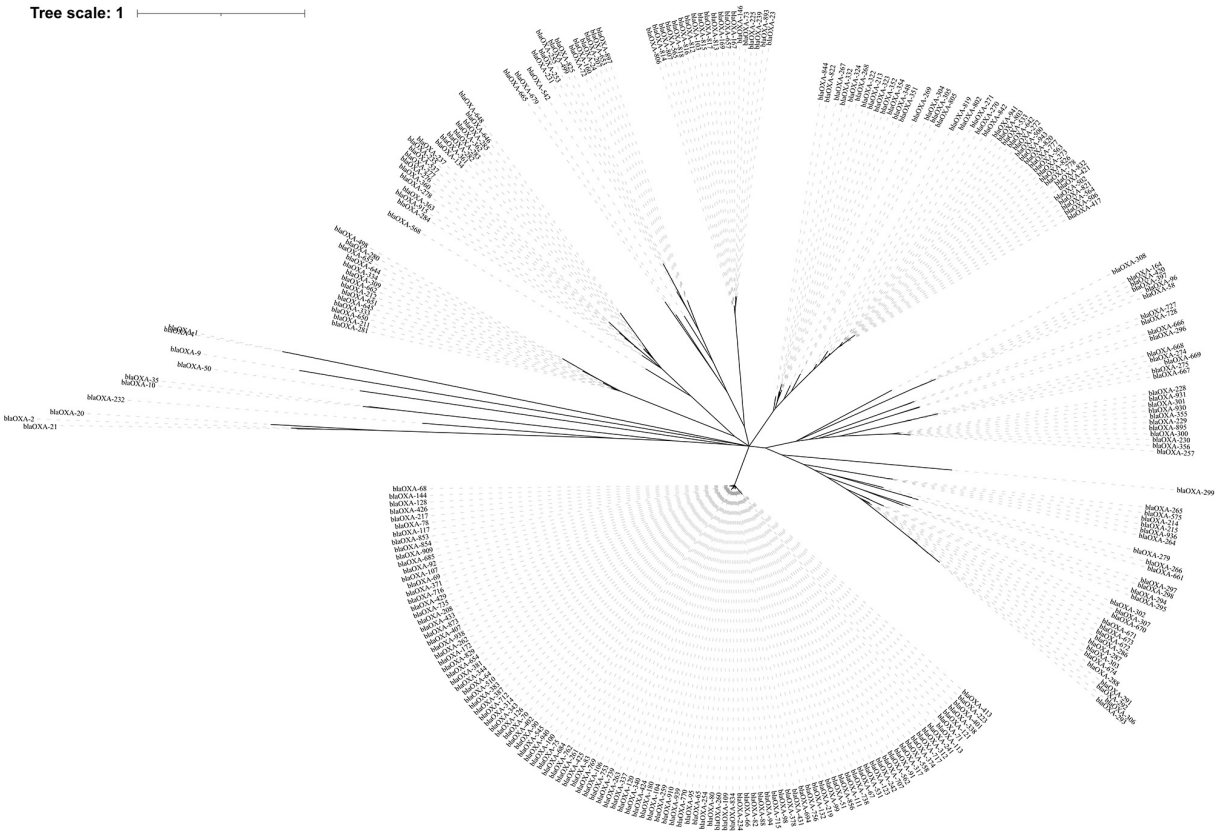

FIGURE 1
The phylogenetic tree of 282 *bla*OXA variants detected across global *Acinetobacter* spp. The phylogenetic tree was constructed using MUSCLE and RAxML software, and finally visualized with iTOL software. The clades were calculated based on the function of OXA variants.

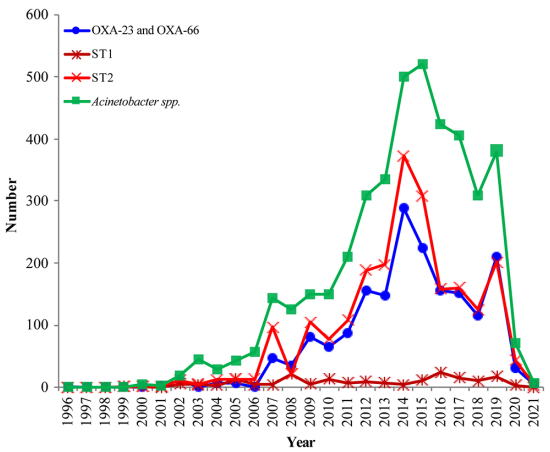

FIGURE 2
The prevalence of OXA-23 and OXA-66 combination in global *Acinetobacter baumannii* (The blue line); The distribution of OXA-producing *Acinetobacter baumannii* ST1 and ST2 during 1999–2021 (The dark red line and light red line); Analysis of the submitted and released genomes of *Acinetobacter* spp. per year (The green line).

strains could not be identified due to limitation in typing strains (Supplementary Table S1). Furthermore, the 5,893 *bla*OXA-carrying *Acinetobacter* spp. were assigned to 52 species, indicating that *bla*OXA was not identified in at least 18 *Acinetobacter* species that included *A. baretiae* (*n* = 2), *A. baylyi* (*n* = 11), *A. brisouii* (*n* = 4), *A. celticus* (*n* = 1), *A. equi* (*n* = 1), *A. guerrae* (*n* = 3), *A. harbinensis* (*n* = 2), *A. lanii* (*n* = 2), *A. larvae* (*n* = 1), *A. nectaris* (*n* = 1), *A. pollinis* (*n* = 4), *A. rathckeae* (*n* = 1), *A. soli* (*n* = 35), *A. terrae* (*n* = 8), *A. terrestris* (*n* = 5), *A. tjernbergiae* (*n* = 2), *A. ursingii* (*n* = 56), and *A. wanghuae* (*n* = 2). Further, we observed that the distribution of *bla*OXAs across *Acinetobacter* species was different, the most common species being *A. baumannii* (*n* = 4,904) followed by *A. pittii* (*n* = 299), which was then followed by *A. bereziniae* (*n* = 47), *A. haemolyticus* (*n* = 39), *A. johnsonii* (*n* = 40), *A. lwoffii* (*n* = 37), *A. oleivorans* (*n* = 34), and *A. radioresistens* (*n* = 43; Table 4). Other species were quite rare, as shown in Supplementary Table S1. Notably, 174 *bla*OXA-carrying *Acinetobacter* spp. could not be assigned to specific species due to the limited type strains in GenBank.

It would be worth noting that the most prevalent *bla*OXA variants were different across the *Acinetobacter* species (Table 4). For example, *bla*OXA-23 and *bla*OXA-66 were the predominant *bla*OXA variants in *A. baumannii*, whereas *bla*OXA-500 and *bla*OXA-506 were the dominant ones in *A. pittii*. Of note, more than two *bla*OXA variants were identified across 3,615 strains, and 2,223 were found to simultaneously carry *bla*OXA-23 and *bla*OXA-66; co-carriage of *bla*OXA-23 and *bla*OXA-66 was only found in *A. baumannii*. The other difference was that *bla*OXA-66 was only identified in *A. baumannii*, whereas *bla*OXA-23 was not only detected in *A. baumannii* (*n* = 3,129), but also in *A. cumulans*

TABLE 1 The carbapenem-hydrolyzing-class-D-β-lactamase with *Acinetobacter spp*.

Enzyme group	Number	Enzyme (s)	Host species
OXA-23-family	20	OXA-23, OXA-73, OXA-103, OXA-146, OXA-167, OXA-169, OXA-225, OXA-239, OXA-565, OXA-657, OXA-806, OXA-807, OXA-812, OXA-813, OXA-814, OXA-815, OXA-816, OXA-817, OXA-818, and OXA-893	*A. baumannii*, *A. seifertii*, *A. cumulans*, *A. wuhouensis*, *A. nosocomialis*, *A. indicus*, *A. radioresistens*, *A. pittii*, *A. wuhouensis*, *A. cumulans*, *A. johnsonii*, *A. seifertii*, and *A. gandensis*
OXA-24-family	6	OXA-24, OXA-72, OXA-160, OXA-207, OXA-653, and OXA-897	*A. baumannii, A. pittii*, and *A. wuhouensis*
OXA-48-family	1	OXA-232	*A. baumannii*
OXA-51-family	108	OXA-51, OXA-64, OXA-65, OXA-71, OXA-75, OXA-78, OXA-80, OXA-82, OXA-83, OXA-88, OXA-90, OXA-92, OXA-94, OXA-95, OXA-98, OXA-99, OXA-100, OXA-104, OXA-106, OXA-107, OXA-109, OXA-111, OXA-113, OXA-117, OXA-120, OXA-121, OXA-123, OXA-126, OXA-128, OXA-132, OXA-144, OXA-172, OXA-180, OXA-208, OXA-217, OXA-219, OXA-223, OXA-241, OXA-242, OXA-254, OXA-259, OXA-260, OXA-263, OXA-312, OXA-314, OXA-317, OXA-337, OXA-338, OXA-340, OXA-343, OXA-344, OXA-371, OXA-374, OXA-378, OXA-381, OXA-383, OXA-387, OXA-401, OXA-402, OXA-407, OXA-413, OXA-424, OXA-426, OXA-429, OXA-431, OXA-433, OXA-510, OXA-531, OXA-545, OXA-558, OXA-562, OXA-654, OXA-684, OXA-685, OXA-694, OXA-707, OXA-712, OXA-715, OXA-717, OXA-735, OXA-738, OXA-739, OXA-753, OXA-756, OXA-762, OXA-769, OXA-770, OXA-829, OXA-834, OXA-853, OXA-854, OXA-856, OXA-873, OXA-909, OXA-910, OXA-938, OXA-939, and OXA-940	*A. baumannii*, *A. johnsonii*
OXA-58-family	5	OXA-58, OXA-96, OXA-164, OXA-397, and OXA-420	*A. baumannii*, *A. colistiniresistens*, *A. johnsonii*, *A. cumulans*, *A. seifertii*, *A. wuhouensis*, *A. bereziniae*, *A. haemolyticus*, *A. rongchengensis*, *A. towneri*, *A. lwoffii*, *A. pittii*, *A. sichuanensis*, *A. defluvii*, *A. chinensis*, *A. variabilis*, *A. nosocomialis*, *A. modestus*, *A. junii*, *A. indicus*, *A. chengduensis*, and *A. tianfuensis*
OXA-134-family	18	OXA-134, OXA-235, OXA-237, OXA-276, OXA-278, OXA-282, OXA-285, OXA-360, OXA-363, OXA-537, OXA-646, OXA-648, and OXA-915	*A. lwoffii*, *A. baumannii*, *A. schindleri*, *Acinetobacter spp*., *A. lwoffii*, and *A. pseudolwoffii*
OXA-143-family	5	OXA-231, OXA-253, OXA-255, OXA-499, and OXA-825	*A. baumannii*, *A. pittii*
OXA-211-family	14	OXA-211, OXA-212, OXA-280, OXA-281, OXA-309, OXA-333, OXA-334, OXA-498, OXA-644, OXA-645, OXA-650, OXA-652, and OXA-662	*A. johnsonii*, *A. towneri*, *A. cumulans*, and *Acinetobacter spp*.
OXA-213-family	42	OXA-213, OXA-267, OXA-273, OXA-304, OXA-305, OXA-322, OXA-324, OXA-332, OXA-348, OXA-351, OXA-352, OXA-354, OXA-417, OXA-421, OXA-500, OXA-502, OXA-506, OXA-533, OXA-563, OXA-564, OXA-642, OXA-777, OXA-778, OXA-802, OXA-803, OXA-805, OXA-819, OXA-822, OXA-826, OXA-832, OXA-842, OXA-844, OXA-941, and OXA-943	*A. calcoaceticus*, *Acinetobacter spp*., *A. lactucae*, *A. pittii*, *A. oleivorans*, *A. geminorum*, and *A. vivianii*,
OXA-214-family	6	OXA-214, OXA-215, OXA-264, OXA-265, OXA-575, and OXA-936	*A. haemolyticus*
OXA-229-family	11	OXA-228, OXA-230, OXA-257, OXA-300, OXA-301, OXA-355, OXA-356, OXA-895, OXA-930, and OXA-931	*A. bereziniae*, *A. bereziniae*, *A. shaoyimingii*, *A. piscicola*, *A. wuhouensis*, *A. rongchengensis*, and *Acinetobacter spp*.
Other carbapenem-hydrolyzing-class-D-β-lactamase	3	OXA-542, OXA-665, and OXA-666	*A. oleivorans*, *A. pittii*, *A. rudis*, *A. albensis*, *A. pullicarnis*, *A. terrestris*, and *Acinetobacter spp*.

(*n* = 2), *A. gandensis* (*n* = 2), *A. indicus* (*n* = 2), *A. johnsonii* (*n* = 1), *A. nosocomialis* (*n* = 4), *A. pittii* (*n* = 6), *A. radioresistens* (*n* = 10), *A. seifertii* (*n* = 3), and *A. wuhouensis* (*n* = 1).

Albeit there was a wide distribution of *bla*OXAs across *A. bereziniae*, *A. haemolyticus*, *A. johnsonii*, *A. lwoffii*, *A. oleivorans*, *A. pittii*, *A. radioresistens*, and *A. baumannii* (Table 4), in general, the prevalence of *bla*OXAs among *A. baumannii* (4,904/4,923, 99.6%) was significantly higher than that of *bla*OXAs among *non-A. baumannii Acinetobacter* spp. (989/1716, 57.6%, $p = 0.000$).

The distribution of carbapenem-hydrolyzing-class-D-β-lactamases showed that *bla*OXA-51 family, the biggest clade, was only found in *A. baumannii* and *A. johnsonii*. Other carbapenemases, including *bla*OXA-134, *bla*OXA-211, *bla*OXA-213, *bla*OXA-214, and *bla*OXA-229 families, as well as other enzymes not assigned into a specific family were not found in *A. baumannii*.

3.4. Sequence types of *bla*OXA-carrying *Acinetobacter baumannii*

A total of 291 distinct STs were identified for 4,904 *bla*OXA-carrying *A. baumannii*. The most identified ST was ST2 (*n* = 3,023), which was classified as clonal complex 2 (CC2), presenting a global distribution trend. ST1 (*n* = 228; clonal complex CC1) and ST25 (*n* = 132), which were the second most common, corresponded to CC2/92 (Pasteur/Oxford scheme; Figure 3A), other distinct STs (ST79, ST78, and ST10) were relatively less common (Figure 3A). The STs of 29 strains were novel (the profile of MLST gene was not assigned into specific ST), and the STs of 125 strains remained unknown (the STs could not be determined since some genes did not match the known MLST loci).

The earliest detected clone in *A. baumannii* was ST2 collected in Rotterdam, Netherlands, in 1982 from *Homo sapiens*, which was further found in 1996 in Singapore. The clonal ST2 was continuously and increasingly detected every year all over the world since its first detection in Beijing, China, in 1999 (Figure 2, The light red line). Additionally, ST1 was consecutively collected almost each year, except in 1985, 1995, and 2001 (Figure 2, The dark red line); however, the number was quite less compared to that of ST2 (Figure 2, The light red line).

Ninety-two distinct STs were identified for *bla*OXA-23-carrying strains, with ST2 (*n* = 2,429) being the most prevalent clone, followed by ST1 (*n* = 154). Meanwhile, 25 different STs were found for 2,663 *bla*OXA-66-carrying *A. baumannii* with ST2 (*n* = 2,425) being the most common. Notably, 2081 *bla*OXA-23 and *bla*OXA-66 co-carrying *A. baumannii* belonged to ST2 while the other 22 STs were also identified.

Obvious differences were also observed among the major STs. *bla*OXA-23 (*n* = 2,429) and *bla*OXA-66 (*n* = 2,374) were the main *bla*OXAs within ST2, whereas *bla*OXA-69 (*n* = 217) and *bla*OXA-23 (*n* = 154) were the predominant *bla*OXAs among ST1, and *bla*OXA-64 (*n* = 128) was the dominant *bla*OXA among ST25 clones (Figures 3B–D).

3.5. Temporal and geographical distribution of global *bla*OXA-carrying *Acinetobacter* spp.

The earliest OXA-producing *Acinetobacter* spp. could be dated back to 1943, and was carried by an *A. baumannii* from *Parthenium argentatum* Gray (guayule shrubs) in the

TABLE 2 The oxacillin-hydrolyzing class D-β-lactamase in *Acinetobacter spp*.

Enzyme groups	Number	Enzyme(s)	*Acinetobacter* species
OXA-1-family	2	OXA-1, OXA-4	*A. colistiniresistens*, *A. venetianus*
OXA-2-family	2	OXA-2, OXA-21	*A. baumannii*, *A. nosocomialis*, and *A. pittii*
OXA-10-family	2	OXA-10, OXA-35	*A. pittii*, *A. baumannii*, and *A. nosocomialis*
Other oxacillinase	3	OXA-9, OXA-20, and OXA-50	*A. baumannii*, *Acinetobacter spp.*

TABLE 3 Class-D-β-lactamase could not be assigned into detailed classification in *Acinetobacter* spp.

Enzyme groups	Number	Enzyme(s)	*Acinetobacter* species
OXA-266-family	2	OXA-266, OXA-661	*A. venetianus*
OXA-274-family	5	OXA-274, OXA-275, OXA-667, and OXA-669	*A. guillouiae*, *A. kanungonis*, *A. guillouiae*, *A. stercoris*, *Acinetobacter spp.*, and *A. tandoii*
OXA-286-family	15	OXA-286, OXA-288, OXA-291, OXA-293, OXA-302, OXA-303, OXA-306, OXA-307, OXA-670, and OXA-674	*A. proteolyticus*, *A. colistiniresistens*, and *Acinetobacter spp.*
OXA-667-family	1	OXA-679	*A. courvalinii*, *Acinetobacter spp.*
OXA-727-family	2	OXA-727, OXA-728	*A. chinensis*, *A. defluvii*, *A. gandensis*, and *Acinetobacter spp.*
Others	9	OXA-279, OXA-294, OXA-299, OXA-308, and OXA-568	*A. halotolerans*, *A. parvus*, *Acinetobacter spp.*, *A. populi*, *A. vivianii*, *A. albensis*, *A. bohemicus*, *A. courvalinii*, *A. pragensis*, *A. bouvetii*, and *A. gerneri*

TABLE 4 The distribution of major OXA-variants among predominant *Acinetobacter* spp.

Strains	Number of OXA-carrying Strains (%)	Number of OXA-variants	The most prevalent OXA-variants		
A. baumannii	4,904/4,923 (99.6%)	136	OXA-23	OXA-66	OXA-82
			(*n* = 3,129)	(*n* = 2,621)	(*n* = 790)
A. pittii	299/305 (98.0%)	36	OXA-500	OXA-506	OXA-421
			(*n* = 146)	(*n* = 27)	(*n* = 25)
A. bereziniae	47/47 (100%)	11	OXA-355	OXA-301	OXA-301/356
			(*n* = 14)	(*n* = 11)	(*n* = 4)
A. johnsonii	40/42 (95.2%)	20	OXA-281	OXA-644	OXA-58
			(*n* = 7)	(*n* = 7)	(*n* = 6)
A. haemolyticus	39/39 (100%)	7	OXA-264	OXA-214	OXA-265
			(*n* = 13)	(*n* = 11)	(*n* = 8)
A. lwoffii	37/40 (92.5%)	12	OXA-283	OXA-282	OXA-362
			(*n* = 15)	(*n* = 7)	(*n* = 3)
A. oleivorans	34/35 (97.1%)	4	OXA-304	OXA-305	OXA-805
			(*n* = 19)	(*n* = 13)	(*n* = 2)
A. indicus	23/130 (17.7%)	4	OXA-58	OXA-146	OXA-23
			(*n* = 14)	(*n* = 6)	(*n* = 2)
A. calcoaceticus	22/22 (100%)	10	OXA-332	OXA-213	OXA-268/351
			(*n* = 8)	(*n* = 4)	(*n* = 2)
A. nosocomialis	16/217 (7.4%)	7	OXA-58	OXA-96	OXA-23
			(*n* = 7)	(*n* = 3)	(*n* = 3)
A. radioresistens	43/44 (97.7%)	11	OXA-23	OXA-816	OXA-813
			(*n* = 10)	(*n* = 7)	(*n* = 5)
A. schindleri	21/21 (100%)	8	OXA-360	OXA-537	OXA-276/277
			(*n* = 7)	(*n* = 3)	(*n* = 3)
Acinetobacter spp.	174/222 (78.4%)	46	OXA-728	OXA-58	OXA-727
			(*n* = 54)	(*n* = 23)	(*n* = 19)

United States. The *bla*OXA-producing *Acinetobacter* spp. was intermittently isolated over the following years: 1948 (*n* = 2), 1951 (*n* = 1), 1953 (*n* = 1), 1982 (*n* = 2), 1984 (*n* = 1), 1985 (*n* = 1), and 1986 (*n* = 1), and was found to continuously increase during 1991 to 2021, although the isolation date for 1,585 strains still remains unknown (Figure 2). Overall, the strains were collected from 79 countries (Figure 2; Supplementary Table S3), of which, United States (*n* = 1,315) and China (*n* = 1,191) submitted the most number of strains, followed by Germany (*n* = 205), India (*n* = 279), South Korea (*n* = 145), Brazil (*n* = 134), and France (*n* = 101; Figure 4; Supplementary Table S3). Unfortunately, the countries from where the other 1,345 strains were collected remain unknown. Furthermore, *Homo sapiens* were the most common host. The *Acinetobacter* spp. isolated only from non-*Homo sapiens* were *A. chengduensis*, *A. chinensis*, *A. cumulans*, *A. gandensis*, *A. kanungonis*, *A. modestus*, *A. piscicola*, *A. populi*, *A. pragensis*, *A. pseudolwoffii*, *A. rongchengensis*, *A. pullicarnis*, *A. shaoyimingii*, *A. sichuanensis*, *A. terrestris*, *A. tianfuensis*, *A. towneri*, *A. variabilis*, and *A. wuhouensis*.

The distribution of *bla*OXAs displayed significant regional differences. *bla*OXA-23 and *bla*OXA-66, in combination, were the main *bla*OXAs in Asian regions, such as China, South Korea, Singapore, Pakistan, Malaysia, Lebanon, Kuwait, Thailand, and India. However, *bla*OXA-23 and *bla*OXA-82 were the predominant ones in United States.

4. Discussion

In this study, we analyzed the data available on the distribution of *bla*OXAs across the genomes of the *Acinetobacter* spp. available in the GenBank, and the evolutionary relationship among the *bla*OXA variants based on the global genomes of *Acinetobacter* spp. Furthermore, the relationship between *bla*OXA variants and STs in *A. baumannii* was analyzed.

First, consistent with the previous report, we found a wide distribution of diverse OXA-type β-lactamases across the *Acinetobacter* spp., especially in *A. baumannii*, indicating that

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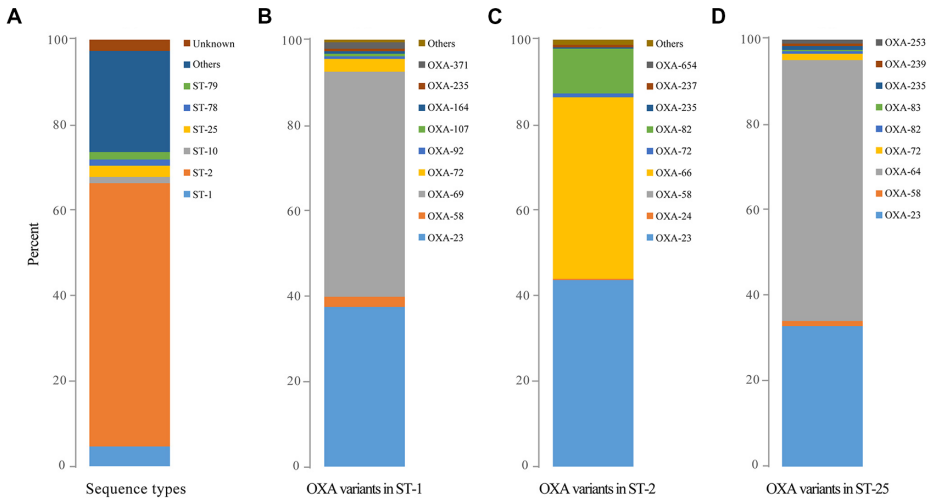

FIGURE 3
The predominant sequence types of global OXA-producing *Acinetobacter baumannii* in 1996, 1999, and during 2001–2021 as well as the distribution of OXA variants among the three dominant clones of *Acinetobacter baumannii*. **(A)** The predominant sequence types of global OXA-producing *Acinetobacter baumannii* in 1996, 1999, and during 2001–2021; **(B)** The distribution of OXA variants among *Acinetobacter baumannii* ST1; **(C)** The distribution of OXA variants among *Acinetobacter baumannii* ST2; and **(D)** The distribution of OXA variants among *Acinetobacter baumannii* ST25.

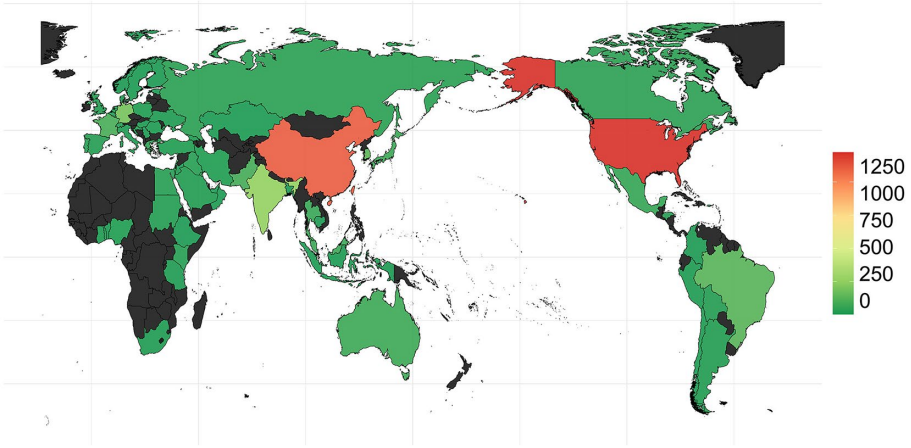

FIGURE 4
Submitted and released genomes of *Acinetobacter* spp. by different countries worldwide.

A. baumannii might be the main host of *bla*OXA genes. It was noteworthy that most of the OXA-type β-lactamase identified in our study belonged to carbapenem-hydrolyzing-β-lactamase, leading to high resistance of *Acinetobacter* spp. to carbapenem in different degrees (Boral et al., 2019; Tamma et al., 2022). Evidently, the wide distribution of *bla*OXA-23 and *bla*OXA-66 across global *A. baumannii*, in our study, suggested that they may be the main enzyme mediating carbapenem resistance, since the expression of *bla*OXA-23 within the *A. baumannii* strain was enough to confer resistance to carbapenems (Evans and Amyes, 2014); however, a much higher turnover rate was observed for imipenem than for meropenem, ertapenem, or doripenem for *bla*OXA-23 (Smith et al., 2013). In addition, a recent study showed that hyperexpression of *bla*OXA-23 β-Lactamase in *A. baumannii* drives significant collateral changes with increased amidase activity, resulting in peptidoglycan integrity and new genetic vulnerabilities (Colquhoun et al., 2021), which may represent novel targets for antimicrobial agents. *bla*OXA-66 is well known as a chromosomally encoded *bla*OXA-51-like β-lactamase, and the most prevalent CHβLs in *A. baumannii* worldwide (Hu et al., 2007). Therefore, the wide co-occurrence of *bla*OXA-23 and *bla*OXA-66 in combination with other diverse carbapenem-hydrolyzing enzymes within *A. baumannii* provides enough explanation for the failure of β-lactam in clinical therapy.

Currently, many studies have shown frequent co-carriage of *bla*OXA-23 and *bla*OXA-66 among clinical *A. baumannii*, with high resistance rates (Al-Hassan et al., 2021; Zhang et al., 2021), and the continuously rising co-prevalence of *bla*OXA-23 and *bla*OXA-66 over the years indicates the importance of the two variants for high resistance of *A. baumannii* to carbapenem.

In our study, *bla*OXA-78 (OXA-51 family) was the earliest *bla*OXA in *A. baumannii* isolated in 1943 in United States. However, searching *bla*OXA-78 as a keyword in PubMed, it was first reported in a clinical multi-drug resistant *A. baumannii* from a hospitalized patient in a major hospital in Kuwait in 2015 (Vali et al., 2015). As is well known, carbapenem was approved for clinical use in 1980s, indicating that class-D-CHßLs had already existed in *A. baumannii* before carbapenem usage. Analysis from our study showed that *bla*OXA-51 family, including *bla*OXA-66, *bla*OXA-69, and *bla*OXA-98, appeared in bacterial genomes submitted in the 1980s, and OXA-213 family, including *bla*OXA-500, *bla*OXA-506, and *bla*OXA-417, emerged in strains collected in the 1990s, although the *bla*OXAs were first reported in 2007 (Zhou et al., 2007), and *bla*OXA-69 in 2005 (Héritier et al., 2005), *bla*OXA-417 in 2014 (D'Souza et al., 2019), and *bla*OXA-500 in 2019 (Sun et al., 2014), indicating the presence of these *bla*OXA before the use of carbapenems. Whether there was an association between the evolution of class D CHβLs and the carbapenem use would require further investigation. Importantly, all the *bla*OXA-48-like CHβLs, were quite rare, except *bla*OXA-232, which was quite popular in *Enterobacterales* (Pitout et al., 2019). This could be due to the good fitness between *bla*OXA-48 and *Enterobacterales*, especially *Klebsiella pneumoniae*.

In our study, the clades of *bla*OXA variants were in accordance with the subgroups categorized by enzyme function, from an overall perspective, and *bla*OXA-51 family included the most members. However, not all the *A. baumannii* isolates contained *bla*OXA-51-family genes, providing evidence that this gene was not omnipresent in the species, but rather distributed in subpopulations of *A. baumannii* (Walther-Rasmussen and Høiby, 2006). Nevertheless, the members of this family were reported to diverge by amino acid modifications in *A. baumannii* (Brown and Amyes, 2005; Héritier et al., 2005), contributing to intrinsic resistance to imipenem (Hu et al., 2007). In addition, *bla*OXA family appears to be evolving quite quickly in recent years as supported by the presence of 282 *bla*OXA variants across all *Acinetobacter* spp. globally, along with more yet-unknown variants, since most of the recently submitted genomes had not been released when the related genomes were downloaded for analysis.

We found quite a wide distribution of ST2, most of them co-carrying *bla*OXA-23 and *bla*OXA-66, indicating an international clonal dissemination of the strains, mainly among *Homo sapiens* in health care centers in 38 countries and six continents across the world, posing a serious threat to global public health. As the clone secondary to ST2, although ST1 clone only accounted for little part of the strains, we not only found an increasing trend of ST1 clone every year, but also a wide distribution across 30 countries, mainly Brazil, United States, and Australia. Interestingly, ST1 was not found in isolates from China. The other multiple STs identified in our study indicated the diversity of *bla*OXA-carrying *A. baumannii* strains.

According to the current WGS identification and typing methods for *Acinetobacter* spp., at least 70 members of this genus were identified. It would be worth mentioning that the naming of at least 27 existing genomes was wrong. For example, "*A. colistiniresistens*" was mistaken as "*A. baumannii*," indicating that most *Acinetobacter* species were misidentified previously, and the underlying reason could be that NCBI started ANI verification-taxonomy nomenclature around 2016, and there was no ANI verification for previously submitted genomes. Moreover, the genomes of typing strains submitted have been increasing, which will also affect their taxonomy verification. If there is no obvious error, the submitted genome with spp. may pass automatically.

There were some limitations in our current study. First, some strains may have been excluded from the analysis, since only the *A. baumannii* strains, whose genomes were submitted to GenBank and released, were analyzed, even though the analysis was based on global data. Second, the resistant profiles of these strains were missing; we could not check the accordance between the phenotype and genotypes of these strains. Third, all the results were acquired based on WGS, and the strains were not available for further confirmation by molecular methods.

In summary, class D β-lactamase *bla*OXA variants in *Acinetobacter* spp. have been rapidly evolving, with CHβLs being the most predominate class D β-lactamase, widely distributed within *Acinetobacter* spp. *bla*OXA-23- and *bla*OXA-66-co-carrying *A. baumannii* ST2 is a predominant international high-risk clone spreading globally that poses potential threat to global public health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JL, YL, and XC performed the bioinformatics analysis and writing. JZ and YZ sorted the data and helped with the writing. HX interpreted the data regarding resistance determinants and plasmid replicons. CLi performed statistical analysis. CLiu and HS designed the work and were major contributors in revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1174200/full#supplementary-material

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Epidemiology and zoonotic transmission of *mcr*-positive and carbapenemase-producing Enterobacterales on German turkey farms

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Introduction: The emergence of carbapenem-resistant bacteria causing serious infections may lead to more frequent use of previously abandoned antibiotics like colistin. However, mobile colistin resistance genes (*mcr*) can jeopardise its effectiveness in both human and veterinary medicine. In Germany, turkeys have been identified as the food-producing animal most likely to harbour *mcr*-positive colistin-resistant Enterobacterales (*mcr*-Col-E). Therefore, the aim of the present study was to assess the prevalence of both *mcr*-Col-E and carbapenemase-producing Enterobacterales (CPE) in German turkey herds and humans in contact with these herds.

Methods: In 2018 and 2019, 175 environmental (boot swabs of turkey faeces) and 46 human stool samples were analysed using a combination of enrichment-based culture, PCR, core genome multilocus sequence typing (cgMLST) and plasmid typing.

Results: *mcr*-Col-E were detected in 123 of the 175 turkey farms in this study (70.3%). *mcr*-Col-E isolates were *Escherichia coli* (98.4%) and *Klebsiella* spp. (1.6%). Herds that had been treated with colistin were more likely to harbour *mcr*-Col-E, with 82.2% compared to 66.2% in untreated herds ($p = 0.0298$). Prevalence also depended on husbandry, with 7.1% *mcr*-Col-E in organic farms compared to 74.5% in conventional ones ($p < 0.001$). In addition, four of the 46 (8.7%) human participants were colonised with *mcr*-Col-E. *mcr*-Col-E isolates from stables had minimum inhibitory concentrations (MICs) from 4 to ≥ 32 mg/l, human isolates ranged from 4 to 8 mg/l. cgMLST showed no clonal transmission of isolates. For one farm, plasmid typing revealed great similarities between plasmids from an environmental and a human sample. No CPE were found in turkey herds or humans.

Discussion: These findings confirm that *mcr*-Col-E-prevalence is high in turkey farms, but no evidence of direct zoonotic transmission of clonal *mcr*-Col-E strains was found. However, the results indicate that plasmids may be transmitted between *E. coli* isolates from animals and humans.

KEYWORDS

colistin resistance (*mcr*), zoonosis, one health, poultry, farmers, personnel, staff

Introduction

Infections with multidrug-resistant bacteria are a major health burden in both human and veterinary medicine. Selective pressure from antimicrobial use in human and animal populations fuels the development of antibiotic resistance (European Food Safety Authority, and European Centre for Disease Prevention and Control, 2022).

The incidence of human infections caused by multidrug-resistant organisms, particularly carbapenem-resistant bacteria such as *Klebsiella pneumoniae* and *Acinetobacter baumannii*, is increasing in Germany (Robert Koch-Institut, 2022). This has prompted a need for alternative therapeutic options. Consequently, the antibiotic colistin has been reintroduced as a last resort treatment option for infections caused by carbapenem-resistant bacteria (Falagas and Kasiakou, 2005). In Germany, systemic colistin therapy was (re-) approved in 2012, in spite of severe adverse effects such as neurotoxicity and nephrotoxicity (Yahav et al., 2012). In 2016, the WHO listed colistin as one of the critically important antimicrobial drugs for human therapy in accordance with the following criteria: colistin is one of a limited number of treatment options in serious bacterial infections, and the bacteria, as well as their resistance determinants, can be transmitted to humans from non-human sources (World Health Organization, 2005). For the same reasons, carbapenems have been prioritised in this group from the first draft of the report (World Health Organization, 2017).

Resistance to colistin is not a new phenomenon: bacteria such as *Proteus* spp. have long been known to be intrinsically resistant (Olaitan et al., 2014). While intrinsic resistance should certainly not be underestimated, the zoonotic transmission of bacteria and their resistance genes between humans and animals or vice versa has been a much greater One Health concern in recent years (Hernando-Amado et al., 2019). Such transmission has been shown to be likely for extended-spectrum β-lactamase (ESBL) producing (Fischer et al., 2017), carbapenemase-producing (CPE) (Elmonir et al., 2021), and colistin-resistant Enterobacterales (Col-E) (Nakano et al., 2021; Viñes et al., 2021).

The discovery of the plasmid-mediated *mcr-1* (mobile colistin resistance) gene has added a new dimension to colistin resistance of gram-negative bacteria both in livestock primary production and human medicine: such newly discovered plasmids could drastically accelerate zoonotic transmission (Liu et al., 2016). To date, 10 MCR-family genes and their variants (*mcr-1* to *mcr-10*) have been identified in various bacteria from humans, animals, food, farms and the environment (Hussein et al., 2021).

On the veterinary side, orally administered colistin is widely used to treat enteric infections in food-producing animals because of its lack of systemic resorption (Catry et al., 2015). In Germany, 42% of the colistin used for veterinary purposes is used for the treatment of poultry (Kietzmann et al., 2019). An increasing prevalence of colistin-resistant gram-negative bacteria in turkeys would be a particular cause for concern, as these animals are most often treated as a group rather than individually. This raises the risk of horizontal transmission of resistance traits among them. Indeed, among livestock and food, Col-E are most common in samples from turkeys (Irrgang et al., 2016; Alba et al., 2018; Zając et al., 2019).

Although carbapenems are not licensed for use in veterinary medicine, carbapenemase-producing bacteria are part of the panel investigated for the annual national monitoring programme in the European Union that covers the entire food chain, and CPE isolates have occasionally been identified in livestock (Fischer et al., 2012, 2013, 2017; Irrgang et al., 2017, 2020).

According to the German national as well as regional surveillance systems for antimicrobial resistance in clinical isolates from humans, resistance of *Escherichia coli* to imipenem or meropenem was <0.1% between 2008 and 2020. In the same period, resistance of *K. pneumoniae* to these drugs increased from <0.1 to 0.5 and 0.3%, respectively (Niedersächsisches Landesgesundheitsamt, 2021; Robert Koch-Institut, 2022). For human medicine, data on resistance to colistin in Germany are rare because Enterobacterales are not routinely tested.

At a methodological level, using conventional culture-based techniques focusing on commensal *E. coli* collected for screening purposes to detect colistin- and carbapenem-resistant Enterobacterales runs the risk of underestimating their prevalence. Enrichment followed by resistance gene pre-screening by PCR is far more sensitive (Irrgang et al., 2019) and sets the stage for sequencing approaches.

This study used this more sensitive combination of methods to investigate the prevalence of Col-E and CPE not only in German turkeys but also in their human contacts. Identical methods were applied to samples from animal faeces and human stool to compare isolates and their genomic profiles by means of microbiological, PCR-based and sequencing strategies.

Methods

Studied populations and sample collection

Between March 2018 and February 2019, local veterinary authorities visited 175 turkey farms in connection with the annual official zoonosis monitoring programme of the European Union based on Directive 2003/99/EC. The programme is designed to include a number of farms that allows statistically significant conclusions at a national level. This number is then divided among the German federal states depending on the percentage of animals kept in each state, making Lower Saxony the largest contributor for both farm- and slaughter-related data on turkeys. The farms in this study kept a minimum of 500 animals per fattening period. They were distributed in the northwestern part of Lower Saxony, Germany. As per the programme guidelines, sampling took place during the 3 weeks before the animals' transport to the slaughterhouse. The standard official sampling form already contained a field for the husbandry system. For the purpose of this study, the form was supplemented by additional tick boxes to record if and how many times the herd had been treated

with colistin. For sampling, two pairs of boot swabs were taken for each farm [i.e., boot swabs were put on the boots and the sample was taken by walking around in the poultry house, ensuring that all sections in a house were represented in a proportionate way (European Commission, 2012)]. The faecal material adherent to the boot swabs was then processed as described below.

During their visits to the farms, staff of the local veterinary authorities acquainted farmers, their employees and relatives with the study. They handed out test kits containing equipment for a self-sampling of stool swabs together with written and pictured instructions for sampling, as well as an informed consent form. In addition, participants were asked to fill in a questionnaire to analyse potential risk factors for colonisation with *mcr*-Col-E. Questions referred to the kind and duration of contact with poultry, contact with other livestock, consumption of antibiotics, hospitalisation, work in medical care and travel. Approval by the Ethics Committee of the University of Münster (No. 2018-008-f-S) had been obtained in advance.

Molecular and culture-based methods targeting *mcr*-positive colistin-resistant Enterobacterales (*mcr*-Col-E)

A harmonised protocol was used to analyse samples from both animal and human sources, although some minor adjustments were required to meet accreditation requirements. Any diagnostic modifications made for the human stool samples are indicated in square brackets as appropriate. For colistin susceptibility testing, faecal material from boot swabs and human stool samples was dissolved in 1–2 ml (depending on the amount of material) of buffered peptone water and mixed thoroughly. Of this mixture, 250 µl were added to 3 ml of buffered peptone water containing 2 mg/l colistin and incubated for 18–22 h at 37 ± 1°C [36 ± 1°C] aerobically. An aliquot of 200 µl was then used for DNA extraction before screening for *mcr-1* as well as *mcr-2* genes with a multiplex PCR method (Cavaco et al., 2016). Samples with positive or ambiguous PCR results were further analysed, streaking 10 µl [1 µl] on chromID® Colistin R (bioMérieux, Marcy-l'Etoile, France), Super Polymixin (ELITechGroup, Puteaux, France) and MacConkey agar (CLED agar, both BD, Franklin Lakes, NJ, United States), respectively. After incubation of the agar plates as described above, one colony of *mcr*-Col-E was selected at random and analysed with MALDI-TOF mass spectrometry (MS) (MALDI biotyper®, Bruker Daltonic, Bremen, Germany) [VITEK®2 Compact, bioMérieux] to confirm the species identification. The colony was then subcultured on Columbia blood agar with a disk containing 10 µg colistin to maintain selection pressure (Oxoid by Thermo Fischer Scientific, Wesel, Germany). To verify the supposed *mcr* gene(s) in the respective isolates, the PCR method indicated above was applied to all isolates, control strains (*mcr-1*: *E. coli* NCTC 13846, *mcr-2: E. coli* RKI 278/17) and negative control (water ad iniectabilia, Braun, Melsungen, Germany) respectively. During the course of the study, additional methods became available. Therefore, 141 isolates of animal origin that had not been analysed at this point were also subjected to a multiplex PCR for *mcr-1* to *mcr-5* (Rebelo et al., 2018). All isolates were stored at −80°C (CRYOBANK®, Mast, Bootle, United Kingdom).

Molecular and culture-based methods targeting CPE

The boot swabs were soaked in 450 ml of buffered peptone water and incubated as indicated above. Additionally, 10 µl were streaked onto ChromID® OXA-48 agar and chromID® CARBA agar (both bioMérieux), respectively. Incubation followed the instructions of the manufacturer.

Overnight cultures of boot swab and human faecal samples and aliquots of the stool suspensions were mixed with glycerol (1:3) and stored at −20°C until further processing. For genotypic detection of CPE, samples were thawed and 100 µl of each aliquot was transferred into a vial with 9 ml buffered peptone water (ReadyTube™ 9 bpW, Merck, Darmstadt, Germany) with 50 mg/l vancomycin and 0.25 mg/l ertapenem and incubated overnight at 37 ± 1°C. From each enrichment, 200 µl were transferred into a 1.5 ml reaction tube. Up to five samples were pooled in one tube and centrifuged at 13,400 rpm for 5 min. DNA was extracted from the pellets using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for 'Purification of total DNA from Animal Tissue.' DNA was stored at 4°C for <24 h or at −20°C for later use. Presence of carbapenemase genes bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP} and bla_{OXA-48} in the pooled samples was checked in an RT-PCR using the Check-Direct CPE Kit (Check-Points B.V., Wageningen, Netherlands). In case of a positive pool sample, individual samples were tested with conventional PCRs to confirm the result. DNA from individual samples was extracted as described above. A set of 5 PCRs was run for each sample using the primers indicated in Table 1. All PCRs were run using the following protocol: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 56°C for 30, 72°C for 1 min and a final elongation for 7 min at 72°C. PCR products were checked on gel (amplicon size see Table 1). Isolates from PCR-positive samples were cultured on ChromID® Carba and ChromID® OXA-48 (both bioMérieux) as well as MacConkey

TABLE 1 Primers and amplicon size for CPE-PCR analysis.

Gene	Forward primer	Reverse primer	Product length	Reference
bla_{NDM}	GGG CAG TCG CTT CCA ACG GT	GTA GTG CTC AGT GTC GGC AT	475 bp	Mushtaq et al. (2011)
bla_{KPC}	ATG TCA CTG TAT CGC CGT CT	TTT TCA GAG CCT TAC TGC CC	950 bp	Schechner et al. (2009)
bla_{VIM}	GAT GGT GTT TGG TCG CAT A	CGA ATG CGC AGC ACC AG	390 bp	Ellington et al. (2007)
bla_{IMP}	GGA ATA GAG TGG CTT AAY TCT C	CCA AAC YAC TAS GTT ATC T	188 bp	Ellington et al. (2007)
bla_{OXA-48}	TTG GTG GCA TCG ATT ATC GG	GAG CAC TTC TTT TGT GAT GGC	810 bp	Poirel et al. (2004)

plates. Species identification was done by MALDI-TOF MS (Microflex® LT, Bruker Daltonik).

Determination of antimicrobial susceptibility

For the determination of colistin minimum inhibitory concentrations (MICs), a commercially available kit (ComASP® Colistin, bestbion, Cologne, Germany) was used according to the manufacturer's instructions.

Molecular typing of isolates and plasmids

mcr-1-positive *E. coli* isolates from stool samples together with isolates from corresponding and additional randomly selected farms were used for molecular typing. As described above, any diagnostic modifications made for the human stool samples are indicated in square brackets as appropriate.

Swabs from each isolate were streaked onto Columbia agar plates containing 5% sheep blood (Oxoid by Thermo Fischer Scientific). Each agar plate was then equipped with a colistin disc (Oxoid by Thermo Fischer Scientific) and incubated at 37 ± 1°C for 24 h. After that, colonies nearest to the colistin disc were transferred into buffered peptone water containing 2 mg/l colistin and incubated at 37 ± 1°C for 24 h.

1.5 ml of these cultures were used for DNA extraction. Genomic DNA of *E. coli* isolates was extracted with the Maxwell® RSC Instrument (an automated nucleic acid purification platform) using the Maxwell® RSC Whole Blood DNA Kit (Promega, Walldorf, Germany) [smart DNA prep kit (Analytik Jena, Jena, Germany)] according to the manufacturer's instructions. Quantus™ fluorometer was used to measure the concentration of genomic DNA following the manufacturer's instructions (Promega, Walldorf, Germany).

Sequencing libraries were prepared using the Illumina® Nextera™ DNA Flex Library Preparation Kit and Nextera™ DNA CD Indexes (96 Indexes, 96 Samples) according to the manufacturer's instructions (Illumina Inc., San Diego, CA, United States). The normalised and pooled DNA libraries were loaded onto the flow cell for sequencing. Sequencing (2 × 250 bp paired-ends) [2 × 150 bp paired-ends] was performed on a MiSeq using the Illumina 500 [300] cycles V2 MiSeq reagent kit (Illumina Inc.).

For genotyping of isolates, raw sequence data were assembled with SKESA v2.3 and allelic profiles created using a task template based on 2,513 cgMLST targets of *E. coli* [based on core genome MLST scheme from EnteroBase[1] in Ridom SeqSphere+ version 8.2.0 (2021–12) (Ridom GmbH, Münster, Germany)]. A minimum spanning tree was created from these profiles using the 'Pairwise ignore missing values' option in SeqSphere+.

For analysis of plasmids, genomic DNA (gDNA) was extracted using the NEB Monarch® Genomic Purification Kit (New England Biolabs, Ipswich, MA, United States). Isolates were sequenced on a PacBio Sequel IIe system (Pacific Biosciences. Menlo Park, CA, United States) using a 20 kb insert size library and the SMRTbell® Express Template Prep Kit 2.0. Raw sequences were *de novo* assembled using the hierarchical genome assembly process (HGAP) and analysed using the SMRT®Link software suite v8 with default parameters for microbial assembly. Final assembly contigs were extracted in FASTA format.

The final assembly files were uploaded to ResFinder v3.2 (Zankari et al., 2012) to determine the contig containing the *mcr* gene. The extracted sequences of the respective contigs were uploaded to PlasmidFinder v2.1 (Carattoli et al., 2014) to predict the respective plasmid replicon type. Plasmid sequences were annotated using a local installation of the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v4.10 (Tatusova et al., 2016). Annotated sequences were aligned and gene content around the *mcr* locus was compared in Mauve v20150226 (Darling et al., 2004). Raw and assembled sequence data have been deposited under the NCBI BioSample number PRJNA934726.

Analysis and statistics

For analysis and visualisation of data, R (R Core Team, 2021), RStudio (RStudio Team, 2021) and the packages tidyverse (Wickham et al., 2019), here (Müller and Bryan, 2021), janitor (Firke, 2021), AMR (Berends et al., 2022), ggprism (Dawson, 2021) and patchwork (Petersen, 2021) were used. Considering the hypothesis that the use of antibiotics leads to higher resistance rates, a one-tailed Fisher's exact test from rstatix (Kassambara, 2021) was applied to test the association between *mcr*-Col-E carriage, colistin treatment and husbandry system. $p < 0.05$ was considered significant. Due to the limited number of samples, no such calculations were performed for the data relating to the human participants.

Results

Epidemiology

The veterinary authorities visited 175 turkey farms. One-hundred fifty-three (87.4%) of these used conventional farming systems, 14 (8.0%) were organic farms, and for eight (4.6%) there was no data on husbandry. Regarding medication, 130 of the 175 herds (74.3%) had not been treated with colistin during the fattening period. Of the 45 herds (25.7%, all conventional) where colistin had been administered, 35 (20.0%) had received one course of treatment, eight (4.6%) had been treated twice, one (0.6%) herd received three courses of treatment, and one (0.6%) more than three. There are no data on the use of antibiotics other than colistin.

For the collection of human samples, 209 test kits were handed out on 126 farms. Forty-six (22%) of these, originating from 31 farms, were returned and fulfilled the inclusion criteria (stool sample, fully completed questionnaire and informed consent).

Table 2 shows that participants were mostly farmers and their relatives. Their ages ranged from 14 to 79 years. Most of them were male (31, 67.4%). Twenty (43.5%) participants reported contact with livestock other than poultry. Ten persons reported having taken antibiotics, four (8.7%) had been hospitalised (solely in Germany); two (4.3%) participants worked in medical care and 13 (28.3%)

1 http://enterobase.warwick.ac.uk

reported having travelled abroad (unspecified where), all within the last 12 months.

mcr genes and colistin-resistant Enterobacterales

The *mcr-1* gene was identified in 123 of the 175 boot swab samples, equivalent to a prevalence of *mcr*-Col-E of 70.3% in the turkey farms included in the study (Figure 1A). Culture of these 123 samples led to 121 *E. coli* isolates (69.1% of all samples and 98.4% of all positive samples). For the remaining two samples, the *mcr-1* gene was harboured by *Klebsiella variicola* and *K. pneumoniae*. The *mcr-2* gene was not detected in any of these samples. After the methods became available, the 141 remaining samples were additionally screened for *mcr-3, mcr-4* and *mcr-5*, and none of them tested positive.

In the 46 human stool samples, seven isolates of *mcr*-Col-E were found. All of them were *E. coli* and stemmed from four different individuals (Figure 1B). One of them harboured three, another person two different isolates, and two participants harboured one isolate each. The two farmers and two family members belonged to two different farms with a conventional husbandry system where *mcr-1*-positive *E. coli* had also been detected in the boot swab samples.

None of these persons had contact with livestock other than poultry, worked in medical care, had taken antibiotics or had been hospitalised within the last 12 months. One person reported an 8- to 10-day-long stay abroad (see Table 2).

There was no connection between colonisation and kind or duration of contact with poultry (dung) (see Table 3).

Minimum inhibitory concentrations were measured for only 120 of the 121 animal *E. coli*-isolates because one was overgrown by *Proteus* spp. A MIC of 4 mg/l was found in 37 (30.8%) isolates, 74 (61.7%) had a MIC of 8 mg/l, five (4.2%) of 16 mg/l, and for four (3.3%) the MIC was ≥32 mg/l. Among the isolates from human stool, five (71.4%) had a MIC of 4 mg/l and two (28.6%) of 8 mg/l (Figure 1C).

Detection of *mcr*-Col-E in samples from turkey stables differed depending on whether the animals had been treated with colistin, with a prevalence of 82.2% in the 45 treated herds compared to 66.2% in the 130 untreated ones ($p = 0.0298$). No organic herd had been treated. For one herd where colistin had been administered, there was no data on husbandry. The remaining 44 treated herds were kept under conventional husbandry systems. The prevalence in these conventional treated herds was 81.8% (36 of 44) compared to 71.6% (78 of 109) in conventional herds without colistin treatment. There was also a difference when comparing husbandry systems regardless of treatment: while *mcr*-Col-E were found in 114 of the 153 conventionally kept herds (74.5%), this was only the case for a single one of the 14 organic herds, amounting to 7.1% ($p < 0.001$, Figure 2).

TABLE 2 Participants and their reported risk factors differentiated by colonisation with *mcr*-Col-E ($n = 46$, multiple risk factors possible).

	mcr-Col-E	
	yes	no
Farmer	2	22
Staff	0	7
Family member	2	12
Unknown	0	1
Risk factors		
Other livestock contact	0	20
Antibiotics	0	10
Hospitalisation	0	4
Working in medical care	0	2
Travelling abroad	1	12

Carbapenem-resistant Enterobacterales and carbapenem resistance genes

No CPE were detected, neither in the 175 animal-derived samples nor the 46 human stool swabs. Screening for carbapenem resistance

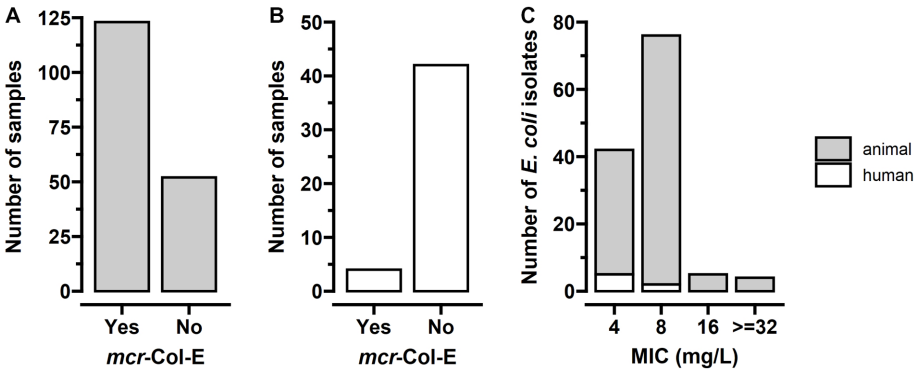

FIGURE 1
mcr-positive colistin-resistant Enterobacterales (*mcr*-Col-E) were highly abundant in the boot swab samples from turkey stables and rare but detectable in human stool samples, with all minimum inhibitory concentrations (MICs) above the epidemiological cut-off value. **(A)** The *mcr-1* gene was present in 123 of the 175 samples from animal faeces (70.3%), harboured by *E. coli* in 121 and *Klebsiella* spp. in two cases ($n = 175$). **(B)** In stool samples from humans, *mcr*-Col-E were present in four samples (8.7%), all *E. coli* ($n = 46$). **(C)** In line with pre-enrichment broth containing 2 mg/l colistin, all MICs were higher than the epidemiological cut-off value of 2 mg/l, with a median MIC of 8 mg/l ($n = 120$ animal and $n = 7$ human isolates). Isolates from turkey stables had higher MICs than those from human stool samples.

TABLE 3 Kind or duration of contact (per week) with poultry of participants differentiated by colonisation with *mcr*-Col-E (n = 46, not all participants responded to every item).

	mcr-Col-E	
	Yes	No
Work with direct contact >10 h	1	27
Work with direct contact <= 10 h	3	15
Cleaning stable >10 h	0	2
Cleaning stable <= 10 h	4	37
Other work inside stable >10 h	0	19
Other work inside stable <= 10 h	4	21
Dung contact outside stable >10 h	0	3
Dung contact outside stable <= 10 h	4	35
Other contact >10 h	0	0
Other contact <= 10 h	3	15

genes bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48} directly in the samples showed that boot swabs from two different farms contained bla_{OXA-48} genes. Culturing these samples demonstrated that the bla_{OXA-48} genes were harboured by isolates belonging to the non-enterobacterial species *Shewanella* and *Aeromonas*.

Comparison of colistin-resistant *Escherichia coli* isolates and *mcr*-plasmids

To find out whether *mcr-1*-positive *E. coli* isolated from turkeys and their human contacts were clonal, suggesting a direct transmission, the isolates were sequenced and compared using cgMLST. Figure 3 shows a minimum spanning tree based on cgMLST allelic profiles of *mcr-1*-positive isolates from 10 turkey farms: two farm isolates and the corresponding seven isolates from human participants on these farms, and eight randomly selected isolates from unrelated farms to investigate the overall diversity of *mcr-1*-positive *E. coli*. Except for two phenotypically different isolates from the same person (Hum3a and Hum3b) which had an identical cgMLST profile, no genotypic clusters were detected. The isolates that were most closely related, Hum4a and Vet4, differed in 46 alleles and originated on different farms. All further isolates differed in more than 1,000 alleles.

To investigate whether a transmission of colistin resistance via the exchange of plasmids may have occurred between animal and human isolates, the plasmids carrying the *mcr-1* gene were identified, and their sequences were analysed.

All 17 *mcr-1* plasmids of both environmental and human origin belonged to one of two plasmid family types: one, with a size of ~33–34 kb, harbouring the IncX4 replicon (n = 6) and one harbouring IncHI2 family replicons and a size ranging between ~172 kb and 291 kb (n = 11). From the latter, four also partially mapped IncQ1 (Table 4).

On farm 2, all human isolates belonged to plasmid type IncHI2 while the animal-associated isolate harboured an IncX4 replicon type plasmid. On farm 1, IncHI2 plasmids were isolated from two persons and the farm environment. A comparison of the region around the *mcr* locus (Figure 4) showed that the human isolate Hum2 and the farm-associated isolate Vet7 were identical. Isolates

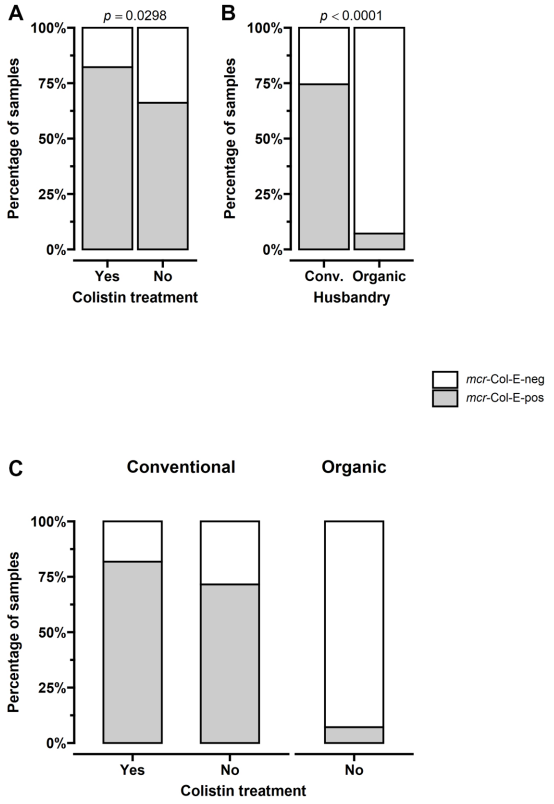

FIGURE 2
The prevalence of *mcr*-positive Enterobacterales was significantly higher in samples from stables where turkeys had been treated with colistin and in those from conventional farms. **(A)** The prevalence of *mcr*-Col-E was 82.2% (37 of 45) in treated herds and 66.2% (86 of 130) in untreated ones (p = 0.0298). **(B)** 74.5% (114 of 153) of conventionally kept herds were *mcr*-Col-E-positive, compared to 7.1% (1 of 14) of organic ones (p < 0.001). **(C)** The combination of both factors shows that no organic herd had been treated and that the prevalence in conventional herds was 81.8% (36 of 44) in those that had been treated compared to 71.6% (78 of 109) in those that had not. For **(A)**: n = all 175 farms (45 treated, 130 untreated), for **(B,C)**: n = 167 farms for which husbandry data was available (153 conventional – 45 treated, 108 untreated- and 14 organic – all untreated).

Hum3a and Hum3b from the same farm were lacking ISApI, an IS30-like family transposase, completely, while all other isolates only lacked a copy downstream of the *mcr-1* cassette. Although isolates Hum4a, Hum4b and Hum4c were taken from the same stool sample and all three harboured IncHI2-type plasmids, they differed in size. Moreover, Hum4b was lacking a kinase close to the *mcr* locus.

Discussion

This study investigated the prevalence and genomic profiles of *mcr*-Col-E and CPE on German turkey farms as well as among farm personnel using a combination of selective culture, detection of antimicrobial resistance genes, cgMLST and plasmid typing.

Following the initial discovery of *mcr-1* as a mobile resistance gene, subsequent studies aimed to determine the prevalence of the plasmid in colistin-resistant Enterobacterales. Often, these studies

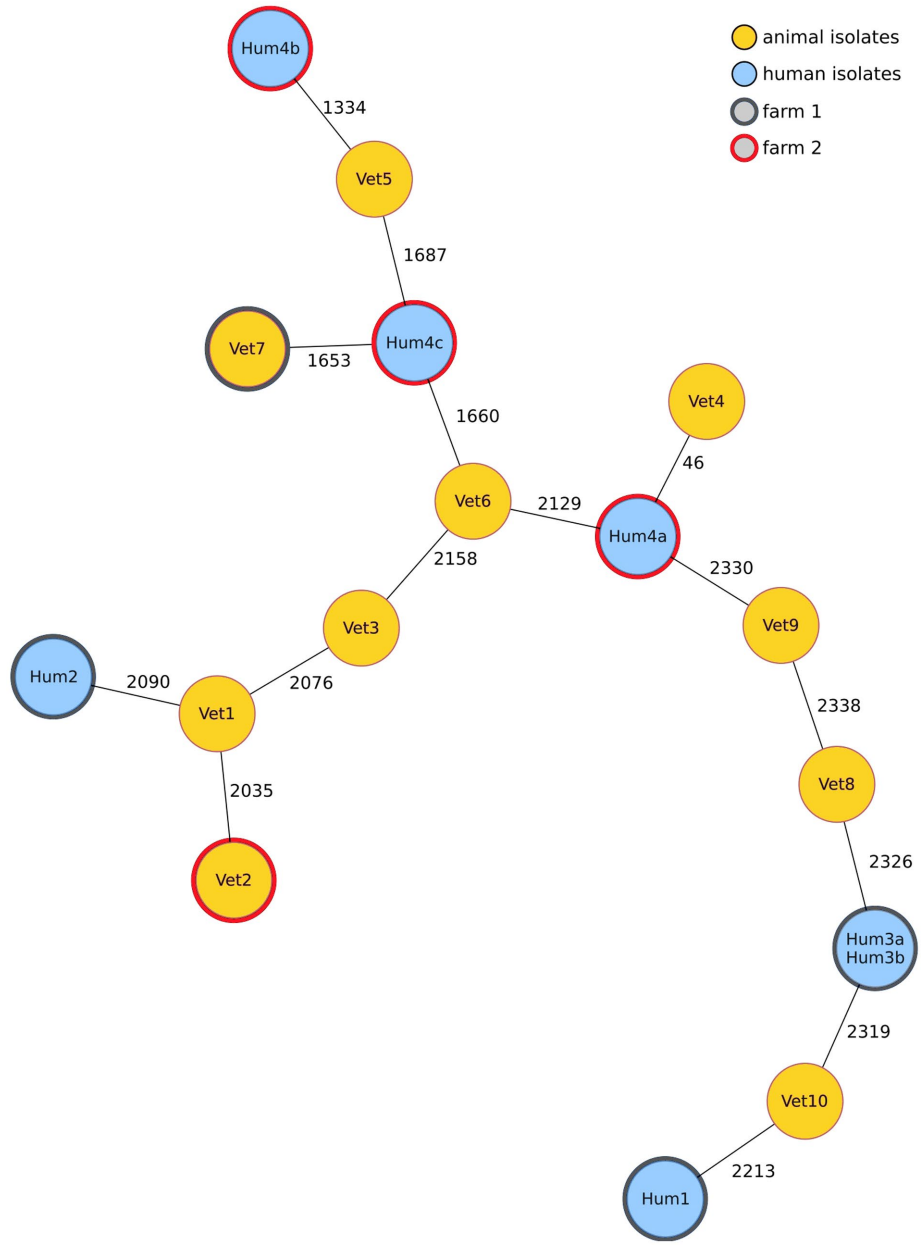

FIGURE 3
A minimum spanning tree based on the cgMLST allelic profiles of *mcr-1*-positive *E. coli* isolates shows that the two most closely related isolates differed in 46 alleles but originated from different turkey farms. Each node represents an allelic profile based on a sequence analysis of 2,513 cgMLST targets (seven isolates from human participants who lived/worked on farm 1 or 2, the two corresponding veterinary isolates from these farms and eight randomly selected isolates from unrelated farms, sample numbers do not correspond to farm of origin). Missing values were ignored during pairwise comparison. The numbers on the connecting lines correspond to the number of cgMLST targets with different alleles. Node colour depends on the sample origin (animal or human), and isolates with the same border colour are from the same farm.

retrospectively analysed existing isolates of indicator *E. coli*. These data revealed turkeys as the species most frequently harbouring *mcr-1*-positive isolates in Germany: between 2010 and 2014, the highest overall prevalence, 11.8%, was found in turkeys (faeces taken at farm level and caeca sampled at slaughter). The proportion of *mcr-1*-positive *E. coli* among colistin-resistant isolates ranged from 89.2 to 100% during this time (Irrgang et al., 2016). From 2016 to 2018, Grobbel et al. (2022) found a comparable prevalence of colistin-resistant isolates in faecal samples obtained from conventional turkey farms, at 9%.

However, all these data are based on a statistical population consisting of commensal *E. coli* collected for screening purposes. This method is particularly useful for monitoring time trends in antimicrobial resistance (Hesp et al., 2022). Nevertheless, a more sensitive approach is needed to gain an estimate of the potential spread of *mcr*-Col-E in turkey farming. Although cultural enrichment does not represent clinical impact, it is ideal for identifying all isolates carrying the traits under scrutiny.

Farm level prevalence data based on selective cultural enrichment of *mcr*-Col-E have to date only been available for swine in Germany:

TABLE 4 Overview of plasmids containing the *mcr-1* gene.

Isolate ID	Farm	Length (bp)	Replicon type
Hum1	1	34,639	IncX4
Hum2	1	172,270	IncHI2, (IncQ1)
Hum3a	1	242,799	IncHI2, (IncQ1)
Hum3b	1	242,799	IncHI2, (IncQ1)
Vet7	1	181,092	IncHI2
Hum4a	2	245,550	IncHI2
Hum4b	2	291,022	IncHI2
Hum4c	2	240,353	IncHI2
Vet2	2	33,303	IncX4
Vet1	\	278,947	IncHI2, (IncQ1)
Vet10	\	34,082	IncX4
Vet3	\	33,310	IncX4
Vet4	\	33,304	IncX4
Vet5	\	33,310	IncX4
Vet6	\	257,485	IncHI2
Vet8	\	217,572	IncHI2
Vet9	\	237,334	IncHI2

Length in bp and detected plasmid replicon types are given for each sequenced isolate (seven isolates from human participants who lived/worked on farm 1 or 2, the two corresponding environmental isolates from these farms and eight randomly selected isolates from unrelated farms).

Effelsberg et al. (2021) found *mcr*-Col-E in 12.3% of farms and 1.4% of farmers. Klees et al. (2020) used the same methodology and detected *mcr* genes in 43% of samples from a poultry slaughterhouse. However, they were not always able to obtain Col-E isolates from the same samples. As expected, the numbers in the current enrichment-based study are much higher than those that have previously been reported for poultry farms based on screening of commensal *E. coli*: with a prevalence of 70.3% for *mcr*-Col-E in environmental boot swabs of turkey faeces, the results greatly exceed the highest prevalence found by Irrgang et al. (2016), namely 17.9% in faeces at farm level in 2011.

In the present study, all *mcr-1*-positive isolates, with the exception of two, were identified as *E. coli*. These exceptions were *K. variicola* and *K. pneumoniae*, which have previously been shown to harbour the *mcr-1* gene in isolates derived from humans, animals and the environment (Liu et al., 2016; Kieffer et al., 2017; Elbediwi et al., 2019).

The isolates from turkeys in this analysis ($n = 120$) had MICs ranging from 4 to ≥32 mg/l, with a median of 8 mg/l, whereas human isolates ($n = 7$) only ranged from 4 to 8 mg/l. All isolates had been pre-enriched in presence of 2 mg/l colistin. Evidence from other studies is inconclusive: Zając et al. (2019) only reported values of 2, 4, and >4 mg/l in *mcr-1*-positive *E. coli* from food-producing animals, whereas Muktan et al. (2020) observed MICs up to 32 mg/l, with similar ranges in both poultry and human specimens.

Colistin was used in 25.7% of all herds included in the study. *mcr*-Col-E were detected in 80.0% of these herds that had been treated with colistin. Since the beginning of mandatory surveillance of antibiotic use in 2011, veterinary colistin sales in Germany have been reduced by 52.8%, from 127 t to 60 t in 2020 (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2021). However, at 8.6% of all sales, polypeptides are currently the fourth most commonly used group of antibiotics in veterinary medicine, after penicillins, tetracyclines and sulfonamides. It has been shown that a reduction in colistin usage can lead to greatly reduced resistance rates (Zhang et al., 2022), and that complete cessation of colistin use may entirely eliminate Col-E after a transitional period (Randall et al., 2018).

According to the findings of Islam et al. (2020), there is a positive correlation between the frequency of colistin administration in chickens and the prevalence of *mcr* and Col-E. Mesa-Varona et al. (2020) also detected such a relationship for broilers but not for turkeys in Germany. It has been suggested that higher resistance rates in turkeys than in chickens may be due to their longer life span, leading to longer exposure to antimicrobials and therefore higher selective pressure (Zając et al., 2019). Selective pressure of colistin on the intestinal microbiota could be high due to its lack of systemic resorption. In addition, colistin has been reported to often be greatly overdosed in poultry, as highly as 12-fold in chickens (Bundesinstitut für Risikobewertung, 2022; Flor et al., 2022). However, at 66.2%, alarmingly high rates of *mcr*-Col-E were also found in herds that had not been treated with colistin at all. In pigs, Randall et al. (2018) also found a high prevalence of Col-E in untreated animals.

One possible explanation is residual colistin in the stables resulting from past treatments of previously housed herds: colistin has been demonstrated to remain chemically intact for at least 60 days solved in water (Li et al., 2003). Moreover, *mcr-1*-carrying IncHI2 plasmids seem to be stable despite their large size (Ma et al., 2018). Plasmids carrying *mcr-1* have likewise been found to remain biologically active even after typical procedures occurring in livestock maintenance such as composting (Le Devendec et al., 2016). Also, the identified plasmids, particularly those of the IncHI2 type, contain a high number of additional antimicrobial resistance genes, potentially adding to a co-selective effect. Furthermore, wild animals may act as a source of Col-E (Franklin et al., 2020). Finally, there could have been cross-transmission of Col-E between herds via bacterial contaminations remaining in the farm environment, or Col-E could have been introduced via poultry that was already colonised when newly transferred to the farm (Majewski et al., 2020). All these circumstances may therefore induce colistin resistance in untreated animals.

Only one of 14 organically farmed turkey herds (7.1%) harboured *mcr*-Col-E, compared to 74.5% of conventionally kept herds. There are no farm-level data on German turkeys for comparison. However, an isolate-level study of commensal *E. coli* detected no colistin resistance in boot swab samples from German organic turkey farms, in contrast to a prevalence of 9% in conventional ones (Grobbel et al., 2022). Like all organically farmed herds in the present study, the *mcr*-Col-E-positive herd had not been treated with colistin, even though colistin is licensed for use in organic turkey farming. On the other hand, prevalence of *mcr*-Col-E was very high (71.6%) in conventional farms without colistin treatment. Therefore, the lack of such treatment alone is not a sufficient explanation. Mughini-Gras et al. (2020) detected higher levels of lysozyme and serum bactericidal activity in organically raised turkeys. As such findings could greatly impact turkey primary production, further studies are needed to determine to what extent the difference can be explained by factors like feed, stocking density and the administration or residues of antibiotics and other substances.

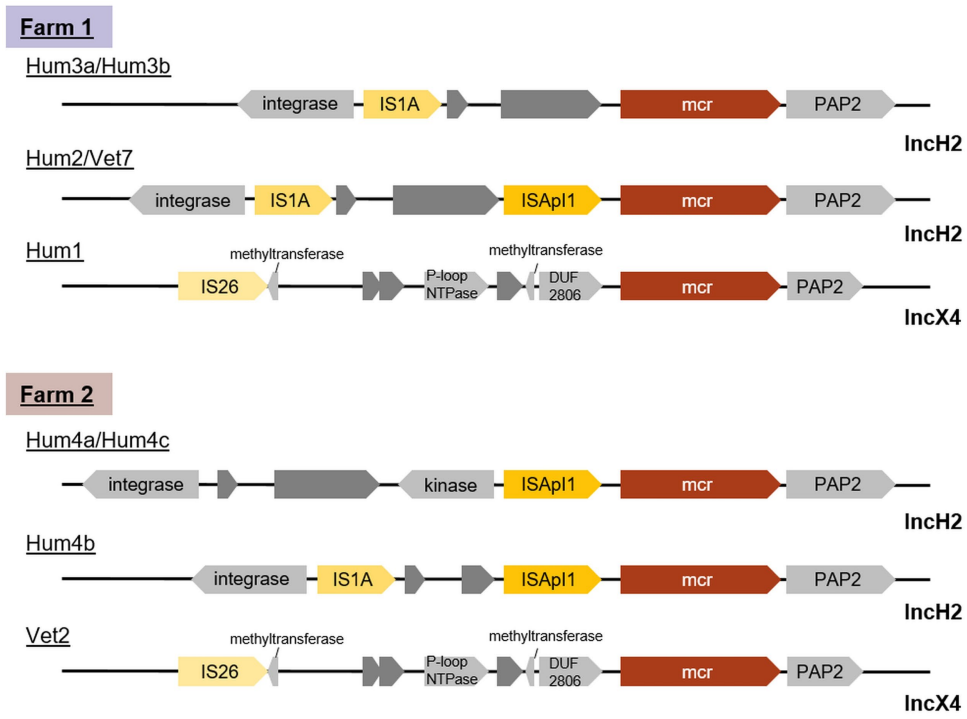

FIGURE 4
Genetic content of *mcr-1* adjacency on plasmids isolated from two turkey farms shows that the farm-associated isolate Vet7 and the human isolate Hum2 from farm 1 were identical. The *mcr* gene is highlighted in red, transposases in yellow, hypothetical proteins in dark gray and other annotated proteins in light gray.

Carbapenemase-producing Enterobacterales were not found in any of the samples examined in this study. Carbapenemases were only detected in oxidase-positive bacteria that have previously been described to harbour $bla_{\text{OXA-48}}$ (Ceccarelli et al., 2017). In German analyses along the food chain, CPE have only rarely been detected in samples originating from pigs and broilers (Fischer et al., 2012, 2013, 2017; Irrgang et al., 2017, 2020), and the human study population had no risk factors associated with CPE-carriage (Köck et al., 2021).

This study found that 8.7% of the 46 poultry farm workers were carrying *mcr*-Col-E. Studies investigating the prevalence of rectal *mcr*-Col-E carriage of humans in Western European countries observed much lower carriage rates (0.0–0.4%; Terveer et al., 2017; Zurfluh et al., 2017; van Dulm et al., 2019). Contact with livestock is a known risk factor for human colonisation with *mcr-1*-positive bacteria: PCR-screening for *mcr-1* of rectal swabs led to a prevalence of 33.0% in Vietnamese chicken farmers exposed to *mcr-1*-positive chickens, a lot higher than the rates in non-farming participants from rural (17.9%) and urban areas (9.1%; Trung et al., 2017).

It is also possible that the risk is not directly associated with contact with livestock itself because on farms, *mcr*-Col-E have also been isolated from dog faeces, stable flies and manure (Guenther et al., 2017). The colistin treatment of the animals appears to be a contributing factor: Evidence from China suggests that banning the use of colistin as a growth promoter greatly reduced human colonisation by colistin-resistant bacteria (Wang et al., 2020). Similarly, in Thailand, the number of farm workers carrying *mcr-1*-positive *E. coli* dropped from 4 in 10 to 0 after cessation of colistin use in pigs, although detection in pigs and wastewater remained possible even 3 years later (Khine et al., 2022).

It should be highlighted that only one *mcr*-Col-E colonised person in this study reported another risk factor for the acquisition of *mcr*-Col-E except for poultry farm contact, i.e., travel, although it was not assessed whether the travel destination was among those countries associated with an increased risk (Von Wintersdorff et al., 2016; Schaumburg et al., 2019). Underreporting of risk factors was decided to be neglected, as the questionnaire items did not refer to personality traits or require long-term recall. Recently, consumption of fish and seafood has also been suggested as a risk factor in healthy humans (Lv et al., 2022).

Molecular typing revealed the *mcr-1*-positive *E. coli* isolates to be highly diverse, with no clonality between human and animal isolates from the same farms. This corresponds to other findings that also reported great diversity in *mcr-1*-positive *E. coli* (Migura-Garcia et al., 2020; Effelsberg et al., 2021). However, it is a clear limitation of the study design that a maximum of two *mcr*-positive *E. coli* isolates per farm were analysed for a comparison of environmental and human isolates. Given the diversity of *E. coli* prevalent in the farm environment, this makes it unlikely to find matching human-environmental pairs and confirm direct transmission.

However, to improve the comparative techniques, plasmid-typing of *mcr*-Col-E isolates from humans and the farm environment was also performed: the *mcr* gene was contained by two different replicon family types: IncX4 and IncHI2. They have both been found among

the most prevalent *mcr*-carrying plasmids not only in Germany (Falgenhauer et al., 2016; Roschanski et al., 2017) and other European countries such as Denmark (Hasman et al., 2015), the Netherlands (Veldman et al., 2016), France (Haenni et al., 2016; Treilles et al., 2022), the United Kingdom (Doumith et al., 2016), Switzerland (Zurfluh et al., 2017), and Spain (Migura-Garcia et al., 2020) but also in Asia (Matamoros et al., 2017). Comparing the plasmids harbouring *mcr* genes from human and environmental isolates from the same farm, there was one case with a similar plasmid backbone and identical *mcr* environment, pointing towards zoonotic transmission. However, considering the lack of clonality of isolates from humans and the farm environment, other risk factors and the technical limitations of this study discussed above, the exact sources for *mcr*-Col-E among the farmers remain unclear.

Besides the limitations already mentioned, further limiting aspects of this study are the following: Due to the use of a more sensitive combination of methods, direct comparison of these turkey prevalence data with other findings, such as those reported by Irrgang et al. (2016), is not possible. Additionally, the study did not include testing for *mcr-6* to *mcr-10* in animal samples or *mcr-3* to *mcr-10* in human samples, as these methods were not available when the protocols were implemented. Also, the study area was limited to Lower Saxony and is hence not representative of Germany. However, Lower Saxony is by far the most significant federal state in the poultry industry: in 2019, 58.5% of turkeys slaughtered in Germany were slaughtered in Lower Saxony (Statistisches Bundesamt (Destatis), 2021), and Lower Saxony regularly provides between 70 to 80% of turkey-associated samples for the national zoonosis monitoring programme, which is based on proportional numbers for all federal states. Additionally, in light of the high prevalence in untreated herds, it would have been desirable to collect data on previous colistin use as well as the use of other antimicrobial agents, even if they had not been applied during the current fattening round. Finally, the number of human participants is rather small. Although various methods of information were used, the motivation on farms to participate in the study was low. This may have been caused not by lack of interest in the study itself but rather by the obstacle of taking a stool sample. Acceptance for taking stool samples can be hard to achieve even for cancer screening (Gordon and Green, 2015). Also, knowledge of colonisation may be perceived as a stigma, and decolonisation is not recommended.

Conclusion

Reduction and careful evaluation of antibiotic usage must be part of the ongoing efforts in both human and veterinary medicine. This study showed a high prevalence of *mcr*-Col-E on German poultry farms even in herds that had not been treated (66.2%). However, the fact that *mcr*-Col-E were found on only one of 14 organic farms (7.1%) indicates that a combination of several factors may have a favourable influence: These herds not only remained untreated, but were also kept according to other principles of organic farming, such as slower fattening and lower stocking density. Further research is needed to identify the specific contributions of such factors. Overall, carriage of *mcr*-Col-E was 8.7% among farm personnel, which exceeded expectations for the general population. In one case, similar plasmids were found in isolates from the farmer and from environmental samples of the respective farm. However, direct clonal transmission was not detected.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA934726.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Münster (No. 2018-008-f-S). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DR, KC, JE, and RK: conceptualisation. MS, DR, KC, ACM, AM, IM, CW, JE, and RK: methodology. KN, MS, and NE: formal analysis. KN, MS, NE, CK, DR, KC, RE, AM, IM, SR, CW, AW, and JE: investigation. KN, MS, CK, RE, and JE: data curation. KN and MS: writing – original draft. KN, MS, NE, CK, DR, KC, RE, ACM, AM, IM, SR, CW, AW, JE, and RK: writing – review and editing. DR, KC, JE, and RK: supervision. CK, DR, KC, JE, RK: project administration. DR, ACM, JE, and RK: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Coexistence of *bla*$_{IMP-4}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-1}$ in *bla*$_{KPC-2}$-producing *Citrobacter freundii* of clinical origin in China

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Purpose: To explore the genetic characteristics of the IMP-4, NDM-1, OXA-1, and KPC-2 co-producing multidrug-resistant (MDR) clinical isolate, *Citrobacter freundii* wang9.

Methods: MALDI-TOF MS was used for species identification. PCR and Sanger sequencing analysis were used to identify resistance genes. In addition to agar dilution, broth microdilution was used for antimicrobial susceptibility testing (AST). We performed whole genome sequencing (WGS) of the strains and analyzed the resulting data for drug resistance genes and plasmids. Phylogenetic trees were constructed with maximum likelihood, plotted using MAGA X, and decorated by iTOL.

Results: *Citrobacter freundii* carrying *bla*$_{KPC-2}$, *bla*$_{IMP-4}$, *bla*$_{OXA-1}$, and *bla*$_{NDM-1}$ are resistant to most antibiotics, intermediate to tigecycline, and only sensitive to polymyxin B, amikacin, and fosfomycin. The *bla*$_{IMP-4}$ coexists with the *bla*$_{NDM-1}$ and the *bla*$_{OXA-1}$ on a novel transferable plasmid variant pwang9-1, located on the integron In*1337*, transposon Tn*AS3*, and integron In*2054*, respectively. The gene cassette sequence of integron In*1337* is *IntI1-bla*$_{IMP-4}$*-qacG2-aacA4′-catB3Δ*, while the gene cassette sequence of In*2054* is *IntI1-aacA4cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1*. The *bla*$_{NDM-1}$ is located on the transposon Tn*AS3*, and its sequence is IS*91-sul*-IS*Aba14-aph (3′)-VI*-IS*30-bla*$_{NDM-1}$*-ble-trpF-dsbD*-IS*91*. The *bla*$_{KPC-2}$ is located on the transposon Tn*2* of plasmid pwang9-1, and its sequence is *klcA-korC*-IS*kpn6-bla*$_{KPC-2}$-IS*kpn27-tnpR-tnpA*. Phylogenetic analysis showed that most of the 34\u00B0*C. freundii* isolates from China were divided into three clusters. Among them, wang1 and wang9 belong to the same cluster as two strains of *C. freundii* from environmental samples from Zhejiang.

Conclusion: We found *C. freundii* carrying *bla*$_{IMP-4}$, *bla*$_{NDM-1}$, *bla*$_{OXA-1}$, and *bla*$_{KPC-2}$ for the first time, and conducted in-depth research on its drug resistance mechanism, molecular transfer mechanism and epidemiology. In particular, we found that *bla*$_{IMP-4}$, *bla*$_{OXA-1}$, and *bla*$_{NDM-1}$ coexisted on a new transferable hybrid plasmid that carried many drug resistance genes and insertion sequences. The plasmid may capture more resistance genes, raising our concern about the emergence of new resistance strains.

KEYWORDS

***Citrobacter freundii*, In*1337*, Tn*AS3*, In*2054*, Tn*2*, *bla*$_{IMP-4}$, *bla*$_{NDM-1}$, *bla*$_{KPC-2}$**

1. Introduction

Citrobacter is a facultative anaerobic Gram-negative bacteria, which exists widely in nature, such as water, soil and food. Today *C. freundii* is regarded as an important nosocomial pathogen and is frequently found in patients' blood, urine, soft tissues, and wounds (Khorasani et al., 2008; Kumar et al., 2013). In some extensive observational studies, *Citrobacter* accounted for approximately 3–6% of *Enterobacteriaceae* isolates in clinical settings (Mohanty et al., 2007). Carbapenems are the primary antimicrobial drugs for treating serious infections caused by ESBL-producing bacteria (Nicolau, 2008). The emergence of *C. freundii* carrying carbapenem-resistant gene (such as bla_{KPC}, bla_{NDM}, and bla_{IMP}) has brought more significant challenges to clinical treatment (Hammerum et al., 2016; Xiong et al., 2016; Xu et al., 2018). Even the coexistence of multiple carbapenemase genes, such as bla_{KPC-2} + bla_{NDM-1}, bla_{NDM-1} + bla_{IMP-14}, bla_{KPC-2} + bla_{NDM-1} + bla_{NDM-5}, undoubtedly makes antibiotic use less selective (Rimrang et al., 2012; Feng et al., 2015; Zheng et al., 2018). Carbapenemase-resistant *C. freundii* may cause an outbreak of nosocomial infection and seriously threaten public health (Pletz et al., 2018; Jung et al., 2020).

Carbapenemases can be divided into two groups according to their degree of cation dependence: serine carbapenemases (zinc-independent: classes A, C, and D) and metallo-beta-lactamases (MBLs; zinc-dependent: class B) (Queenan and Bush, 2007). Of the latter, VIM, IMP, and NDM types are the most prevalent types of carbapenemases globally (Nordmann, 2014).

IMP-type MBLs are the earliest transferable carbapenemases reported in Gram-negative bacteria. The bla_{IMP-1} was first discovered in *Pseudomonas aeruginosa* in Japan in 1991, and then quickly appeared in countries around the world (Watanabe et al., 1991). Over time, many variants of IMP have appeared, such as bla_{IMP-8}, bla_{IMP-26}, and have also been found in various *Enterobacteriaceae* bacteria, such as *Enterobacter hormaechei*, *Klebsiella pneumoniae* (Zheng et al., 2015; Gou et al., 2020; Guo et al., 2021). So far, 97 bla_{IMP} variants have been identified (November/2022).[1] The bla_{IMP-4} has been the most reported IMP variant, frequently found in class 1 integrons and carried by multiple plasmid types (such as HI2, L/M, A/C, and N) for horizontal transfer (Roberts et al., 2020). However, unlike NDM-type MBLs, bla_{IMP} is not common in CREs from China (Han et al., 2020).

NDM-type MBLs were widespread worldwide, and 44 NDM-type variants have been identified (November/2022) (see Footnote 1). Among the 44 NDM-type variants, NDM-1 has the broadest host spectrum discovered so far, and has been found in many species of 11 bacterial families, of which *K. pneumoniae* and *Escherichia coli* being the main carriers of bla_{NDM} (Zheng et al., 2011; Wu et al., 2019). Most bla_{NDM} are located on plasmids, and most plasmids carrying bla_{NDM} belong to the limited replicon type (IncX3, IncFII, and IncC) (Kumarasamy et al., 2010; Baraniak et al., 2016). NDM-positive strains can cause various infections that have been reported to be associated with high mortality (Guducuoglu et al., 2018).

According to the SMART global surveillance program, KPC is now the most widespread carbapenemase in the world. The bla_{KPC-2} was first detected in *K. pneumoniae* in North Carolina in 2001, spreading rapidly around the world (Yigit et al., 2001; Zheng et al., 2020). Now, 144 KPC-type variants have been identified (November/2022).[2]

KPC is a serine enzyme that can be inhibited by β-lactamase inhibitors, such as avibactam, clavulanic acid, etc. MBLs degrades almost all beta-lactam antibiotics, and its activity cannot be suppressed by clinically available beta-lactamase inhibitors, including avibactam, relebactam and vaborbactam (Boyd et al., 2020). Moreover, strains carrying bla_{NDM} and bla_{IMP} were also resistant to ceftazidime/avibactam (Bush and Bradford, 2019). The clinical treatment options for pathogens that carry serine enzymes or metalloenzymes are quite different. However, once the same strain of metalloenzymes and serine enzymes coexist, the clinical treatment options will be more challenging to choose.

At present, the study of the multidrug resistance in *C. freundii* has been reported sporadically, especially few studies has been conducted on resistance plasmids that carry multiple carbapenemase genes (coexistence of bla_{NDM-1}, bla_{OXA-1}, and bla_{IMP-4}). The research on the in the same transferable plasmid is not enough. We found a strain of *C. freundii* carrying bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-1}, and bla_{IMP-4} from cerebrospinal fluid, and through further research, we found that bla_{NDM-1}, bla_{OXA-1}, and bla_{IMP-4} coexist in a novel hybrid plasmid variant. We conducted in-depth research on the drug resistance mechanism, plasmid structure, horizontal transfer and epidemiology of *C. freundii*.

2. Materials and methods

2.1. Collection of bacterial strains and identification of antibiotic resistance genes

We continuously collected carbapenem-resistant Gram-negative bacilli from a tertiary hospital in Henan, China from 2018 to 2022. The antimicrobial susceptibility of the strains was preliminarily tested by VITEK®2 Compact (BioMerieux, Marcy l'Etoile, France), and then identified by MALDI-TOF MS (Bruker, Bremen, Germany) (Bizzini and Greub, 2010). We used PCR to identify common carbapenemase-encoding genes, such as bla_{KPC} (F: ATGTCACTGTATCGCCGTC; R: TTACTGCCCGTTGACGCC), bla_{IMP} (F: GTTTATGTTCATACW TCG; R: GGTTTAAYAAAACAACCAC), bla_{NDM} (F: ATGGAATTGC CCAATATTATGCAC; R: TCAGCGCAGCTTGTCGGC), and bla_{OXA-48} (F: TTGGTGGCATCGATTATCGG; R: GAGCACTTCTTTTGT GATGGC). We then used Sanger sequencing analysis to verify the PCR results (Yang et al., 2022). Supplementary Table S1 displays the pertinent primer sequences.

2.2. Antimicrobial susceptibility

Agar dilution and broth microdilution methods were used for antimicrobial susceptibility testing (AST), and *Escherichia coli* ATCC® 25922™ was used as the control. AST results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) 2021 standards. Tigecycline and colistin, whose

1 http://bldb.eu/BLDB.php?prot=B1

2 http://bldb.eu/BLDB.php?prot=A

clinical breakpoints were based on the 2022 EUCAST.[3] Supplementary Table S2 has been updated with the pertinent material information and details of antimicrobial susceptibility methodology.

2.3. Plasmid characterization and southern blotting and hybridization

S1-PFGE was undertaken on the CHEF-DR III system (Bio-Rad. Hercules, CA, United States), and patterns were evaluated and interpreted according to the published guidelines (Wang et al., 2019). The adjusted bacterial suspension was mixed with 1% Seakem Golden agarose and 1% sodium dodecyl sulfate (SDS), digested with proteinase K for 2 h at 56°C and then digested with S1 enzyme. The electrophoresis time was 16 h, the pulse time was from 2.16 s to 63 s, and a *Salmonella* serotype Braenderup strain (H9812) digested by Xba-I was used as the Marker. Then we used digoxigenin-labeled $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{KPC-2}}$ probes made the dig-high prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Swiss Confederation) to determine the location of plasmids harboring $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{KPC-2}}$ via southern blotting and hybridization. $bla_{\text{IMP-4}}$ (F: GTAGCATGCTACACCGCAGCAG; R: TCGTTAACCC TTTAACCGCC), $bla_{\text{NDM-1}}$ (F: TGCCCAATTATGCACCCG; R: CCACGGTGATTTTCACTG), and $bla_{\text{KPC-2}}$ (F: ATGTCACTGTAT CGCCGTC; R:TTACTGCCCGTTGACGCC).

2.4. Conjugation assays

The transferability of plasmids was investigated by using, a NaN_3-resistant standard strain, as the recipient for conjugation assays. To culture wang1, wang9, and *E. coli* J53, shake them and let them grow in the broth for 6 h until they reach the logarithmic growth phase. Then, add 100 microliters of wang1 and 200 microliters of *E. coli* J53 to the broth, and add 100 microliters of wang9 and 200 microliters of J53 to the broth. Culture both samples overnight at 37°. Subsequently, transconjugants carrying $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{KPC-2}}$ were first selected using Mueller-Hinton agar (OXOID, Hampshire, United Kingdom) plates containing both 1 mg/L meropenem and 200 mg/L NaN_3. Further, the selected transconjugant were confirmed by MALDI-TOF/MS, PCR identified the $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{KPC-2}}$ genes, and AST was used to verify the expression of antimicrobial resistance genes.

2.5. Plasmid stability assays

Briefly, the isolated wang1 and wang9 strains were cultured in LB broth with shaking (180 rpm) at 37°C and then serially passaged daily at a dilution of 1:1,000 in antibiotic-free LB broth for 5 days. After 5 days, the cultures were inoculated on MH agar plates without antibiotics, and 188 single colonies were selected for PCR identification after culturing at 37° overnight.

3 http://www.eucast.org

2.6. Whole genome sequencing and *in silico* analyses

Genomic DNA was extracted using a Genomic DNA Isolation Kit (QIAGEN, Hilden, Germany) and sequenced using Illumina Novaseq 6000 (Illumina, San Diego, CA, United States) and Oxford Nanopore platforms (Oxford Nanopore Technologies, Oxford, United Kingdom). RAST 2.0 and Prokka were used to annotate the draft genomes obtained by SPAdes version 3.9.1 and Uncycler (Aziz et al., 2008; Seemann, 2014; Wick et al., 2017).[4] ISfinder was used to detect insertion sequence elements and integrons.[5] Antimicrobial resistance genes (ARGs) were identified by ResFinder.[6] Plasmid identification were identified by PlasmidFinder 2.1.[7] We found the plasmid sequence with the highest consistency with the plasmid pwang9-1 and pwang9-2 in this study using the NCBI blast tool. Different plasmid genome sequences were compared using the BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). The figures about the genetic context surrounding the antibiotic resistance genes were drawn by Easyfig 2.3 (Sullivan et al., 2011). Whole-genome sequencing data were imported into an online website, and MLST analysis was performed based on seven housekeeping genes.[8] A report on both the quality of the sequences (wang1, Supplementary Figure S1; wang9, Supplementary Figure S2) and the quality of the assembly (Supplementary Table S3) has been included in the Supplementary material.

2.7. Phylogenetic analysis

We downloaded all *C. freundii* genomes ($n = 42$), plus 16 valid species of Citrobacter strains reference genomes,[9] from China from public data on NCBI and conducted core genes research through Roary (Page et al., 2015). For the reliability of the data, we use average nucleotide identity (ANI) analysis for all data.[10] Phylogenetic analysis was performed with these genomes, plus wang1 and wang9, by the maximum likelihood method on MEGA X. The resulting phylogenetic tree was modified by iTOL.[11]

3. Results

3.1. Species confirmation of strains

We isolated two carbapenem-resistant *C. freundii* strains from the cerebrospinal fluid of a 12-year-old patient with a meningococcal infection, post-pineal tumor surgery. Two strains of *C. freundii* were identified by MALDI-TOF-MS and WGS (ANI analysis, the ANI values for wang1 and wang9 were both similar to the reference genome of *C. freundii*, with values of 0.988 and 0.988, respectively, Supplementary Table S2), and designated as wang1 and wang9,

4 http://rast.nmpdr.org/
5 https://www-is.biotoul.fr/
6 https://cge.food.dtu.dk/services/ResFinder/
7 https://cge.food.dtu.dk/services/PlasmidFinder/
8 https://cge.food.dtu.dk/services/MLST/
9 https://lpsn.dsmz.de/genus/citrobacter
10 https://github.com/widdowquinn/pyani
11 https://itol.embl.de/

respectively. Wang1 carried bla_{KPC-2}, wang9 carried bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-1}, and bla_{IMP-4}. This phenomenon aroused our curiosity, and we conducted in-depth research on wang1 and wang9, respectively.

3.2. AST of *Citrobacter freundii* wang1, wang9 and transconjugants wang1J1 and wang9J2

We identified the transconjugants by MALDI-TOF-MS and PCR, in which wang9J1 carried bla_{KPC-2}, bla_{IMP-4}, and bla_{NDM-1}, while wang1J1 and wang9J2 carried bla_{KPC-2}. The isolates wang1 and wang9 both displayed resistance to most of the antibiotics, for example penicillins, cephalosporins, carbapenems, amino-glycosides, fluorquinolones etc. classes. They also both displayed susceptibility to amikacin, fosfomycin and polymyxin B. For tigecycline, wang1 and wang9 were determined as intermediate. For gentamicin, imipenem and meropenem, the MIC values of wang9 were significantly higher than those of wang1. This is also proved by comparing the AST results of wang9J1 with wang9J2 and wang1J1. The results of the AST of *C. freundii* wang1, wang9 and transconjugants are shown in Table 1.

3.3. MLST and genome of *Citrobacter freundii* isolates wang1 and wang9

According to the WGS results, wang1 and wang9 were shown by MLST to carry the genes *arc*A (18), *aspC* (151), *clpX* (14), *dnaG* (9), *fadD* (33), *lysP* (11), *mdh* (29), confirming its typing as ST415.

As mentioned above, the results of S1-PFGE and WGS showed that isolates wang1 and wang9 both carried two plasmids of different sizes. We searched the whole genome of wang1 and wang9 by ResFinder and PlasmidFinder. Specific information is displayed in Table 2. Both wang1 and wang9 have genomes of 5,232,707 bp, 5,232,708 bp, respectively. Wang1 has three plasmids, named pwang1-1, pwang1-2, and pwang1-3, with sizes of 149,719 bp, 65,148 bp, and 4,782 bp, respectively. Wang9 contains three plasmids, designated pwang9-1, pwang9-2, and pwang9-3, with sizes 223,404 bp, 149,719 bp, and 4,782 bp, respectively. According to WGS data analysis, pwang1-1 and pwang9-2, pwang1-3, and pwang9-3, are exactly the same. The G+C contents of pwang1-2, pwang1-3, pwang9-1, and pwang9-2 were 54.4, 52.6, 49.1, and 52.6%, respectively. The pwang9-1 carried both bla_{IMP-4} and bla_{NDM-1}, and the pwang9-2 carried bla_{KPC-2}.

3.4. S1-PFGE and southern blotting and hybridization

The S1-PFGE results demonstrated that that there are two plasmids in wang1, with sizes of 150 kb ~ and 70 kb~, whereas wang9 possesses two plasmids, with sizes of 230 kb ~ and 150 kb~, respectively. Interestingly, the second plasmid present in wang1 and the first plasmid of wang9 are of almost the same size.

Southern blotting and hybridisation results showed that bla_{IMP-4} and bla_{NDM-1} were both located on a 230 k ~ plasmid, and bla_{KPC-2} was located on a 150 kb ~ plasmid (Supplementary Figure S3). It was revealed by the results that wang1 carried bla_{KPC-2}, whereas wang9

TABLE 1 MIC values of antimicrobials for *C. freundii* wang1 and wang9, recipient strain J53, transconjugants wang1J1, wang9J1, and wang9J2, and control strain *E. coli* ATCC® 25922™.

Antimicrobials	MIC values (mg/L)		wang1J1	wang9J1	wang9J2	J53	ATCC® 25922™
	wang1	wang9					
Aztreonam	>128 (R)	>128 (R)	128 (R)	128 (R)	128 (R)	0.125 (S)	0.125 (S)
Imipenem	8 (R)	32 (R)	8 (R)	32 (R)	8 (R)	0.25 (S)	0.125 (S)
Meropenem	16 (R)	32 (R)	16 (R)	32 (R)	8 (R)	0.03 (S)	0.015 (S)
Ceftriaxone	>128 (R)	>128 (R)	128 (R)	>128 (R)	>128 (R)	0.06 (S)	0.125 (S)
Cefotaxime	>128 (R)	>128 (R)	128 (R)	>128 (R)	>128 (R)	0.25 (S)	0.25 (S)
Ceftazidime	>128 (R)	>128 (R)	>128 (R)	>128 (R)	>128 (R)	0.06 (S)	0.06 (S)
Levofloxacin	16 (R)	16 (R)	8 (R)	16 (R)	8 (R)	0.06 (S)	0.03 (S)
Ciprofloxacin	4 (R)	8 (R)	4 (R)	8 (R)	4 (R)	0.03 (S)	0.015 (S)
Amikacin	2 (S)	2 (S)	2 (S)	2 (S)	2 (S)	2 (S)	2 (S)
Gentamicin	16 (R)	64 (R)	16 (R)	64 (R)	16 (R)	2 (S)	1 (S)
P/T	128/4 (R)	>128/4 (R)	128/4 (R)	128/4 (R)	128/4 (R)	2/4 (S)	4/4 (S)
Fosfomycin	64/25 (S)	64/25 (S)	64/25 (S)	64/25 (S)	64/25 (S)	0.25/25 (S)	0.25/25 (S)
Chloromycin	128 (R)	128 (R)	128 (R)	128 (R)	128 (R)	4 (S)	4 (S)
T/S	8/152 (R)	8/152 (R)	8/152 (R)	8/152 (R)	8/152 (R)	0.25/4.75 (S)	0.25/4.75 (S)
AMC	>128/64 (R)	>128/64 (R)	128/64 (R)	>128/64 (R)	>128/64 (R)	2/1 (S)	4/1 (S)
Cefepime	>128 (R)	>128 (R)	>128 (R)	>128 (R)	>128 (R)	0.06 (S)	0.06 (S)
Tigecycline	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)	0.06 (S)	0.03 (S)
Polymyxin B	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)

R, resistant; S, susceptible; I, intermediate; T/S, Trimethoprim/Sulfamethoxazole; AMC, Amoxicillin-clavulanic acid; P/T, Piperacillin/Tazobactam.

10.3389/fmicb.2023.1074612

TABLE 2 Plasmid and genome information of *C. freundii* wang1 and wang9.

	Sizes (bp)	Type	G+C%	ARGs
wang1				
genome	5,232,707	ST415	51.8%	bla_{CMY-48}
pwang1-1	149,719	IncFII	52.6%	bla_{KPC-2}
pwang1-2	65,148	IncFIB (K)	54.4%	*sul1*, *aph(3″)-Ib*, *aph(6)-Id*, *aac(3)-IV*, *aph(4)-Ia*, *dfrA12*, *qacE*, *aadA2*
pwang1-3	4,782	Col	52.6%	–
wang9				
genome	5,232,708	ST415	51.8%	bla_{CMY-48}
pwang9-1	223,404	IncHI1B	49.1%	bla_{IMP-4}, bla_{NDM-1}, bla_{SFO-1}, $bla_{TEM-206}$, bla_{OXA-1} *sul1*, *aph(3″)-Ib*, *aph(6)-Id*, *aac(6′)-Ib3*, *aph(3′)-VI*, *qacE*, *aac(3)-IId*, *mph(A)*, *mph(E)*, *aac(6′)-Ib-cr*, *catB3*, *msr(E)*, *arr-3*
pwang9-2	149,719	IncFII	52.6%	bla_{KPC-2}
pwang9-3	4,782	Col	52.6%	–

carried bla_{KPC-2}, bla_{IMP-4}, and bla_{NDM-1} simultaneously. The results were consistent with the WGS sequencing analysis.

3.5. Plasmid stability assays

The selected 188 colonies of wang1 and wang9 were subjected to PCR validation of bla_{KPC-2} and bla_{IMP-4}. It was found that the preservation rate of pwang9-1 was 98.4%, while the preservation rate of pwang9-2 was 96.27% (Supplementary Table S4). This provides conclusive evidence that the resistant plasmid is capable of enduringly coexisting with and multiplying alongside the host bacteria, guaranteeing its stable existence and sustained expression over an extended period.

3.6. Structural characterization of the transferable plasmid

The plasmid pwang1-2 was identified as an IncFIB(K) type plasmid by PlasmidFinder analysis, while pwang9-1 and pwang9-2 could be classified into any of the known incompatibility groups. When we lowered the threshold for minimum % identity and the minimum % coverage of PlasmidFinder, pwang9-1 and pwang9-2 could have IncHI1B (the threshold for 75.22% identity and the 99.82% coverage) and IncFII (the threshold for 91.86% identity and the 96.09% coverage), respectively. The pwang9-1 carried bla_{IMP-4}, bla_{OXA-1}, and bla_{NDM-1}. We found a replicon FIB downstream of IS*5075* in the plasmid and a replicon repAciN interrupted by IS*Kpn26* downstream of IS*15*, indicating that pwang9-1 might be a hybrid plasmid. Through ISfinder and INTEGRONF, we noticed that pwang9-1 has a lot of insertion sequences, transposons and integrons, such as Tn*AS3*, IS*15*, IS*26*, In*2054*, In*1337* and so on. The bla_{IMP-4} coexists with bla_{NDM-1} and bla_{OXA-1} on a novel transferable plasmid variant pwang9-1, located on integron In*1337* and transposon Tn*AS3*, and integron In*2054*, respectively. The gene cassette sequence of integron In*1337* is *IntI1-*bla_{IMP-4}*-qacG2-aacA4′-catB3Δ*, while the gene cassette sequence of In*2054* is *IntI1-aacA4cr-*bla_{OXA-1}*-catB3-arr3-qacEΔ1-sul1*. The bla_{NDM-1} is located on the transposon Tn*AS3*, and its sequence is IS*91-sul-*IS*Aba14-aph(3′)-VI-*IS*30-*bla_{NDM-1}*-ble-trpF-dsbD-*IS*91*. The most similar plasmids identified by NCBI blast are as follows: pKP1814-1 from *Klebsiella pneumoniae* (GeneBank: KX839207, with 90% query coverage and 99.85% nucleotide identity) and pA from *Klebsiella quasipneumoniae* (GeneBank: CP068445, with 86% query coverage and 99.85% nucleotide identity). BLAST Ring Image Generator (BRIG) generated a circular image of multiple plasmid comparisons, as demonstrated in Figure 1A. We found that the main differences are concentrated in several gaps, and there were basically insertion sequences or transposons upstream and downstream of the gaps, such as IS*Kpn21*, Tn*2* and so on.

At the same time, we used Easyfig 2.3 to study the upstream and downstream environments of major antibiotic resistance genes. Among these, the transposon Tn*As3* carrying bla_{NDM-1} is highly conserved (Figure 2A). The integrase of integron In*1337* and group II intron reverse transcriptase/maturase (*ItrA*) were both interrupted into two contiguous sequence fragments (Figure 2B). The sequence of the integron In*2054* is also highly conserved, but group II *ItrA* is inserted between *catB3* and *arr-3*. By comparing CP70436, groups II intron reverse transcriptase/maturase are not exactly the same (Figure 2C). The pwang9-1 could not be analyzed using oriTfinder, but the success of the conjugation experiment proved that it could conjugate autonomously and transfer across species.

The pwang9-2 carried only one antibiotic resistance gene, bla_{KPC-2}, which is located on the transposon Tn*2*. The most similar plasmids identified by NCBI blast were follows: pKP19-3,023-142 K from *K. pneumoniae* (GeneBank: CP063749, with 95% query coverage and 100% nucleotide identity) and pkp18-2,110-2-2 from *K. pneumoniae* (GeneBank: CP084988, with 95% query coverage and 99.99% nucleotide identity). BLAST Ring Image Generator (BRIG) generated a circular image of multiple plasmid comparisons (Figure 1B). We found that the main differences were concentrated in one gap, and there was a Tn*3* family transposase (IS*Ec63*) downstream of the gap. At the same time, the upstream and downstream environment of bla_{KPC-2} was studied, and it was found to be located on the transposon Tn*2*. The sequence was highly conserved (Figure 2D). The sequence of Tn*2* is *klcA-korC-*IS*kpn6-*bla_{KPC-2}*-*IS*kpn27-tnpR-tnpA*. At the same time, it was analyzed by oriTfinder that it has an autonomous conjugation module and can conjugate and transfer autonomously, which is consistent with the results of the conjugation experiment (Supplementary Figure S4). We also found class 1 integron In*27* on pwang1-2, whose gene cassette sequence is *IntI1-dfrA12-gcuF-aadA2-qacEΔ1-sul1-orf5*.

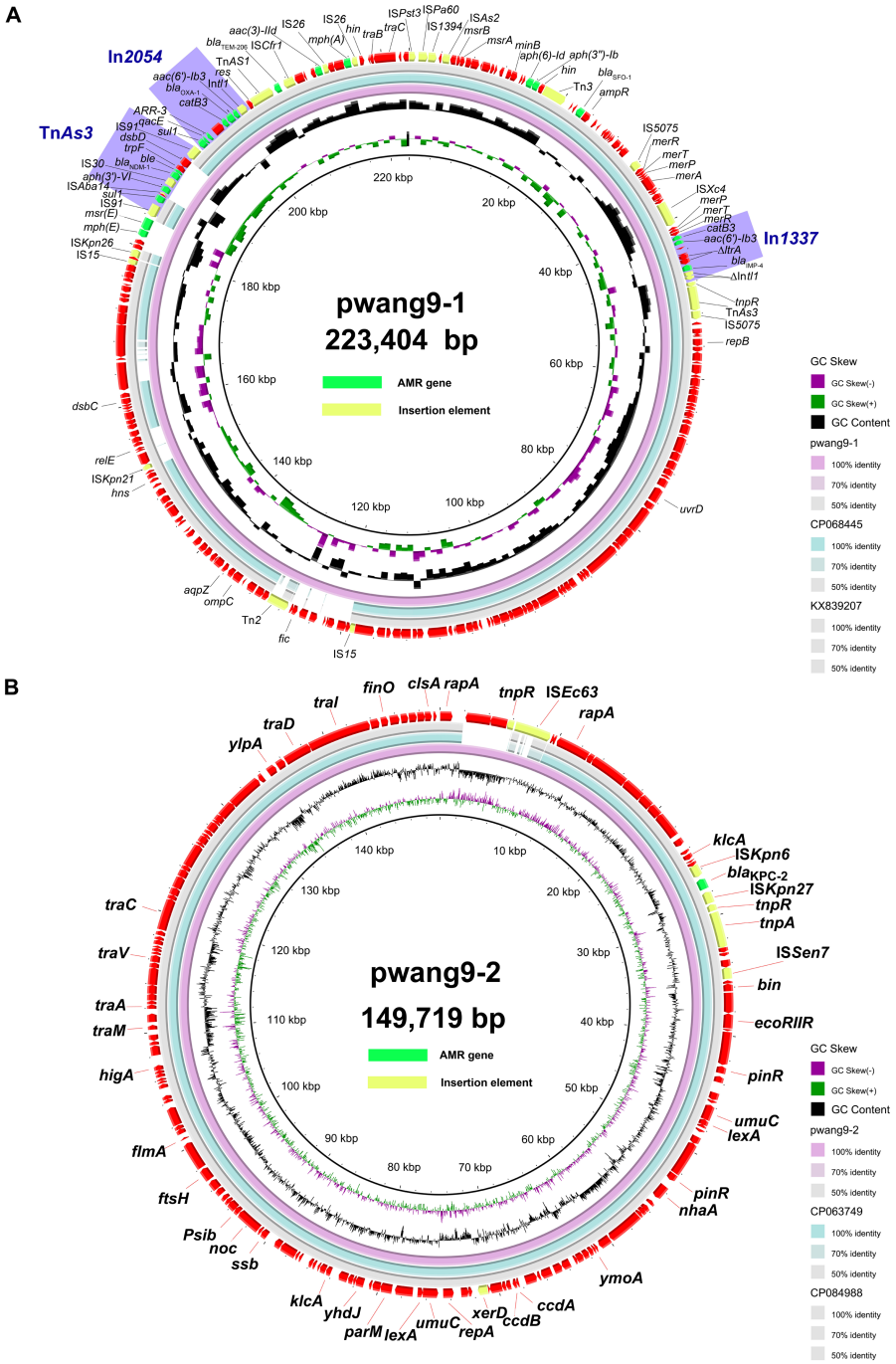

FIGURE 1
Genomic analyses of plasmid pwang9-1 **(A)** and pwang9-2 **(B)**. The comparative plasmid circular map of pwang9-1 **(A)** and pwang9-2 **(B)**, generated using BLAST Ring Image Generator (BRIG), shows the genes and their locations.

Although both wang1 and wang9 contain a 4,782 bp plasmid, pwang1-3, it is too small to contain any antibiotic resistance genes or virulence genes, therefore, is considered unimportant. The plasmid pwang1-3 was identified as an Col type plasmid by PlasmidFinder analysis. Because it does not contain any antibiotic resistance genes, the transferability of this plasmid cannot be checked by conjugation assays. We used oriTfinder to analyze the plasmid and found that it did not include type IV secretion system or type IV coupling protein (Supplementary Figure S5). We believe that this means it cannot be transferred.

3.7. Phylogenetic analysis

Based on the ANI results analysis, we believe that 10 strains are not part of *C. freundii* (Supplementary Figure S6, Supplementary Table S5). So, these 10 bacterial strains were excluded from the phylogenetic analysis.

Phylogenetic analysis showed all 34 strains of *C. freundii* isolated from China were divided into three clusters (Figure 3). Among them, nine isolates were from Guangdong, nine isolates were isolated from Jiangsu, seven isolated from Zhejiang, and only two isolated from Henan. Overall, environmental and clinical isolates showed a clear

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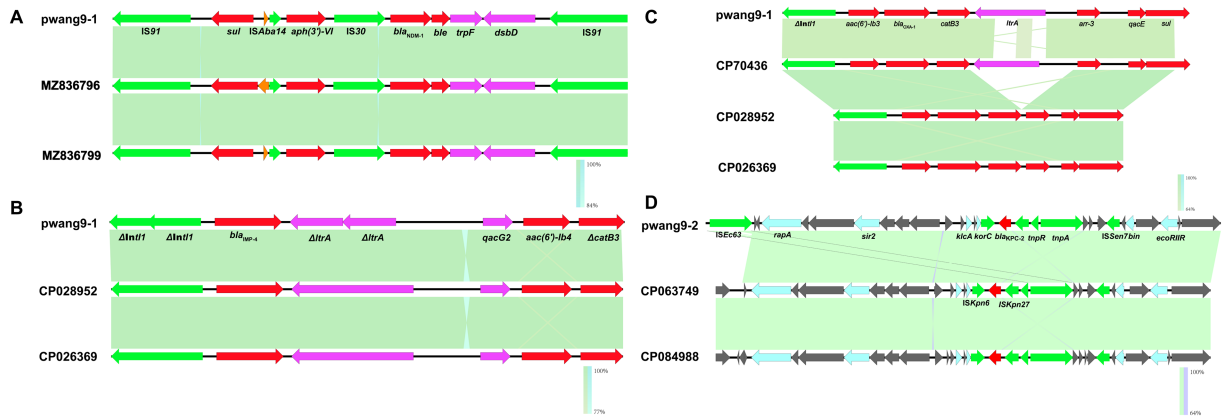

FIGURE 2
Genetic context of bla_{NDM-1} **(A)**, bla_{IMP-4} **(B)**, bla_{OXA-1} **(C)** on pwang9-1 and bla_{KPC-2} **(D)** on pwang9-2. Arrows denote genes. Genes, mobile elements, and other features are colored based on their functional classification.

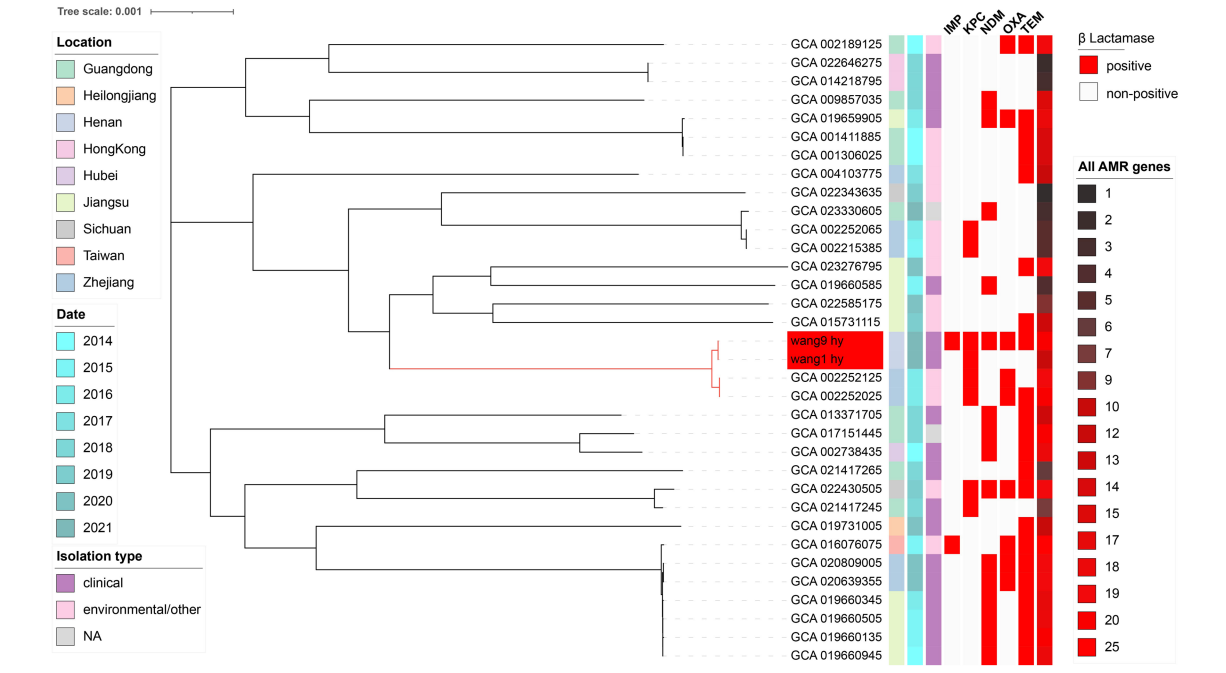

FIGURE 3
The phylogenetic tree of 32 strains of *C. freundii* from public data on NCBI and conducted core genes research through Roary. Phylogenetic analysis was performed with these genomes, plus wang1 and wang9, by the maximum likelihood method on MEGA X, and iTOL modified the resulting phylogenetic tree. We used different colors to represent different meanings. We marked wang1 and wang9 in red.

segmental distribution. The data demonstrates that there are several small groups of *C. freundii* carrying genes for antibiotic resistance (bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA}, and bla_{TEM}) and all AMRs, which are in significantly greater numbers than other isolates. Among them, wang1 and wang9 belong to the same cluster as two strains of *C. freundii* from environmental samples from Zhejiang.

4. Discussion

Citrobacter freundii has become an important pathogen causing nosocomial infections, but its related research is not deep enough, especially *C. freundii* carrying multiple carbapenemases genes. We continuously collect CREs for a large tertiary teaching hospital in Henan, China. A strain of *C. freundii* carrying bla_{KPC-2}, bla_{IMP-4}, bla_{OXA-1}, and bla_{NDM-1} was discovered in the cerebrospinal fluid of a 12-year-old post-operative brain tumor patient. After a pineal tumor surgery, the patient was treated with ceftriaxone for long-term anti-infective therapy. However, 8 days after surgery, the patient developed a meningococcal infection. Levofloxacin was added to the existing therapy to combat the infection. Nevertheless, the patient's condition progressively worsened, and multidrug-resistant *C. freundii* was isolated 20 days post-surgery. The patient remained comatose for an extended period, and her condition further

deteriorated 32 days after surgery. The family ultimately decided to discontinue treatment.

Our research on *C. freundii* has found that some isolates only carried bla_{KPC-2}, but not bla_{IMP-4} or bla_{NDM-1}. We hypothesized that the plasmids carrying bla_{IMP} and bla_{NDM} were lost and named the *C. freundii* carrying bla_{KPC-2} as wang1, and the *C. freundii* carrying bla_{IMP-4}, bla_{NDM-1}, bla_{KPC-2} as wang9. The WGS results and ANI analyze (Supplementary Figure S7, Supplementary Table S6) show that the chromosomal genomes of wang1 and wang9 are completely consistent, and pwang1-1 and pwang9-1 are also completely consistent. We also found that wang1 carries pwang1-2. Upon comparing it with pwang9-1, we found that it is very different, meaning that it is a plasmid unrelated to pwang9-1. We posited that the same *C. freundii* strain obtained different drug resistance plasmids during clinical treatment, thus manifesting different drug resistance profiles. Compared with wang1, and wang9, the MIC values of IMP, MEM and Gentamicin were significantly increased, which proved the coexistence of bla_{KPC-2}, bla_{NDM-1} and bla_{IMP-4} would significantly enhance the drug resistance of the strains. The antimicrobial susceptibility results of the transconjugants wang9J1 and wang9J2 also supported the conclusion. For tigecycline, wang1 and wang9 were determined to be intermediate. The isolates wang1 and wang9 both displayed sensitivity to amikacin, fosfomycin and polymyxin B.

Plasmids can capture, assemble, maintain and disseminate genes associated with antibiotic resistance, heavy metal resistance, and virulence (Tarabai et al., 2021; Okoye et al., 2022). It is speculated that plasmids carrying multiple drug-resistance genes may impose higher adaptation costs on the host strain. The stability of the resistance plasmids was assessed to determine their potential to remain functional under antibiotic-free conditions. The plasmid pwang9-1, which contains the genes bla_{IMP-4} and bla_{NDM-1}, showed high stability after serial passage for 5 days, with a retention rate of 98.4%. At the same time, pwang9-1 was analyzed by BRIG, and it was found to carry a large number of insertion sequences and drug-resistance genes. It is believed that this strain is likely to capture more drug-resistance genes, thereby enhancing its drug resistance. The main differences between pwang9-1 and CP068445, and KX839207 are located in a few sections containing transposons and insertion sequences. It is possible that the variation between pwang9-1 and the other two genomes is due to the capture of new and different genes by the insertion sequences and transposons. The upstream and downstream regions of bla_{NDM-1}, bla_{IMP-4}, and bla_{OXA-1} share a significant similarity, suggesting that transposons and integrons are key players in disseminating drug resistance. These results suggest that strains carrying pwang9-1 may be able to persist for long periods without affecting their fitness. Although oriTfinder could not analyze pwang9-1, the success of conjugation assays suggests that it can be horizontally spread across species. This raises concerns about an emerging drug resistance in the clinical setting.

The pwang9-2 is a plasmid that cannot be classified and carries bla_{KPC-2}. The conjugation assays and oriTfinder analysis show that it can autonomously transfer and conjugate across species. The pwang9-2 plasmid, which contains the bla_{KPC-2} gene conferring resistance, showed high stability after 5 days of continuous passage, with a retention rate of 96.27%. This suggests that it can not only be autonomously transferred to different species, but also that the plasmid is highly stable once the transfer is successful, allowing it to spread widely among strains. This issue further compounds drug resistance and makes clinical treatment more difficult.

The results of the phylogenetic analysis showed that multidrug-resistant *C. freundii* infections are becoming more prevalent in China and that the drug resistance levels of both environmental and clinical strains have increased significantly. wang1 and wang9 form a subcluster with GCA 002252125.1 and GCA 002252025.1, suggesting that the environment is a reservoir for multidrug-resistant strains, and bacteria can spread to each other between the environment and the human body, which is consistent with previous research (Bi et al., 2015; Ji et al., 2019; Zheng et al., 2019). Although only 34 strains of *C. freundii* were isolated from China, our conclusions may not be sufficient. Although there have been articles reporting *C. freundii* carrying bla_{IMP-4}, bla_{NDM-1}, and bla_{KPC-2}, a query of the strain information uploaded by NCBI reveals that the strain P10159 is not *C. freundii*, but *Citrobacter portucalensis*.

5. Conclusion

To the best of our knowledge, we are the first to find *C. freundii* carrying bla_{IMP-4}, bla_{NDM-1}, bla_{OXA-1} and bla_{KPC-2}. Its drug resistance mechanism and molecular transfer mechanism have been studied in depth. We found that bla_{IMP-4}, bla_{OXA-1}, and bla_{NDM-1} coexist on a new transferable hybrid plasmid that carries many insertion sequences and drug-resistance genes. This may further capture more drug resistance genes and lead to the development of new drug resistance. The emergence of new drug-resistant strains is a cause for concern.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SAMN29992768; https://www.ncbi.nlm.nih.gov/, SAMN29992269.

Author contributions

The experiments were conceived and designed by JG and BZ. The samples and experiments were collected and performed by JQ, YC, HG, RL, CL, and RC. The data was analyzed by HX and XG. The manuscript was written by JQ and revised by BZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1074612/full#supplementary-material

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Genomic and functional portrait of multidrug-resistant, hydrogen sulfide (H_2S)-producing variants of *Escherichia coli*

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Atypical *Escherichia coli* forms exhibit unusual characteristics compared to typical strains. The H_2S-producing variants of some atypical *E. coli* strains cause a wide range of illnesses in humans and animals. However, there are sparse reports on such strains worldwide. We performed whole-genome sequencing (WGS) and detailed characterization of four H_2S-producing *E. coli* variants from poultry and human clinical sources in Dhaka, Bangladesh. All four isolates were confirmed as *E. coli* using biochemical tests and genomic analysis, and were multidrug-resistant (MDR). WGS analysis including an additional Chinese strain, revealed diverse STs among the five H_2S-producing *E. coli* genomes, with clonal complex ST10 being detected in 2 out of 5 genomes. The predominant phylogroup detected was group A (n=4/5). The bla_{TEM1B} (n=5/5) was the most predominant extended-spectrum beta-lactamase (ESBL) gene, followed by different alleles of bla_{CTX-M} (bla_{CTX-M} -55,-65,-123; n=3/5). Multiple plasmid replicons were detected, with IncX being the most common. One *E. coli* strain was classified as enteropathogenic *E. coli*. The genomes of all five isolates harbored five primary and four secondary function genes related to H_2S production. These findings suggest the potential of these isolates to cause disease and spread antibiotic resistance. Therefore, such atypical *E. coli* forms should be included in differential diagnosis to understand the pathogenicity, antimicrobial resistance and evolution of H_2S-producing *E. coli.*

KEYWORDS

atypical H2S-producing *E. coli* variants, genomics, epidemiology, functional characterization, differential diagnosis, *E. coli* identification algorithm

Introduction

Escherichia coli is one of the most genetically diverse and versatile organisms, varying from commensal/avirulent to highly specialized pathogenic strains. *E. coli* can thrive in several niches, including hosts and in the environment (Kaper et al., 2004; Braz et al., 2020). The variant strains of *E. coli* may act as facultative or obligate pathogens (Köhler and Dobrindt, 2011). The facultative strains of pathogenic *E. coli* survive in the intestinal tract

and often cause opportunistic infections when reaching suitable extraintestinal sites (Nataro and Kaper, 1998; Kaper et al., 2004). In contrast, enteric obligate pathogens can cause infections in different conditions that range from moderate to severe diarrhea, and can sometimes cause lethal gastrointestinal infections (Nataro and Kaper, 1998).

Pathogenic variants of *E. coli* are responsible for infections in a variety of animals, more commonly in humans and poultry (Bélanger et al., 2011; Hussain et al., 2017). Pathogenic *E. coli* has been reported in livestock, including poultry, cattle, and swine (Bélanger et al., 2011). Animal reservoirs of pathogenic *E. coli* are responsible for diseases in animals, but can spread the infections to humans, including antimicrobial resistant (AMR) strains (Bélanger et al., 2011). Traditionally, biochemical tests have been used for differentiating and identifying members of Enterobacteriaceae, including hydrogen sulfide (H_2S) gas (Zabransky et al., 1969). H_2S can be synthesized by bacteria such as *Campylobacter*, *Salmonella*, *Citrobacter*, and *Erwardsiella* and *Proteus* species on TSI or KIA media (Blachier et al., 2019). This distinct characteristic feature of H_2S production by certain bacteria within Enterobcateriacea is used as a bacterial identification test in diagnostic microbiology. *E. coli* generally does not produce H_2S, which differentiates it from the other members of Enterobacteriaceae (Percival et al., 2004). However, a few studies worldwide have reported the presence of atypical H_2S-producing *E. coli* forms in humans and animals (Darland and Davis, 1974; Maker and Washington, 1974; Magalhães and Vance, 1978). They have been isolated from poultry, swine and clinical human urine specimens.

The enzyme 3-mercaptopyruvate sulfurtransferase (3MST) is reported to be mainly responsible for the synthesis of endogenous H_2S in Enterbacteriaceae (Mironov et al., 2017). Some studies have also demonstrated the transmissibility of H_2S-producing traits between strains via plasmids (Jones and Silver, 1978; Magalhães and Vance, 1978). Although the physiological function of endogenously produced hydrogen sulphide is not clearly defined, recent studies have pointed out its role in protecting bacteria against antibiotics and host defence systems (Mironov et al., 2017; Rahman et al., 2020). A plausible explanation for this is that the antibiotics induce oxidative stress in bacteria by increasing the levels of reactive oxygen species; in response to this, the bacteria produces H_2S which in turn stimulates enzymes such as superoxide dismutase (SOD) and catalase that alleviates the effect of reactive oxygen species, and thereby reduces the efficacy of antibiotics contributing to AMR (Eswarappaápradeep, 2017). Also, studies have demonstrated the role of bacterial H_2S production in defence against host immunity by making them resistant to leukocytes- mediated killing *via* unknown mechanisms (Toliver-Kinsky et al., 2019; Rahman et al., 2020).

The accurate identification of H_2S-producing variants of *E. coli* in diagnostic laboratories is an important step for initiating effective infection management. There is a need to raise awareness of this unusual type of *E. coli* form that occurs frequently but differs in its inability to produce H_2S compared to the typical *E. coli* forms. Therefore, this study aimed to perform bacteriological, biochemical and genomic characterization of H_2S-producing variants of *E. coli* from healthy poultry and human clinical sources in Dhaka, Bangladesh. We present the first report on the genomic characterization of H_2S-producing variants of *E. coli* from Bangladesh and that from South Asia.

Materials and methods

Ethics statement

The study protocols were approved by the Research Review committee and Ethics Review Committee of icddr,b, Dhaka, Bangladesh (PR-23045).

Bacterial strains

A surveillance study was conducted between 2019 and 2021 to investigate the genomic-based epidemiology of AMR Enterobacteriaceae in healthy poultry and human clinical samples in Dhaka, Bangladesh (Mazumder et al., 2020a, 2021, 2022). During that study, we detected four lactose fermenting *E. coli* colonies but with an atypical biochemical feature of H_2S production. These were confirmed to be *E. coli* by the methods described hereafter. Three (BD7, BD8, BD9) of these H_2S-producing *E. coli* originated from raw poultry meat and one isolate (BD_CL10) was cultured from a urine sample of a suspected urinary tract infection patient in Dhaka, Bangladesh. Thus, from a collection of 96 poultry *E. coli* isolates and 204 human clinical *E. coli* study isolates, we could obtain three and one H_2S-producing *E. coli* isolates, respectively. These four H_2S-producing *E. coli* isolates then formed the basis of this study, and underwent various tests and whole genome sequencing (WGS). One H_2S positive *E. coli* genome from China (Biswas et al., 2020) was used for the in-silico analysis together with the four studied H_2S positive *E. coli* genomes sequenced in this study.

Biochemical characterization and antimicrobial susceptibility

The complete bacteriological and biochemical characteristics of H_2S-producing variants of *E. coli* strains are summarized (Table 1). The biochemical identification included the following tests; kligler iron agar (KIA) test, motility, indole and urease (MIU) test, citrate and acetate utilization test, catalase test, oxidase test, vogas-proskauer test, gelatin liquefaction and ONPG tests. In addition, colonies were plated on Muller-Hinton agar containing 0.68% of sodium thiosulfate plus 0.08% of ferric ammonium sulfate as previously described (Park et al., 2015). The isolates that mimic *E. coli* in all aspects except H_2S-production in Kligler iron agar (KIA) and Muller-Hinton agar (with sodium thiosulfate and ferric ammonium sulfate) were carried forward in this study. These preliminary identified 4 H_2S-positive *E. coli* isolates were subjected to additional tests, including fermentation of sugars and decarboxylation reaction of amino acids (Mazumder et al., 2022). Further, the possibility of *Salmonella* spp. was ruled out by slide agglutination test using O, O1 polyvalent and VI *Salmonella* antisera (Denka Seiken Co. Ltd. Tokyo, Japan). The API 20E kit (bioMérieux) was used to generate the analytical profile index (Table 1). Haemolysis was evaluated using 5% sheep blood agar plates. Disk diffusion method was employed to determine the antimicrobial susceptibility. The Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein, 2019) were followed. Twenty commercially available antibiotic disks (Oxoid, US) covering 11 antimicrobial classes were tested (see Table 2). The intermediate susceptibility was described as non-susceptible. Isolates were termed multi-drug resistant (MDR)

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TABLE 1 Biochemical and growth characteristics of H_2S-producing *Escherichia coli* from Dhaka, Bangladesh.

Biochemical tests performed	Test results			
	Isolate BD7	Isolate BD8	Isolate BD9	BD_CL10
Gram stain	Gram negative bacilli	Gram negative bacilli	Gram negative bacilli	Gram negative bacilli
Catalase test	+	+	+	+
Oxidase test	–	–	–	–
TSI agar				
a. Acid production in slant	+	+	+	+
b. Acid production in butt	+	+	+	+
c. Hydrogen sulfide production (H_2S)	+	+	+	+
d. Gas production	+	+	+	+
Motility indole ureas test (MIU)				
a. Motility	+	+	+	+
b. Indole Production	+	+	+	+
c. Urea hydrolysis	–	–	–	–
Simmons citrate reaction test	–	–	–	–
Acetate	+	+	+	+
Mueller Hinton agar + sodium thiosulfate with ferric ammonium sulfate	Produce H_2S	Produce H_2S	Produce H_2S	Produce H_2S
Sugar fermentation				
a. Glucose	+	+	+	+
b. Lactose	+	+	+	+
c. Sucrose	+	+	–	+
d. Maltose	+	+	+	+
e. Mannose	+	+	+	+
f. Arabinose	+	+	+	+
g. Sorbitol	+	+	+	+
h. Mannitol	+	+	+	+
i. Inositol	–	–	–	–
Nitrate Reduction	+	+	+	+
Gelatine liquefaction	–	–	–	–
ONPG	+	+	+	+
Vogas-proskauer	–	–	–	–
Lysine decarboxylase	+	+	+	+
Ornithine decarboxylase	–	–	+	–
Arginine dihydrolase	+	+	–	+
Haemolysis on blood agar	–	–	–	–
Growth characteristics				
a. MacConkey agar	PC[a]	PC[a]	PC[a]	PC[a]
b. SS agar agar	PC[a]	PC[a]	PC[a]	PC[a]
c. CHROMagar™ Orientation	DPC[b]	DPC[b]	DPC[b]	DPC[b]
d. Blood agar	WC[c]	WC[c]	WC[c]	WC[c]
e. Gelatin agar	WC[c]	WC[c]	WC[c]	WC[c]
Growth Temperature	26–42°C	26–42°C	26–42°C	26–42°C
API Number (Detect *E. coli* with 99% probability)	5,544,512	5,544,512	5,544,552	5,544,512

[a]PC, pink color colony.
[b]DPC = Dark pink color colony.
[c]WC = White colony.

TABLE 2 Antimicrobial susceptibility profiles of H_2S-producing *Escherichia coli* isolates from Dhaka, Bangladesh.

Classes	Antibiotics	BD7	BD8	BD9	BDCI-10	China_H2S
Aminoglycosides	Amikacin (AK)-30 μg	S	S	S	S	DA
	Gentamicin (CN)-10 μg	R	S	R	S	R
β-Lactams (Penicillin)	Ampicillin (Amp)-10 μg	R	R	R	R	R
β Lactams (Cephalosporins)	Cefepime (FEP)-30 μg	S	R	R	R	DA
	Cefixime (CFM)-5 μg	S	R	R	R	DA
	Cefotaxime (CTX)-30 μg	S	R	R	R	S
	Ceftazidime (CAZ)-30 μg	S	R	R	R	DA
	Ceftriaxone (CRO)-30 μg	S	R	R	R	DA
	Cefuroxime (CXM)-30 μg	S	R	R	R	R
Phenicols	Chloramphenicol (C)-30 μg	R	S	R	S	R
Fluoroquinolones	Ciprofloxacin (CIP)-5 μg	R	R	R	R	R
	Nalidixic Acid NA-30 μg	R	R	R	R	R
Polymyxins	Colistin (CT)-10 μg	13.7*	13.8*	13.8*	13.6*	R
Trimethoprim/Sulfonamides	Trimethoprim-sulfamethoxazole (SXT)-1.25/ 23.75 μg	R	R	R	R	R
Tetracyclines	Doxycycline (DO)-30 μg	R	R	R	R	R
Phosphonic antibiotic	Fosfomycin (FOS)-50 μg	R	S	R	I	S
Carbapenems	Imipenem (IPM)-10 μg	S	S	S	S	S
	Meropenem (MEM)-10 μg	S	S	S	S	S
Nitrofuran derivatives	Nitrofurantoin (F)300 μg	R	S	I	I	DA
Glycylcycline	Tigecycline (TGC)-15 μg	R	R	R	R	DA

R, resistance; S, susceptible, I, intermediate, DA, data absent, *zone of inhibition in mm.

if refractory to at least one antibiotic from three or more antimicrobial classes (Magiorakos et al., 2012).

Whole-genome sequencing

Total bacterial DNA was extracted using the Maxwell RSC Instrument and Culture Cell DNA extraction Kit (Promega) for gram-negative bacteria with an additional RNaseA treatment (Baddam et al., 2020; Mazumder et al., 2020b,c). The DNA QC was assessed by Nanodrop spectrophotometer (Thermo Fisher Scientific, US), Quantus Fluorometer (Promega, US) and by 1% agarose gel electrophoresis. The paired-end bacterial WGS libraries were constructed from 220 to 250 ng of genomic DNA using the Illumina DNA Prep kit as per the manufacturer's instructions (Mazumder et al., 2021). The pooled libraries thus obtained were sequenced at the icddr,b Genome Centre on Illumina NextSeq 500 system to obtain 100- to 150-fold coverage for each genome using a NextSeq v2.5 Mid Output reagent kit (2 × 150 bp) (Mazumder et al., 2022; Monir et al., 2023).

Sequence assembly and annotation

WGS data quality was examined using FastQC (Andrews, 2010). Trimmomatic software (v0.36) (Faircloth, 2013) was used to extract adapters and poor-quality bases (<Q30) from the unprocessed sequencing reads using the following parameters described elsewhere (Mazumder et al., 2020c, 2021, 2022). Deconseq software (v4.3) was used to eliminate contaminated sequences (Schmieder and Edwards, 2011). The processed reads were used to create *de novo* assemblies of each genome using SPAdes software (v3.11.1) (Bankevich et al., 2012). QUAST (v5.0) (Gurevich et al., 2013) was used to evaluate the assembly metrics of scaffold fasta files. The genomes were annotated using Prokka (v1.12) (Seemann, 2014) using *E. coli* MG1655 as the reference genome (GenBank accession number NC 000913.3). The genomic features of H_2S-producing *E. coli* strains are summarized (Table 3).

In silico sequence analysis

The reads of H_2S-producing *E. coli* were uploaded to the KmerFinder v3.2 (Hasman et al., 2014; Larsen et al., 2014) for species confirmation. The phylogenetic groups were ascertained using Clermon Typing tool (Beghain et al., 2018). The sequence types (STs), clonal complex and pathovars were predicted employing the Achtman7 seven-locus scheme at EnteroBase v1.1.3[1] web tool. The O and H

1 https://enterobase.warwick.ac.uk/

TABLE 3 Genomic features, the status of CRISPR-CAS system and prophage sequences in H_2S-producing *Escherichia coli* isolates.

Strain Name		BD7	BD8	BD9	BD-Cl10	H_2S *E.coli*_ China
Pathogenicity Score (No. of Pathogenic Families)		0.94 (666)	0.93 (635)	0.933 (585)	0.941 (548)	0.934 (567)
Human Pathogenicity		Yes	Yes	Yes	Yes	Yes
Genomic features of H_2S producing *E. coli* isolates	Genome Size (bp)	5,050,301	5,198,676	4,990,709	4,525,004	4,501,832
	Genome coverage	102X	127X	125X	133X	206X
	Contig no. (>500 bp)	149	248	135	125	122
	GC %	50.37%	50.43%	50.63%	50.87%	50.71%
	No. of Coding Sequences	4,809	5,069	4,757	4,224	4,216
	Accession No.	JAGINC000000000	JAGIND000000000	JAGINE000000000	JAODTH000000000	Not found
	SRA	SRX11616412	SRX11616413	SRX11616414	SRX17654297	SRX6956426
	Bio-project	**PRJNA714244**			**PRJNA882002**	**PRJNA576077**
Characteristic features of CRISPR-Cas system	Subtype	I-E, I-A	I-A, I-E	I-A, I-E	I-A, I-E	I-A
	Cas Proteins	Cas3, DEDDh, Csa3, Cas8e, Cse2gr11, Cas7, Cas5, Cas6e, Cas1, Cas2	Cas3, DEDDh, Csa3, Cas8e, Cse2gr11, Cas7, Cas5, Cas6e, Cas1, Cas2	Cas8e, DEDDh, Cas3, Cas2, Cas1, Cas6e, Cas5, Cas7, Cse2gr11, Cas3	Cas3, DEDDh, Csa3, Cas8e, Cse2gr11, Cas7, Cas5, Cas6e, Cas1, Cas2	Csa3, DEDDh, Csa3
	No. of loci	1	1	1	1	1
	No. of repeats	12	14	7	19	5
	Average length of repeats	29	29	29	29	29
	No. of spacers	11	13	6	18	4
	Average length of spacers	32	32	32	32	32
	Questionable CRISPR*	+	+	+	+	+
Completeness of prophage sequences#	Intact	2	3	3	4	0
	Incomplete	4	8	7	1	5
	Questionable	2	2	0	0	0
	Total prophage regions	**8**	**13**	**10**	**5**	**5**
	Intact prophage Region Length	26.9Kb; 37.6Kb	26Kb, 34.8Kb, 12.3Kb	49.7Kb, 46.8Kb, 100.2Kb	38.6Kb, 32.3Kb, 39.2Kb, 35.5Kb	ND[a]
Intact Phage Name based on highest number of hits		Enterobacteria phage SfI-13 Klebsiella phage 4 LV-2017	Yersinia phage L413C Shigella phage SfII Enterobacteria phage HK544	Enterobacteria phage P88 Salmonella phage118970_sal3 Salmonella phage SSU5	Enterobacteria phage Lambda Klebsiella phage 4 LV-2017 Escherichia phage 500,465–1 Shigella phage SfII	ND[a]

ND[a], not detected.

serotypes were determined employing SerotypeFinder v2.0 (Joensen et al., 2015). FimH and FumC types were determined by CH typer 1.0 (Roer et al., 2018). AMR determinants, virulence factors, and plasmid types were screened using the ABRicate tool v1.0.1 (Seemann, 2018), ResFinder (Zankari et al., 2012), Virulence Factor Database (VFDB) (Chen et al., 2005), and PlasmidFinder (Carattoli et al., 2014) databases, respectively. We used a cut-off of 80% query coverage and 98% identity for screening genes in the genomes analysed. Mobile Element Finder (v1.0.3) was utilized to identify mobile genetic elements linked with acquired antimicrobial resistance genes. Mutations encoding fluoroquinolone resistance were detected by PointFinder (Zankari et al., 2017). IntegronFinder (v2.0) was used to identify integrons (Néron et al., 2022). The chromosomal or plasmid origin of ESBLs genes were analysed by *BLAST*n analysis of contigs against NCBI

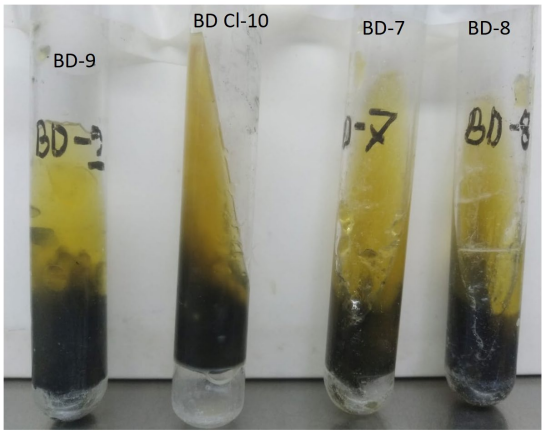

FIGURE 1
Hydrogen Sulfide (H_2S)-producing variants of *Escherichia coli* strains: BD7, BD8, BD9, and BD-CL10 showing black precipitate of H_2S on Kligler Iron Agar (KIA) tubes.

database. Prophage sequences in *E. coli* genomes were determined using the Phage Search Tool Enhanced Release (PHASTER). Prophage regions were classified as intact, questionable, and incomplete based on prophage sequence scores of ≥90, 70–90, and ≤70, respectively. (Arndt et al., 2016). CRISPR-Cas system of H_2S-producing *E. coli* strains were characterized using CRISPRone tool[2] (Zhang et al., 2012). Cysteine-degradation genes in *E. coli* were identified based on genes described previously (Braccia et al., 2021). A threshold of 100% coverage and 98% identity were used. The pathogenic pontential of strain was predicted using the web-server PathogenFinder (Cosentino et al., 2013). Default parameters were used for the *in-silico* analysis unless otherwise stated.

Single nucleotide polymorphism-based core genome phylogeny

We used Snippy (v4.4.0) software (Seemann, 2015) with default parameters to obtain the reference-guided multi-fasta consensus alignment of 5 H_2S-producing *E. coli* genomes using *E. coli* MG1655 as the reference. Gubbins software (v3.2 5) (Croucher et al., 2015) was used to filter true point mutations from those arising from recombination. The phylogenetic tree was determined using RaxML (v8.2.12), utilizing the Generalized Time Reversible substitution model and a GAMMA distribution to account for rate heterogeneity (Stamatakis, 2014). Finally, the phylogenetic tree was displayed using IToL (Letunic and Bork, 2016).

Accession numbers

The four genomes that were sequenced for this study can be identified by their GenBank accession numbers: JAGINC000000000 (BD7), JAGIND000000000 (BD8), JAGINE000000000 (BD9) and JAODTH000000000 (BD_CL10) (Table 3).

Results

Bacterial characteristics

Four H_2S-positive *E. coli* variants were identified that formed a black precipitate after overnight incubation in an aerobic environment in the Kligler iron agar (KIA) and Mueller Hinton agar medium enriched with both sodium thiosulfate and ferric ammonium sulfate (Figure 1). Attempts to agglutinate the strains with polyvalent *Salmonella* antisera yielded negative results. When streaked on CHROMagar Orientation media, *E. coli* produced small, pink-red colonies that were characteristic of the species. Routine biochemical tests identified the strains as *E. coli*, except for their ability to reduce thiosulfate to H_2S (Table 1). All four isolates were gram-negative rods, motile, oxidase-negative, catalase-positive and indole positive. All isolates tested showed a lack of urease and Voges-Proskauer reaction, and they did not grow on the Simmons Citrate agar medium. Nonetheless, all isolates exhibited a positive result for O-nitrophenyl-beta-D-galactopyranoside (ONPG) and carried out fermentation of glucose and lactose sugars, leading to gas production (Figure 1). The API results revealed two distinct profiles, 5,544,512 ($n = 3$), and 5,544,552 ($n = 1$) and confirmed isolates as *E. coli* with 99% certainty (Table 1). The optimum growth temperate ranged between 26° to 42°C and they produced gamma-haemolysis on sheep blood agar.

Molecular and phylogenomic analysis of H_2S-positive *E. coli* genomes

This analysis included the four in-house strains and a genome of H_2S-producing *E. coli* reported from China (China_H_2S). WGS-based species identification confirmed all the isolates as *E. coli*. Across the five H_2S-producing *E. coli* strains, the average genome size was 4,853,304 bp (range 4,501,832 to 5,198,676) with an average GC content of 50.6% (range: 50.4 to 50.9%). The genome assemblies had an average coverage of 138-fold, with a range of 102 to 206-fold (Table 3). They had five distinct STs, which comprised ST10, ST48, ST12434, ST189, and ST12066. We detected four clonal complexes that included CC10 (two strains from human sources) followed by CC155, CC165 and CC206, representing one strain each (Figure 2). We identified four isolates (80%) belonging to commensal phylogroup A and one isolate (20%) to B1 phylogroup. All H_2S-producing *E. coli* isolates exhibited distinct serotypes and CH types. A phylogenetic tree was constructed for five H2S-producing *E. coli* genomes using the MG1655 genome as a reference, by aligning the core genome single nucleotide polymorphisms (SNPs). The studied H_2S-producing *E. coli* strains were found to be relatively diverse. However, the three poultry H_2S-positive strains from Bangladesh clustered together, with human strains adjacent to this cluster. The molecular characteristics did not correlate with the source of origin or the phylogenetic clustering of the H_2S-producing *E. coli* isolates (Figure 2).

2 http://omics.informatics.indiana.edu/CRISPRone

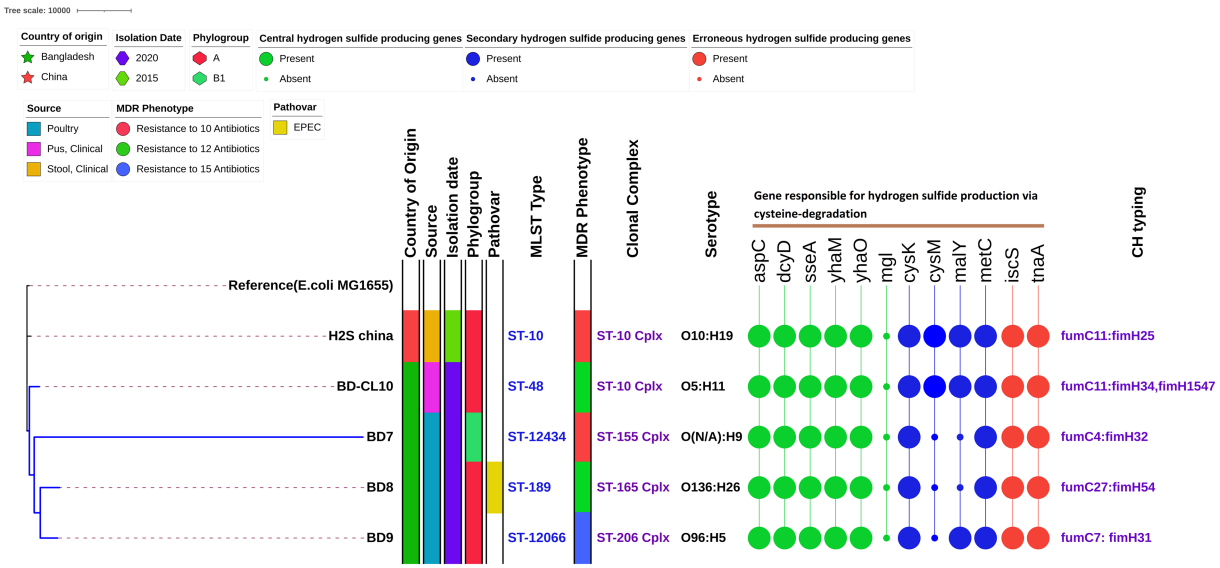

FIGURE 2
Phylogenetic relationships among sequenced Hydrogen Sulfide (H_2S)-Producing *Escherichia coli* genomes. The maximum likelihood phylogenetic tree is based on the alignment of detected core genomes with the MG1655 genome strain as a reference genome. The country of origin, isolation date, sample source, phylogroups, pathovar, MDR status, STs, clonal complex, serogroups, genes responsible for hydrogen sulfide production via cysteine-degradation and CH types are shown next to the tree.

TABLE 4 Cysteine-degradation genes and their location in the H_2S-producing *Escherichia coli* genomes.

Cysteine-degradation based H2S producing Gene		Genome locus				
		BD7	BD8	BD9	BD-Cl 10	China_H2S
aspC	Cysteine aminotransferase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
dcyD	Cysteine desulfhydrase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
sseA	3-mercaptopyruvate sulfurtransferase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
yhaM	yhaOM operon	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
yhaO		Chromosome	Chromosome	Chromosome	Chromosome	chromosome
mgl	Methionine gamma-lyase	Absent	Absent	Absent	Absent	Absent
cysK	Cysteine synthase A	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
cysM	Cysteine synthase B	Absent	Absent	Absent	Chromosome	chromosome
malY	Cystathionine beta-lyase like; repressor of maltose regulon	Absent	Absent	Chromosome	Chromosome	chromosome
metC	Cystathionine beta-lyase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
iscS	Cysteine desulfurase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome
tnaA	Tryptophanase	Chromosome	Chromosome	Chromosome	Chromosome	chromosome

H_2S-producing genes

All five H_2S-producing *E. coli* genomes harbored five primary hydrogen sulfide-producing genes; cysteine aminotransferase (*aspC*), cysteine desulfhydrase (*dcyD*), 3-mercapto pyruvate sulfurtransferase (*sseA*), yhaOM operon (*yhaM, yhaO*). Whereas the methionine gamma-lyase (*mgl*) gene was completely absent. Two of the four secondary function genes, cysteine synthase A (*cysK*) and cystathionine beta-lyase (*metC*) were found in all four strains. However, the other genes such as cysteine synthase B (*cysM*), and cystathionine beta-lyase-like repressor of maltose regulon (*malY*) are sparingly present in poultry isolates. The erroneous H_2S-producing genes, including cysteine desulfurase (*iscS*) and tryptophanase (*tnaA*) were observed in all isolates. As expected, all three class of cysteine-degradation genes were found on chromosomes (Figure 2; Table 4).

Plasmid replicon types

PlasmidFinder identified 20 unique plasmid replicon groups (Table 5). All five isolates harbored multiple plasmid replicons. The plasmid replicons identified include IncFII (pHN7A8), IncFII

TABLE 5 Plasmid replicon, integrons, ESBL genes and genetic context of ESBL genes in the H_2S-producing *Escherichia coli* isolates.

Strain	Plasmid replicon	Integrons	ESBLs producing gene		
			ESBLs gene	Genome locus	MGEs
BD7	IncHI2, IncHI2A, IncN, IncQ1, IncX2, p0111	Class 1 integron	bla_{TEM-1B}	Plasmid	–
			$bla_{TEM-106}$	Plasmid	–
			$bla_{TEM-126}$	Plasmid	–
			$bla_{TEM-135}$	Plasmid	–
			$bla_{TEM-220}$	Plasmid	–
BD8	ColE10, ColRNAI, IncFII (pHN7A8), IncFII (pSE11), IncN, IncX1, IncY	Class 1 integron	bla_{TEM-1B}	Plasmid	ISKra4
			$bla_{CTX-M-55}$	Plasmid	–
BD9	Col (MG828), Col (pHAD28), IncFIB (K), IncFIB (pLF82PhagePlasmid), IncI (Gamma), IncN, IncX2	Class 1 integron	bla_{TEM-1B}	Plasmid	–
			$bla_{CTX-M-65}$	Plasmid	–
BD-Cl 10	ColRNAI, IncFIA (HI1), IncFIB (K), IncFIB (pB171)	Class 1 integron	bla_{TEM-1B}	Plasmid	IS6100R
			$bla_{CTX-M-123}$	Chromosome	ISEcp1
H2S _China	IncR, IncX1	Class 1 integron	bla_{TEM-1B}	Plasmid	–
			mcr-1.1	Plasmid	–

"-" = not detected.

(pSE11), IncFIA (HI1), IncFIB (K), IncFIB (pLF82-PhagePlasmid), IncFIB (pB171), IncHI2, IncHI2A, IncI (Gamma), IncN, IncQ1, IncR, IncX2, IncX1, IncY, ColE10, ColRNAI, Col (MG828), Col (pHAD28) and p0111 (Table 5). The majority of isolates (4/5; 80%) harbored the IncX, followed by (3/5; 60%) IncF (FII, FIB, FIA), IncN and Col. The H_2S-producing *E. coli* strains that were positive for the bla_{CTX-M} gene were significantly linked to IncF-type replicons (specifically FIA, FIB, and FII) and CoI plasmids.

Prophage analysis

We detected intact prophages in the Bangladeshi H_2S-producing *E. coli* strains, but not in the Chinese isolate. The poultry strains harbored two to three intact prophage sequences, while the human clinical strains carried four intact prophage sequences. Incomplete prophage sequences ranged from four to seven in poultry strains and one in human strains, while questionable prophage sequences ranged from zero to two in poultry strains and were absent in human strains identified in Bangladesh. However, five incomplete prophage sequences were detected in H_2S-producing *E. coli* from China. The most common phages in the H_2S-producing *E. coli* strains were *Klebsiella* phage 4 LV-2017 (2/5) and *Shigella* phage SfII (2/5), with both phages present together in a single H_2S-producing human clinical *E. coli* strain (Table 3).

CRISPR-CAS system

The CRISPR-CAS system subtype I-A and I-E were found to be the most prevalent in the five H_2S-producing *E. coli* genomes. All H_2S-producing *E. coli* strains obtained from both poultry and human sources had only one CRISPR locus. The number, nucleotide sequence, and average length of repeats and spacers were similar in all H_2S-producing *E. coli* strains, but they varied in the quantity of repeats and spacer units. The human clinical strain BDCl_10 was comparable to poultry strains, except that it had a higher number of repeats and spacers than the poultry strains (as shown in Table 3).

Virulome

The virulome analysis of H_2S-producing *E. coli* isolates revealed the predominance of virulence factors (VFs) (Figure 3). The H_2S-producing *E. coli* isolates showed a 93% mean probability of being human pathogens using the PathogenFinder web-server. The isolate BD8 harbored the highest number of VFs (57), followed by the isolates BD9 (48), BD7 (40), BD-Cl10 (32) and China_H2S (30). All isolates (5/5) harbored the type I fimbriae genes *fim* (A, C–D, E–H, l). All of the isolates showed the presence of invasin of brain endothelial cells locus B (*ibeB*) and invasin of brain endothelial cells locus C (*ibeC*) genes, which belong to the invasin virulence factor category (Figure 3). The *E. coli* laminin-binding fimbriae genes (ELF) *elfA, elfC, elfD, elfG* were also present (5/5) in all *E. coli* isolates. However, hemorrhagic *E. coli* pilus (HCP) genes associated with the production of type IV pili were highly prevalent of which *hcpA* gene was most predominant (100%, 5/5) followed by *hcpC* (80%, 4/5). Non-LEE encoded T3SS (Type III Secretion System) related genes specifically *espL1, espL4, espR1, espX1, espX4, espX5* were observed in almost all H_2S-producing *E. coli* isolates. The autotransporter genes such as *aatA, cah, ehaB* were also prevalent (80%, 4/5) (Figure 3). The *hlyE/clyA*, a pore-forming toxin was observed in 60% (3/5) of isolates. One H_2S-producing *E. coli*

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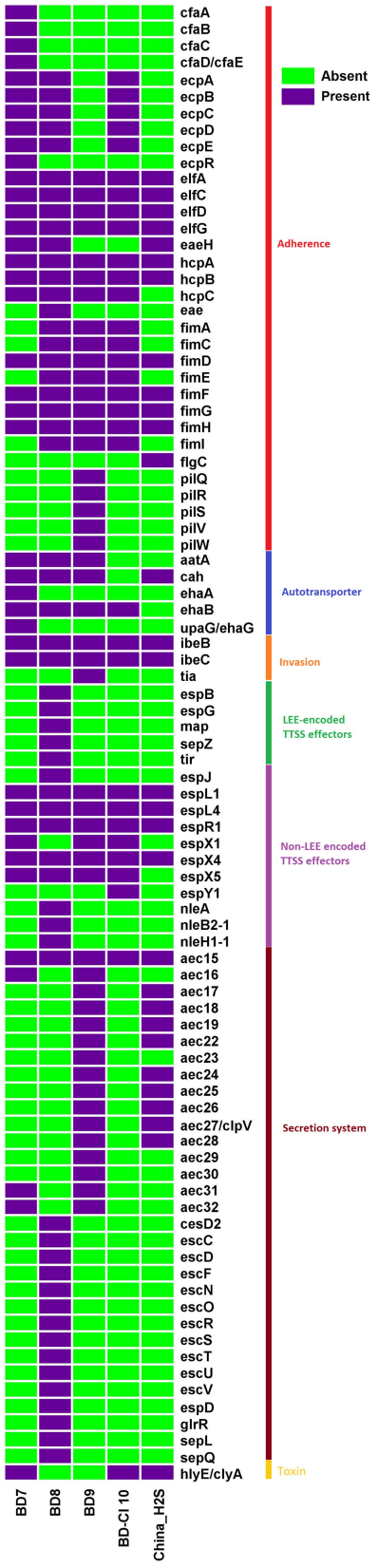

FIGURE 3
Heat map depicting the distribution of 89 virulence genes among 5 Hydrogen Sulfide (H_2S)-producing *Escherichia coli* genomes. Dark violet blocks represent the presence, and light green blocks represent virulence gene absence.

strain BD8 harbored the *Intimin* related *eae* gene and was classified as Enteropathogenic *E. coli* (EPEC). Overall, the poultry *E. coli* isolates (87) harbored higher number of VFs than human isolates (44).

Antimicrobial resistance phenotypes and genotypes

All four (100%) H_2S-producing *E. coli* isolates from Bangladesh were resistant to ampicillin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim, doxycycline, and tigecycline. While they were all sensitive to amikacin, imipenem, meropenem, and colistin. However, three isolates (75%) were resistant to ceftazidime, cefotaxime, cefepime, cefuroxime, and ceftriaxone. Whereas 50% of isolates were resistant to chloramphenicol, fosfomycin, and gentamicin. All four H_2S-producing *E. coli* isolates were classified as MDR.

We identified 43 distinct AMR gene alleles belonging to various classes (Figure 4). A minimum of seven AMR genes per genome were detected with some variation across strains (poultry *E. coli* 13–25; human *E. coli* 7–12). All (5/5) the H_2S-producing *E. coli* genomes harbored beta-lactamase genes. All isolates were positive for the bla_{TEM1B} gene (100%). The bla_{CTX-M} gene alleles ($bla_{CTX-M-55}$, $bla_{CTX-M-65}$, and $bla_{CTX-M-123}$) were detected in 3 out of 5 H_2S-producing *E. coli* genomes (Figure 2). The bla_{TEM1B} and bla_{CTX-M} variants coexisted in three isolates (60%, 3/5). Among the 14 aminoglycoside resistance genes identified, *aadA1* was predominant (60%, 3/5) followed by *aadA2*, *aph* (*3′*)-*Ia*, *aph* (*3″*)-*Ib*, and *aph* (*6*)-*Id* genes detected in 2 genomes (2/5). In addition, *aadA5*, *aac* (*3*)-*IId*, *aac* (*3*)-*IV*, and *aac* (*6′*)-*Ib3* genes were found in one genome (20%, 1/5). All *E. coli* genomes harbored a *tet* (*A*) gene encoding tetracycline resistance. One isolate (BD7) harbored both *tet (A)* and *tet (M)* genes. The predominant sulfamethoxazole resistance gene was *sul3* (4/5) followed by *sul2* (1/5). Among the 4 different trimethoprim resistance genes identified, *dfrA12* was predominant (40%, 2/5). Macrolide-associated resistance gene *mph* (*A*) was commonly detected (4/5). Phenicol resistance gene *floR* was predominantly (60%,3/5) found, followed by *cmlA1* (40%, 2/5) gene. The efflux, small multidrug resistance transporter gene, *qacL*, was also detected in a poultry isolate (BD7). None of the isolates harbored carbapenemase genes and did not show phenotypic resistance to carbapenem antibiotics. Overall, the average number of AMR genes per genome was highest in poultry *E. coli* compared to human *E. coli* isolates (Table 2). The probable genome locus of the bla_{TEM1B} and bla_{CTX-M}-group genes were plasmids for two strains (Table 3). In BD-Cl10 isolate, the bla_{CTX-M} gene was found on a chromosome with insertion element ISEcp1. The bla_{TEM1B} gene in the BD8 strain and BD-Cl10 isolate was linked with insertion elements ISKra4 and IS6100R, respectively (Table 5). We identified amino acid substitutions in *gyrA* at codon positions S83L (4/5) and D87N (1/5), and in *parC* at S80I (2/5), S80R (1/5) and A56T (1/5). There was a significant correlation between the *gyrA* S83L mutation and resistance to ciprofloxacin. The ESBLs genes bla_{TEM1B}, bla_{CTX-M}-group, and *gyrA* S83L were associated with H_2S-producing *E. coli* strains. Additionally, all the isolates harbored PMQR genes, including *Qnrs1* (2/5), *Qnrs4* (1/5) and *Qnrs13* (1/5). The *Qnrs13* gene in the BD8 strain and *Qnrs4* in the BD-Cl10 strain consisted of the insertion element ISKra4 (Table 3). The β-lactamase genes, PMQRs and QRDRs were all strongly associated with the MDR phenotype.

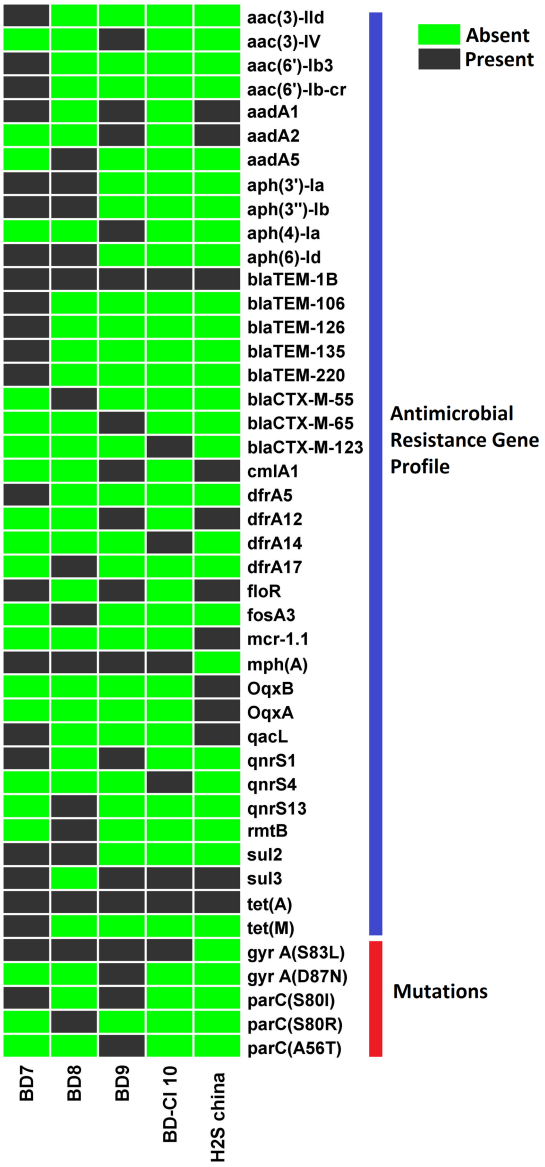

FIGURE 4
Heat map demonstrating the antimicrobial resistance (AMR) genes profile of 5 Hydrogen Sulfide (H_2S)-producing *Escherichia coli* genomes. Dark black blocks represent the presence of AMR genes, and light green blocks represent the absence of particular genes.

Discussion

Production of hydrogen sulphide (H_2S) is seen in many members of *Enterobacteriaceae*. However, it is well established that *E. coli* strains are H_2S non-producers. H_2S non-production is one of the key characteristics used to identify *E. coli* in laboratory tests. Nonetheless, a fraction of H_2S-producing *E. coli* variants has previously been identified in animal and human infections (Maker and Washington, 1974; Sogaard, 1975; Magalhães and Vance, 1978). By studying H_2S-producing *E. coli*, researchers can better understand the biology and behavior of such variants and develop improved diagnostic tests. Further, a comprehensive characterization of H_2S-producing *E. coli* including analysis of genomic features was needed. To address this, we conducted a thorough investigation of four H_2S-producing *E. coli* variants by utilizing whole-genome sequencing (WGS) in combination with comprehensive microbiological and biochemical testing.

The four bacterial isolates were recovered from poultry and human clinical samples in Dhaka, Bangladesh, as part of a larger surveillance study. These isolates biochemically mimic typical *E. coli* for all reactions except for one reaction, the H_2S production. The prevalence of H_2S-producing variants in our study can be estimated at 3% (3/96) in poultry and 0.5% (1/204) in clinical *E. coli* isolates. However, this may not reflect the true prevalence figures, as in this study, the primary specimens were not screened targeting H_2S-producing *E. coli*. But only the archived *E. coli* isolates were tested. However, our estimates of prevalence are similar to those previously reported (Maker and Washington, 1974; Sogaard, 1975; Weber et al., 1981). Our and other reports reveal that H_2S-positive strains of *E. coli* are not uncommon among poultry and human clinical samples (Braunstein and Mladineo, 1974; Maker and Washington, 1974; Sogaard, 1975; Traub and Kleber, 1975; Magalhães and Vance, 1978; Weber et al., 1981; Barbour et al., 1985). Many such variants are probably misidentified in laboratories, such as *Citrobacter*, *Arizona* and *Salmonella* (Darland and Davis, 1974). This misidentification stems from the production of black precipitate on KIA or TSI medium. It is also possible that acid production sometimes masks H_2S production due to lactose fermentation (Magalhães and Vance, 1978). Muller-Hinton agar supplemented with sodium thiosulfate and ferric ammonium sulfate media is considered superior to KIA agar media for identifying H_2S production. The utility of the same has been demonstrated in this study. However, the CHROMagar Orientation media could not differentiate between typical *E. coli* and H_2S-producing *E. coli* variants. Primary screening with this media can effectively screen typical *E. coli* and H_2S-producing *E. coli* variants in a single step.

The studied H_2S-producing *E. coli* strains mainly belonged to the commensal phylogenetic groups A (80%,4/5) and B1 (20%, 1/5). Several reports confirm that phylogroups A and B1 were the most prevalent among *E. coli* isolates, particularly in the gut microbiome (Li et al., 2010; Stoppe et al., 2017). The H_2S-producing *E. coli* strains of human origin, isolated from Bangladesh and China, belonged to the worldwide predominant clonal complex CC10. CC10 group of strains belong to emerging clone of extra-intestinal pathogenic *E. coli* (ExPEC) (Manges et al., 2019). They are isolated from a wide range of niches including clinical settings, food animals and environment (Manges et al., 2019). They are also known to be associated with wide range of AMR and virulence genotypes (Massella et al., 2021). This group of *E. coli* needs close monitoring to safeguard public health (Hussain et al., 2023). We identified 8–13 prophage regions in H_2S-producing *E. coli*, of which 2–4 were found intact. *Klebsiella* phage 4 LV-2017 and Shigella phage SfII were the predominant bacteriophages detected. The existence of a higher number of phage elements (8 to 13) in poultry strains compared to the human clinical strain (5) may indicate more horizontal gene transfer (HGT) events that brought in more toxin genes in poultry strains than in the human clinical strain. The CRISPR-Cas system confers immunity against viruses and plasmids (Horvath and Barrangou, 2010). Investigation of the CRISPR-Cas system in H_2S-producing *E. coli* strains indicated that it

was conserved in both poultry and human clinical H_2S-producing *E. coli* isolates.

Previous work has identified cysteine-degradation genes in H_2S-producing bacteria and classified them into primary, secondary and erroneous categories based on their functions (Braccia et al., 2021). Most primary producer genes (*aspC, dcyD, sseA, yhaOM* operon) were present in all H_2S-producing *E. coli* strains. In the case of secondary producer genes, we observed inconsistent results. But all erroneous genes were present in the study isolates. We found all genes related to H_2S production on chromosomes, which is in line with the previous report (Braccia et al., 2021).

The patterns of antibiotic resistance were similar for human and poultry isolates. High resistance rates were observed for ampicillin, ciprofloxacin, nalidixic acid, trimethoprim and sulfamethoxazole, doxycycline and cephalosporin. Our findings show partial agreement with the previous report on H_2S-producing *E. coli* (Braunstein and Mladineo, 1974; Maker and Washington, 1974; Sogaard, 1975; Traub and Kleber, 1975; Magalhães and Vance, 1978; Weber et al., 1981; Barbour et al., 1985; Park et al., 2015). The H_2S-producing *E. coli* isolates contained multiple plasmids. The major replicon types were IncX (4/5; 80%) and IncF (3/5; 60%). As per earlier reports, these plasmid replicons were associated with fluoroquinolone resistance and $bla_{\text{CTX-M-group}}$ in humans and livestock *E. coli* (Phan et al., 2015; Sun et al., 2017). As healthy animals and humans were found to harbor H_2S-producing *E. coli* (Sogaard, 1975; Biswas et al., 2020), the presence of these plasmids may contribute as careers of antibiotic resistance in microbiomes. The results of our study suggests that aminoglycosides and carbapenem antibiotics are effective candidates against these strains. However, this cannot be generalized due to several limitations of our study and it is always better to initiate evidence-based treatment of diseases arising from infectious agents.

All isolates were predicted as human pathogens as per their pathogenicity score determined by *in silico* analysis. The studied H_2S-producing *E. coli* isolates harbored at least 30 virulence factors. Among them, poultry isolates had more virulence genes (40–57 VFs) than human samples. The H_2S-producing *E. coli* isolates harbored a wide range of virulence factors encoding *E. coli* laminin-binding fimbriae (ELF) (*elfA,C,D,G*), Hemorrhagic *E. coli* pilus (HCP) (*hcpA-B*), Type I fimbriae (*fimD, fimF, fimG, fimH*) and Non-LEE encoded TTSS effectors (*espL1, espL4, espR1, espX4*). The intimin (*eae*) gene, a marker for enteropathogenic *E. coli,* was observed in one H_2S-producing *E. coli* isolate (BD8) belonging to ST189. This indicates that *E. coli* pathotypes also exhibit H_2S production features or vice versa. Therefore, virulence genes play an important role in the pathogenicity of H_2S-producing *E. coli* strains. Also, the convergence of wide range of AMR and virulence genotypes is a cause of great concern (Massella et al., 2021). These observations warrant studying the role of H_2S-producing *E. coli* isolates in different infections for developing effective treatments and preventive measures.

In conclusion, this study investigated H_2S-producing *E. coli* variants recovered from poultry and human clinical samples in Dhaka, Bangladesh. The isolates were confirmed as *E. coli* by routine biochemical tests and WGS-based species identification. The H_2S-producing isolates exhibited relatively diverse molecular characteristics with no correlation between the source of origin or the phylogenetic clustering of the isolates. The study also found high rates of AMR and extensive virulence gene repertoire in these isolates. The findings of this study highlight that the genomic features, antibiotic resistance and virulence potential of H_2S-producing *E. coli* resemble the typical *E. coli* forms. Therefore, we suggest the need for continued surveillance and genomic characterization of atypical *E. coli* forms like H_2S-producing *E. coli* to better understand the characteristics of such variants and improve diagnostics and treatment outcomes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA882002, PRJNA714244.

Author contributions

RM designed the study and conducted all microbiological tests, and whole genome sequencing. RM and AH carried out the bioinformatics analyses interpretation of results, prepared tables and figures, and drafted the manuscript. MR and RM performed the sample collections and initial sample processing. AH contributed to the discussions, manuscript writing, editing, and proofreading. AA, JP, SC, TC, and DM contributed to the discussions and reviewed the manuscript. DM managed the funds and supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin

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Healthy, untreated cows of nine dairy herds from the Swiss Canton Tessin were analyzed three times within one year to identify the most abundant species of the intramammary bacteriome. Aseptically collected milk samples were cultured and bacteria identified using MALDI-TOF. Of 256 cows analyzed, 96% were bacteriologically positive and 80% of the 1,024 quarters were positive for at least one bacterial species. 84.5% of the quarters were healthy with somatic cell counts (SCC) < 200,000 cells/mL, whereas 15.5% of the quarters showed a subclinical mastitis (SCC ≥ 200,000 cells/mL). We could assign 1,288 isolates to 104 different bacterial species including 23 predominant species. Non-*aureus* staphylococci and mammaliicocci (NASM) were most prevalent (14 different species; 73.5% quarters). *Staphylococcus xylosus* and *Mammaliicoccus sciuri* accounted for 74.7% of all NASM isolates. To describe the intramammary resistome, 350 isolates of the predominant species were selected and subjected to short-read whole genome sequencing (WGS) and phenotypic antibiotic resistance profiling. While complete genomes of eight type strains were available, the remaining 15 were *de novo* assembled with long reads as a resource for the community. The 23 complete genomes served for reference-based assembly of the Illumina WGS data. Both chromosomes and mobile genetic elements were examined for antibiotic resistance genes (ARGs) using in-house and online software tools. ARGs were then correlated with phenotypic antibiotic resistance data from minimum inhibitory concentration (MIC). Phenotypic and genomic antimicrobial resistance was isolate-specific. Resistance to clindamycin and oxacillin was most frequently observed (65 and 30%) in *Staphylococcus xylosus* but could not be linked to chromosomal or plasmid-borne ARGs. However, in several cases, the observed antimicrobial resistance could be explained by the presence of mobile genetic elements like *tetK* carried on small plasmids. This represents a possible mechanism of transfer between non-pathogenic bacteria and pathogens of the mammary gland within and between herds. The-to our knowledge-most

extensive bacteriome reported and the first attempt to link it with the resistome promise to profoundly affect veterinary bacteriology in the future and are highly relevant in a One Health context, in particular for mastitis, the treatment of which still heavily relies on antibiotics.

KEYWORDS

One Health, mastitis, intramammary bacteria healthy cows, antimicrobial resistance genes, whole genome sequencing, type strains, plasmids, antibiotics

1. Introduction

Bovine mastitis, also referred to as bovine intramammary infection (IMI) caused by pathogens, is the most important and costly disease of dairy cows worldwide (Seegers et al., 2003). Severe economic losses in dairy cattle herds are caused by four main factors: reduced milk yield, unsuitability of the milk for consumption, antibiotic treatment costs, and culling of animals in case of treatment failure (Ruegg, 2017). In the frame of the One Health concept, which advocates a general view of human, animal and environmental health, research aimed at describing the bacterial diversity of both commensals and potential pathogens in animal food production systems is highly relevant (Aslam et al., 2021). Such research is expected to considerably reduce the use of antibiotics and thereby the associated dangers of transfer of antibiotic resistance genes (ARGs) and spreading of multi-resistant strains (McEwen and Collignon, 2018). While IMI by pathogens is well understood, very little is known about the bacteria present in the mammary gland of healthy and untreated cows and their antimicrobial resistances (AMR) (= intramammary resistome, IR). A few studies of the milk microbiota, relying both on culture-dependent and culture-independent approaches have been carried out in recent years [reviewed in Quigley et al. (2013) and Oikonomou et al. (2020)]. They arrived at the preliminary conclusion that milk of healthy udders is not a sterile matrix, but instead, harbors a complex microbial community composed of different microorganisms (Addis et al., 2016).

In subclinical IMI, non-*aureus* staphylococci (NAS) and *Streptococcus uberis* are the most frequently isolated bacteria (De Visscher et al., 2015). Despite NAS being considered less pathogenic than *Staphylococcus aureus*, they can carry virulence factors, toxins and antibiotic resistance genes and are able to generate biofilms (Vanderhaeghen et al., 2014; Taponen et al., 2016). However, their potential pathogenicity needs to be further clarified and evaluated in more detail. Potentially, they could just represent commensal microorganisms of the normal flora in the mammary gland (De Buck et al., 2021). A recent Swiss bacteriome study by Sartori et al. (2018) reported that NAS were the main bacteria colonizing healthy cows with *Staphylococcus xylosus* and *Staphylococcus chromogenes* representing the most frequent isolates from the milk of the selected herds. However, the IR of healthy cows was not assessed in that study.

Only recently in 2020, five species belonging to the NAS were reclassified in the novel genus *Mammaliicoccus* due to an evolutionary study based on 16S sequencing conducted by Madhaiyan et al. (2020). Nowadays, the staphylococci and mammaliicocci are indicated with the acronym of non-*aureus* staphylococci and mammaliicocci (NASM) (Rosa et al., 2022).

In Switzerland, the application of antibiotics (AB) in agriculture has been decreasing over the last years (2010–2019), evidenced by a 52% reduction of the sales of ABs used in livestock animals since 2010 (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). In part, this reduction can be attributed to the fact that critical antibiotic classes for human medicine (fluoroquinolones, macrolides, and 3rd/4th generation cephalosporins) were banned to be given for stocks due to the Ordinance on Veterinary Medicinal Product, in line with the aims of the One Health approach (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2018). However, in contrast to this overall reduction, the use of antimicrobials licensed for treatment of IMI was relatively stable during 2010–2019 (Switzerland in fact has the highest use of intramammary products in Europe) (European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption, 2021). Overall, 70% of all antimicrobials administered concern antibiotics for the treatment of mastitis during lactation. The main antibiotic is penicillin followed by aminoglycosides and cephalosporins. This high rate of applications could represent an important reason for the development of resistances in pathogens inducing mastitis episodes and in bacteria colonizing the healthy mammary gland (Oliver and Murinda, 2012).

Unfortunately, at the European level only resistance data of NASM strains responsible for clinical mastitis are available which displayed a high resistance to penicillin G and oxacillin (29.1 and 43.9%, respectively) (El Garch et al., 2020). In contrast, no phenotypic resistance data are available regarding the intramammary bacteriome of healthy cows except for one Swiss study from Frey et al. (2013). In this study, NASM were isolated from control milk, i.e., milk from healthy cows previously positive to mastitis, subjected to treatment, with the characteristic to have somatic cell counts (SCC) <150,000 cells/mL. The results of this study showed a prevalence of phenotypic resistance between 17 to 40% to the antibiotics oxacillin, fusic acid, tiamulin, penicillin, tetracycline, and streptomycin. Increasing research on comparing the resistance profiles of isolates from different countries could provide relevant insights into treatment strategies of affected herds.

Advances in whole genome sequencing (WGS) and the availability of online tools supporting researchers in the identification of antimicrobial resistance genes (ARGs) are important pre-requisites for studying not only the abundance and dissemination of AMR (Hendriksen et al., 2019), but also the potential transfer of ARGs from species colonizing animals to species infecting humans (Wendlandt et al., 2015). The transfer of ARGs commonly involves mobile genetic elements (MGEs). The most prevalent ones are plasmids, i.e., extrachromosomal DNA molecules that encode genes that play a role,

among others, in virulence, antibiotic resistance, tolerance to heavy metals, and metabolism of carbon sources (Malachowa and Deleo, 2010). The classification of plasmids based on the replicon protein (Rep) is an important approach that can be used to examine the distribution of such MGEs in the environment (Orlek et al., 2017).

In the present study, more than 1,200 bacterial isolates were identified allowing to describe 23 predominant species of the intramammary bacteriome of healthy cows. Furthermore, WGS and phenotypic profiling was carried out for 350 isolates from the 23 most abundant species to infer the resistome (IR) at the phenotypic and the gene level and attempt to link phenotype and genotype information. The results are discussed under the aspects of diagnostic and clinical importance as well as of the One Health approach.

2. Materials and methods

2.1. Study design and sample collection

Nine different herds were randomly selected in the Swiss Canton of Tessin. Quarter milk samples were collected aseptically 3 times during winter 2017–2018 (time point 0), late spring 2018 (time point 1, sampling was performed before the cows were sent for common pasturing on alps during the summer season), and winter 2018–2019 (time point 2) from at least 10 randomly selected lactating cows (unless stated otherwise; Table 1), following the guidelines of the National Mastitis Council [National Mastitis Council (NMC), 2016]. Prerequisites for inclusion in the study were that the cows (i) did not receive any antibiotic therapy within the previous five days, (ii) did not show any clinical signs of mastitis or teat injuries, (iii) appeared visually normal, and (iv) their milk was suitable for human consumption according to Swiss legislation (VHyMP 2020[1]). Data regarding age, lactation number, and stage of lactation of the cows were collected. Considering the stage of lactation, we referred to three different stages divided in early (14–100 days after calving), mid (100–200 days after calving), and late lactation (>200 days after calving). For lactation number, we divided the cows into three different groups: (i) 1st lactation (primiparous), (ii) 2nd and 3rd lactation, and (iii) >3 lactations. For herd 6, only samples from the 1st and 2nd sampling could be collected as the farm was given up later.

2.2. Analysis of somatic cell counts

Somatic cell counts (SCC) in individual quarter milk samples (identical to those used for bacteriological analyses, see below) were used to differentiate between healthy quarters and those with subclinical mastitis (cows and quarters with clinical forms of mastitis were strictly excluded from the study). According to the International Dairy Federation (IDF), a quarter was considered healthy if SCC were < 200,000 cells/mL, independent on number and stage of lactation (International Dairy Federation, 2022). Values above were considered as a quarter with subclinical mastitis. Total SCC were analyzed in frozen milk samples using the recently published flow cytometry method by Widmer et al. (2022). For analysis, the samples were defrosted at room temperature and gently mixed by inversion. The impact of freezing on SCC using this method was tested with 120 raw milk samples and the average decrease in cell numbers was 6.3%.

2.3. Bacteriological analyses and identification

Bacteriological analyses were performed following the *Laboratory Handbook on Bovine Mastitis* of the National Mastitis Council [National Mastitis Council (NMC), 2017]. In brief, 10 µL of each milk sample was streaked out on sheep blood agar (BA) plates (Biomèrieux Suisse SA, Geneva, Switzerland) and bacterial colonies obtained after 24 and 48 h of aerobic incubation at 37°C were evaluated based on their morphology. Samples were considered "contaminated" and not included in the study, if more than 3 morphologically different bacterial colonies could be identified (Wyder et al., 2011). Representatives of each colony type were selected for bacterial identification using MALDI-TOF MS according to the protocols of the manufacturer (Bruker Daltonics GmbH, Bremen, Germany). Analysis was performed by a Microflex LT MALDI-TOF instrument using the MALDI Biotyper (MBT) Compass Library 7,311 (both Bruker Daltonics GmbH). Isolates with a score ≥ 2.2 were identified at the species level. All distinct isolates were conserved in skim milk at-20°C for subsequent analyses.

2.4. Selection of isolated bacterial species for resistome analysis

By analyzing all morphologically distinct colonies for each milk sample by MALDI TOF MS, a total of 1,288 isolates were obtained. To reduce the number of isolates and to restrict it to the most relevant bacteria for each herd and sampling, the following selection procedure was performed: (1) For each BA plate, all morphologically different colonies with an abundance ≥5 were taken, identified, and the corresponding bacteria registered in a frequency table. The table was then sorted in descending order according to the observed frequencies. Starting from top, bacteria were selected until the sum of their frequencies resulted in ≥85% of the total frequency. (2) For each of these selected, relevant bacteria, 5 isolates (if available) were then randomly chosen resulting overall in 350 isolates that were later subjected to phenotypic AMR testing and bioinformatic ARGs analysis after whole genome sequencing (WGS).

2.5. Phenotypic antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) were determined for all 350 isolates using different antibiotics panels that accounted for the respective characteristics of the bacterial species. All tests were performed according to the manufacturer's instructions of the Microscan System (Beckman Culture Microbiology, West Sacramento, CA). For details see the Supplementary Methods Section in the Supplementary materials.

1 https://www.fedlex.admin.ch/eli/cc/2005/824/de

TABLE 1 Overview of the within-herd bacterial positivity for the three sampling time points.

Number of quarters positive per farm and sampling																											
	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5			Farm 6		Farm 7			Farm 8			Farm 9			Total
Sampling	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	0	1	2	0	1	2	0	1	2	
Type of Bedding	Straw, sawdust			Straw, sawdust			Straw			Straw, ferus			Straw			Straw		Straw			Straw			Manure			
N. cow sampled	11	10	10	9	8	9	10	10	10	10	10	10	10	10	10	9	10	10	10	10	10	10	10	10	10	10	256
N. cow bacteriologically positive	11	10	10	9	8	8	10	10	10	10	10	7	10	10	9	9	10	10	10	10	10	10	9	10	10	10	250
N. quarter sampled	44	40	40	36	32	36	40	40	40	40	40	40	40	40	40	36	40	40	40	40	40	40	40	40	40	40	1,024
N. quarter bacteriologically positive	43	31	31	32	31	15	38	34	34	39	37	12	29	37	23	27	35	30	36	26	39	40	24	36	31	34	824
Quarters positive only with one bacterial species	18	24	18	4	22	6	15	17	18	26	15	10	19	14	15	17	15	20	18	17	20	15	18	21	19	19	440
Quarters positive with 2 bacterial species	24	5	10	23	8	7	17	11	12	11	13	1	9	17	6	8	17	10	15	8	14	24	5	11	8	11	305
Quarters positive with 3 bacterial species	1	2	3	5	1	2	6	6	4	2	9	1	1	6	2	2	3	0	3	1	5	1	1	4	4	4	79
Quarters positive only by one species NASM	18	17	15	4	22	4	14	15	16	10	8	6	9	7	10	7	12	14	13	8	15	14	17	7	1	4	287
Quarters positive only by two species NASM	24	4	10	23	8	6	17	11	11	10	12	1	5	12	4	5	12	7	15	7	13	23	5	7	2	3	257
Quarters positive only by three species NASM	1	2	3	5	1	2	6	6	3	1	8	1	1	6	1	2	3	0	3	1	5	1	1	3	2	1	69
NASM and *S. aureus*																											
Staphylococcus xylosus	30	14	25	20	9	6	21	17	11	4	20	7	10	19	8	6	22	21	29	10	2	0	1	3	3	5	323
Mammaliicoccus sciuri	18	6	8	29	24	10	7	23	0	2	9	0	2	15	0	0	5	1	16	3	31	38	4	11	2	1	265
Staphylooccus succinus	17	2	1	5	1	1	17	1	5	1	2	0	0	0	0	3	6	2	0	3	3	0	0	2	1	0	73
Staphylococcus equorum	0	0	0	0	0	0	0	0	7	0	0	0	3	0	7	1	0	0	0	0	1	0	13	0	0	0	32
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	7	3	2	3	0	0	0	11	2	0	32
Mammaliicoccus vitulinus	0	0	0	0	0	1	9	0	15	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	28

(Continued)

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TABLE 1 (Continued)

Number of quarters positive per farm and sampling																											
	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5			Farm 6		Farm 7			Farm 8			Farm 9			Total
Sampling	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	0	1	2	0	1	2	0	1	2	
Type of Bedding	Straw, sawdust			Straw, sawdust			Straw			Straw, ferus			Straw			Straw		Straw			Straw			Manure			
Staphylococcus chromogenes	0	1	1	0	0	0	0	2	3	0	0	0	0	0	3	0	0	0	0	0	2	0	2	1	1	1	17
Staphylococcus haemolyticus	0	1	2	0	0	0	0	2	0	0	0	2	0	0	0	1	0	1	0	4	0	0	2	0	0	0	15
Staphylococcus warneri	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	13
Others[1]	1	0	0	3	0	0	1	3	0	3	2	0	0	0	0	3	0	1	0	2	0	0	2	0	0	0	21
Bacillus cereus group[2]	0	3	2	0	3	0	2	3	2	1	13	1	1	13	4	0	3	1	2	0	5	18	0	17	12	9	115
Others *Bacillus*[3]	0	2	0	0	0	1	0	1	0	0	2	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	9
Acinetobacter spp.[4]	0	0	4	0	0	0	0	0	1	2	1	0	1	0	0	1	3	0	0	0	0	2	1	1	4	2	23
Aerococcus viridans	0	0	0	0	0	3	1	0	0	2	0	0	4	0	0	4	1	2	0	0	2	0	2	0	0	0	21
Arthrobacter spp.[5]	0	0	0	0	0	0	0	1	1	0	3	1	4	1	4	0	0	0	0	0	0	0	0	2	0	7	24
Enterococcus spp.[6]	0	0	0	0	1	0	1	0	0	0	3	0	1	0	0	0	0	0	0	1	2	0	0	4	8	21	42
Escherichia coli	0	1	0	3	0	0	0	3	0	0	0	1	0	2	0	0	1	0	0	1	5	4	0	0	7	1	29
Lactococcus spp. [7]	0	0	0	0	0	0	0	0	0	1	2	0	4	2	0	10	6	0	0	0	0	0	0	0	0	0	25
Streptococcus spp.[8]	0	0	1	0	0	2	2	0	0	8	6	1	0	0	4	0	0	1	3	6	1	0	0	1	1	0	37
Others[9]	1	6	1	2	0	0	5	0	6	14	5	1	5	11	3	5	4	3	2	2	7	4	0	1	1	3	92
Not reliable identification	2	4	2	3	3	2	1	0	3	3	0	1	5	2	0	1	0	4	3	1	2	0	2	1	4	3	52
Total																											1,288

Additional relevant information is provided, including the type of bedding used by the herds, the number of cows and the number of quarters. [1]*Staphylococcus epidermidis* (2), *Staphylococcus fleuretti* (2), *Staphylococcus gallinarum* (5), *Staphylococcus hyicus* (1), *Staphylococcus lentus* (3), *Staphylococcus simulans* (2), *Staphylococcus spp.* (6). [2]In this group are included *Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis* (115). [3]*Bacillus altitudinis* (1), *Bacillus licheniformis* (2), *Bacillus pumilus* (3), *Bacillus simplex* (1), *Bacillus subtilis* (1), *Bacillus spp.* (1). [4]*Acinetobacter towneri* (1), *Acinetobacter calcoaceticus* (4), *Acinetobacter guillouiae* (6), *Acinetobacter johnsonii* (3), *Acinetobacter lwoffii* (8), *Acinetobacter spp.* (1). [5]*Arthrobacter arilaitensis* (4), *Arthrobacter gandavensis* (8), *Arthrobacter histidinolovorans* (1), *Arthrobacter ilicis* (1), *Arthrobacter mysorens* (2), *Arthrobacter nicotinovorans* (1), *Arthrobacter protophormiae* (5), *Arthrobacter spp.* (2). [6]*Enterococcus faecium* (2), *Enterococcus faecalis* (5), *Enterococcus gallinarum* (1), *Enterococcus saccharolyticus* (34). [7]*Lactococcus garvieae* (16), *Lactococcus lactis* (9). [8]*Streptococcus agalactiae* (11), *Streptococcus dysgalactiae* (1), *Streptococcus parauberis* (7), *Streptococcus uberis* (17), *Streptococcus spp.* (1). [9]*Aeromonas spp.* (3), *Carnobacterium maltamaticum* (2), *Citrobacter spp.* (5), *Enterobacter cloacae* (3), *Erwinia persicina* (1), *Klebsiella spp.* (10), *Kluyvera spp.* (2), *Kocuria spp.* (2), *Lelliotta amnigena* (2), *Lysinibacillus sphaericus* (3), *Microbacterium arboriscens* (1). *Micrococcus luteus* (1), *Moraxella spp.* (1), *Neisseria macacae* (1), *Paenibacillus spp.* (1), *Pantoea agglomerans* (8), *Proteus spp.* (9), *Providencia rettgeri* (2), *Pseudomonas spp.* (21), *Rahnella aquatilis* (1), *Raoultella spp.* (4), *Serratia marcescens* (5), *Trueperella pyogenes* (2), *Vagococcus fluvialis* (1), *Vibrio cincinnatiensis* (1).

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2.6. Whole genome sequencing

Details concerning the extraction of genomic DNA for short and long read sequencing platforms to then first *de novo* assemble, polish and annotate complete genomes of type strains (15 of 23 that lacked a complete genome at NCBI) are described in detail in the Supplementary Methods, along with the respective strategies to assemble the reads even from highly repeat-rich and complex strains (Schmid et al., 2018). The complete type strain genomes served as a basis for reference-based assembly for the 350 WGS sequenced isolates (Illumina HiSeq platform) (Supplementary Tables S1–S4).

The 350 isolates were plated on sheep blood agar (BA) plates (Biomèrieux Suisse SA, Geneva, Switzerland) and incubated aerobically at 37°C for 18 h (h). Two to four single colonies were picked, resuspended in 5 mL BHI (Brain Heart Infusion Broth, Merck KGaA, Darmstadt, Germany) and incubated under the same conditions for 18 h. Subsequently, 200 µL of the pre-cultures were added to 100 mL of fresh BHI and incubated aerobically at 37°C for 18 h under constant shaking, before 50 mL were collected and centrifuged (18,000 × *g* for 5 min at 4°C). The supernatant was discarded, the pellet resuspended in 600 µL of buffer A1 (NucleoSpin® 8 Plasmid kit, Macherey-Nagel AG, Oensingen, Switzerland) and DNA isolated according to the manufacturer's protocol. The total amount and quality of DNA were evaluated by spectroscopy assessing the OD_{260}/OD_{280} ratio (QuickDrop; Molecular Devices, San Jose, CA) and a quantitative analysis (Qubit assay; Thermo Fisher Scientific, USA).

2.7. WGS and assembly

DNA samples ($n = 350$) were sequenced by Eurofins Genomics GmbH (Ebersberg, Germany) on the HiSeq sequencing platform (Illumina, San Diego, CA). The reads were first assembled using the complete genome of the type strain of the corresponding species as reference (Supplementary files *Illumina and long reads sequencing (PacBio/ONT) of type strains*) using SeqMan NGen 16 software (default settings) from the DNASTAR Lasergene 16 software package (DNASTAR Inc., Madison, WI). The unassembled reads were *de novo* assembled with the *de novo* task (with the parameters deactivated 'repeat handling' option, minimum read overlap match of 93%, and contigs longer than 1,000 nucleotides). The assembled chromosomes and contigs were next annotated with the RAST pipeline.[2]

2.8. *In silico* identification of antimicrobial resistance genes

The assembled genomes (chromosomes and contigs) of the 350 isolates were analyzed for the presence of antimicrobial resistance genes (ARGs) using three different approaches: an in-house manually curated fasta database for AMR genes of *Staphylococcus* spp. together with CM software (Supplementary file), and two online software tools, i.e., ResFinder[3] (Bortolaia et al., 2020) and Resistance Gene Identifier RGI[4] (Alcock et al., 2020, Comprehensive Antibiotic Resistance Database, CARD, 2019). An additional manual check to evaluate the functionality of the ARGs was performed (Clone Manager v9.51; CM9; Sci Ed Software, Westmister, CO) by comparing the reference gene to that of the respective isolate. To leverage the benefits of curated databases (SIB, Swiss Institute of Bioinformatics Members, 2016) all ARGs were compared with CARD to establish an association between the genes and the respective antimicrobial compounds against which their encoded products act.

2.9. Analysis of mutations in the *mecA1* gene of *Mammaliicoccus sciuri* isolates

For 20 of the 83 *Mammaliicoccus sciuri* isolates, the promoter region of the *mecA1* genes was analyzed manually using Clone Manager v9.51 software (CM9Sci Ed Software, Westmister, CO). A sample was considered positive, when a *mecA1* promoter mutation was detected at position-10 as reported in previous studies (Wu et al., 2001; Frey et al., 2013).

2.10. *In silico* analysis of plasmids

The assembled chromosomes and contigs of all bacterial species were analyzed for the presence of plasmids using the PlasmidFinder[5] software (Carattoli et al., 2014); if positive, they were further analyzed using the curated database PLSDB[6] (Schmartz et al., 2022).

2.11. Additional analysis of tetracycline AMR

All reads from isolates that exhibited matches to the *tetK* gene were mapped against the *tetK* reference gene (1,380 bp; NCBI, GenBank: S67449.1) downloaded from the CARD database using SeqMan NGen 16 (default settings). Alignments were manually checked using the Clone Manager 9.51 software (CM9; Sci Ed Software, Westmister, CO). Additionally, plasmid SPADES and Unicycler were used to circularize the plasmids of four randomly selected isolates that carried the *tetK* gene. Subsequently, all reads of isolates positive for the *tetK* gene were assembled with the closed plasmids as references and compared with Clone Manager 9.51.

2.12. Statistics

Descriptive statistics for bacterial prevalence at the cow and quarter level were performed using Microsoft Excel 2016 (Microsoft Corporation). Additionally, descriptive statistical analyses were achieved to evaluate the percentage of healthy and inflamed

2 https://rast.nmpdr.org

3 https://cge.cbs.dtu.dk

4 https://card.mcmaster.ca

5 https://cge.food.dtu.dk/services/PlasmidFinder

6 https://ccb-microbe.cs.uni-saarland.de/plsdb

(subclinical mastitis) quarters that showed a monoinfection with *S. xylosus* and *M. sciuri*, respectively.

A Fisher's exact test was used to test if there was non-random association between the bacteria and the distribution in different herds. Additionally, a generalized version of the Fisher's exact test for k x m tables was performed to test the association between herds and resistance to the main antibiotics involved (azithromycin, clindamycin, oxacillin, penicillin, and tetracycline). The same test was further performed to evaluate a possible association between milk sampling and lactation stage of the sampled cows.

To assess the impact of the stage of lactation on the intramammary presence of *S. xylosus* and *M. sciuri*, for each bacterium a loglinear model was computed, both at the cow and at the quarter level. The models included the factors bacterium, stage of lactation, farms, and their interactions. The very same procedure was used to assess the impact of the lactation number on intramammary *S. xylosus* and *M. sciuri*. For both *S. xylosus* and *M. sciuri*, their overall presence was evaluated including mono-and co-cultures with other bacteria.

For all statistical analyses except stated the Systat 13.1 software (Systat Software, San Jose, CA) was used. A value of $p < 0.05$ was considered significant.

2.13. Data availability

The complete genomes sequences (and sequences of the plasmids) for 15 type strains that were *de novo* assembled and used as reference genomes are available under Bioproject PRJNA936091.[7] The raw Illumina reads obtained from WGS of 350 isolates are publicly available from NCBI GenBank under Bioproject PRJNA859642.[8] Our manually curated database of *Staphylococcus* spp. ARGs is released as Supplementary Word file.

3. Results

The results are organized along the main two themes of the present study, i.e., the intramammary bacteriome and resistome, respectively (Figure 1).

3.1. Composition of the intramammary bacteriome

The composition of the intramammary bacteriome and distribution of individual species of each single herd at the three sampling time points is summarized in Table 1 and Figure 2. An additional figure showing the distribution of the different bacteria during sampling time (T0, T1, T2) is included in the Supplementary material (Supplementary Figure S1). Overall, a total of 1,024 milk samples (from 256 cows) collected aseptically from each quarter were analyzed. For each herd and time point, 10 randomly selected healthy cows were sampled (unless otherwise stated, see Materials and methods). The average age of the cows ranged between 4.4 to 8.9 years. 78.4% ($n = 200$) of the cows were multiparous, while 21.6% ($n = 55$) were primiparous. No data was available for two cows. Another parameter collected was the lactation stage; 108 cows were in late lactation, 76 in mid, and 70 in early lactation. These data are listed in a table integrated in the Supplementary material (Supplementary Table S5).

To assess the impact of the stage of lactation on the intramammary presence of *S. xylosus* and *M. sciuri*, the most frequently observed bacteria in this study, a loglinear model was computed for each bacterium, both at the cow and at the quarter level. The models included the factors bacterium, stage of lactation, farms, and their interactions. The variable sampling time (T0, T1, T2) was omitted from the model as a strong association was observed between this variable and stage of lactation ($p < 0.001$). Indeed, at T1 most cows were in mid (29%) and late lactation (62%). The 4 loglinear models were also used to assess the impact of the lactation number on intramammary *S. xylosus* and *M. sciuri*. The results showed that at the cow level the intramammary presence of *S. xylosus* was independent on the stage of lactation ($p = 0.840$) but dependent on the farm ($p < 0.001$). Different was the situation at the quarter level where a dependency for stage of lactation was observed ($p = 0.004$). Regarding *M. sciuri*, intramammary presence at the cow level was farm dependent ($p < 0.001$), but was independent on the stage of lactation ($p = 0.515$). At the quarter level, intramammary *M. sciuri* positivity was farm ($p < 0.001$) and lactation stage dependent ($p = 0.001$). Considering lactation number, it did not affect intramammary presence of *S. xylosus* at the cow level ($p = 0.652$), whereas an effect was observed at the quarter level ($p = 0.015$). For *M. sciuri*, the loglinear model showed no dependency as well as at the cow ($p = 0.131$) as at the quarter level ($p = 0.076$).

The SCC data demonstrated overall a high number of quarters (84.5%) with SCC below of 200,000 cells/mL and were, therefore, considered healthy. The remaining 15.5% were considered as quarters with subclinical mastitis. For *S. xylosus* explicitly, 81% of the quarters showed values below 200,000 cells/mL, with a median SCC of 36,740 cells/mL, while for *M. sciuri*, 92% of the quarters were considered healthy (SCC < 200,000 cells/mL) with a median SCC of 18,920 cells/mL.

250 of the 256 cows were bacteriologically positive for at least one quarter (98%): nineteen cows were positive for one quarter (7%), 28 cows for 2 quarters (11%), and 64 (26%) and 139 (56%) for 3 or 4 quarters, respectively. At the quarter level, 824 of the overall 1,024 milk samples were bacteriologically positive (80%). The prevalence of positive quarters in the different herds ranged from 30 to 100% (Table 1). The median values for the sampling time point T0, T1, and T2 were 87, 89, and 63%, respectively. Overall, 440 quarters were colonized by one bacterial species (53%), 305 by 2 bacterial species (37%), and 79 (10%) by 3 different species. In 613 quarters (74%) *Staphylococcus* spp. or *Mammaliicoccus* spp. were detected: 287 quarters were positive for one species (47%), 257 (42%) for 2 different species, and 69 (11%) for three different species (Table 1). The percentage of bacteriologically positive quarters differed between the herds. For the one farm that used extracted and compressed manure particles, 5 of 31 quarters (16%) were positive for NASM in the second sampling. For the other 8 farms that used straw and sawdust for bedding, the median prevalence for NASM at the quarter level was 79%.

7 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA936091

8 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA859642/

10.3389/fmicb.2023.1183018

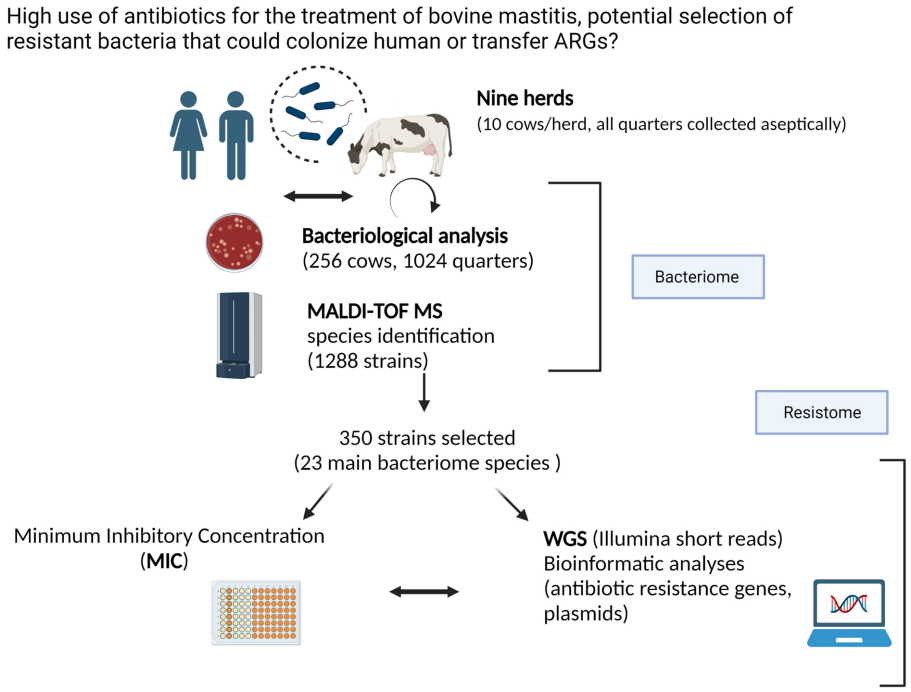

FIGURE 1
Graphical abstract representing an overview of the on-farm sampling (nine dairy cow herds from the Canton Tessin) and the experimental laboratory procedure to describe the bacteriome and resistome of healthy cows (created in Biorender.com). Confirmation of Publication and Licensing Rights.

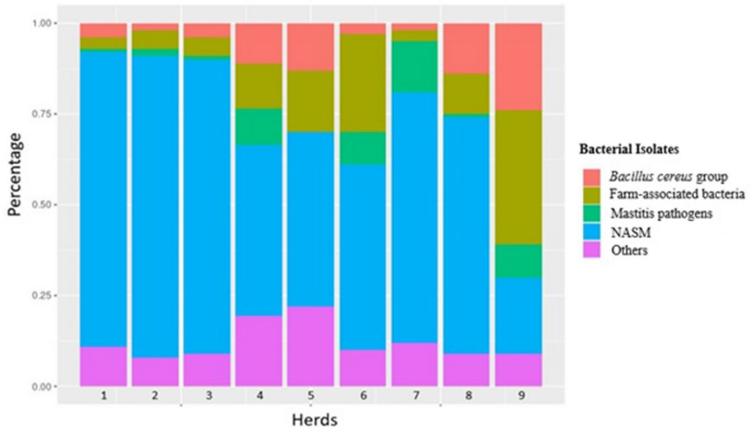

FIGURE 2
Distribution of the main bacterial groups identified in the nine different herds over the three sampled time points.

S. xylosus was the most frequently identified isolate; it was detected in all herds and sampling time points except for herd 8 at T2. In herds 8 and 9, a much lower prevalence compared to the other herds was recorded (Table 1). In herds 1, 5, and 7, *S. xylosus* represented the main species in all three samplings, while in herd 2, *M. sciuri* was predominantly detected. For the other herds, different patterns were found. In herd 3, NASM were the main bacteria, mainly represented by *S. xylosus*, *M. sciuri* and *Mammaliicoccus vitulinus* with differences within the samples. In herd 4, the first sampling was colonized by *Staphylococcus warneri*, while in the second and third sampling, *S. xylosus* was mainly identified. Farm number 6 was sampled only twice. The first sampling included mainly *Lactococcus* spp. and the second was mainly composed of *S. xylosus*. At farm number 8, the first 2 sampling included *S. xylosus*, while in the third *Staphylococcus equorum* was mainly detected. Cows from herd 9 displayed a completely different pattern of

isolates. In the first and second sampling mainly bacteria from the *Bacillus cereus* group were isolated. In the third sampling *Enterococcus* spp. was predominantly detected. To evaluate a non-random association between the herds and groups of isolated bacteria (*S. xylosus*, *M. sciuri*, *Bacillus cereus* group, farm-associated bacterial, and mastitis pathogens), a Fisher exact test was performed. The Exact test uncovered a statistically significant association ($p < 0.05$) between the herds and the bacteria isolated (*S. xylosus*, *M. sciuri*, *Bacillus cereus* group, farm-associated bacterial, mastitis pathogens) implying a distinct distribution of the groups in the different herds.

The 1,288 isolates that were isolated and identified, belonged to 104 different bacterial species (Table 1). The intramammary bacteriome compositions displayed a herd-specificity with an overall very high prevalence for NASM (Figure 3). The percentage of bacteria known to cause mastitis [*S. aureus* (2.5%), *S. uberis* (1.3%), and *Streptococcus agalactiae* (0.85%)] was lower in relation to the other categories listed above. *S. xylosus* (323 isolates) and *M. sciuri* (265 isolates) were by far the most prevalent detected bacteria in milk (for a total of 46%, 588 isolates) (distribution of the different NASM explained in the Supplementary Figure S2), followed by the *Bacillus cereus* group (9%). *S succinus* (6%) and *Enterococcus saccharolyticus* (3%), and *Escherichia coli* (2.2%) represented further potentially pathogenic bacteria. 52 isolates (4%) could not be identified by MALDI-TOF MS typing. Figure 3 shows a graphical distribution of all bacterial isolates.

3.2. Intramammary resistome

Among all isolates, 350 isolates were selected to be analyzed in more detail with respect to their genomic sequence and phenotypical antimicrobial resistance profile. To study the AMR genes and the phenotypical resistome, we chose a subset of isolates that represented the most abundant species as described in the Materials and Methods section. The data which species were identified in the study and the corresponding number of isolates are listed in Table 2.

3.2.1. Phenotypic AMR

Clindamycin and oxacillin resistance were most often observed among the 350 isolates with a total of 227 isolates (65%) resistant to clindamycin and 105 isolates (30%) to oxacillin (Table 3). The main resistant isolates were assigned to the NASM, and *Bacillus cereus* families. In total, 18% of the isolates were sensitive to all tested antibiotics, 25% of the isolates (88) were resistant to one and 28% (97) to two different antibiotics. Fifteen percent of the isolates (54) displayed AMR to more than four antibiotics. These isolates mainly belonged to the multi-resistant *Bacillus cereus* group. The distribution of the antibiotic resistances and percentage of the phenotypic resistances are listed in Tables 3, 4 (Graphical representation heatmaps Figure 4). The data of the phenotypic results for all species analyzed are listed in Supplementary Table S7.

3.2.1.1. *Staphylococcus* spp.

For 17 of the 31 antibiotics tested in this study, we identified at least one isolate that was resistant. Eighteen percent of the *Staphylococcus* spp. isolates were sensitive to all tested antibiotics, 34% were resistant to one, 30% to two antibiotics, and the remaining 18% showed multiple resistances for up to six antibiotics. Resistance to clindamycin (116, 73%), oxacillin (31, 20%), tetracycline (26, 16%), azithromycin (25, 16%), and penicillin (14, 9%) were detected most often. In detail, *S. xylosus* were phenotypically resistant to clindamycin in 83% of the isolates, oxacillin (31%), and tetracycline (25%). Fifteen percent of the *S. xylosus* isolates were multi-drug resistant (MDR) for more than 3 classes of antibiotics. For *Staphylococcus succinus* 41% of the isolates showed resistance to clindamycin and 36% to penicillin. *Staphylcoccus equorum* isolates mainly exhibited resistance to azithromycin and erythromycin (macrolides) (69%), and 50% of the isolates were resistant to clindamycin. *S. warneri* isolates only showed resistance to fosfomycin. *Staphylococcus haemolyticus* isolates were resistant to azithromycin and teicoplanin. All *S. chromogenes* isolates showed resistance to clindamycin; interestingly, one isolate was also resistant to macrolides (azithromycin and erythromycin) and tetracycline. In summary, in *Staphylococcus* spp., a high resistance rate to clindamycin, oxacillin, tetracycline and azithromycin was detected.

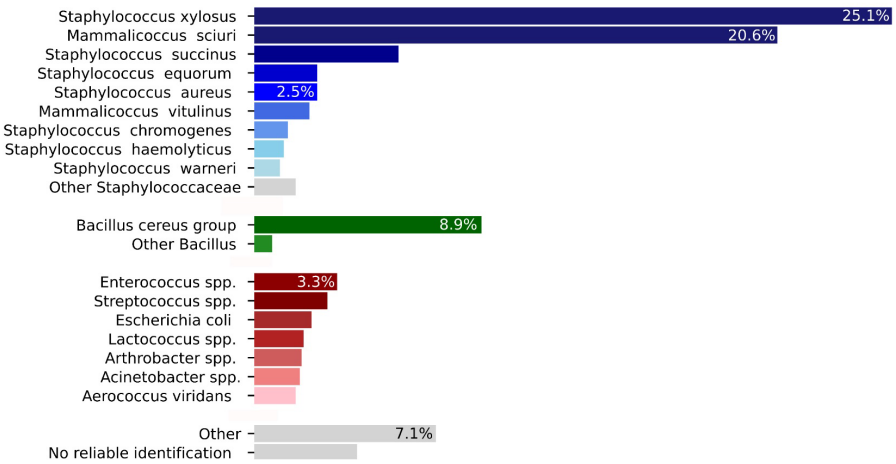

FIGURE 3
Graphical summary of the distribution of the 1,288 bacterial isolates from Table 1 included in the main text (total number and percent). The bacterial species are divided accordingly with the main table.

TABLE 2 Bacteria selected for Illumina-based WGS and phenotypic antibiotic analysis.

Bacterial species isolates	Milk T0	Milk T1	Milk T2	Sum per species
Staphylococcus xylosus#	35	38	28	101
Mammaliicoccus sciuri#	27	40	16	83
*Staphylococcus succinus**	13	4	5	22
*Staphylococcus equorum**	3	0	13	16
Staphylococcus aureus#	4	0	5	9
*Mammaliicoccus vitulinus**	5	0	4	9
*Staphylococcus warneri**	5	0	0	5
*Staphylococcus haemolyticus**	2	0	1	3
*Staphylococcus chromogenes**	0	0	3	3
*Acinetobacter lwoffii**	2	0	0	2
*Aerococcus viridans**	6	0	3	9
*Arthrobacter gandavensis**	2	0	0	2
Bacillus cereus group#	10	25	0	35
*Enterococcus faecalis**	0	1	0	1
*Enterococcus faecium**	0	2	0	2
*Enterococcus saccharolyticus**	3	3	0	6
Escherichia coli #	4	9	0	13
*Lactococcus garvieae**	5	2	0	7
*Lactococcus lactis**	4	3	0	7
Streptococcus agalactiae #	6	5	0	11
Streptococcus uberis #	0	0	4	4
Total number				350

The respective frequency of the bacterial species isolated at three different sampling time points is listed. Hash-tag symbols denote species for which a complete type strain genome was available at the NCBI. Asterisks denote species for whose type strain a *de novo* assembly was carried out combining data from 3rd generation long read sequencing technologies (PacBio and/or ONT) and the Illumina short read platform (see Supplementary Table S2).

TABLE 3 Distribution of the overall number of phenotypic antibiotic resistances exhibited by the different bacterial isolates analyzed in this study.

Bacteria isolates	No R	R 1 AA	R 2 AA	R 3 AA	R 4 AA	R > 4 AA
Staphylococcus xylosus	11	**37**	34	15	4	
Mammaliicoccus sciuri	4	27	**35**	14	1	2
Staphylococcus succinus	7	7	**8**			
Staphylococcus equorum	2	2	5	**6**	1	
Staphylococcus aureus	**4**	1	1	1		2
Mammaliicoccus vitulinus	**6**	3				
Staphylococcus warneri	**3**	2				
Staphylococcus haemolyticus	1	**2**				
Staphylococcus chromogenes	**2**				1	
Acinetobacter lwoffii	**2**					
Aerococcus viridans	1	1	2	1		**4**
Arthrobacter gandavensis	**2**					
Bacillus cereus group					1	**34**
Enterococcus faecalis						**1**
Enterococcus faecium			**2**			
Enterococcus saccharolyticus			**6**			
Escherichia coli	**10**	2				1
Lactococcus garvieae			1			**6**
Lactococcus lactis		2				**5**
Streptococcus agalactiae	**11**					
Streptococcus uberis	**3**		1			
Total	69	86	95	37	8	55

The table shows the number of resistances observed for the isolates, which could range from resistance to one antibiotic (AB) up to resistance to over four ABs. R, resistance; AB, antibiotics. The meaning of the bold values represent the majority of the samples resistance to the antibiotics.

3.2.1.2. *Mammaliicoccus* spp.

M. sciuri and *M. vitulinus* were mainly resistant to clindamycin (87%) and oxacillin (52%). Of 83 *M. sciuri* isolates, 91% were resistant to clindamycin, 54% to oxacillin, and 8% to tetracycline. Twenty-nine isolates presented a resistance to both clindamycin and oxacillin (35%), and 32% were limited to clindamycin. Additional AMRs to daptomycin (6 isolates: 7%), azithromycin (4 isolates: 5%) and

TABLE 4 Percentage of phenotypic resistance observed against different antibiotics (based on the MIC assays).

Bacteria isolates species	Gm[1]	To[1]	Etp[2]	Imp[2]	Mer[2]	Cpe[3]	Cft[3]	Crm[3]	Cax[3]	Cp[4]	Mxf[4]	NA[4]	Nxn[4]	Tei[5]	Cd[6]	Lin[6]	Dap[7]	Azi[8]	E[8]	Mup[9]	Fd[10]	Lzd[11]	Am[12]	Aug[12]	Ox[12]	P[12]	C[13]	Fos[14]	Cl[15]	Rif[16]	Sts[17]	Prs[17]	Syn[18]	Min[19]	Te[19]	T/S[20]	Total N. isolates
ST_XY			1												83		1	7	2	2	1				31	7	3	1		1					25		101
MA_SC	1						1			1	1				98		7	5	2	4					54	1	2	1		2					8		83
ST_SU														9	41		9	9								36										22
ST_EQ															50		13	69	69									6						6		16
ST_AU	11														22			33	11				22			22										9
MA_VI															33			11							22											9
ST_WA																												40								5
ST_HA														33				33																		3
ST_CH															100			33	33															33		3
AC_LW																																				2
AE_VI					11	33	22	33																		55	22			11				22	44	11	9
AR_GA																																				2
BAC_CER_GR	6	9	6	3	6	100	100	100							91		40	20		100			97	14	86	100		3		6					9	6	35
EN_FAECALIS																100			100											100	100	100		100		1
EN_FAECIUM																100													100							2
EN_SA																100																100				6
ES_CO												8	8					8			8								15								13
LA_GA						71	86	86	86						57				29				43			86				100				29	29		7
LA_LA						14	71	71	71																	57				100							7
STR_AG																																				11
STR_UB																																	25	25		4
Total isolates																																				350

The table displays the percentage of the resistant isolates, i.e., compared to the total number of isolates per species. The antibiotics were arranged according to their classification into different classes (superscript numbers 1 to 20; see legend). Bacterial species listed: ST_XY: *Staphylococcus xylosus*, MA_SC: *Mammaliicoccus sciuri*, ST_SU: *Staphylococcus succinus*, ST_EQ: *Staphylococcus equorum*, ST_AU: *Staphylococcus aureus*, MA_VI: *Mammaliicoccus vitulinus*, ST_WA: *Staphylococcus warneri*, ST_HA: *Staphylococcus haemolyticus*, ST_CH: *Staphylococcus chromogenes*, AC_LW: *Acinetobacter lwoffii*, AE_VI: *Aerococcus viridans*, AR_GA: *Arthrobacter gandavensis*, BAC_CER_GR: *Bacillus cereus group*, EN_FAECALIS: *Enterococcus faecalis*, EN_FAECIUM: *Enterococcus faecium*, EN_SA: *Enterococcus saccharolyticus*, ES_CO: *Escherichia coli*, LA_GA: *Lactococcus garvieae*, STR_AG: *Streptococcus agalactiae*, STR_UB: *Streptococcus uberis*. Classification of different antibiotics: 1 Aminoglycoside (Gm: gentamycin, To: tobramycin), 2 Carbapenem (Etp: ertapenem, Imp: imipenem), 3 Cephalosporins (Cpe: cefepime, Cft: cefotaxime, Crm: cefuroxime, Cax: ceftriaxone) 4 Fluorochinolones (Cp: ciprofloxacin, Mxf: moxifloxacin, NA: nalidixic acid, Nxn: norfloxacin) 5 Glycopeptides (Tei: teicoplanin), 6 Lincosamides (Cd: clindamycin, Lin: lincomycin) 7 Lipopeptides (Dap: daptomycin), 8 Macrolides (Azi: azithromycin, E: erythromycin), 9 Mupirocins (Mup: mupirocin), 10 Nitrofurantoin (Fd: nitrofurantoin), 11 Oxazolidinones (Lzd: linezolid), 12 Penicillin (Am: ampicillin, Aug: amoxacillin/ K clavulanate, Ox: oxacillin, P: penicillin) 13 Phenicols (C: chloramphenicol), 14 Fosfomycins, (Fos: fosfomycin)15 Polymyxins (CI: colistin), 16 Rifampicins (Rif: rifampin), 17 Streptogramins (Sts: streptomycin, Prs: pristamycin), 18 Synercyd (Syn), 19 Tetracyclines (Min: minocycline, Te: tetracycline), 20 Trimethoprim/sulfamethoxazole (T/S).

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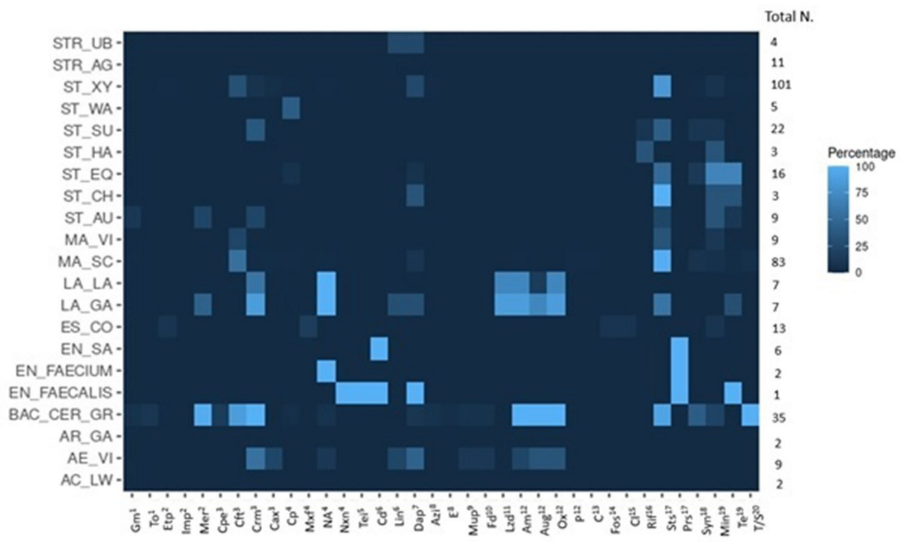

FIGURE 4
Graphical heatmap showing the results displayed in the Table 4. The different color scale represents the percentage of isolates resistant to the different antibiotics checked.

mupirocin (3 isolates: 4%) were observed. 5% of the *Mammaliicoccus* spp. isolates were sensitive to all antibiotics analyzed in this study. For *M. vitulinus*, five out of nine isolates were sensitive to all antibiotics (56%), one isolate was resistant to azithromycin and two displayed a resistance to oxacillin.

The Exact test revealed a highly significant association between the NASM species (*S. xylosus, M. sciuri, S. equorum, S. succinus*) and the five main antibiotic resistances to azithromycin, clindamycin, oxacillin, penicillin, and tetracycline; for all species analyzed ($p < 0.001$). For *S. xylosus* and *M. sciuri*, the two most prominent species, the Exact test showed only a significant association for *S. xylosus* between the herds and tetracycline resistance ($p < 0.001$). A resistance to tetracycline was detected in six out of the nine herds.

3.2.1.3. *Bacillus cereus* group

The isolates of the *Bacillus cereus* group were mostly multi-drug resistant isolates. All isolates were resistant to more than six antibiotics, except one isolate, which was only resistant to four antibiotics. All 35 isolates exhibited resistances to cefepime, cefotaxime, cefuroxime, and penicillin; additionally, 34 out of 35 were resistant to ampicillin. These results indicated a high resistance to β-lactam antibiotics. Only three isolates were resistant to tetracycline (9%), but a high proportion was resistant to clindamycin (32 isolates, 91%).

3.2.1.4. Farm associated bacteria

Further research on antibiotic resistance was performed with bacterial isolates from *Acinetobacter lwoffii*, *Aerococcus viridans*, *Arthrobacter gandavensis*, *Enterococcus* spp., *E. coli*, *Lactococcus* spp., and *Streptococcus* spp.

For *Acinetobacter lwoffii* and *Arthrobacter gandavensis*, no resistance to any of the tested antibiotics was detected. Regarding *Aerococcus viridans*, the breakpoints were not defined for all tested antibiotics. Based on the EUCAST guidelines for *Aerococcus* spp., only the resistance to amoxicillin/K clavulanate, ampicillin, levofloxacin, meropenem, penicillin, rifampin, and vancomycin could be defined. Based on this observation, we isolated one isolate resistant to meropenem and another one to rifampin. Additionally, five isolates from two different farms were resistant to penicillin (55%). We identified additional resistances to cephalosporins, chloramphenicol, tetracycline (44%) and trimethoprim/sulfamethoxazole. All bacteria of the *Lactococcus* spp. displayed resistance to rifampin and to different categories of β-lactam antibiotics.

All *Enterococcus* species were resistant to lincomycin. Two isolates of *Enterococcus faecium* were additionally resistant to rifampin, while six isolates of *Enterococcus saccharolyticus* were also resistant to synercid. The isolates of *Enterococcus faecalis* displayed multi-resistances to erythromycin, gentamicin, pristinamycin, streptomycin, synercid, and tetracycline.

Ten out of 13 analyzed *E. coli* isolates were sensitive to all the antibiotics tested; two isolates were resistant to colistin and one isolate was resistant to aztreonam, nalidixic acid, nitrofurantoin, and norfloxacin.

3.2.1.5. Mastitis pathogens

Among the nine *S. aureus* isolates, two isolates showed only resistance to azithromycin and clindamycin, one isolate to azithromycin and the β-lactams ampicillin and penicillin. Additionally, one isolate was MDR. Considering the 15 *Streptococcus* spp. (11 *S. agalactiae* and four *S. uberis*), 14 were sensitive to all antibiotics (93.3%). One isolate of *S. uberis* was exclusively resistant to tetracyclines (minocycline and tetracycline).

3.3. Whole genome sequencing

We set out to study the resistome using a collection of 23 type strains for the most abundant species that were uncovered by our extensive bacteriome analysis. For fifteen of these strains, no complete genome was available, and we thus set out to *de novo* assemble their complete genomes as a reference for the community and to avoid missing antibiotics resistance-relevant genes in fragmented Illumina assemblies (Varadarajan et al., 2020). A combination of long reads from third generation long read sequencing platforms (PacBio or ONT) and short read Illumina sequences (for polishing and to identify potential small plasmids) was used and *de novo* assembled (Supplementary Methods). All 350 isolates were sequenced with Illumina HiSeq (Supplementary Methods). A first reference-based assembly was done using the 23 complete genomes of the respective type strain of each species as a reference. The median, minimum, and maximum coverage and median total sequence lengths are listed in Supplementary Table S6. Importantly, for the type strains, we also tried to assemble plasmid sequences (Supplementary Methods; Supplementary Table S2).

3.4. Prevalence of antibiotic resistance genes

To assess the presence and prevalence of ARGs, three different bioinformatic methods were applied. Combining the results of all methods, 66% of the 350 isolates carried at least one ARG. In total, 96 different ARGs were detected; based on the literature, 53 genes were classified as specific for resistance against one antibiotic molecule, 43 were implied in causing resistances to more than one molecule. The high number of identified ARGs largely results from the big number of genes detected by the CARD database for *S. aureus* and *E. coli* species. The complete results for the ARGs, including the functionality of the main ARGs detected in the isolates (Supplementary Tables S8, S9), can be found in the Supplementary material. The main ARGs detected in the 350 isolates are summarized in Table 5 and Figure 5.

3.4.1. *Staphylococcus* spp.

For *S. xylosus*, 64 isolates (63%) were negative for ARGs, while in the 37 remaining isolates (37%) at least one ARG was detected. The *tetK* gene was the most prevalent gene observed in 25 isolates (25%). Other less prevalent genes were *fosD* (6 isolates), *str* (6), *cat* (4), *mphC* (4), *erm* (44) (1), and *msrA* (1). One isolate carried the ARGs *blaZ* and *mecC2*. The isolates of *S. chromogenes*, *Staphylococcus haemolyticus*, and *S. succinus* did not carry any known ARG. For *S. equorum*, most of the isolates (13%) harbored the *mphC* gene, in three isolates (19%) the *str* gene was detected. In one isolate *mphC, str* and *tetK* were observed simultaneously. *S. warneri* isolates were always positive for *gyrB*.

3.4.2. *Mammaliicoccus* spp.

In all 83 *M. sciuri* isolates subjected to WGS, the *mecA1* and *salA* genes were detected. Additionally, two isolates (~2%) carried *cat*, *lnu (A)*, *str*, *tetK* and *tetL*. Only one isolate carried 6 ARGs: *mecA*, *salA*, *aac (6′)-aph (2″), lnuA, str* and *tetL*. Regarding *M. vitulinus,* seven out of nine isolates (77.8%) were *mphC* positive. One isolate additionally carried the *msrA*. Additional bioinformatic promoter analyses for the *mecA1* gene did not reveal any mutation in the promotor region in any of the 20 analyzed isolates when compared to the promoter region of the *M. sciuri* type strain (Wu et al., 2001; Frey et al., 2013).

3.4.3. *Bacillus cereus* group

A high prevalence of β-lactams antibiotic resistance genes was detected in the *Bacillus cereus* group. The *Bc* gene was found in 24 isolates (68.6%) and the *BcII* gene that encodes a zinc metallo β-lactamase was found in all the isolates. In addition, most of the isolates were positive for *fosB1* (77.1%). In one case, the tetracycline resistance gene, *tetL*, was also detected.

3.4.4. Farm-associated bacteria

Further analyses of the ARGs were done with the other 11 bacterial species, which were less prevalent in the milk samples. For *Arthrobacter gandavensis* and *Enterococcus saccharolyticus* bacteria, no ARGs were detected. The investigation of the *E. coli* isolates showed different results depending on the bioinformatics tools used. The analysis done with the ResFinder software revealed the presence of *mdfA* in all of the isolates. In one isolate, this gene was present together with *fosA7*. Notably, the analysis performed with the CARD database showed a different picture; more than 30 different genes were found in all 13 *E. coli* isolates and they seemed to be an intrinsic part of the *E. coli* genome but, based on our phenotypic profiling, not always expressed in the bacterial isolates.

For the other bacteria that belong to the bacteriome but that were less prevalent, a more detailed analysis for look regarding the presence of ARGs is explained in the Supplementary Table S8.

3.4.5. Mastitis pathogens

For all streptococci, at least one ARG was present. All the *S. agalactiae* isolates carried *mre(A)* and *mprF*. All isolates of *S. uberis* carried the *patB* gene and in one isolate *tetM* was additionally observed. Some *S. aureus* isolates carried at least four resistance genes *mepR*, *mgrA*, *arlR*, and *glpT* (three isolates). All nine *S. aureus* isolates were positive for *arlR*, *mgrA* and *mepR*. In the remaining six isolates, more than four ARGs were detected. Only two isolates were positive for *blaZ*, while three different ARGs were detected specifically for fosfomycin resistance (*fosB, glpT*, and *murA*).

A graphical heatmap shows the discrepancy between phenotypic resistant and the presence of ARGs in the same isolates (Figure 6). The results showed for several classes of antibiotics a low correlation between phenotypic and genomic results (aminoglycosides and β-lactams). Differently, in all the phenotypically resistant isolates the presence of the tetracycline ARGs was detected.

3.5. ARGs and plasmids

After *de novo* assembly, 104 (30%) out of the 350 isolates examined were positive for at least one plasmid-based replicon (*rep*) gene, in 40 cases (39%) the gene was located on a contig harboring at least one ARG (Table 6). In *S. xylosus*, *rep7* was mainly found in contigs carrying ARGs for tetracycline (*tetK*), chloramphenicol (*cat*), and streptomycin (*str*). Association of *rep7* with ARGs was also detected in *M. sciuri* (*str*, *cat*, *tetK*, and *lnuA*), in *S. equorum* (*str*, *str* plus *tetK*), and in *S. warneri* (*tetK*). Additionally, *rep7* was detected in three of nine *Aerococcus viridans* isolates that carried as well the *str* and *cat* gene. In *M. sciuri*, the gene *lnuA* was associated with 3 different *rep* types: *rep7*, *rep13*, and

TABLE 5 Main antibiotics resistance genes (ARGs) identified.

Bacteria	*aac(6')-aph(2'')*	*aac(6')-Ii*	*Bc or BcII*	*blaZ*	*cat*	*eatAv*	*erm(44)*	*fosB1*	*fosD*	*gyrB*	*lnuA*	*lmrD*	*lsa(D)*	*mecA1*	*mecC2*	*mre(A)*	*mphC*	*mprF*	*msrA*	*msrC*	*OXA-362*	*patB*	*salA*	*str*	*tetk*	*tetL*	*tetM*	*tetS*	Total N. Strains
ST_XY				1	4		1		6						1		4		1					6	**25**				101
MA_SC	1				3						3			**83**									**83**	5	5	2			83
ST_EQ																	**11**							4	1				16
MA_VI																	**8**		1										9
ST_WA										**5**															1				5
AC_LW																					2								2
AE_VI					2						1													4			4		9
BAC_CER_GR			35	1				**28**																		1			35
EN_FAECIUM		**2**				1														**2**									2
LA_GA													**3**														1	1	7
LA_LA												**4**																	7
STR_AG																**11**		**11**											11
STR_UB																						**4**					1		4

The corresponding phenotypic antibiotic resistance correlated with the genes are listed in Supplementary Table S7. The isolates *Staphylococcus succinus*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes*, *Arthrobacter gandavensis*, and *Enterococcus saccharolyticus* were negative for the detection of ARGs. The isolates with more than three ARGs [*Staphylococcus aureus* (ST_AU), *Enterococcus faecalis* (EN_FAECALIS), and *Escherichia coli* (ES-COLI)] are described in detail in the Supplementary Table S7. The bold values represent the majority of the strains carried the antimicrobial resistance genes.

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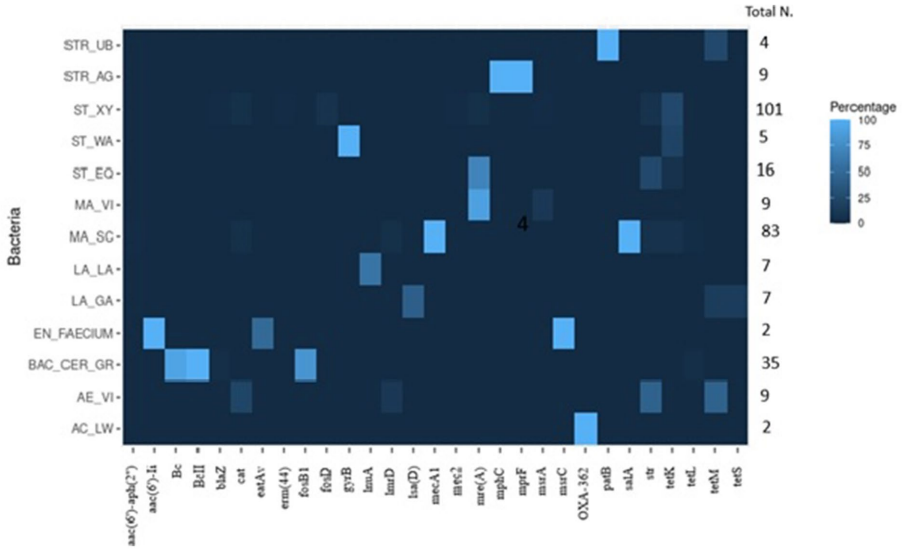

FIGURE 5
Graphical heatmap showing the results displayed in the Table 5. The different color scale represents the positivity of isolates resistant to the different ARGs.

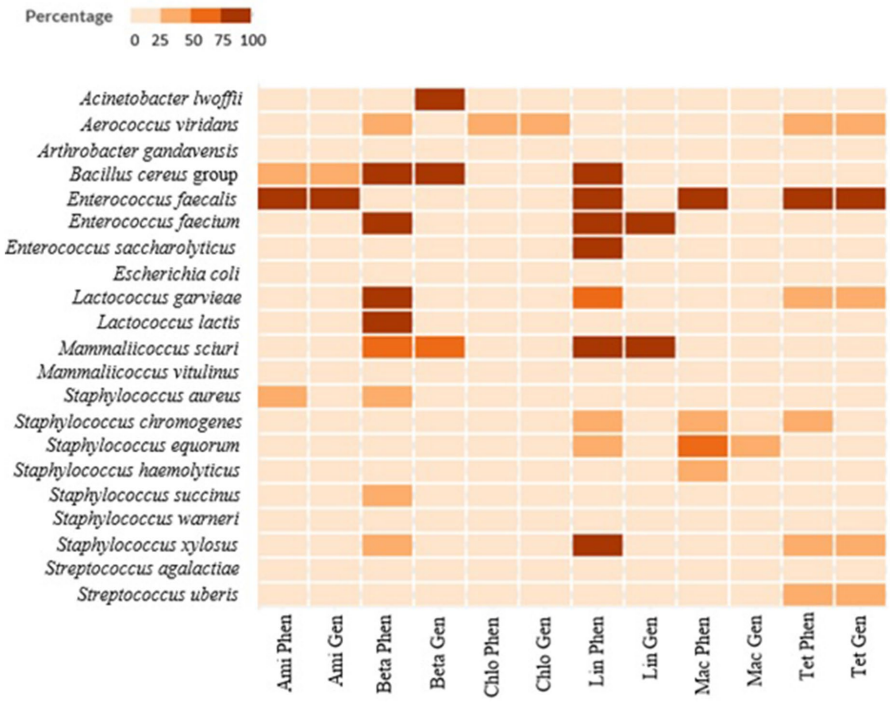

FIGURE 6
Correlation between phenotypic AMR resistance (in percent) and underlying genomic information for six classes of antibiotics (aminoglycosides, β-lactams, chloramphenicol, lincosamides, macrolides, and tetracyclines). The table displays the percentage of resistant isolates (phenotypic analysis) compared with the number of isolates where the ARGs correlated to the resistance to the antibiotics were identified. Ami Phen, Aminoglycoside phenotypic percentage; Ami Gen, Aminoglycoside genomic percentage; BetaPhen, β-lactams phenotypic percentage; Beta Gen, β-lactams genomic percentage; Chlo Phen, Chloramphenicol phenotypic percentage; Chlo Gen, Chloramphenicol genomic percentage; Lin Phen, lincosamides phenotypic percentage; Lin Gen, lincosamides genomic percentage; Mac Phen, Macrolides phenotypic percentage; Mac Gen, Macrolides genomic percentage; Tet Phen, tetracycline phenotypic percentage; Tet Gen, tetracycline genomic percentage.

TABLE 6 Results from the plasmid identification with PlasmidFinder (Center for Genomic Epidemiology, 2019).

Bacterial isolates	N. isolates positive (% on the total)	N. replicons	N. Isolates	Replicon proteins (number of isolates identified)
Staphylococcus xylosus	30 (30%)	1	27	***rep7a*** (15), *rep19c* (9), *rep 21* (3)
		2	3	***rep7a***, *rep19c* (2),***rep7a***, *rep21* (1)
Mammaliicoccus sciuri	17 (20%)	1	14	***rep7a*** (8), *rep13* (4), *rep21* (2)
		2	3	***rep7a***, *rep13* (3)
Staphylococcus succinus	10 (45%)	1	9	*rep16* (5), *rep19c* (2), *rep20* (1), *rep21* (1)
		2	1	*rep16, rep21* (1)
Staphylococcus equorum	7 (43%)	1	7	***rep7a*** (4), *rep16* (1), *rep19* (2)
Staphylococcus aureus	6 (67%)	1	5	***rep7a*** (4), *rep13* (1)
		2	1	*rep16, rep19c* (1)
Mammaliicoccus vitulinus	1 (11%)	1	1	*rep19b* (1)
Staphylococcus warneri	1 (20%)	1	1	***rep7a*** (1)
Aerococcus viridans	3 (33%)	1	3	***rep7a*** (3)
Bacillus cereus group	1 (3%)	1	1	*rep22* (1)
Enterococcus faecalis	1 (100%)	4	1	*rep2, rep8b, repUS11, repUS41* (1)
Enterococcus faecium	2 (100%)	1	1	*rep1* (1)
		2	1	*rep1, repUS15* (1)
Enterococcus saccharolyticus	5 (83%)	1	4	*rep1* (2), *repUS21* (2)
		2	1	*rep1, repUS21* (1)
Escherichia coli	10 (77%)	1	2	*IncFCI* (2)
		2 3	4 4	*IncFIA, IncFIB* (2), *IncFIA, IncFII* (1), *IncX1, IncFIC* (1) *IncFIA, IncFIB, IncFII* (3), *IncFIB, IncFIC, Inc1-I* (1)
Lactococcus garvieae	3 (43%)	1	3	*rep32* (1), *rep33* (2)
Lactococcus lactis	5 (71%)	1	5	*repUS33* (5)
Streptococcus uberis	1 (25%)	1	1	*repUS43* (1)

rep21. *Rep*13 was also observed in one contig (each) of *M. sciuri* and *S. aureus* carrying the *str* and *ermT* genes, respectively. The search for the *rep* genes was performed using PlasmidFinder. For comparison, identical analyses were also executed using PLSBD. In this case, 37 (35.6%) out of the 104 *rep* genes detected by PlasmidFinder were found.

For all NASM isolates phenotypically resistant to tetracycline including *S. xylosus* ($n = 21$), *M. sciuri* ($n = 5$), *S. equorum* ($n = 1$), and *S. warneri* ($n = 1$), a small plasmid could be identified always carrying the same *tetK* gene (100% similarity among strains) and the same *rep7a* replication initiation factor gene (Supplementary Table S10). Two of four plasmids circularized with plasmid Spades and Unicycler were, with 4,440 bp and 4,439 bp, very similar in size and were both composed by the three genes *rep7a*, *tetK*, and a plasmid recombination gene (5′-3′). The similarity was 99.7%. The other two circularized plasmids were larger in size amounting to 4,666 bp and 4,804 bp. They showed the same gene structure as the smaller plasmids, but the non-coding part was larger. The remaining 24 plasmids matched best with the 4,440 bp circularized plasmid (= reference) and showed a size between 4,435 bp and 4,448 bp; 19 of them exhibited a size of 4,440 bp with a similarity between 98.7 and 100.0% (toward the reference). Interestingly, 13 of those plasmids showed an identical similarity of 99.7% when compared to the reference. They were mostly observed in *S. xylosus* ($n = 11$), but also in *M. sciuri* ($n = 1$), and *S. warneri* ($n = 1$) and were repeatedly found in isolates within different farms.

4. Discussion

The composition of the intramammary bacteriome of healthy, untreated cows is extremely relevant in order to assess the prevalence of commensals and potential pathogens and to determine what kind of antibiotics resistance traits are encoded by these species. Enabled by large advances in next generation sequencing, we here describe the most extensive bovine intra-mammary bacteriome study to date, which we expect to have far-reaching implications for veterinary bacteriology practice and for diagnostics (Figure 1). These aspects are becoming increasingly important in the context of the One Health concept (McEwen and Collignon, 2018), where a large reduction of the use of antibiotics is envisaged, including their wide-spread use in animal husbandry and livestock production. Moreover, knowledge

about the routes of transmission of mobile antibiotics resistance elements from animals to humans is also critical. To extend on the very detailed bacteriome, we thus also analyzed 350 isolates from the most abundant species by WGS [informed by a collection of 23 type strains, 15 of which we here assembled *de novo* relying on our expertise (Schmid et al., 2018)] and the knowledge that, compared to complete genomes, Illumina assemblies can miss genes relevant for antibiotics resistance (Varadarajan et al., 2020) and phenotypic profiling. This was done in order to (i) determine their resistome and (ii) to attempt to link phenotypic profiling data with antibiotics resistance genes, an area that requires further developments including more comprehensive and better curated databases of ARGs.

The current study highlights the presence of mainly NASM bacteria in mammary glands from healthy cows of nine Swiss herds. Overall, the bacterial isolates were found to be highly resistant to clindamycin and oxacillin. Genomic analyses revealed some consistent patterns regarding the presence of antibiotic resistance genes, for example the presence of *mecA1* and *salA* in all *M. sciuri* isolates. The tetracycline resistance was related to *tetK,* encoded on a small plasmid, which implicated a possible horizontal gene transfer between different NASM. For various phenotypic AB resistances observed, however, no ARGs were detected. As a consequence, further analyses should be performed to identify new ARGs or *in vitro* studies regarding the antibiotic resistance of veterinary bacterial isolates to actualize the current breakpoint of the MIC broth microdilution.

4.1. Bacteriome

In recent years, a few studies have investigated the bovine intramammary bacteriome using culture-dependent and culture-independent approaches (metagenomics) (Oikonomou et al., 2014; Cremonesi et al., 2018; Metzger et al., 2018; Sartori et al., 2018; Al-Harbi et al., 2021). Based on their findings, the bovine mammary gland is considered to contain a spectrum of different bacterial species. The results of the present study, using a culture-dependent approach, support these recent findings, but they show a much broader and more complex diversity of bacteria than expected. Bacteria from the NASM (61.1%) and the *Bacillus cereus* group (9%) were most frequently identified. Bacteria commonly present in the farm environment were less abundant [*Acinetobacter* spp. (1.8%), *Aerococcus viridians* (1.6%), *Arthrobacter* spp. (1.9%), *Enterococcus* spp. (3.2%), *E. coli* (2.2%), and *Lactococcus* spp. (1.9%)]. Further, a low percentage of known mastitis pathogens were detected in healthy cows [*S. aureus* (2.4%), *S. agalactiae* (0.8%), and *S. uberis* (1.3%)]. Indeed, in the present study 15.5% of the analyzed quarters showed a subclinical mastitis as defined by IDF (SCC ≥ 200,000 cell/mL) and these pathogens definitely contributed to the prevalence although it was also observed in quarters with monocultures of *S. xylosus* (19%) and *M. sciuri* (8%) (International Dairy Federation, 2022). Rarely, increased SCC were also observed for other NASM and bacteria and for co-cultures (data not shown). For *S. xylosus*, its prevalence is substantial, whereas for *M. sciuri* it is lower.

The present study further demonstrates that the intramammary presentence of *S. xylosus* and *M. sciuri* is highly farm dependent but is independent on the cow's stage and number of lactations at the cow level. At the quarter level, however, a significant association was established for *S. xylosus* and both variables indicating that quarters of older cows and in progressed lactation are more susceptible to intramammary *S. xylosus*. For *M. sciuri*, a significant association between stage of lactation and intramammary presence was observed at the quarter level demonstrating that intramammary *M. sciuri* is more common during later lactation.

4.1.1. Non-*aureus* staphylococci and mammaliicocci

Staphylococcaceae represented by *S. xylosus* (25%) and *M. sciuri* (20.6%) were the most frequently detected bacteria, followed by *S. succinus* (5.7%). Interestingly, *S. xylosus* and *M. sciuri* were frequently (40%) co-isolated from the same quarter. These results confirm previous studies where *S. xylosus* and *M. sciuri* were also the most frequently isolated bacteria (Malinowski et al., 2011; Frey et al., 2013; Xu et al., 2015; De Visscher et al., 2016; Condas et al., 2017; Sartori et al., 2018; Valckenier et al., 2020). In the present study, however, with 73.5% of milk quarter samples being positive for NASM, the detection rate was higher, particularly when compared to studies that have analyzed milk samples from healthy cows (Porcellato et al., 2020; Al-harbi et al., 2021). It has been shown that the prevalence and distribution of NASMs is influenced by regional and environmental factors (Vanderhaeghen et al., 2015; Rowbotham and Ruegg, 2016; Alanis et al., 2021). Indeed, we detected, a clear association between intramammary bacteria and the bedding: mammary glands of cows kept on straw contained a higher number of NASM; while in one farm, in which manure was used as bedding, *Enterobacteriaceae* and *Enterococcaceae* were predominant. However, this finding needs further investigations.

4.1.2. *Bacillus cereus* group

An important aspect is the high prevalence of bacteria belonging to the *Bacillus cereus* group in milk samples of healthy cows (9%). This bacterial group, including twelve closely related species, is commonly found in environmental and food products accounting for between 11 to 47% of isolates, particularly in raw milk from cows and buffalo (Liu Y. et al., 2015; Owusu-Kwarteng et al., 2017; Baldwin, 2020; Radmehr et al., 2020; Zhao et al., 2020; Bartoszewicz and Czyżewska, 2021). Due to heat-resistant spores, these bacteria survive the pasteurization process and could cause spoilage of dairy products and even intoxication of human consumers (Gopal et al., 2015). All of these strains carried multiple ARGs.

4.1.3. Farm associated bacteria

The prevalence of these bacteria was low and accounted for a range between 1.6 to 3% of all isolates. They included *Acinetobacter lwoffii, Arthrobacter gandavensis, E. coli, Enterococcus faecalis, Enterococcus faecium, Enterococccus saccharolyticus, Aerococcus viridans, Lactococcus garvieae* and *Lactococcus lactis*. *E. coli* and enterococci are typical fecal representatives [National Mastitis Council (NMC), 2017], whereas lactococci are commonly isolated from raw milk with the ability to persist in a farm environment and the cows (Werner et al., 2014). In addition, *Lactococcus garvieae* and *Lactococcus lactis* were previously identified as pathogens inducing chronic subclinical mastitis in cows, mostly during late lactation (Wyder et al., 2011). *Acinetobacter lwoffii* is mainly isolated from human skin and infections, from soil and plants, but also from other sources (Adewoyin and Okoh, 2018). In contrast, *Arthrobacter gandavensis* and *Aerococcus viridans* are largely found on bovine teat skin and in milk (Wyder et al., 2011; Verdier-Metz et al., 2012). These results

demonstrate that farm-associated bacteria can be part of the intramammary bacteriome, but compared to NASM, they play a minor or even a negligible role.

4.1.4. Mastitis pathogens

With an observed frequency of 2.8%, mastitis pathogens were rarely observed. Their presence was farm specific (especially for Farm 4) and included *S. aureus*, *S. agalactiae*, and *S. uberis*. For the streptococci, our findings were very similar to a previous Swiss study that had screened whole herds for *S. aureus* (Moret-Stalder et al., 2009). The low presence of pathogens in the present study is not astonishing as only healthy cows with healthy udders were included, whose milk was suitable for human consumption according to Swiss legislation. Nevertheless, they were detected, raising the question why they were observed. All observed bacteria are known to be frequently involved in subclinical bovine mastitis [National Mastitis Council (NMC), 2017], meaning that the udder and milk are grossly normal so that the farmer was unaware that an IMI was present. Alternatively, it may be possible that the isolates represent apathogenic subtypes, as has been previously shown for *S. aureus* (Fournier et al., 2008).

4.2. Clinical and diagnostic implication

For decades, veterinarians have been convinced that the bovine mammary gland is sterile, and all bacteria isolated from milk have been considered a result of an intramammary infection and mastitis. This has been increasingly questioned in recent times, when it turned out that particularly NASM could regularly be isolated from milk samples of the same, healthy quarter over time (Sartori et al., 2018). So far, NASM have been considered as minor pathogens causing subclinical forms of mastitis (Sears and McCarthy, 2003; Taponen and Pyörälä, 2009; Condas et al., 2017) and are the most common bacteria isolated from clinical milk samples sent in for diagnostic analysis by veterinarians [National Mastitis Council (NMC), 2017]. NASM infections are regularly treated with AB, at least in Switzerland, based on the assumption that the bovine mammary gland is free from bacteria. In contrast to almost all other studies, and certainly in contrast to the clinical work in the field where milk samples are only taken and bacteriologically examined after an udder health problem has been detected, the present study, however, focuses on healthy quarters. Importantly, the same standard diagnostic culturing methods were used as they are routinely applied. And suddenly the very same NASM were found at the species level, a fact that definitely questions the role of NASM in the context of bovine mastitis. Are they really minor pathogens or do they have a more protective function? Even the finding that NASM are found together with increased amounts of inflammatory cells in the milk does not necessarily mean that they are the cause of the observed inflammation. It is well known that inflammation of the mammary gland can also result from inappropriate milking procedures leading to mechanical tissue irritation (Giesecke et al., 1986), the NASM could merely be a by-product as, at the time of sampling, they were in the quarter anyway. As a consequence, and clearly based on the present study, it is no longer possible to interpret the finding of NASM isolated from a diseased quarter in clinical terms. This is particularly true for *S. xylosus* and *M. sciuri* which first of all had been both isolated from healthy quarters. Under field conditions, a positive culture for these bacteria should no longer be interpreted in the way that a subclinical mastitis is present in the corresponding quarter. This is only possible if the milk of the quarter shows SCC ≥ 200,000 cells/mL or a positive California mastitis test.

Indeed, the clinical significance of NASM present in the mammary gland remains to be further elucidated. Potentially, the most abundant NASM such as *M. sciuri* and *S. xylosus* need to be further subtyped to tease out some relevant differences.

4.3. Resistome

In Switzerland, between 2018 and 2019 approximately 70% of all antimicrobials used for IMI were products applied for the treatment of mastitis during lactation. Penicillin followed by aminoglycosides are the most predominantly used antibiotics according to the Swiss Antibiotic Resistance Report 2020 (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). Over the last years, an increase of antimicrobial resistant bacteria has been reported which is at least in part due to the misuse of antibiotics in agriculture (Manyi-Loh et al., 2018; Mann et al., 2021). Concomitantly, ARGs were detected in different environments including milk samples (Pol and Ruegg, 2007; Saini et al., 2013).

4.3.1. *Staphylococcus* spp.

Although their pathogenicity and epidemiology are still under debate (Nyman et al., 2018; De Buck et al., 2021), IMI caused by NASM are regularly treated with antibiotics in Switzerland (BLV, 2019). This requires a good understanding of AB resistance patterns and the mechanisms of action to offer an optimal therapy. In this study, they exhibited the highest resistance to clindamycin and oxacillin, which has increased compared to a previous Swiss study (Frey et al., 2013). The reason of the clindamycin resistance increase could be the recent decrease and change in the resistance breakpoint (from >0.5 to >0.25 mg/mL) in the EUCAST guidelines [The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2022]. The percentage of penicillin resistant isolates was higher (23.3%) than the percentage for the *Staphylococcus* spp. isolates in the Frey work (8%). Both studies from Switzerland recognized a high number of isolates resistant to oxacillin, 20 and 47%, respectively. A tetracycline resistance rate between 12 to 38% was detected in *S. xylosus* bacteria in a previous Swiss study of different food products such as fermented sausages, cheeses, and meat starter cultures (Leroy et al., 2019). Our study, with a percentage of 25% *S. xylosus* isolates, confirm a high resistance prevalence for this antibiotic.

4.3.2. *Mammaliicoccus* spp.

Previous studies involving *Mammaliicoccus* spp., showed a high variability of tetracycline resistant isolates. In Switzerland, two previous studies analyzed the presence of *M. sciuri* in pigs, cattle, poultry, and different food matrixes as bulk tank milk, minced meat, and abattoir employees demonstrated the presence of resistant *M. sciuri* (Huber et al., 2011; Nemeghaire et al., 2014). The low resistance to aminoglycosides found in our study, agrees with a study by Hauschild et al. (2007) where the resistance of 204 *M. sciuri* isolates was evaluated. Recently, Lienen et al. (2022), revealed a high resistance prevalence of 26 *M. sciuri* isolates against five to twelve antimicrobial substances including methicillin. Additionally, a high prevalence of

penicillin resistance was detected (90%), much higher than in our study (52%). The high number of resistant isolates can be attributed to a selection of the *Mammaliicoccus* isolates belonging to herds where MRSA isolates were detected. Interestingly, 15% of *S. xylosus* and 21% of *M. sciuri* were MDR. The prevalence of multiple resistant isolates was considerable and can become problematic, if the ARGs would be transferred to a pathogenic bacterium such as *S. aureus*.

4.3.3. *Bacillus cereus* group

The *Bacillus cereus* group showed a high prevalence of resistance to β-lactams, in accordance with a recent paper (Bartoszewicz and Czyżewska, 2021) that had isolated *Bacillus* isolates from raw milk and which showed more often resistance than isolates from natural environments; the authors hypothesized that the higher resistance could be due to residual amounts of antibiotics in the milk. To our knowledge, and as described before, a high antibiotic resistance could be associated with intrinsic phenotypic resistance (Owusu-Kwarteng et al., 2017; Mills et al., 2022).

4.3.4. Farm-associated bacteria

The two *Acinetobacter* isolates analyzed in our study were sensitive to all antibiotics and did not show any known ARGs. In contrast, Gurung et al. (2013) found that *Acinetobacter* were highly resistant to different antibiotics tested.

The nine *Aerococcus viridans* isolates were mainly resistant to β-lactams (5) and tetracycline (4). These results partially agree with those of Sun et al. (2017) showing only a partial resistance for tetracycline and no resistance for the β-lactams. A high resistance prevalence to trimethoprim/sulfamethoxazole was found in previous works (Martín et al., 2007; Liu G. et al., 2015; Sun et al., 2017) while in this study, only one isolate was resistant to this antibiotic. Notably, the differences between the studies could be due to the different methods used; the earlier studies mainly used disk diffusion while our study used the microdilution broth (MIC).

The Enterococci of the present study (*Enterococcus faecalis, Enterococcus faecium, Enterococcus saccharolyticus*) were commonly resistant to lincomycin. This is in line with the results described by Różańska et al. (2019). All *Enterococcus saccharolyticus* and all *Enterococcus faecalis* were resistant to synercid. No isolates were resistant to vancomycin, which represents an example of an actual increasing clinical problem in humans with infections caused by *Enterococcus* spp. Our enterococci isolates showed less antibiotic resistant isolates compared to those isolated from humans (Rogers et al., 2021). This is most likely due to the fact that the majority of enterococci found in our study were *Enterococcus saccharolyticus,* which were susceptible to all AB tested except to lincosamides and synercid.

A general low resistance prevalence to antibiotics was found in *E. coli* isolates in this study (three isolates out of 13). Colistin resistance, a last resort antibiotic, was found in two isolates. This is in contrast to the Swiss antibiotic report (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020) where all *E. coli* isolates causing bovine mastitis were susceptible to this AB.

Most *Lactococcus* spp. isolates, were multidrug-resistant. This result was in contrast to the Swiss study by Walther et al. (2008), who demonstrated that *Lactococcus lactis* isolates were sensitive to all 17 antibiotics tested. In that study, the main resistances were observed to tetracycline, clindamycin, and erythromycin, respectively (14.6, 7.3, and 7.3%). In contrast, our work showed a high resistance to rifampin (100%), penicillin, and cephalosporins (71%). Another *Lactococcus* species, *Lactococcus garvieae*, exhibited resistance to clindamycin (four out of seven isolates). Additionally, resistance to rifampin and β-lactam AB was prominent, disagreeing with previous Swiss, and international studies (Walther et al., 2008; Devirgiliis et al., 2013). However, the higher β-lactams resistance observed in the current study could well be associated with the large use of this class of antibiotics at the intramammary level.

4.3.5. Mastitis pathogens

The resistance profile of bovine streptococci has been shown to be strongly influenced by the geographical origin of the sample and the species (Saed and Ibrahim, 2020; Kabelitz et al., 2021). In our study, all *S. agalactiae* isolates were sensitive to all the antibiotics used in the MIC assay. This supports the results of a recent study of bovine mastitis isolates in which the resistance to antibiotics in *S. agalactiae* isolated from different European countries were studied (El Garch et al., 2020). The tetracycline resistance rate of *S. uberis* agreed with the results obtained by El Garch et al. (2020) and with Swiss data (Federal Office of Public Health and Federal Food Safety and Veterinary Office, 2020). Interestingly, although penicillin is the most frequently used antibiotic against *Streptococcus* causing mastitis, no resistant isolates were found. This observation agrees with the work of Käppeli et al. (2019). The percentage of penicillin resistance of *S. aureus* isolates is comparable to results reported in two other European studies (El Garch et al., 2020; Ivanovic et al., 2023).

4.4. *In silico* analysis of ARGs and association with phenotypic results

Advances in DNA sequencing technologies and the availability of various bioinformatics tools and curated databases of ARGs have revolutionized diagnostic microbiology and microbial surveillance (Hendriksen et al., 2019). In our study, we used bioinformatics tools to try to find the ARGs causing the observed resistance. In particular, the genomes were evaluated for ARGs using two of the most popular online tools, Comprehensive Antibiotic Resistance Database (CARD) and ResFinder (Center for Genomic Epidemiology, 2019); they are very effective and have sustainable curation strategies (Lal Gupta et al., 2020). Additionally, an in-house, BLAST based program (GBlast) together with a manually curated database for *Staphylococcus* spp. containing 105 ARGs was used to specifically search for fragmented genes.

The present study demonstrates that there still is a big gap between the phenotypic and genotypic findings. In fact, for many bacteria and many ABs tested, no ARGs were found that could explain the observed phenotype. Obviously, the genetic basis of the mechanisms leading to phenotypic AMR in the bacteria present in the surrounding of cows is less well understood compared with the main pathogens causing mastitis. Nevertheless, *S. xylosus* plays an important role as a fermentative agent in food industry and *Bacillus cereus* has been known for its role in food poisoning for a long time. Particularly striking is the poor association between phenotype and genotype for NASM although recent genomics methods and databases were used. From an evolutionary point of view, NASM are close to *S. aureus*, which has been intensively investigated for AMR during the last decades (Madhaiyan et al., 2020). Based on these considerations,

we expected to find the NASM chromosomal ARGs orthologous to those of *S. aureus* that contribute to intrinsic AMR. Furthermore, we expected to find the same plasmid based ARGs that were previously observed in *S. aureus,* as a possible plasmid transfer between the two species was observed (Fišarová et al., 2019). Except for *tetK* (see below), few of these assumptions turned out to be correct. The reasons for this discrepancy remain largely unclear. For orthologous genes, the similarity between those of NASM and *S. aureus* used as the target genes is probably too low so that the NASM genes cannot be detected by the software tools applied. Considering the plasmid transfer, it is rare between *S. aureus* and NASM in the environment of cows although *S. aureus* is a major mastitis pathogen and is commonly observed on dairy farms (Leuenberger et al., 2019). From a technical point of view, the discrepancy between phenotypic and genotypic findings is hardly the result of inappropriate genomic methods, since the same were successfully applied in our recent publication to predict phenotypic penicillin resistance in *S. aureus* by WGS (Ivanovic et al., 2023).

Importantly, the present work demonstrates that only using genomic approaches might not be adequate to infer phenotypic AMR. Additional genomic and wet laboratory investigations are necessary, and a more comprehensive overview of the mechanisms of actions of different antibiotics.

4.4.1. *Staphylococcus* spp.

Except for tetracycline, no or little genomic information was found explaining the observed resistances in *Staphylococcus*. This is particularly true for β-lactam AB (penicillins and cephalosporins), lincosamides, and in part for macrolides (limited explanation for *S. equorum*). These results demonstrate that NASM may have developed their own mechanisms of resistance. Maybe some of these mechanisms rely on yet undiscovered genes orthologous to known ARGs, but it is also possible that they are based on completely distinct and so far unknown mechanisms and genes. Tetracycline resistance could be fully explained in all resistant isolates by the presence of the *tetK* gene. It was always present on a small plasmid (approx. 4,440 bp after closing) and displayed a 99% similarity to the plasmid pSX10B1, previously found in *S. xylosus* isolated from fermented sausage and linked to a possible, albeit low, transfer of this resistance to other *S. xylosus* strains (Leroy et al., 2019).

The missing association between phenotypic and genotypic results for β-lactam ABs has largely to do with the fact that no known ARGs were found explaining penicillin and oxacillin resistance in *S. xylosus* and *S. succinus*. In fact, no *blaZ* or *mecA, mecA1* or *mecC* genes were observed except in one case. The lack of these genes in *S. xylosus* agrees with the PCR findings reported by Frey et al. (2013), who were also unable to detect them. In the case of *S. equorum*, 75% of the isolates were positive for the *mphC* gene, explaining their observed resistance to macrolides. Although this association is substantial, still 30% of the isolates rely on genes other than *mphC*. Interestingly, this gene was located on the chromosome and was not associated with a mobile element suggesting that it is an intrinsic part of the *S. equorum* chromosome.

4.4.2. *Mammaliicoccus* spp.

The *mecA1* and *salA* genes were observed in all isolates analyzed. Both were located on the chromosome and were not associated with a mobile element. This means both genes are part of the core genome of *M. sciuri* and contribute to the intrinsic AMR of this bacterium. A high association was observed between clindamycin resistance (lincosamides) and the presence of *salA,* as all resistant isolates (93%) also carried the gene. In contrast, as previously found by Cai et al. (2021), although 100% of the isolates carried the *mecA1* gene, only 55% of the isolates showed resistance to oxacillin. For *M. sciuri* it is known that high-level *mecA1* expression is required to observe oxacillin resistance in these bacteria. Overexpression is associated with a *mecA1* promoter mutation at position-10 (Wu et al., 2001; Frey et al., 2013). In the present study, however, none of the 20 isolates analyzed showed the indicated mutation independent whether they were oxacillin resistant or not. Furthermore, all 20 genes could be translated *in silico* into a full-length protein suggesting that the protein function was not harmed by a mutation. These findings suggest that a different mechanism than the previously observed overexpression of *mecA1* accounts for the observed oxacillin resistance.

Even more controversial was that all oxacillin resistant isolates were penicillin susceptible although the low-affinity penicillin-binding proteins (PBP2A) encoded by the *mec* genes including *mecA1* cause resistance to all β-lactam AB (Schwendener and Perreten, 2022). All these findings suggest to re-consider the function of *mecA1* and its expression in future studies. The fact that resistance to β-lactam AB at the genomic level is still incompletely understood in *Staphylococcaceae* is further illustrated by our results for *M. vitulinus*: two isolates were oxacillin resistant, but no ARG was found. Considering tetracycline resistance and *M. sciuri*, all phenotypically positive isolates harbored either the *tetK* ($n = 5$) or the *tetL* genes ($n = 2$). The *tetK* gene was always found on the same small plasmid as observed in *S. xylosus*. For the *tetL* gene, a location on a new, larger plasmid or a correlation with a transposon could be assumed but this will require a further, detailed follow-up study.

4.4.3. *Bacillus cereus* group

The majority of the *Bacillus cereus* group isolates contained *fosB1*; confirming previous reports that found this ARG in isolates from vegetables (Fiedler et al., 2019) and humans (Bianco et al., 2021). Just in one case we could find an association with the phenotypic results.

All *Bacillus cereus* isolates carried the gene *BcII*, previously shown to be involved in β-lactam resistance (Bartoszewicz and Czyżewska, 2021). Additionally, in agreement with the study of Fiedler et al. (2019), a lower prevalence of the *Bc* gene was detected (68%). In our study, when at least one of the two genes was present, the resistance to β-lactams could be always detected.

4.4.4. Farm-associated bacteria

A recent review about the resistomes of *Acinetobacter* non-*baumannii* strains demonstrated that penicillin resistance mediating ARGs were described to be the most prominent resistance genes in these bacteria (Baraka et al., 2020). This study supports these results, with the presence of the gene *Oxa-362*.

A perfect agreement between some phenotypic results and the ARGs was found in *Aerococcus viridans*: all isolates were resistant to tetracycline and, concomitantly, the *tetM* gene was detected. The same was true for the chloramphenicol resistant isolates, which encoded the corresponding *catA8* gene. In contrast, no ARGs were

found that could explain oxacillin resistance, and the presence of the *str* gene was not accompanied by streptomycin resistance.

With a total of 49 genes, a rather high number of ARGs were detected in *E. coli*. Thirty-one genes were observed in all isolates, a finding that confirmed the results of Tyson et al. (2015). In addition, 18 ARGs were found in 8 to 92% of the isolates. Despite the large number of ARGs, all isolates except three were susceptible to all ABs tested. This discrepancy could be easily explained by the fact that for clinical use, and implemented on the commercial resistance plate, only those AB classes for which inherent genes and AMR known mechanisms were contained. The lack of clinical relevance is probably also the reason why most ARGs found in CARD for *E. coli* were missing in the ResFinder database (Center of genomic Epidemiology), an observation that had previously also been made by Jeamsripong et al. (2021).

Lactococcus garvieae and *Lactococcus lactis* were mainly positive for the ARGs *lsa(D)* and *lmrD*, respectively. These genes confer resistance to lincosamides, streptogramins, and pleuromutilins (Shi et al., 2021). In the present study, however, both species were susceptible to these ABs. Further discrepancies between phenotype and genotype were also found for other ABs: although all *Lactococcus garvieae* and *Lactococcus lactis* isolates were resistant to rifampicins and the majority to cephalosporins, no ARGs were detected for both species.

4.4.5. Mastitis pathogens

All *S. agalactiae* isolates carried the ARGs *mprF* and *mreA*. Both genes are commonly present in this species and are always found on the chromosome. *MprF* confers resistance to peptide-based AB while *mreA* to macrolides (Clancy et al., 1997; Ernst et al., 2009). However, in the present study, no phenotype antibiotic resistance results were observed. This fact could be correlated with an intrinsic presence of the genes on the chromosome.

In *S. uberis* isolates, a variant of the gene *patB* was found. In contrast, the *patA* gene, which is correlated with the mechanism of resistance to quinolone, was not identified (El Garch et al., 2010). As a consequence, the resistance to fluoroquinolones could not be detected.

In all *S. aureus* isolates, *arlR, arlS, lmrS, mepR, mgrA,* and *norA* were identified. All these ARGs are intrinsically located on the chromosome and are considered to contribute to basic AMR of *S. aureus* (CARD database). In the present study, however, none of the isolates showed the corresponding phenotype. This is emphasized in case of AMR to quinolones as each isolate harbored the ArlR/ArlS and the MgrA/NorA systems. The same was also true for the *lmrS* gene that was present in all isolates, but no AMR was observed for aminoglycosides, linezolid, macrolides, and phenicols. Additionally, four isolates were *fosB* positive, but sensitive to fosfomycin. In contrast, the observed AMR to penicillin/ampicillin and azithromycin/erythromycin could be explained by the presence of *blaZ* and *ermT* genes, respectively. In *S. aureus*, both genes are normally plasmid based (Kadlec and Schwarz, 2010; Ivanovic et al., 2023) leading to the question whether the current MIC methods are appropriate to assess AMR resulting from mechanisms whose ARGs are intrinsically located on the chromosome. Alternatively, the ARG may not be expressed in the media that we used. In general, transcriptomic or proteomic analyses of the expression levels of certain ARGs (and under different conditions or media) would add additional relevant information for the aim to be able to link genotype and phenotype data.

In summary, the association between phenotype and genotype is missing for various ABs analyzed in the present study. This was observed for β-lactam, lincosamide, and macrolide ABs where the gap can be highlighted. Reasons for this discrepancy may be that the orthologous ARGs of these less commonly studied bacteria were not detected by the current bioinformatics methods, that the corresponding proteins were not expressed, or that, as a consequence of convergent evolution, other AMR mechanisms than the known ones were involved in expression of the observed phenotype. Creating complete genome assemblies for the 350 isolates was beyond the financial possibilities of this study, and might contribute to the fact that some ARGs were missed as well.

4.5. Plasmids and their transfer of ARGs

Plasmids are an important source for the exchange of ARGs between different species and have been reported for *Staphylococcus* species (Malachowa and Deleo, 2010; Mlynarczyk-Bonikowska et al., 2022).

Based on the analyses of circular plasmids, we can contemplate a possible horizontal gene transfer mechanism, when *S. xylosus* and *M. sciuri* co-exist in the same farm. The bacteria apparently transfer small plasmids involved in the antibiotic resistance mechanisms as for tetracycline (*tetK*). The plasmid harboring the *tetK* gene was found in *S. xylosus* (approx. 4,440 bp) and displayed a 99% similarity to the plasmid pSX10B1, previously isolated in *S. xylosus* from fermented sausage (Leroy et al., 2019). In this study, the small plasmid mediated the mechanisms of mobilization, but was rarely transferred to others *S. xylosus* bacteria. The small plasmids are non-conjugative mobilizable, which means that they are not able to be transferred without some helper elements as conjugative plasmids or transposons (Francia et al., 2004; Ramsay et al., 2016). In our study, the completely identical small plasmid found in *S. xylosus* was isolated also from *M. sciuri* and one isolate of *S. warneri* and *S. equorum*. The presence of the same small plasmids circulating in different species could suggest an exchange of the plasmids between the four NASM species. Additional to the small plasmids carrying the tetracycline resistance, other plasmids, carrying *cat* (chloramphenicol resistance) and *str* (aminoglycosides resistance), for instance were detected. These results, although limited to only some of the bacterial species, highlight a possible transfer of ARGs mainly through small plasmids.

In summary, our study finds a high prevalence of bacteria in aseptically collected milk samples from healthy cows. The composition of the intramammary bacteriome displayed a farm-and bedding-dependency: the predominant isolated species were *S. xylosus* and *M. sciuri*, especially in herds that used a straw bedding system. The physiological significance of the NASM in the mammary glands, however, remains to be elucidated in further studies. In contrast, species belonging to the *Bacillus cereus* group or other mastitis pathogens were only rarely detected. It is essential to get more knowledge about the bacteriome of the mammary glands of healthy and diseased cows to understand and preserve the

physiologically normal microbiota, hinder pathogens to gain a foothold and, in the long term, prevent the development and spread of resistances. Further studies addressing the phylogeny of the isolates from milk and herd environment need to be done to understand the origin of the isolates. NASM displayed individual species-specific ARG profiles. Not all phenotypic resistances were based on the presence of known ARGs. WGS represents an important tool for detecting ARGs but still needs to be associated with phenotypic analysis and with gene and/or protein expression analyses. Screening for new genes associated with AMR and an increase of the ARG databases will be essential, especially for the One Health concept.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: All the reads of the different bacteria isolated were uploaded in NCBI under the Bioproject PRJNA859642, https://www.ncbi.nlm.nih.gov/bioproject/859642. The genome and the plasmids of the 15 de novo assembled type strains were uploaded in NCBI under the Bioproject PRJNA936091, https://www.ncbi.nlm.nih.gov/bioproject/936091. Our manually curated database of 105 Staphylococcus spp. ARGs is released as Supplementary material Data Sheet 2.

Author contributions

HUG designed and wrote the initial project application. AR, LS, and MV performed the sampling of the herds. AR, II conducted experiments. JW and LE performed the SCC measurements. TS, MS, and CHA performed *de novo* genome assembly of the type strains (with long read data contributed by DF and JF), identified additional plasmids and closed them. AR and HUG analyzed the data. AR and HUG wrote the first draft of the manuscript with substantial input from CHA. LV, MD, and AS contributed to the conceptualization of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1183018/full#supplementary-material

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Unusually high clarithromycin resistance in *Mycobacterium abscessus* subsp. *abscessus* isolated from human gastric epithelium

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Mycobacterium abscessus subsp. *abscessus* is a rapidly growing facultative intracellular pathogen that usually infects human lung and skin epithelium. Recently, we and another group have shown that it also has the potential to colonize human gastric epithelium, but its significance with respect to gastric diseases remains unclear. Although *Helicobacter pylori* still remains the only definite gastric pathogen, recent studies have shown that *M. abscessus* subsp. *abscessus* also has the potential to colonize human gastric epithelium. *M. abscessus* subsp. *abscessus* is known to exhibit multidrug resistance and clarithromycin has been used as the drug of choice. We aimed to determine the clarithromycin resistance profile of 117 (74 rough and 43 smooth) gastric *M. abscessus* subsp. *abscessus* strains and to detect the point mutations in *rrl* and *erm* (41) genes conferring the resistance. Our data showed 79.48% (19 smooth and 74 rough) of *M. abscessus* subsp. *abscessus* strains were resistant to clarithromycin ($MIC_{90} \leq 512$ μg/mL), while 20.51% (24 smooth) were susceptible ($MIC_{90} \leq 8$ μg/mL). Nucleotide sequence analysis of the *rrl* gene with reference strains of *M. abscessus* subsp. *abscessus* did not show any mutation that is relevant to the clarithromycin resistance. However, analysis of *erm* (41) gene showed that *M. abscessus* subsp. *abscessus* strains, which were susceptible to clarithromycin had C, C, G, and C at their nucleotide positions 28, 159, 238, and 330, respectively, while the resistant strains showed T, T, A, and A at the same positions. Based on antibiogram and sequence analysis data we recommend further studies involving genomic analysis to identify the other genes involved in high clarithromycin resistance in gastric *M. abscessus* subsp. *abscessus* along with the mechanisms involved.

KEYWORDS

gastric diseases, *Mycobacterium abscessus* subspecies *abscessus*, *Helicobacter pylori*, clarithromcycin resistance, erm (41), antibiotic resisitance

Introduction

Mycobacterium abscessus subspecies *abscessus* (*Mycobacterium abscessus* subsp. *abscessus*) is a non-tuberculous mycobacteria (NTM) and is known for its rapid growth and resistance to multiple drugs. It is known to cause pulmonary infection and skin and soft tissues infections (mostly nosocomial) in humans (Lee et al., 2015). *M. abscessus* subsp. *abscessus* infection is difficult to treat because of its intrinsic resistance to most macrolide and other antibiotics including the classical anti-tuberculous drugs (Nessar et al., 2012; Griffith and Daley, 2022).

Recently, we and another group have isolated *M. abscessus* subsp. *abscessus* from human gastric epithelium. Interestingly, in Trivandrum, Kerala, India, the prevalence of gastric *M. abscessus* subsp. *abscessus* is even higher than the prevalence of *Helicobacter pylori*, a well-known gastric pathogen, which causes gastric cancer and peptic ulcer (Al-Momani et al., 2017; Chouhan et al., 2019). The common treatment regimen for *H. pylori* related gastric diseases is a proton pump inhibitor (e.g., Lansoprazole) and antibiotics. Because of the indiscriminate use of metronidazole to prevent amoebiasis in diarrhoea-endemic places in India and other countries, most *H. pylori* strains are resistant to metronidazole and clarithromycin is mostly the drug of choice against *H. pylori* (Safavi et al., 2016; Gonzales et al., 2019; Shetty et al., 2019). The significance of gastric colonization of *M. abscessus* subsp. *abscessus* with respect to gastric diseases is unknown at present, but the potential of this bacterium to cause diseases should not be neglected. Therefore, the resistance profile of the gastric *M. abscessus* subsp. *abscessus* strains against clarithromycin are worth studying for effective management of gastric diseases.

In 1990s, clarithromycin was the choice of drug to eradicate *M. abscessus* subsp. *abscessus* (Mushatt and Witzig, 1995; Brown-Elliott and Wallace, 2002). Clarithromycin was not effective against *M. abscessus* subsp. *abscessus* with point mutations at A_{2058}G, C and A_{2059}G, C (*Escherichia coli* numbering) or $A_{2270} \rightarrow$ G or C and $A_{2271} \rightarrow$ G or C (*M. abscessus* subsp. *abscessus* numbering) positions in the *rrl* gene that encodes the peptidyltransferese domain of 23S rRNA of bacterial ribosome (Wallace et al., 1996). Apart from mutations in *rrl* gene, another mechanism confers resistance against macrolides in NTM. The *M. abscessus* subsp. *abscessus* strains with functional *erm* (41) gene show an inducible resistance against clarithromycin upon prolonged incubation (14 days), while the *M. abscessus* subsp. *abscessus* strains with non-functional *erm* (41) gene show susceptibility to clarithromycin. In the *erm* (41) gene, a substitution of T-to-C at position 28 (T_{28}C), which leads to the alteration of Trp to Arg at the 10thamino acid of the peptide, was found to be associated with loss of function and susceptibility to clarithromycin (Nash et al., 2009). The isolates of *M. abscessus* subsp. *massiliense* have not shown any inducible macrolide resistance because of a 397-bp deletion in *erm* (41) gene, which results in a non-functional *erm* (41) gene (Kim et al., 2010).

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the gastric *M. abscessus* subsp. *abscessus* strains against clarithromycin and to understand the genetic basis of the resistance.

Materials and methods

Ethics statement

The study was approved by the Institute Human Ethics Committee of Rajiv Gandhi Centre for Biotechnology (Approval Number IHEC/01/2017/18) and by the Human Ethics Committee of Govt. Medical College, Trivandrum (Approval Number IEC.No.05/07/2016/MCT). Patients between the age of 20 and 70 years were recruited for the study and written informed consents were obtained from all patients. Trivandrum is in the southern part of India and is the capital city of Kerala, mostly the part of western ghats with high humidity.

Mycobacterium abscessus subsp. *abscessus* culture and characterization

A total of 117 *M. abscessus* subsp. *abscessus* (rough and smooth) strains isolated from human gastric biopsies were used in this study. The *M. abscessus* subsp. *abscessus* strains were grown on Brain Heart Infusion (BHI) agar plates containing calf serum (7%) and were incubated at 37°C in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). *M. abscessus* subsp. *abscessus* colonies were identified at the species level based on the growth rate, colony morphology (rough and smooth), and pigmentation as well as 16S rRNA gene sequence analysis. Partial nucleotide sequencing of the *hsp65* gene was used for further confirmation of gastric *M. abscessus* subsp. *abscessus* strains to distinguish them from closely related *M. bolletti, M. chelonae* and *M. massiliense*. Phylogenetic analysis was done by using BioEdit software (version 7.2.6.1).

Antibiotic susceptibility

M. abscessus subsp. *abscessus* strains were tested for clarithromycin (macrolide antibiotic) susceptibility (from 0.125 μg/mL to 512 μg/mL) by agar dilution and broth microdilution assay. BHI plates were prepared using newborn calf serum (7%) and required concentrations of antibiotics, and BHI broth was prepared similarly for the microdilution assay and finally supplemented with antibiotic as per the desired concentration. After 3, 7, and 14 days of incubation with clarithromycin in the microwell, 10 μL liquid culture from each treated well was applied on the BHI plates and were incubated in microaerobic incubator for 3–7 days. To verify the inducible resistance of gastric *M. abscessus* subsp. *abscessus* against clarithromycin, gastric *M. abscessus* subsp. *abscessus* were pre-treated with clarithromycin (0.1 μg/mL) for 3 days and then the MIC was determined. *M. abscessus* subsp. *abscessus* clarithromycin breakpoint (MIC_{90} > 8 μg/mL) was determined according to the Clinical and Laboratory Standards Institute (CLSI) guideline published in 2011. Broth microdilution-based methodology for antimicrobial susceptibility testing of nontuberculous mycobacteria has been considered the gold standard (Woods et al., 2011).

Bacterial DNA isolation

The bacterial DNA was isolated as previously described (Berg et al., 1997). In brief, the bacterial colonies were harvested in 500 μL PBS and

centrifuged at 5,000 rcf for 10 min. The bacterial pellet was resuspended in 200 µL GTE (glucose/tris/EDTA) buffer. The bacterial suspension was then treated with lysozyme (10 mg/mL) at 37°C for 1 h. After enzymatic digestion, bacterial cells were lysed using TES (tris/EDTA/SDS) buffer. Proteinase K (50 µg/mL) and RNase (20 µg/mL) were then added and the tubes were incubated at 55°C for 2 h. The digested bacterial proteins were removed by phenol: chloroform: isoamyl alcohol and then by chloroform: isoamyl alcohol treatments. The bacterial DNA was precipitated using 3 M sodium acetate (pH 5.2) and chilled absolute ethanol. The precipitated DNA was washed with 70% ethanol and the dried pellet was dissolved in 1X TE (tris-EDTA) buffer of pH 8.

PCR amplification and sequencing of the antimicrobial resistance genes of gastric *Mycobacterium abscessus* strains

Genomic DNA of *M. abscessus* subsp. *abscessus* was used for PCR with primers 16S rRNA V3-V5 F2- (5'-GCC TAC GGG AGG CAG CAG-3′) and V3-V5 R2 (5′-ATT ACC GCG GCT GCT GG-3′) for bacterial 16S rRNA gene (Chouhan et al., 2019); primersHSPF3 (5'-ATC GCC AAG GAG ATC GAG CT-3′) and HSPR4 (5′-AAG GTG CCG CGG ATC TTG TT-3′) for *hsp65* gene sequencing to distinguish *M. abscessus* subsp. *abscessus* from other members of the NTM group (Kim et al., 2005). A total of 46 (31 resistant and 15 susceptible) *M. abscessus* subsp. *abscessus* strains were used to detect antibiotic associated mutations in *M. abscessus* subsp. *abscessus erm* (41) gene, following primers were used:*erm*F (F-GAC CGG GGC CTT CTT CGT GAT-3′) and *erm*R1 (5'-GAC TTC CCC GCA CCG ATT CC-3′) to amplify and *erm* (41)-4 (5′-CCGGCCCGTAGCG TCCAATG-3′) and *erm*F were used for cycle sequencing (Brown-Elliott et al., 2016). Another set of primers ERM1f (5'-CGC CAA CGA GCA GCT CG-3′) and MC823 (5'-GAC TTC CCC GCA CCG ATT CCA C-3′) were used to amplify *erm* (41) gene and to evaluate polymorphism in *erm* (41) gene (Nash et al., 2009; Bastian et al., 2011). To detect mutations in *rrl* gene for acquired resistance in *M. abscessus*, primer (18F 5′-AGT CGG GAC CTA AGG CGA G-3′ and 21R 5'-TTC CCG CTT AGA TGC TTT CAG-3′) were used for amplification and sequencing (Meier et al., 1994). The resulting amplicon of 16S rRNA, *hsp65, erm* (41), and *rrl* gene were purified using Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and were sequenced using BigDye termination v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). Sequencing PCR products were purified by ethanol precipitation and washed with 70% ethanol. The purified products were sequenced using a 3730XL DNA analyser (Thermo Fisher Scientific, Waltham, Massachusetts, US). For the identification of the bacteria, 16S rRNA gene sequence homology analysis was done using BLAST. For phylogenetic classification, multiple *hsp65* gene sequences were assembled and alignment was carried out using ClustalW and phylogenetic tree was constructed as mentioned in Chouhan et al. (2019). To determine the single nucleotide polymorphism (SNP) in *erm* (41) and *rrl* gene of gastric *M. abscessus* strains, the amplified sequence were compared with *M. abscessus* ATCC 19977 genome (GenBank accession number NC_010397.1). We also compared the sequence with *erm* (41) gene of *M. abscessus* strain ATCC19977 (T28 sequevar., GenBank accession number FJ358483.1) and CR5701 (C28 sequevar., GenBank accession number HQ127366.1). For amino acid based protein sequence analysis *M. abscessus* reference strains (GenBank accession number ADM33801.1) were used. All sequences were aligned using ClustalW multiple sequence alignment and were analysed for the mutations at nucleotide as well as amino acid levels using BioEdit software (version 7.2.6.1).

Nucleotide sequence accession numbers

The *erm* (41) gene sequence of resistant *M. abscessus* subsp. *abscessus* rough (*Mabs* R), resistant *M. abscessus* subsp. *abscessus* smooth (*Mabs* S-A), susceptible *M. abscessus* subsp. *abscessus* smooth (*Mabs* S-B) were submitted to GenBank and accession numbers are MW147115,MW147113, MW147114, respectively. The low molecular weight sequence of *erm* (41) gene was also submitted to GenBank and the accession number is MW142321. Similarly *rrl* gene sequences of resistant *M. abscessus* subsp. *abscessus* rough (*Mabs* R), resistant *M. abscessus* subsp. *abscessus* smooth (*Mabs* S-A), susceptible *M. abscessus* subsp. *abscessus* smooth (*Mabs* S-B) were submitted to GenBank and accession numbers are MW148480, MW148478, and MW148479, respectively.

Results

Colony morphologies and clarithromycin resistance patterns of the gastric *Mycobacterium abscessus* strains

The *M. abscessus* subsp. *abscessus* strains isolated from individuals with various gastric diseases have two distinct colony morphologies: smooth and rough. A total of 117 gastric *M. abscessus* subsp. *abscessus* (74 rough and 43 smooth) strains were tested for clarithromycin resistance using agar dilution and broth microdilution based assays. The MIC_{90} for gastric *M. abscessus* subsp. *abscessus* rough morphotypes was ≤256 µg/mL after 14 days of incubation, while *M. abscessus* subsp. *abscessus* rough morphotypes grown in 0.1 µg/mL clarithromycin exhibited an induced increase in MIC_{90} showed MIC_{90} of ≤512 µg/mL after 14 days of treatment (Table 1). Similarly, clarithromycin treatment (uninduced and induced) was carried out for the 24 smooth *M. abscessus* subsp. *abscessus* morphotypes. We observed that all 24 *M. abscessus* subsp. *abscessus* smooth morphotypes had MIC_{90} ≤ 4 µg/mL after 14 days of incubation in uninduced conditions, while in induced conditions, the *M. abscessus* subsp. *abscessus* smooth morphotypes showed MIC_{90} ≤ 8 µg/mL after 14 days of incubation (Table 1). Based on clarithromycin sensitivity pattern we have 3 different types of gastric *M. abscessus* subsp. *abscessus* strains-(a) resistant *M. abscessus* subsp. *abscessus* rough (*Mabs*-R) (b) resistant *M. abscessus* subsp. *abscessus* smooth type A (*Mabs*-S-A) and (c) susceptible *M. abscessus* subsp. *abscessus* smooth type B (*Mabs*-S-B) by considering MIC_{90} ≤ 8 µg/mL as a cut-off in induced as well as uninduced conditions as per the Clinical and Laboratory Standards Institute (CLSI) guideline published in 2011.

Erm (41) PCR based analysis of *Mycobacterium abscessus* subsp. *abscessus* sensitive and resistant strains

In order to investigate the molecular basis of clarithromycin resistance in gastric *M. abscessus* subsp. *abscessus* strains, a 670 bp

TABLE 1 Clarithromycin MIC for gastric *Mycobacterium abscessus* subsp. *abscessus* strains.

M. abscessus morphotype (Cla susceptibility)	Clarithromycin MIC_{90} (Uninduced) (μg/mL)	Clarithromycin MIC_{90} (Induced) (μg/mL)	*M. abscessus* (117)
Mabs R (resistant)	≤256	≤512	74 (63.24%)
Mabs S-A (resistant)	≤256	≤512	19 (16.23%)
Mabs S-B (sensitive)	≤4	≤8	24 (20.51%)

Mabs-R: *M. abscessus subsp. abscessus* rough, *Mabs*-S-A: *M. abscessus subsp. abscessus* smooth type A, *Mabs*-S-B: *M. abscessus subsp. abscessus* smooth type B and Cla: clarithromycin.

region of the *erm* (41) gene was amplified by PCR. Once *erm* (41) gene was amplified from *M. abscessus* subsp. *abscessus* (rough and smooth) resistant and sensitive strains, the amplified products were visualized on 1.5% of agarose gel. An additional ~180 bp amplicon was observed only for the sensitive strains along with the expected band of 670 bp and the results were consistent for all total 117 strains irrespective of smooth and rough morphotypes (Figure 1A). The additional low molecular weight amplicon (180 bp) has not been reported previously. However, with a different set of primer targeting the *erm* (41) gene, only a single amplicon of 764 bp was observed (Figure 1B).

Sequence analysis of the *erm (41)* and *rrl* genes

As mentioned above, we obtained two amplicons (670 bp and 180 bp) in sensitive and one amplicon (670 bp) in resistant strains. The amplicons were purified and sequenced separately to confirm the association of clarithromycin resistance profiles with single-nucleotide polymorphism (SNP) in the *erm* (41) gene. Earlier reports suggest that SNP at 28 (C to T) nucleotide position is associated with inducible resistance of *M. abscessus* subsp. *abscessus*. We observed all clarithromycin susceptible *M. abscessus* subsp. *abscessus* strains (Pfister et al., 2004) had nucleotide C (GenBank accession number MW147114) and all resistant strains (93) strains had nucleotide T (GenBank accession number MW147113 and MW147115) at the position 28 of *erm* (41) gene (Table 2; Figure 2A). Along with the $T_{28}C$ SNP, we also observed nucleotide C at position 159 ($T_{159}C$), nucleotide G at position 238 ($A_{238}G$), and nucleotide C at position 330 ($A_{330}C$) in all susceptible *M. abscessus* strains (GenBank accession number MW147114), but these mutations were absent in all resistant strains of *M. abscessus* (GenBank accession number MW147113 and MW147115), irrespective of smooth and rough morphotypes. The *erm* (41) genes nucleotide sequences were converted to amino acid sequences for both resistant and susceptible *M. abscessus* subsp. *abscessus* strains and was compared with reference strains. We observed Arginine (Arg) and Valine (Val) at position 10 and position 80, respectively only in susceptible strains, while for all resistant strains, Tryptophan (Trp) and Isoleucine (Ile) were observed which is similar to the reference strain (Table 2; Figure 2B). We also amplified *rrl* gene of gastric *M. abscessus* subsp. *abscessus* and sequenced the nucleotides to confirm SNPs in *rrl* gene but we did not observe any SNPs at $_{2270}$A to G or C and $_{2271}$A to G or C (*M. abscessus* numbering) in the *rrl* gene of *M. abscessus* resistant (GenBank accession number MW148480 and MW148478) and susceptible strains (GenBank accession number MW148479) as mentioned in Table 2; Supplementary Figure S1.

Sequence analysis of the low molecular weight amplicon of *Mycobacterium abscessus subsp. abscessus*

The low molecular weight amplified product (180 bp) of *oxidoreductase* gene from *M. abscessus* subsp. *abscessus* susceptible strains were sequenced using forward and reverse primers. As shown in Supplementary Figures S3A,B, the chromatograms of Sanger sequencing for both forward and reverse primers showed very distinct peaks.

Multiple sequence alignment did not show any match of these sequences with the *erm* (41) gene of *M. abscessus* reference strain as well as the gastric *M. abscessus* subsp. *abscessus* strains. To find out the identity of these sequences sequence homology analysis was performed using the BLAST algorithm on the NCBI platform. The BLAST analysis confirmed the identity of these sequences is not *erm* (41) gene but oxidoreductase gene (GenBank: CP029073.1 and CU458896.1) of *M. abscessus* strain G122 and *M. abscessus* ATCC19977. Oxidoreductase of *M. abscessus* contains a total of 3,552 nucleotides, which encode a 1,183 amino acid containing protein. As it was shown in Figure 3, the 180 bp amplicon shows 100 percent similarity with *M. abscessus* G122 strain, starting from G_2991 to C_3158 (GenBank accession number MW142321) which covers 167 bp (Figure 3A). The amino acid sequence starts from valine at the 133 position and the match ends at amino acid valine at the 187 position, which covers a total of 54 amino acids (Figure 3B). With respect to the reference strain *M. abscessus* ATCC19977, the oxidoreductase gene of the gastric *M. abscessus* subsp. *abscessus* showed 4 point mutations at $C_{3007} \rightarrow G$, $C_{3042} \rightarrow T$, $C_{3109} \rightarrow G$ and $A_{3135} \rightarrow G$ (Supplementary Figure S2). Altogether, these results confirmed that the low molecular weight (180 bp) band obtained in the *erm* (41) PCR is amplified from the oxidoreductase gene present in gastric *M. abscessus* subsp. *abscessus* strains.

Discussion

M. abscessus subsp. *abscessus* is a rapidly growing *Mycobacterium* species responsible for pulmonary and soft tissue infections. Although rare, the bacteria also cause disseminated infection, especially in immunocompromised individuals (Lee et al., 2015). *M. abscessus* subsp. *abscessus* is a non-tuberculous mycobacteria (NTM) showing a high level of antibiotic resistance and poses a serious challenge to disease management (Nessar et al., 2012). Various antibiotics which are found to be effective against *M. abscessus* subsp. *abscessus*, like azithromycin, amikacin, meropenem, ciprofloxacin, imipenem, trimethoprim/sulfamethoxazole, and clarithromycin. Among these antibiotics, clarithromycin remains to be the drug of choice to treat *M. abscessus* subsp. *abscessus* related infections (Griffith et al., 2007;

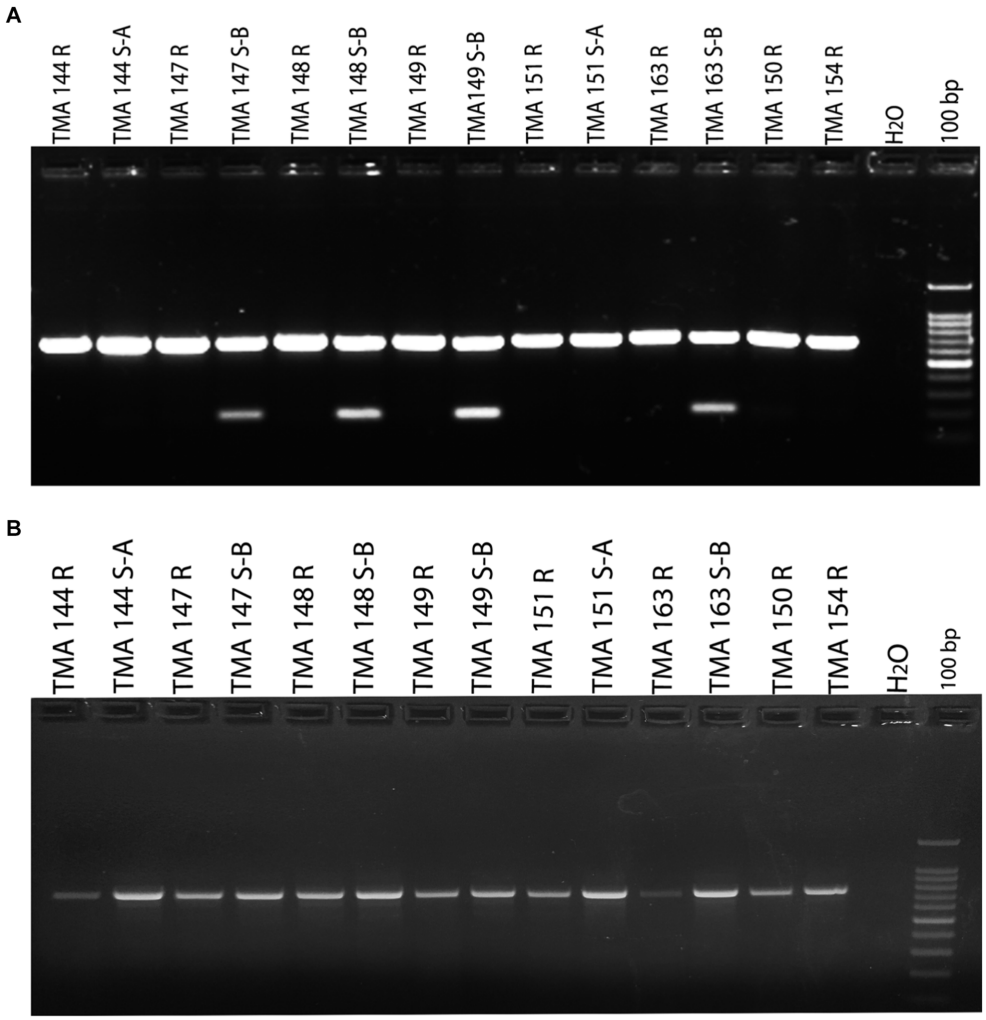

FIGURE 1
Detection of susceptible and resistant gastric *Mycobacterium abscessus* subsp. *abscessus* isolates using *erm* (41) gene PCR. **(A)** *M. abscessus* subsp. *abscessus* strains (TMA 144 R, 144 S-A, 147 R, 148 R, 149 R, 151 R, 151 S-A, 163 R, 150 R and 154 R) showing single amplicon (670 bp) represent resistant phenotype. *M. abscessus* subsp. *abscessus* strains (TMA 147 S-B,148 S-B, 149 S-B and 163 S-B) showing two amplicon (670 bp and 180 bp) represent susceptible phenotype. **(B)** *erm* (41) gene amplification with another set of primer showing single amplicon for gastric *M. abscessus* subsp. *abscessus* strains. (Mention as '100 bp ladder' in the figure instead of just '100 bp).

TABLE 2 ***rrl*** **and** ***erm*** **(41) genotype of susceptible and resistant gastric** ***M. abscessus*** **subsp.** ***abscessus*** **strains.**

M. abscessus morphotype (117)	*rrl* gene mutation $A_{2270} \rightarrow G$ or C $A_{2271} \rightarrow G$ or C	*erm*(41) mutation $T_{28} \rightarrow C$ $T_{159} \rightarrow C$ $A_{238} \rightarrow G$ $A_{330} \rightarrow C$	Erm(41) mutation $Trp_{10} \rightarrow Arg$ $Ile_{80} \rightarrow Val$
Mabs R (resistant) (74)	0	0	0
Mabs S-A (resistant)	0	0	0
Mabs S-B (sensitive)	0	24	24

Mabs-R: M. abscessus subsp. abscessus rough, *Mabs*-S-A: *M. abscessus subsp. abscessus* smooth type A and *Mabs*-S-B: *M. abscessus subsp. abscessus* smooth type B.

Nessar et al., 2012). Recently we reported the presence of *M. abscessus* subsp. *abscessus* in gastric epithelium of patients with various gastric diseases (Chouhan et al., 2019). These gastric diseases are commonly associated with *H. pylori* infection. Recommended treatment option for the eradication of *H. pylori* and the management of related gastric diseases is standard clarithromycin-based triple therapy. In this study, we determined the clarithromycin resistance patterns of gastric *M. abscessus* subsp. *abscessus* strains.

The clarithromycin susceptibility for gastric *M. abscessus* subsp. *abscessus* strains were determined by clarithromycin broth microdilution assay and agar dilution method, where the bacteria was incubated in various concentrations of clarithromycin for 3, 7, and

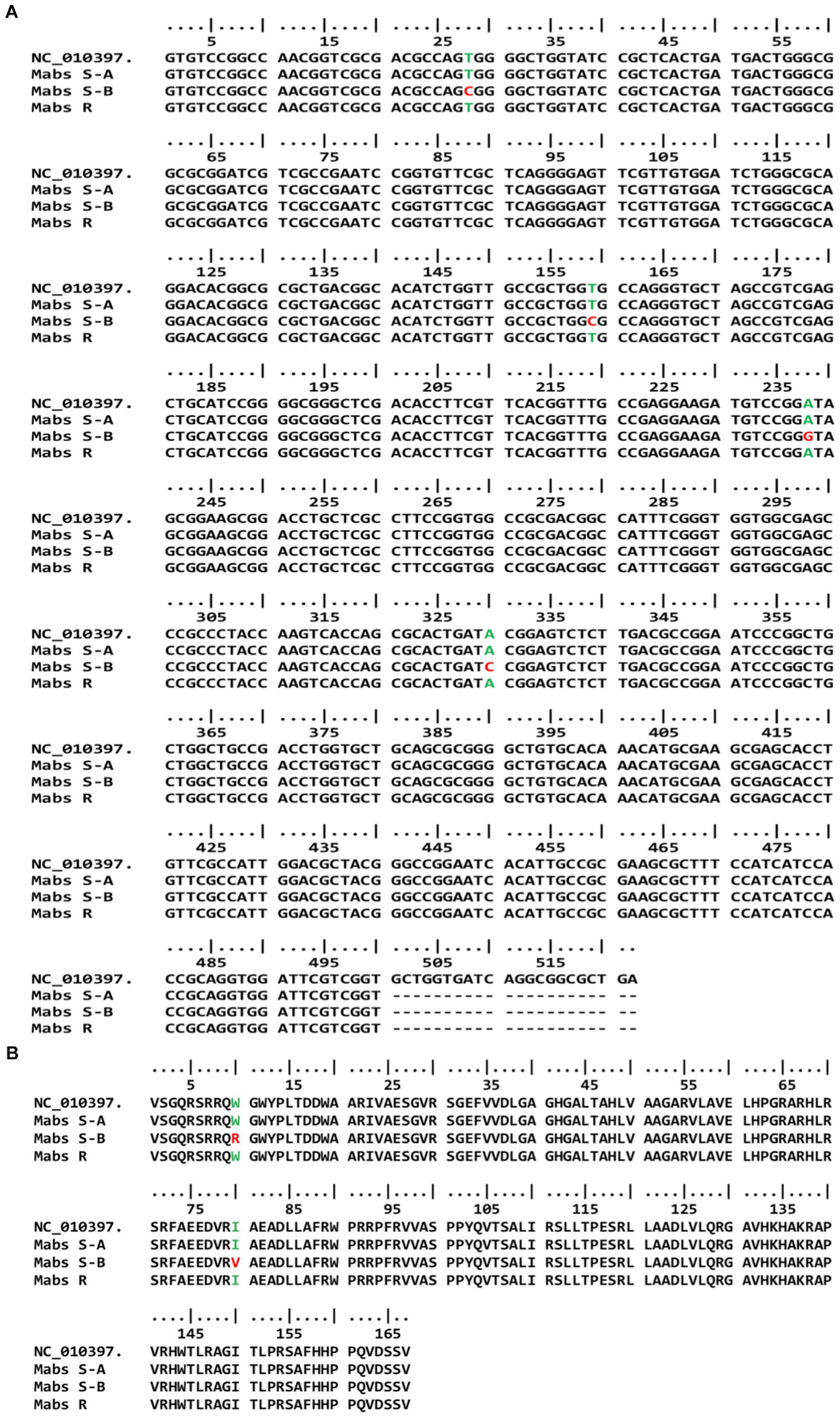

FIGURE 2
erm (41) genotype of susceptible and resistant gastric *M. abscessus* subsp. *abscessus* strains. **(A)** Gene sequence of *erm* (41) showing mutations at 28, 159, 238 and 330 positions in susceptible gastric *M. abscessus* subsp. *abscessus* strains (*Mabs* S-B), while resistant strains (*Mabs* R, *Mabs* S-A) have wild type phenotype. **(B)** Amino acid sequence of Erm (41) protein showing mutations at 10 and 80 amino acid position in susceptible and resistant gastric *M. abscessus* subsp. *abscessus* strains.

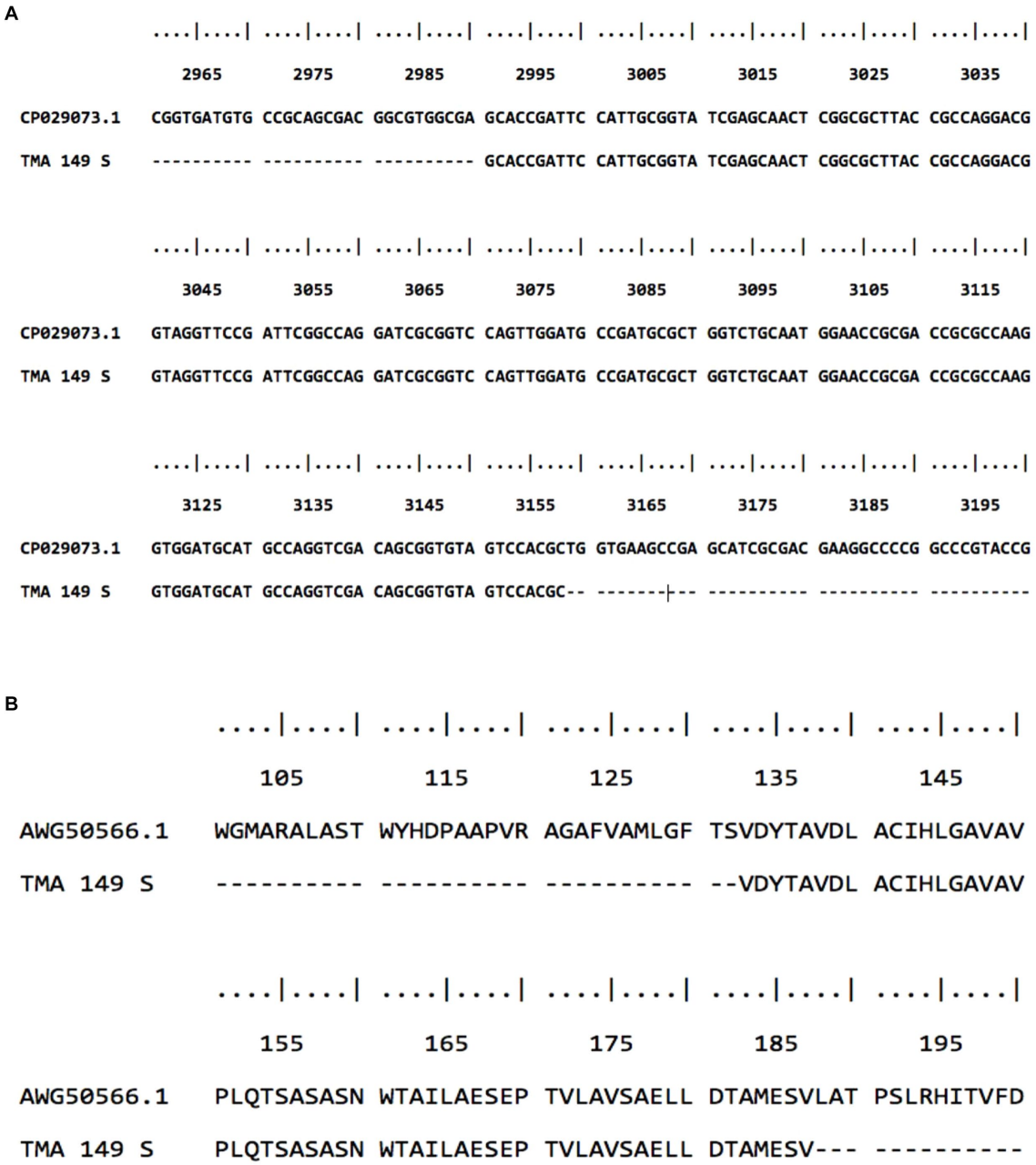

FIGURE 3
Sequence alignment of *M. abscessus* subsp. *abscessus* strains with low molecular weight amplicon. **(A)** Gene sequence of low molecular weight amplicon shows 100 percent similarity with the oxidoreductase gene of *M. abscesus* subsp. *abscessus* strain G122 chromosome. **(B)** low molecular weight amino acid sequence showed 100 percent similarity with oxidoreductase protein.

14 days followed by inspecting bacterial viability. After incubation for 14 days, the growth (viability) pattern of the susceptible and resistant strains were determined. The microdilution-based antibiotic susceptibility test has been recommended by CLSI, because of its reproducibility (Reller et al., 2000; Woods et al., 2011). The clarithromycin susceptibility data obtained in our study of 117 gastric *M. abscessus* subsp. *abscessus* strains demonstrate that the phenotype of clarithromycin susceptibility was fully concordant with *erm* (41) gene SNPs. Our data suggest that of the 117 *M. abscessus* subsp. *abscessus* strains, 93 (79.48%) strains were resistant to clarithromycin, while 24 (20.51%) strains were susceptible to clarithromycin.

The point mutation (C to T) at position 28 of *erm* (41) gene is known to be associated with inducible clarithromycin resistance by *M. abscessus* subsp. *abscessus*. *M. abscessus* subsp. *abscessus* strains showed susceptibility at day 3 of treatment but gradually acquired resistance after 7 days to 14 days of incubation (Nash et al., 2009; Kim et al., 2010; Bastian et al., 2011). Surprisingly we observed there were 19 smooth *M. abscessus* subsp. *abscessus* strains which were resistant to clarithromycin and had wild-type *erm* (41) genotype as like *M. abscessus* subsp. *abscessus* rough morphotype, while susceptible *M. abscessus* subsp. *abscessus* smooth morphotype had SNPs in their *erm* (41) gene. We observed after 14 days of incubation *M. abscessus*

subsp. *abscessus* strains type *Mabs* R showed MIC of 256 μg/mL when they were not induced. However, upon induced with clarithromycin, the MIC raised to 512 μg/mL of clarithromycin. *Mabs*S-A strains were susceptible to 256 μg/mL of clarithromycin in uninduced conditions and MIC was 512 μg/mL in induced conditions. On the other hand, *Mabs*S-B showed MIC of 8 μg/mL after induction, when compared to 4 μg/mL of clarithromycin in uninduced conditions. We observed resistant phenotype with wild type *erm* (41) gene with T, T, A, and A nucleotide base at the 28, 159, 238, and 330 positions, respectively but strains with C, C, G, and C at the 28, 159, 238, and 330 nucleotide positions respectively, exhibited susceptible phenotype (Tables 1, 2). Acquired and inducible resistance for clarithromycin in *M. abscessus* has already been reported. Acquired resistance for clarithromycin has been associated with point mutations at $A_{2270} \rightarrow G$ or C and $A_{2271} \rightarrow G$ or C (*M. abscessus* numbering system) of *rrl* gene (Pfister et al., 2004; Lipworth et al., 2018). To our surprise, we did not observe any mutations in gastric *M. abscessus* subsp. *abscessus* strains at position $A_{2270} \rightarrow G$ or C and $A_{2271} \rightarrow G$ or C (*M. abscessus* numbering system) of *rrl* gene. In conclusion, no association was observed between *rrl* gene and clarithromycin MIC for the resistant and sensitive phenotype of gastric *M. abscessus* subsp. *abscessus* strains.

Mutation from isoleucine (Ile) to valine (Val) at the 80th position has been associated with macrolide drug resistance in *M. abscessus* strains isolated from Korea. Nash et al. has shown that Erm41 protein with Trp10 was associated with resistance while Arg10 was associated with susceptible phenotype, as the protein harbouring Arg10 was non-functional (Nash et al., 2009; Lee et al., 2014). Our amino acid sequence analysis for Erm (41) protein suggests that all susceptible strains of *M. abscessus* subsp. *abscessus* had Arginine (Arg) amino acid at 10th position, while Tryptophan (Trp) was present in resistant strains of *M. abscessus* subsp. *abscessus*. We also observed mutation at position 80, where Valine (Val) was replaced by Isoleucine (Ile) in resistant strains of gastric *M. abscessus* subsp. *abscessus*. Our *erm* (41) gene analysis for gastric *M. abscessus* subsp. *abscessus* strains correlated with susceptibility pattern of *M. abscessus* subsp. *abscessus* strains irrespective of smooth and rough morphotypes. The sequence of low molecular weight (180 bp) amplicon did not show any homology with *erm* (41) gene of *M. abscessus* strains but BLAST analysis identified that 180 bp amplicon belongs to oxidoreductase gene of *M. abscessus* strain G122 with 100 percent match. On the other hand, when compared with *M. abscessus* ATCC19977 strain, the 180 bp amplicon sequence displayed 4 point mutations, $C_{3007} \rightarrow G$, $C_{3042} \rightarrow T$, $C_{3109} \rightarrow G$ and $A_{3135} \rightarrow G$ in oxidoreductase gene (Supplementary Figure S2). Most of the antibiotics also exert their bactericidal effect by generating reactive oxygen species (ROS) or targeting bacterial redox systems. The bacterial oxidoreductase gene has been linked to antibiotic resistance through neutralizing toxic molecules, detoxification of antibiotics, and repair damage caused by antibiotics. Recent studies have revealed that *M. abscessus* subsp. *abscessus* induces efflux pump encoding genes in response to antibiotic stress specifically antibiotic targeting ribosome (Egorov et al., 2018; Mudde et al., 2022; Schildkraut et al., 2022). A mutation in the oxidoreductase gene leading to a non-functional oxidoreductase enzyme may exert a detrimental effect on bacteria during antibiotic treatment. In this study, we observed a possible link between the oxidoreductase gene of *M. abscessus* subsp. *abscessus* in clarithromycin resistance. Further study involving whole genome sequencing of gastric *M. abscessus* subsp. *abscessus* strains are needed to understand the antibiotic resistance gene pool present in the strains along with genotypes and the nature of resistance (acquired or induced). To conclude, we determined the clarithromycin resistance profile of the gastric *M. abscessus* subsp. *abscessus* strains and studied the genetic basis of clarithromycin resistance. We have also demonstrated induced clarithromycin resistance in the isolated gastric *M. abscessus* subsp. *abscessus* strains. Our finding also suggests that the C to T mutation at the 28th nucleotide position of the *erm* (41) gene has an important role in conferring clarithromycin resistance and *erm* (41) gene sequence analysis can light on the mechanism of clarithromycin resistance in of gastric *M. abscessus* subsp. *abscessus*. The results of our study will be helpful while designing efficient strategies to combat multi-drug resistant strains of *M. abscussus* subsp. *abscessus* as well as for the management of associated gastric diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number (s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by Institute Human Ethics Committee of Rajiv Gandhi Centre for Biotechnology (Approval Number IHEC/01/2017/18) and by the Human Ethics Committee of Govt. Medical College, Trivandrum (Approval Number IEC.No.05/07/2016/MCT). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MP conceptualized the idea. DC, TD, KD, SD, and RR performed the experiments. DC, SC, KD, GN, and MP analyzed the data. DC, SC, RR, KD, GN, and MP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1193380/full#supplementary-material

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