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Global dissemination and evolution of epidemic multidrug-resistant gram-negative bacterial pathogens: Surveillance, diagnosis and treatment

volume II

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Global dissemination and evolution of epidemic multidrug-resistant gram-negative bacterial pathogens: Surveillance, diagnosis and treatment volume II

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Table of contents

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Molecular epidemiology and carbapenem resistance characteristics of *Acinetobacter baumannii* causing bloodstream infection from 2009 to 2018 in northwest China

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Bloodstream infection (BSI) caused by *Acinetobacter baumannii* poses a serious threat to health and is correlated with high mortality in patients with hospital-acquired infections, so the molecular epidemiology and antimicrobial resistance characteristics of this pathogen urgently need to be explored. *A. baumannii* isolates from BSI patients were collected in three tertiary hospitals in northwest China from 2009 to 2018. Antimicrobial susceptibility testing was used to determine the MICs of the *A. baumannii* isolates. Whole-genome sequencing based on the Illumina platform was performed for molecular epidemiological analyses and acquired resistance gene screening. The efflux pump phenotype was detected by examining the influence of an efflux pump inhibitor. The expression of efflux pump genes was evaluated by RT-PCR. In total, 47 *A. baumannii* isolates causing BSI were collected and they presented multidrug resistance, including resistance to carbapenems. Clone complex (CC) 92 was the most prevalent with 30 isolates, among which a cluster was observed in the phylogenetic tree based on the core genome multi-locus sequence type, indicating the dissemination of a dominant clone. BSI-related *A. baumannii* isolates normally harbour multiple resistance determinants, of which oxacillinase genes are most common. Except for the intrinsic $bla_{\text{OXA-51}}$ family, there are some carbapenem-resistant determinants in these *A. baumannii* isolates, including $bla_{\text{OXA-23}}$, which is encoded within the Tn*2006*, Tn*2008* or Tn*2009* transposon structures and $bla_{\text{OXA-72}}$. The transfer of $bla_{\text{OXA-72}}$ was suggested by XerC/D site-specific recombination. The AdeABC efflux pump system contributed to carbapenem resistance in *A. baumannii* isolates, as evidenced by the high expression of some of its encoding genes. Both the clone dissemination and carbapenem resistance mediated by oxacillinase or efflux pumps suggest an effective strategy for hospital infection control.

KEYWORDS

***Acinetobacter baumannii*, bloodstream infection, cgMLST, oxacillinase, XerC/D**

Introduction

Acinetobacter spp. are opportunistic pathogens observed during clinical infections, among which *Acinetobacter baumannii* is the most clinically significant (Wong et al., 2017). *A. baumannii* normally colonises on the surface of the skin, mucosa, throat and respiratory tract, causing severe infections including bloodstream infections (BSIs), respiratory infections, skin and soft tissue infections, urinary tract infections, meningitis (Harding et al., 2018; Ramirez et al., 2020). The high mortality associated with BSI caused by *A. baumannii* reached up to 45% in a previous study and is a major concern for nosocomial infection control (Zhou et al., 2019; Gu et al., 2021).

Carbapenems have been known as last-resort antibiotics for *A. baumannii* infections, but unfortunately, carbapenem-resistant *A. baumannii* (CRAB) has spread worldwide and the positive rate observed during clinical screening has continued to increase in recent decades, from 1% in 2003 to 58% in 2008, resulting in a major threat to human health and clinical settings (Reddy et al., 2010). The average positive rate for CRAB in China was 53.7% in 2020, as determined using the CARSS surveillance data.[1]

The resistance mechanism of *A. baumannii* against carbapenems is closely related to the hyperproduction of *β*-lactamases, including some AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs) and carbapenemases (Patel and Bonomo, 2013; Stewart et al., 2019). In *A. baumannii*, the most prevalent mechanism responsible for carbapenem resistance is the production of carbapenem-hydrolysing Ambler class D β-lactamases, such as the OXA-23, OXA-24/40, OXA-58, OXA-143 and OXA-235 types (Peleg et al., 2008; Hammoudi and Ayoub, 2020), among which OXA-23-type carbapenemases are most common in CRAB strains spreading worldwide in nosocomial environments (Potron et al., 2015). Carbapenemase-encoding genes are normally located on chromosomes and/or plasmids, and most of them correspond to mobile genetic elements (MGEs), such as insertion sequences (ISs), integrons and transposons. MGEs are responsible for acquiring, transferring or regulating resistance genes within the host. Several studies have described the dissemination of carbapenem resistance genes by MGEs in *A. baumannii*, resulting in difficulties in treating infectious diseases (Roca et al., 2012; Cornejo-Juarez et al., 2020). Sometimes plasmid-borne carbapenemases are flanked by short DNA sequences providing potential recognition sites for the host XerC and XerD site-specific tyrosine recombinases, contributing to the translocation of these resistance genes (Cameranesi et al., 2018). Furthermore, some multidrug efflux systems, such as the resistance-nodulation-cell division (RND) family efflux pump AdeABC, mediate multidrug resistance, including resistance to carbapenems (Coyne et al., 2011).

The goal of this study was to explore the molecular epidemiology and resistance mechanism of *A. baumannii* isolated from BSI patients, providing an efficient therapy choice and reducing the mortality due to BSI caused by *A. baumannii*.

Materials and methods

Strains

In total, 47 *A. baumannii* isolates from BSI patients were collected, including four strains collected from Shaanxi Provincial People's Hospital in 2018, 25 strains from the First Affiliated Hospital of Xi'an Jiaotong University from 2015 to 2018 and 18 strains from the 3201 Hospital from 2009 to 2018. The species of all the isolates were identified by MALDI-TOF (Bruker, Germany) and confirmed by 16S rDNA sequencing and whole-genome sequencing. This study was approved by the Ethics Committees of 3,201 Hospital of Xi'an Jiaotong University School of Medicine (2020005) with a waiver of informed consent because of the retrospective nature of the study.

Antimicrobial susceptibility testing

The microbroth dilution method was employed to determine the MICs of 47 *A. baumannii* isolates against several antimicrobial agents, including piperacillin/tazobactam, ampicillin/sulbactam, cefepime, ceftazidime, ceftriaxone, cefotaxime, meropenem, imipenem, colistin, gentamicin, amikacin, levofloxacin, ciprofloxacin, tigecycline and cefoperazone/sulbactam. The susceptibility breakpoint was interpreted as recommended by the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2019), except the breakpoint of tigecycline, which was as recommended by the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls.

Whole-genome sequencing

Total genomic DNA from the 47 *A. baumannii* isolates was extracted using the QIAamp DNA Minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The whole genomes of all isolates were sequenced on the Illumina HiSeq X Ten platform (Illumina, San Diego, CA, United states) *via* the 2 × 150 bp paired-end protocol and were subsequently assembled by using CLC genomic workbench version 8.0, and the draft genome contigs were screened for acquired resistance genes by using ResFinder 2.1 on the CGE server.[2] The genetic structure

1 http://www.carss.cn/

2 https://cge.cbs.dtu.dk/

surrounding the resistance gene was annotated by BLAST.[3] The XerC/D-specific recombination site was recognised by PdifFinder.[4]

Molecular epidemiology based on genome sequence

Multi-locus sequence typing (MLST) analysis was performed by screening the assembly contig sequences of each genome using MLST tool version 2.0 on the CGE website.[5] The Oxford MLST allele scheme was employed for typing with seven housekeeping genes, including *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*. Ridom SeqSphere+ software version 4.1.9 (Ridom GmbH Münster, Germany) was used to illustrate the MLST and core genome (cg) MLST relationship. cgMLST analysis was carried out using Paul G. Higgins' scheme, which employed 2,390 genes in the *A. baumannii* genome as core genes. The paired isolates that differed by less than ten core genes were deemed closely related. A phylogenetic tree based on cgMLST was generated by using the minimum spanning tree (AST) algorithm.

Detection of the efflux pump phenotype

Overexpression of the efflux pump phenotype is usually observed when there is a significant increase in carbapenem susceptibility when an isolate is incubated with a carbapenem and the appropriate efflux pump inhibitor (Mmatli et al., 2020). Carbonyl cyanide m-chlorophenylhydrazine (CCCP), phenylalanine-arginine *β*-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) were used as inhibitors to assess the potential decrease of MIC of carbapenem in BSI-related *A. baumannii* isolates.

The expression of the genes *adeA* and *adeB*, which belong to the multidrug efflux pump AdeABC, was assessed in efflux phenotype-positive isolates by RT-PCR. RNA from the isolates was extracted by using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, United States) in the exponential growth period of bacterial cells and was subsequently reverse transcribed to cDNA by the PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan). The gene expression level was evaluated by using TB Green™ Premix Ex Taq™ (Takara, Kyoto, Japan) in a LightCycler 480 system (Roche, Rotkreuz, Switzerland) with triplicate samples for each isolate, and three replicates were performed independently using the $2^{-\Delta\Delta CT}$ method with previously reported primers. Genes for which the fold change in expression was greater than 2 were considered to be differentially expressed. The housekeeping gene *rpoB* was used as the internal reference, and the strain ATCC17978 was used as a reference control.

3 https://blast.ncbi.nlm.nih.gov/

4 http://pdif.dmicrobe.cn/pdif/analysis/

5 https://cge.cbs.dtu.dk/services/MLST/

TABLE 1 Antimicrobial susceptibility testing results of 47 *Acinetobacter baumannii* isolates causing bloodstream infection (BSI).

Antimicrobial agents	Resistance rate (%)	Intermediate rate (%)	Susceptible rate (%)
Ampicillin/sulbactam	55.3	6.4	38.3
Piperacillin/tazobactam	72.3	6.4	21.3
Cefoperazone/sulbactam	61.7	6.4	31.9
Ceftazidime	27.7	0	72.3
Ceftriaxone	72.3	19.1	8.5
Cefotaxime	63.8	6.4	29.8
Cefepime	72.3	6.4	21.3
Meropenem	72.3	0	27.7
Imipenem	72.3	0	27.7
Levofloxacin	66.0	4.3	29.8
Ciprofloxacin	70.2	0	29.8
Tigecycline	72.3	8.5	19.1
Colistin	2.1	0	97.9
Gentamicin	70.2	0	29.8
Amikacin	61.7	2.1	36.2

Results

Antimicrobial susceptibility testing

All 47 *A. baumannii* isolates causing BSI presented high-level resistance against most antimicrobial agents, including β-lactams/β-lactamase inhibitors, third/fourth generation cephalosporins, quinolones, tetracyclines, aminoglycosides and even carbapenem, the last-resort antibiotic for severe infections caused by Gram-negative bacteria. The resistance rates for piperacillin/tazobactam, ceftriaxone, cefepime, meropenem, imipenem, ciprofloxacin, tigecycline and gentamicin were greater than 70%. Colistin showed the highest susceptibility rate (97.9%) among all the tested antimicrobial agents, followed by ceftazidime (Table 1). During the decade of strain collection, we further selected two periods that possessed relatively more isolates to observe the trend of carbapenem resistance. The early period had 13 isolates and was from 2013 to 2015, and the later period had 33 isolates from 2016 to 2018. We found that the carbapenem resistance rate of BSI-related *A. baumannii* increased from 61.5% during the early stage to 72.7% during the later stage.

Molecular epidemiology

All 47 *A. baumannii* isolates were distributed into 22 STs based on the Oxford MLST scheme, among which ST195 was

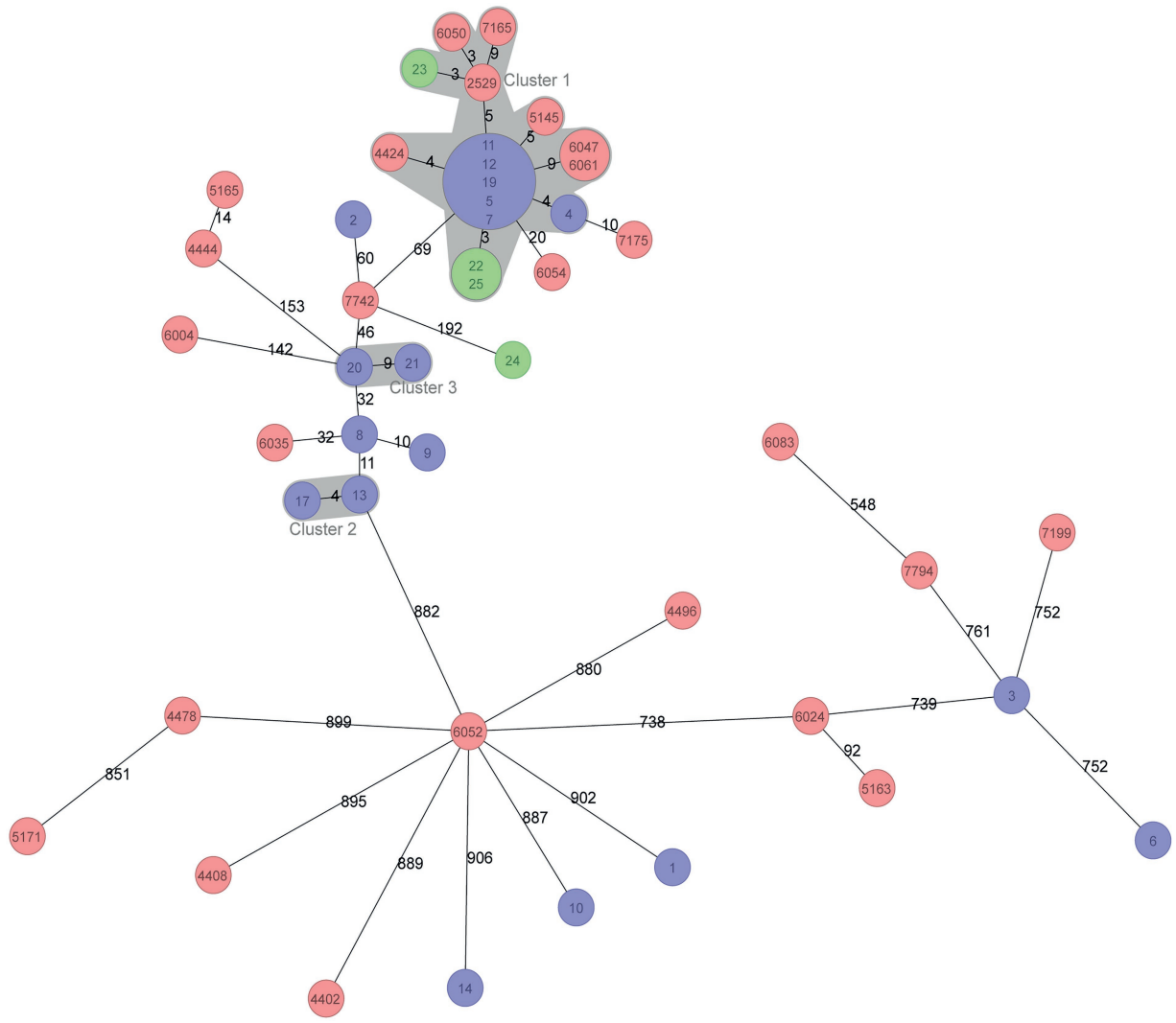

FIGURE 1
Minimum spanning tree of 47 BSI *Acinetobacter baumannii* isolates based on cgMLST. cgMLST profiles are represented by circles, and the isolate name is marked on the circle. The size of the circle is proportional to the number of isolates with an identical cgMLST profile. The different colours in the circle represent the three hospitals from which the isolates were collected. The number on the line connecting the cgMLST circles is the number of core genes that differ between the isolates within the circles. The grey zone surrounding a group of circles represents the closely related isolates that differed by less than ten core genes.

0the most dominant, with 17 isolates (36.2%), followed by ST208 with 8 isolates (17.0%). Except for two ST218 and two ST1818 isolates, each of the remaining isolates belonged to a single ST. Clone complex (CC) 92 was the most prevalent with 30 isolates (63.8%) and encompassed six STs, including ST195 and ST208. Strikingly, all of the CC92 isolates (30/30, 100%) exhibited resistance against carbapenems, whereas 4 of 17 (23.5%) of the other ST isolates were carbapenem-resistant. cgMLST analysis was subsequently performed to assess the phylogenetic relationship of these BSI-related *A. baumannii* isolates with higher resolution based on genome sequences. There was a large relevant cluster (cluster 1 in Figure 1) observed in the phylogenetic tree, the isolates in which originated from all three different hospitals and belonged to ST195, indicating that a dominant clone was disseminated among the hospitals.

Resistome analysis

Most of these BSI-related *A. baumannii* isolates (46/47) harboured intrinsic $bla_{\text{OXA-51-like}}$ or $bla_{\text{OXA-213-like}}$ genes, including $bla_{\text{OXA-51}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-80}}$, $bla_{\text{OXA-88}}$, $bla_{\text{OXA-106}}$, $bla_{\text{OXA-111}}$, $bla_{\text{OXA-120}}$, $bla_{\text{OXA-132}}$, $bla_{\text{OXA-430}}$, $bla_{\text{OXA-273}}$, $bla_{\text{OXA-421}}$, $bla_{\text{OXA-500}}$, $bla_{\text{OXA-526}}$ and $bla_{\text{OXA-533}}$, by which the oxacillinase expression did not mediate carbapenem resistance. Several other OXA-type genes, such as $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-72}}$, were mainly responsible for carbapenem resistance. The $bla_{\text{OXA-23}}$

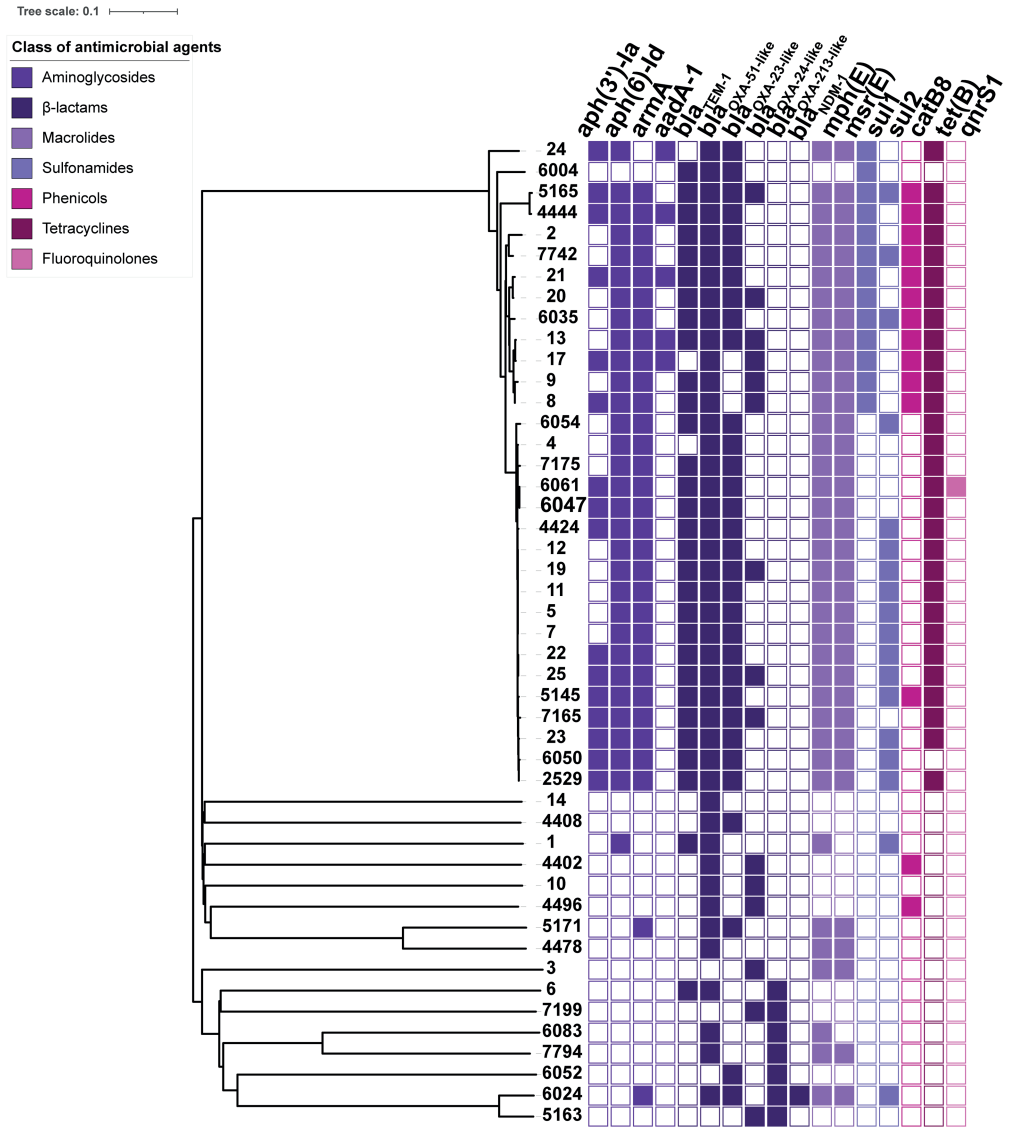

FIGURE 2
Phylogenetic relationship based on the core genome among 47 BSI *A. baumannii* isolates and the heatmap of resistance determinants. In the heatmap, colour-filled rectangles indicate the positive resistance determinants, and colour-empty rectangles indicate the negative resistance determinants. Different colours represent the classes of antimicrobial agents to which the isolate confers resistance.

carbapenemase gene was most common and was present in 32 isolates, one of which co-harboured another metal-β-lactamase, $bla_{\text{NDM-1}}$. There were also 15 $bla_{\text{OXA-72}}$-positive isolates. Moreover, 31/47 isolates harboured the 16S rRNA methylase gene *armA*, which was responsible for high-level resistance against aminoglycosides. Aminoglycoside-modifying enzymes that commonly mediate low-or medium-level resistance to aminoglycosides, such as *aph(3′)-Ia*, *aph(6)-Id* and *aadA-1*, were detected in 32 *A. baumannii* isolates. Among other antimicrobial resistance genes, we also screened the macrolide resistance genes *mph(E)* and *msr(E)*, sulphonamide resistance genes *sul1* and *sul2*, tigecycline resistance gene *tet(B)*, phenicol resistance gene *catB8* and fluoroquinolone resistance gene *qnrS1* (Figure 2).

Genetic structure of carbapenemase genes

The genetic structure of the acquired carbapenemase genes was further analysed. The spread of the carbapenemase gene $bla_{\text{OXA-23}}$ is normally mediated by several known transposons, such as Tn*2006*, Tn*2007*, Tn*2008* and Tn*2009*, among which the distinct genetic structure was found to be distributed on IS*Aba1*/IS*Aba4* or the dual-copy insertion of IS*Aba1*. The known transposon structures were screened in all $bla_{\text{OXA-23}}$-positive isolates, and the comparison results indicated that 21/32 (65.6%) were connected to Tn*2006*, 8/32 (25.0%) were Tn*2009* and the remaining 3/32 (9.4%) were connected to Tn*2008*. Conjugation assays showed that the $bla_{\text{OXA-23}}$ gene was

located on the plasmid in at least 9/32 bla_{OXA-23}-positive isolates, which successfully acquired the bla_{OXA-23}-harbouring plasmid.

The bla_{OXA-40} variant bla_{OXA-72} belonged to the bla_{OXA-24} cluster and was identified in 15/47 (31.9%) *A. baumannii* isolates. The bla_{OXA-72}-containing contig sequences were extracted from the genome data, among which six contigs from isolates 8, 9, 13, 17, 20 and 7,199 were greater than 2 kbp in size and were screened for homology against the GenBank database. The results showed that these six bla_{OXA-72}-containing contigs were approximately 6–15 kb in size and 100% identical or partly similar to plasmids pA2503 (MN495626) and pA2485 (MN495625), which are both 15,405 bp in size. Notably, no mobile element, such as a transposon or IS, was found surrounding the bla_{OXA-72} gene (Figure 3). However, interestingly, the genetic structure comparison illustrated that several insertions, deletions or inversions occurred among these plasmid segments, and on the border of the fragment, we found some pairs of XerC/XerD-like sites, which could provide active pairs for site-specific recombination mediating horizontal gene transfer. For example, a pair of XerC/XerD-like sites were found at the border of a 5 kbp inversion between isolate 8 and isolate 13 (or 17). Similarly, XerC/XerD-like sites also emerged at the border of in/del segments between isolates 20 and 9, isolates 9 and 8, isolates 7,199 and 17, etc. Crucially, the bla_{OXA-72} gene was observed as a segment flanked closely by XerC/XerD-like sites, suggesting that XerC/XerD-like site-mediated recombination may be responsible for mobilisation

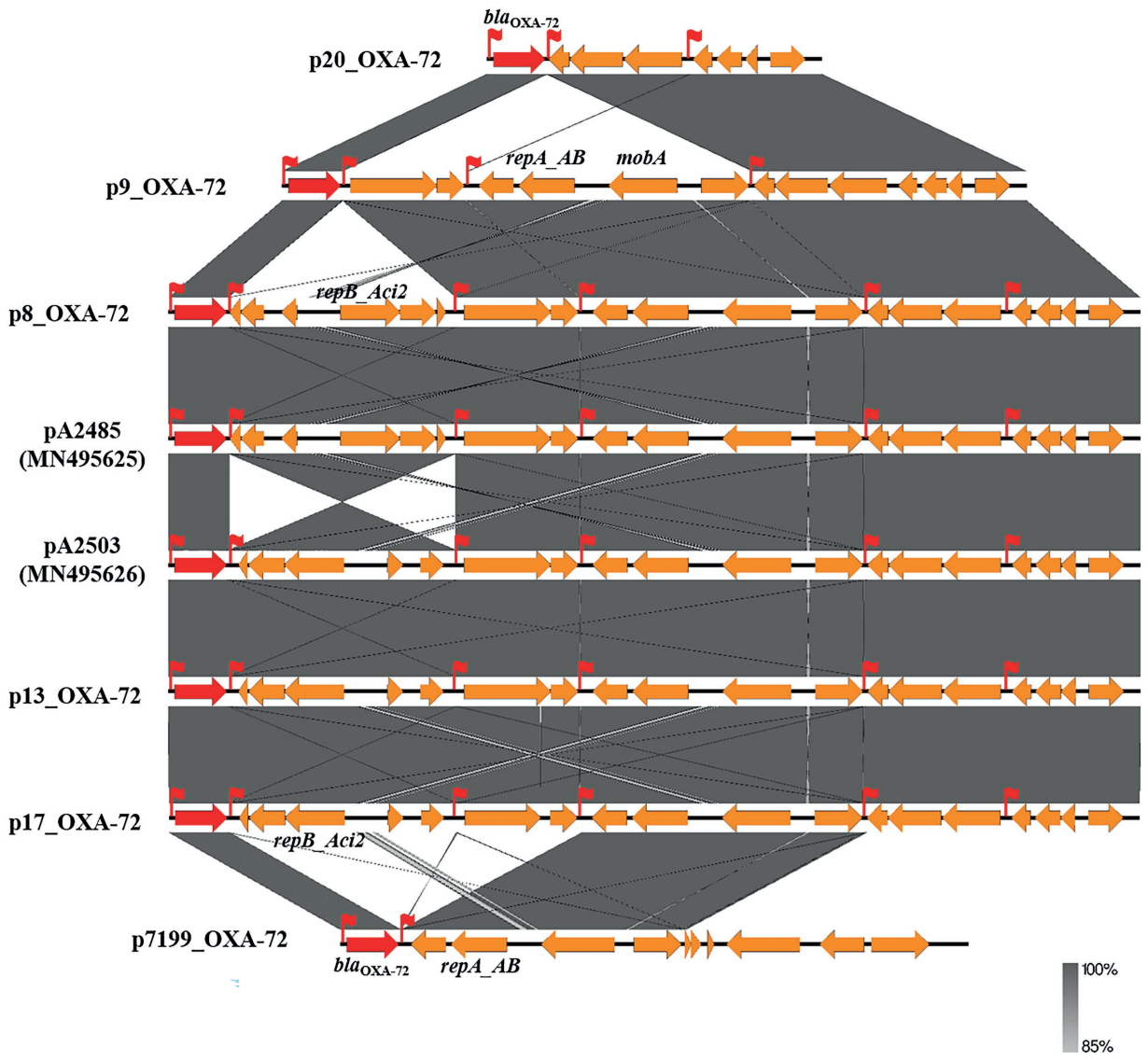

FIGURE 3
Sketch and comparison of the genetic structure of bla_{OXA-72}-harbouring large contigs. Orange rectangular arrows on the line represent the open reading frames (ORFs) encoded by the contig sequence, and the red arrows represent the bla_{OXA}-gene. The dark grey shadows indicate the 72 identical sequence segment between two contigs. The red flags represent the XerC/XerD-like combination site.

of the bla_{OXA-72} gene in the BSI-related *A. baumannii* isolates in our study (Figure 3).

Detection of the efflux pump phenotype

In total, 25/47 (53.2%) isolates presented an efflux pump phenotype that decreased MIC of meropenem or imipenem by 4-fold or more. Most (23/25, 92.0%) of the efflux pump positive isolates were identified under the CCCP inhibitor, and six isolates tested positive under PAβN. The inhibitor NMP did not induce an efflux pump phenotype in any of the isolates. Eighteen isolates that showed a more than 64-fold decrease in the MIC of meropenem were selected for subsequent evaluation of the expression of the genes *adeA* and *adeB* in the multidrug efflux pump system AdeABC. The results showed that 17/18 (94.4%) isolates presented higher expression of *adeA* or *adeB* than the reference strain ATCC 17978, among them 16 isolates presented a more than 2-fold (significant) increase in expression of at least one pump gene (Figure 4). The last isolate exhibited no expression of either the *adeA* or *adeB* gene due to deletion of the AdeABC efflux pump, which was confirmed by PCR on chromosome DNA.

Discussion

BSI caused by *A. baumannii* is associated with high patient mortality and is one of the most urgent threats to public health. In this study, 47 *A. baumannii* isolates causing BSI were collected in three tertiary hospitals over a decade. Antimicrobial susceptibility testing and whole-genome sequencing were performed to assess the molecular epidemiology and antimicrobial resistance characteristics of the isolates. Multidrug resistance, especially resistance against carbapenem, was observed in these BSI-related *A. baumannii* isolates, leaving limited choice for therapy. Colistin, a cationic polypeptide belonging to the polymyxin family, has been introduced into clinical practice as an important therapeutic option for carbapenem-resistant Gram-negative bacterial infections (Falagas et al., 2011). A previous study indicated that colistin monotherapy was associated with a better outcome than colistin-meropenem combination therapy (Dickstein et al., 2019). However, using colistin as a therapy choice still depends on PK/PD and underlying complications due to its potential renal toxicity.

Molecular epidemiology based on genomic data facilitated the investigation of the dissemination and phylogenetic relationship of BSI-related *A. baumannii* isolates with higher distinguishability. CC92 clone dissemination was observed in

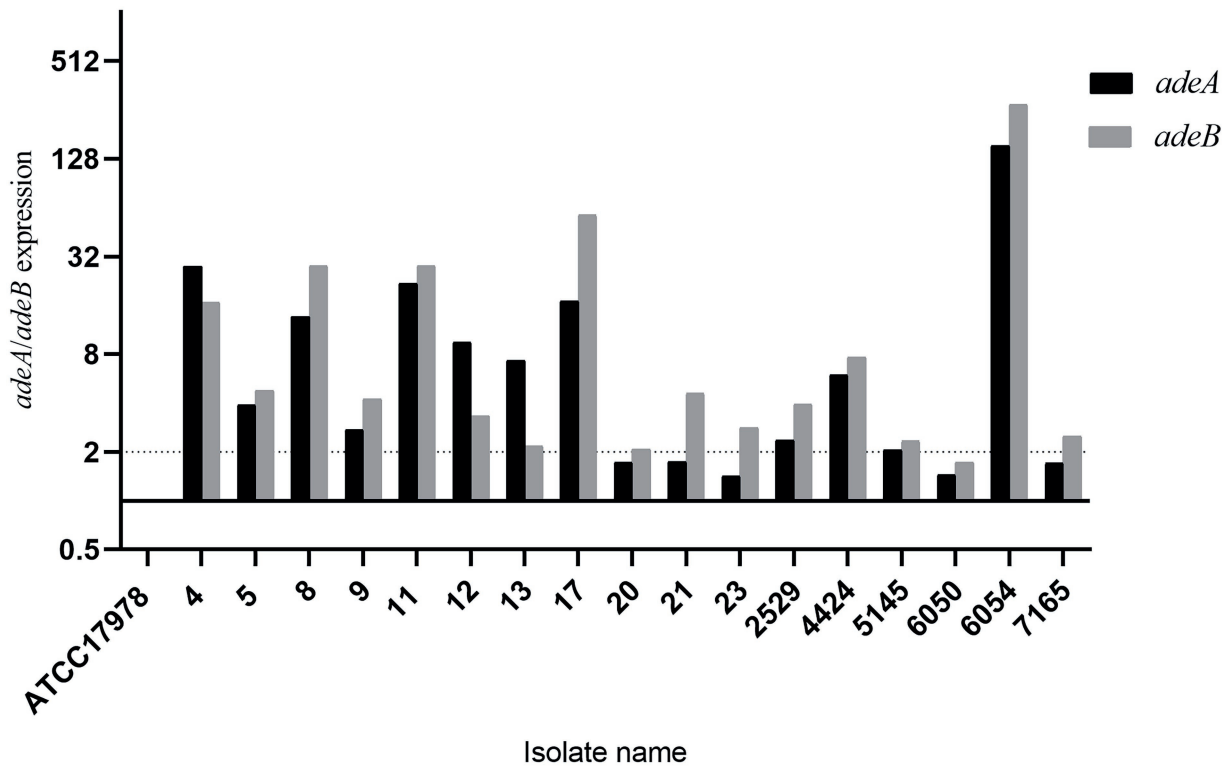

FIGURE 4
Expression of the AdeABC efflux pump genes *adeA* and *adeB*. ATCC17978 was used as a baseline.

this study accounting for more than half of the BSI-related *A. baumannii* isolates. CC92 is the most common clone complex in *A. baumannii* from China (Karah et al., 2012). More importantly, all isolates belonging to the CC92 clone were carbapenem-resistant, which was one of the crucial reasons they survived under high antibiotic pressure and spread widely, consequently inducing BSI. cgMLST was used to further discover the diffusion of a dominant clone among three different hospitals in northwest China, as evidenced by the presence of a cluster comprising genetically indistinguishable isolates.

The BSI-related *A. baumannii* isolates harboured multiple resistance determinants, among which the oxacillinase genes were the most common. The OXA-type enzymes in *A. baumannii* are normally divided into four clusters based on their genetic similarities, namely, the OXA-51, OXA-23, OXA-24 and OXA-58 clusters (Peleg et al., 2008). The enzymes of the OXA-51 cluster are naturally occurring enzymes in *A. baumannii* given their chromosomal location and minimal effect on carbapenem susceptibility. More than ten variants of $bla_{\text{OXA-51}}$ or $bla_{\text{OXA-213}}$ were observed in 46 of the BSI-related *A. baumannii* isolates, indicating the diversity of the intrinsic oxacillinase gene cluster.

In the *Acinetobacter* genus, the acquired carbapenem hydrolysing oxacillinases contribute to carbapenem resistance. OXA-23 was the first identified carbapenemase, and its role in carbapenem-hydrolysis appeared to be elevated in the presence of the upstream IS*Aba1* element. In our study, the $bla_{\text{OXA-23}}$-positive isolates were associated with several known transposons, Tn*2006*, Tn*2008* and Tn*2009*, and all of them encompassed the IS*Aba1* element that possibly mediated their high-level carbapenem resistance (Chen et al., 2017). Yang's study reported 58 *A. baumannii* strains carrying $bla_{\text{OXA-23}}$ gene in China, 47 isolates (47/58, 81.0%) were associated with Tn*2009* and 8 isolates (8/58,13.8%) were associated with Tn*2006* (Yang et al., 2019). Another study reported by Cerezalesa showed that 51 carbapenem-resistant *A. baumannii* strains carried the $bla_{\text{OXA-23}}$ gene in transposon Tn*2008* (Cerezales et al., 2019). In our study, the transposons that harboured the $bla_{\text{OXA-23}}$ gene in BSI-related *A. baumannii* in Shaanxi province presented diverse, and the Tn*2006* was the most common (65.6%) in our report, suggesting a distinction from the results of previous studies.

The $bla_{\text{OXA-40}}$ variant $bla_{\text{OXA-72}}$ belongs to the $bla_{\text{OXA-24}}$ cluster, and its presence has been reported in several previous studies on CRAB (Kuo et al., 2013; Dortet et al., 2016; Chen et al., 2018). Analysis of the $bla_{\text{OXA-72}}$ genetic environment revealed a potential transfer mechanism corresponding to a recombination site. In *A. baumannii* and most bacteria, dimers are resolved to monomers by site-specific recombination, which is a process that is performed by two chromosomally encoded tyrosine recombinases (XerC and XerD). Several studies have reported that plasmid-borne bla_{OXA}-containing structures are bordered by short sequences exhibiting homology with the 28-nucleotide dif motif located at the bacterial chromosome replication terminus and are recognised by XerC/D site-specific recombinases, leading to the hypothesis that their mobilisation could be mediated by site-specific recombination (Merino et al., 2010). In our study, all $bla_{\text{OXA-72}}$-containing contigs were found to have a pair of 28-nucleotide XerC/XerD-like sites that closely flanked the $bla_{\text{OXA-72}}$ gene, indicating that recombination through the Xer system likely occurs to mediate transfer of the carbapenem-resistance gene to the nosocomial environment.

Efflux pumps often play a crucial role in multidrug resistance in *A. baumannii*, including by mediating a possibly significant increase in carbapenem susceptibility (Abdi et al., 2020). In this study, the carbapenem susceptibility in more than half of the BSI-related *A. baumannii* isolates was influenced by efflux pump inhibitors. The inhibitor CCCP seemed more efficient for the majority of isolates with an efflux pump phenotype, and in contrast, the inhibitor NMP showed no impact. The AdeABC efflux pump is a member of the RND family and can pump out multiple antibiotics, and overexpression of the AdeABC efflux pump may confer high-level resistance to carbapenems (Zhu et al., 2013). The majority of efflux pump phenotype-positive isolates showed higher expression of AdeABC efflux pump genes, except one isolate, which lacked these genes. The presence of other functional efflux pumps in that isolate could potentially explain the efflux pump-positive phenotype.

In conclusion, this study of molecular epidemiology and antimicrobial resistance characteristics revealed that *A. baumannii* isolates causing BSI presented clone dissemination and multidrug resistance. The multidrug-resistant clone CC92 had spread among distinct hospitals in northwest China over decade-long period of our study. The BSI-related *A. baumannii* isolates consistently exhibited resistance against carbapenems, which was attributed to the wide distribution of oxacillinases OXA-23 and OXA-72. In addition to the carbapenemases produced, the efflux pump harboured by the *A. baumannii* isolates also plays an important role, and the efflux pump genes were suggested to exhibit significantly increased expression. BSI caused by *A. baumannii* isolates poses a serious threat to health and is correlated with high mortality in patients with hospital-acquired infections. Additional strategies for nosocomial infection control urgently needed to prevent these multidrug-resistant *A. baumannii* clones from becoming endemic.

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/, PRJNA844406.

Author contributions

JX conceived and designed this study. YG, JL, and LZ collected strains of bacteria. YG, JL, and JT collected the isolates and clinical data. WZ and HX performed the antimicrobial susceptibility testing. WZ, HW, JT, MD, and MZ carried out whole genome sequencing and analysis. YG and RW structured the variables and performed the statistical analyses. YG 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

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Prevalence and antibiotics resistance of *Ureaplasma* species and *Mycoplasma hominis* in Hangzhou, China, from 2013 to 2019

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Ureaplasma spp. and *Mycoplasma hominis*, frequent colonizers in the lower urogenital tract, have been implicated in various infections, with antibiotic resistance growing and varying regionally. This study aims to investigate the prevalence and antibiotic resistance profiles of *Ureaplasma* spp. and *M. hominis* in outpatients in Hangzhou, China, from 2013 to 2019. A total of 135,263 outpatients were examined to determine the prevalence of *Ureaplasma* spp. and *M. hominis*, including 48,638 males and 86,625 females. Furthermore, trends in antibiotic susceptibility of *Ureaplasma* spp. and *M. hominis* during 1999–2019 were analyzed. The cultivation, identification, and antibiotic susceptibility of the bacteria (ofloxacin, ciprofloxacin, erythromycin, clarithromycin, azithromycin, josamycin, tetracycline, doxycycline, and pristinamycin) were determined using the Mycoplasma IST2 kit. Our study indicated that the overall prevalence of total *Ureaplasma* spp./*M. hominis* was 38.1% from 2013 to 2019. *Ureaplasma* spp. were the most frequently isolated species (overall prevalence, 31.3%), followed by *Ureaplasma* spp./*M. hominis* coinfection (6.0%) and single *M. hominis* infection (0.8%). The prevalence of *Ureaplasma* spp. and *M. hominis* was significantly higher in females than in males, and the highest positive rates of total *Ureaplasma* spp./*M. hominis* were observed in both female and male outpatients aged 14–20years. During 2013–2019, josamycin, tetracycline, doxycycline, and pristinamycin maintained exceptionally high activity (overall resistance rates, <5%) against both *Ureaplasma* spp. and *M. hominis*, but ofloxacin and ciprofloxacin showed limited activity (overall resistance rates, >70%). During 1999–2019, the rates of resistance to ofloxacin and ciprofloxacin increased against both *Ureaplasma* spp. and *M. hominis* but decreased to erythromycin, clarithromycin, azithromycin, tetracycline, and doxycycline against *Ureaplasma* spp. In conclusion, our study demonstrates a high prevalence of *Ureaplasma* spp. compared to *M. hominis* and *Ureaplasma* spp./*M. hominis*, and their distribution was associated with sex and age. Josamycin, doxycycline, and tetracycline are promising antibiotics that have remarkable activity against *Ureaplasma* species and *M. hominis*.

KEYWORDS

***Ureaplasma* spp., *Mycoplasma hominis*, prevalence, antibiotic resistance, activity**

Introduction

Ureaplasma species and *Mycoplasma hominis*, members of the class Mollicutes, are the smallest self-replicating and free-living organisms known, and are routinely identified as common commensal bacteria in the lower urogenital tract of healthy individuals. They are, however, sometimes implicated in various types of infections, such as chorioamnionitis, infertility, adverse pregnancy outcomes, and neonatal diseases (Waites et al., 2005; Taylor-Robinson and Lamont, 2011; Huang et al., 2015; Kletzel et al., 2018). Genital mycoplasmas can be identified in cervicovaginal and urethral specimens of 40–80% healthy humans. But they are relatively common in the urogenital tracts of sexually active adults with clinical manifestations, where *Ureaplasma* spp. and *M. hominis* can be found, with *Ureaplasma* spp. being the most prevalent (Song et al., 2014; Kasprzykowska et al., 2018; Beeton and Jones, 2019; Piscopo et al., 2020; Doroftei et al., 2021).

Both *Ureaplasma* spp. and *M. hominis* lack cell wall; thus, antibiotic therapies are restricted to those that prevent DNA replication (e.g., fluoroquinolones) and protein synthesis (e.g., macrolides and tetracyclines). The prevalence and antibiotic susceptibility profiles vary geographically, depending on antibiotic use and history of previous antibiotic exposure. Antibiotic resistance has been increasing in recent years probably due to the inappropriate use of antibiotics, which is most likely acquired through gene mutation or the acquisition of resistance determinants (Yang et al., 2020; Chalker et al., 2021). Therefore, it is critical to monitor the change of antibiotic susceptibility regularly to provide guidelines for the treatment of *Ureaplasma* spp. and *M. hominis* infections. The objective of this study was to determine the prevalence and antibiotic susceptibility of *Ureaplasma* spp. and *M. hominis* in outpatients in Hangzhou, China, from 2013 to 2019.

Materials and methods

Study participants

During the period of January 2013 to December 2019, a total of 135,263 outpatients were examined in the clinical laboratory at Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, China. Of these, 48,638 were males aged 14–89 years, and 86,625 were females aged 14–94 years. Furthermore, 804 *Ureaplasma* spp. isolates were collected from outpatients between March and June of 1999–2004, and 1,278 isolates were obtained from outpatients with genital manifestations, such as vaginal or cervical discharge, painful or burning urination, dysuria, frequent urination, and other symptoms, between January 2005 and December 2012, to determine the trend in the antibiotic susceptibility of *Ureaplasma* spp. (Xie and Zhang, 2006; Song et al., 2014). Additionally, 267 *M. hominis* isolates recovered from outpatients between January 2005 and December 2012 were included to determine the trend in the antibiotic susceptibility of *M. hominis* (Kong et al., 2016).

Sample collection, culture, and antibiotic susceptibility testing

Urethral specimens of male patients were obtained by inserting Dacron swabs 2–3 cm into the urethra and spinning for 5 s, and cervicovaginal specimens of female patients were obtained from the cervical area after exocervical mucus was cleansed with a swab. A commercial Mycoplasma IST2 assay (bioMe'rieux, Marcy-l'E' toile, France) was used for the identification, semi-quantification of the concentration, and antibiotic susceptibility testing of *Ureaplasma* spp. and *M. hominis*. The specimens were inoculated and incubated according to the manufacturer's instructions. Briefly, urethral and cervical swabs were inoculated in R1 medium, and the mixture was added to R2 medium and vortexed until the pellet dissolved. Then, the rehydrated R2 growth medium was distributed into wells on the Mycoplasma IST2 strip and protected from drying with mineral oil. The strip and the remaining broth were incubated at 37°C for 48 h and color changes were recorded at 24 h for *Ureaplasma* spp. and 48 h for *M. hominis*. Positive results were noticed when the color of the broth changed from yellow to red with an estimated density of each organism $\geq 10^4$ CFU.

Antibiotic susceptibility testing was performed for the following antibiotics: ofloxacin, ciprofloxacin, erythromycin, clarithromycin, azithromycin, josamycin, tetracycline, doxycycline, and pristinamycin. The antibiotic resistance breakpoints for the above nine antibiotics (mg/L) were as follows: ofloxacin, resistant (R) ≥ 4; ciprofloxacin, $R \geq 2$; erythromycin, $R \geq 4$; clarithromycin, $R \geq 4$; azithromycin, $R \geq 4$; josamycin, $R \geq 8$; tetracycline, $R \geq 8$; doxycycline, $R \geq 8$; and pristinamycin, $R \geq 2$ (Kenny and Cartwright, 2001).

Statistical analysis

The SPSS Statistics for Windows v.21.0 was used to analyze the prevalence and occurrence of resistance to the nine antibiotics tested based on the Chi-square test and Fisher's exact test. p-values of <0.05 were considered significant statistically.

Results

Prevalence of *Ureaplasma* spp. and *Mycoplasma hominis* from 2013 to 2019

Among the 135,263 specimens tested, the overall positive rate of total *Ureaplasma* spp./*M. hominis* was 38.1% (51,504 out of 135,263). *Ureaplasma* spp. infection was more common than *Ureaplasma* spp./*M. hominis* coinfection (31.3% vs. 6.0%,

$p < 0.001$) and *M. hominis* infection (31.3% vs. 0.8%, $p < 0.001$). Of the 48,638 specimens obtained from male outpatients, 12,266 (25.2%) were positive for *Ureaplasma* spp., 216 (0.4%) for *M. hominis*, and 1970 (4.1%) for both *Ureaplasma* spp. and *M. hominis*. Females had a significantly higher prevalence of *Ureaplasma* spp. and *M. hominis* than males ($p < 0.001$). Of the 86,625 specimens obtained from female outpatients, 30,044 (34.7%) were positive for *Ureaplasma* spp., 886 (1.0%) for *M. hominis*, and 6,122 (7.1%) for both *Ureaplasma* spp. and *M. hominis*.

Trends in the prevalence of *Ureaplasma* spp. and *M. hominis* during the test period are shown in Figure 1. *Ureaplasma* spp. infection rates were ranged from 23.1 to 27.1% in males and from 32.7 to 39.9% in females, which were higher than those of *M. hominis* infection and *Ureaplasma* spp./*M. hominis* coinfection (*M. hominis*, 0.3–0.6% for males and 0.8–1.4% for females; coinfection, 3.1–5.1% for males and 5.9–8.2% for females).

Distribution of *Ureaplasma* spp. and *Mycoplasma hominis* in different age groups from 2013 to 2019

The distribution of *Ureaplasma* spp. and *M. hominis* according to the age group from 2013 to 2019 is presented in Table 1. The overall positive rate of total *Ureaplasma* spp./*M. hominis* was highest in male patients aged 14–20 years (33.6%) and lowest in male patients aged ≥51 years (25.1%), with a declining trend as age increased. Similarly, the overall positive rate of total *Ureaplasma* spp./*M. hominis* was highest in female patients aged 14–20 years (58.0%), followed by 46–50 years (54.7%), and lowest in female patients aged 50–94 years (36.7%). For *Ureaplasma* spp. infection, the highest positive rates were found in both male and female patients aged 14–20 years, with 28.2% for males and 40.2% for females. For *M. hominis* infection, the detection rate was highest in males aged 14–20 years (1.1%) and ≥ 51 years (0.9%), but it occurred most commonly in females aged 46–50 years (2.2%) and ≥ 51 years (2.2%). Notably, among the patients with *Ureaplasma* spp./*M. hominis* coinfection, the highest detection rate was found in females aged 14–20 years (16.8%) and 46–50 years (14.4%); however, close detection rates were found in males of different age groups, ranging from 3.8 to 4.9%.

Antibiotics effectiveness from 2013 to 2019

The overall resistance rates of *Ureaplasma* spp. and *M. hominis* from 2013 to 2019 are shown in Table 2. Josamycin, tetracycline, doxycycline, and pristinamycin maintained high activity against *Ureaplasma* spp. and *M. hominis*, with resistance rates all <5%. Erythromycin, clarithromycin, and azithromycin were effective against the majority of *Ureaplasma* spp. isolates (resistant rates, <3%). In comparison, ofloxacin and ciprofloxacin displayed

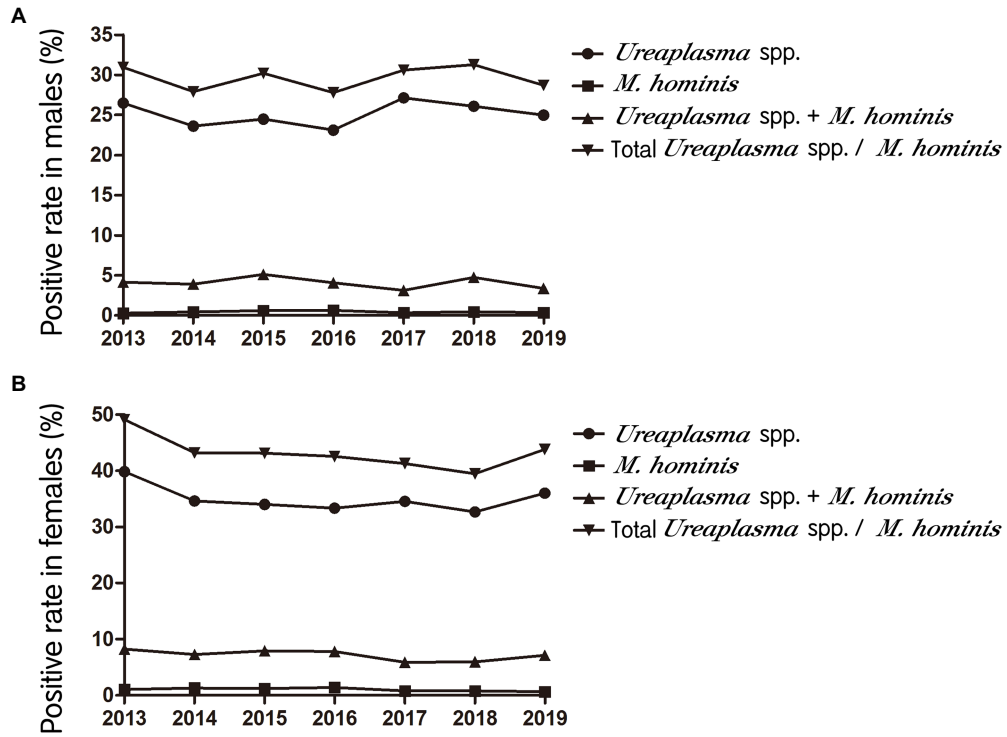

FIGURE 1
Trends in the prevalence of *Ureaplasma* spp. and *Mycoplasma hominis* in males **(A)** and females **(B)** from 2013 to 2019.

TABLE 1 Distribution of *Ureaplasma* spp. and *M. hominis* in the two sexes by age from 2013 to 2019.

Specimen	Distribution [no. (%)]			
	***Ureaplasma* spp. (*n* = 42,310)**	***M. hominis* (*n* = 1,102)**	***Ureaplasma* spp. + *M. hominis* (*n* = 8,092)**	**Total *Ureaplasma* spp./*M. hominis***
Male (year)				
14–20 (*n* = 372)	105 (28.2)	4 (1.1)	16 (4.3)	125 (33.6)
21–25 (*n* = 2,932)	727 (24.8)	15 (0.5)	111 (3.8)	853 (29.1)
26–30 (*n* = 14,201)	3,645 (25.7)	61 (0.4)	557 (3.9)	4,263 (30.0)
31–35 (*n* = 15,589)	3,961 (25.4)	57 (0.4)	616 (4.0)	4,634 (29.7)
36–40 (*n* = 8,508)	2,149 (25.3)	36 (0.4)	347 (4.1)	2,532 (29.8)
41–45 (*n* = 4,048)	1,014 (25.0)	22 (0.5)	199 (4.9)	1,235 (30.5)
46–50 (*n* = 1,651)	393 (23.8)	9 (0.5)	72 (4.4)	474 (28.7)
≥51 (*n* = 1,337)	272 (20.3)	12 (0.9)	52 (3.9)	336 (25.1)
Total (*n* = 48,638)	12,266 (25.2)	216 (0.4)	1970 (4.1)	14,452 (29.7)
Female (year)				
14–20 (*n* = 572)	230 (40.2)	6 (1.0)	96 (16.8)	332 (58.0)
21–25 (*n* = 9,439)	3,697 (39.2)	111 (1.2)	791 (8.4)	4,599 (48.7)
26–30 (*n* = 31,218)	11,089 (35.5)	258 (0.8)	1915 (6.1)	13,262 (42.5)
31–35 (*n* = 25,467)	8,241 (32.4)	239 (0.9)	1,537 (6.0)	10,017 (39.3)
36–40 (*n* = 11,697)	4,046 (34.6)	131 (1.1)	896 (7.7)	5,073 (43.4)
41–45 (*n* = 4,747)	1717 (36.2)	65 (1.4)	428 (9.0)	2,210 (46.6)
46–50 (*n* = 1,560)	594 (38.1)	34 (2.2)	225 (14.4)	853 (54.7)
≥51 (*n* = 1,925)	430 (22.3)	42 (2.2)	234 (12.2)	706 (36.7)
Total (*n* = 86,625)	30,044 (34.7)	886 (1.0)	6,122 (7.1)	37,052 (42.8)

TABLE 2 Overall resistance rates of *Ureaplasma* spp. and *M. hominis* isolates from 2013 to 2019.

Antimicrobial agents	Resistance rate [*n* (%)]	
	***Ureaplasma* spp. (*n* = 42,310)**	***M. hominis* (*n* = 1,102)**
Ofloxacin	30,331 (71.7)	923 (83.8)
Ciprofloxacin	37,303 (88.2)	834 (75.7)
Erythromycin	981 (2.3)	/
Clarithromycin	624 (1.5)	/
Azithromycin	554 (1.3)	/
Josamycin	89 (0.2)	19 (1.7)
Tetracycline	629 (1.5)	47 (4.3)
Doxycycline	321 (0.8)	9 (0.8)
Pristinamycin	82 (0.2)	16 (1.5)

limited effectiveness against both *Ureaplasma* spp. and *M. hominis* (resistant rates, >70%).

Antibiotic susceptibility patterns of *Ureaplasma* spp. and *Mycoplasma hominis* over 20years

The antibiotic susceptibility of *Ureaplasma* spp. isolates collected during 2013–2019 was compared to those collected during 1999–2004 and during 2005–2012 (Figure 2). Ofloxacin resistance of *Ureaplasma* spp. increased from 1999 (resistance rate, 24.1%) to 2019 (resistance rate, 71.9%), whereas ciprofloxacin resistance maintained high from 2001 to 2019, with resistance rates ranging from 64.2 to 93.2% ($p < 0.001$). Resistance to erythromycin, clarithromycin, and azithromycin decreased, with the exception of josamycin, which maintained extremely low (resistance rates, 0–2.8%) during the test period. Resistance to tetracycline and doxycycline increased from 1999 (tetracycline, 4.6%; doxycycline, 3.7%) to 2001 (tetracycline, 12%) or 2002 (doxycycline, 11.3%), then decreased to 2019 (tetracycline, 1.3%, $p < 0.001$; doxycycline, 0.6%, $p < 0.001$). Additionally, resistance rates to pristinamycin were low, ranging from 0 to 4.6%.

The trend in the antibiotic susceptibility of *M. hominis* isolates during 2005–2019 is shown in Figure 3. Resistance to ofloxacin and ciprofloxacin rose from 2005 (ofloxacin, 47.1%; ciprofloxacin, 41.2%) to 2019 (ofloxacin, 81.3%; ciprofloxacin, 65.4%), with peaks in 2017 for ofloxacin (87.1%, $p < 0.001$) and 2014 for ciprofloxacin (83.6%; $p < 0.001$). Resistance rates to josamycin, tetracycline, doxycycline, and pristinamycin remained low, ranging from 0 to 8.7%.

Discussion

Ureaplasma species and *M. hominis* are frequent colonizers in the urogenital tract of adults but are sometimes associated with a variety of diseases. This study aimed to evaluate the prevalence

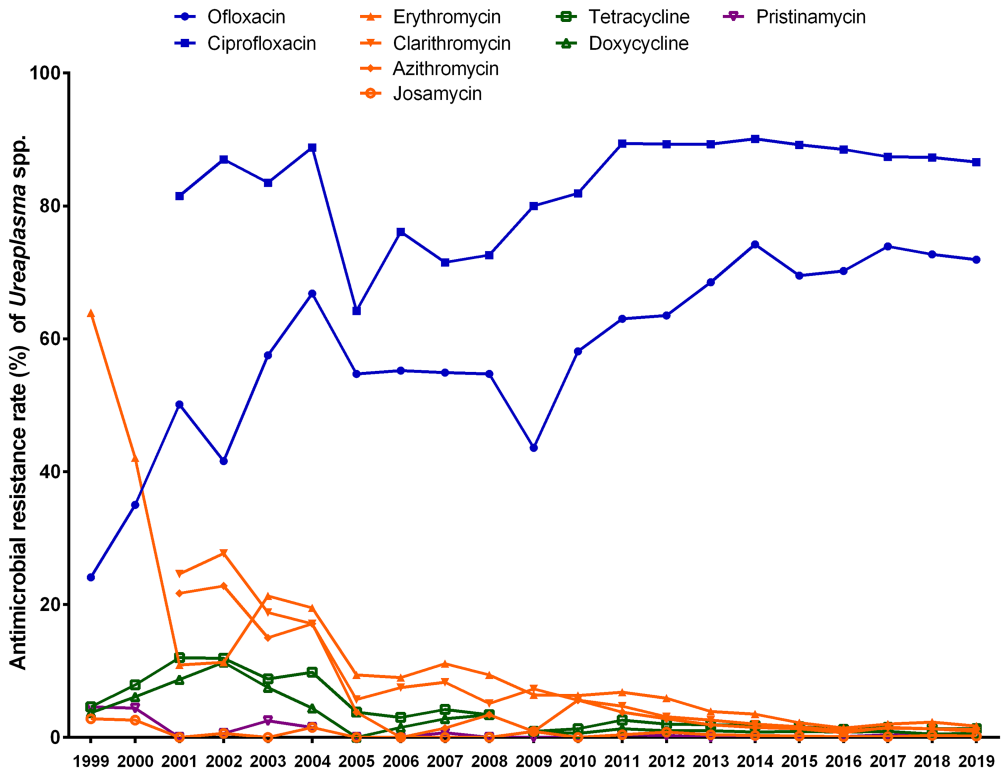

FIGURE 2
Trends in the antibiotic resistance rates of *Ureaplasma* spp. to nine antibiotics from 1999 to 2019.

and antibiotic susceptibility of these species between 2013 and 2019. Our findings identified a high prevalence of *Ureaplasma* species and *M. hominis* (overall prevalence, 38.1%). *Ureaplasma* spp. infection was the most common (31.3%), followed by *Ureaplasma* spp./*M. hominis* coinfection (6.0%) and single *M. hominis* infection (0.8%).

Compared to our previous study analyzing the period of 2005 to 2013, the positive rates of both *Ureaplasma* spp. and *M. hominis* were decreased in females but increased in males in this study (Song et al., 2014). High prevalence of genital mycoplasmas was also observed in other provinces of China. The positive rates of genital mycoplasmas were detected in 33.9% of female outpatients in Beijing, 38.7% of infertile men in Shanghai, and 47.11% of outpatients for gynecologic healthcare screening or the presence of urogenital infection symptoms in Xi'an (Wang et al., 2016; Zeng et al., 2016; Zhou et al., 2018). Similar results were reported in South Korea, Russia, and Romania (Rumyantseva et al., 2019; Lee and Yang, 2020; Doroftei et al., 2021), but relatively lower positive rates were reported in Poland, Italy, and Brazil (Ponyai et al., 2013; Foschi et al., 2018; Piscopo et al., 2020), which could be explained by the discrepancy in socioeconomic conditions, living standards, and the experimental methods used. Notably, the identification of *Ureaplasma* spp. and *M. hominis* in clinical specimens depends on a variety of commercial Mycoplasma testing kits based on molecular or culture methods, the sensitivity and specificity of which are mostly unknown. Moreover, an unequal prevalence between sexes was observed, in which the detection rates of *Ureaplasma* spp. and *M. hominis* were higher in the female population than in the male population. The higher occurrence of *Ureaplasma* spp. and *M. hominis* in females appears to be a general trend, as evidenced by an increasing number of studies (Ponyai et al., 2013; Zeng et al., 2016; Foschi et al., 2018; Kasprzykowska et al., 2018).

Our study also indicated that the prevalence of *Ureaplasma* spp. was higher in younger individuals and declined with age, but we cannot ignore the fact that the number of both male and female patients aged 14–20 years was considerably lower than that of any other age group. However, *M. hominis* was more frequently isolated from the older individuals, with an increasing trend as age increased, especially in female patients. This result is consistent with our previous study, which showed that *M. hominis* was more prevalent in male patients aged 56–60 years, and in female patients aged 61–65 years and 46–50 years (Kong et al., 2016). Lee et al. reported that *Ureaplasma* spp. were most commonly found in female patients aged 18–29 years, but *M. hominis* was more common in females aged 60–89 years, followed by 30–39 years, in Seoul, South Korea (Lee and Yang, 2020). However, Zhou et al. showed that *Ureaplasma* spp. and *M. hominis* occurred mostly in infertile men aged 26–30 years and 21–25 years, respectively, in Shanghai, China (Zhou et al., 2018). These findings suggest that *Ureaplasma* spp. are more likely to be detected in younger patients, but further studies are required to determine the association between *M. hominis* prevalence and age.

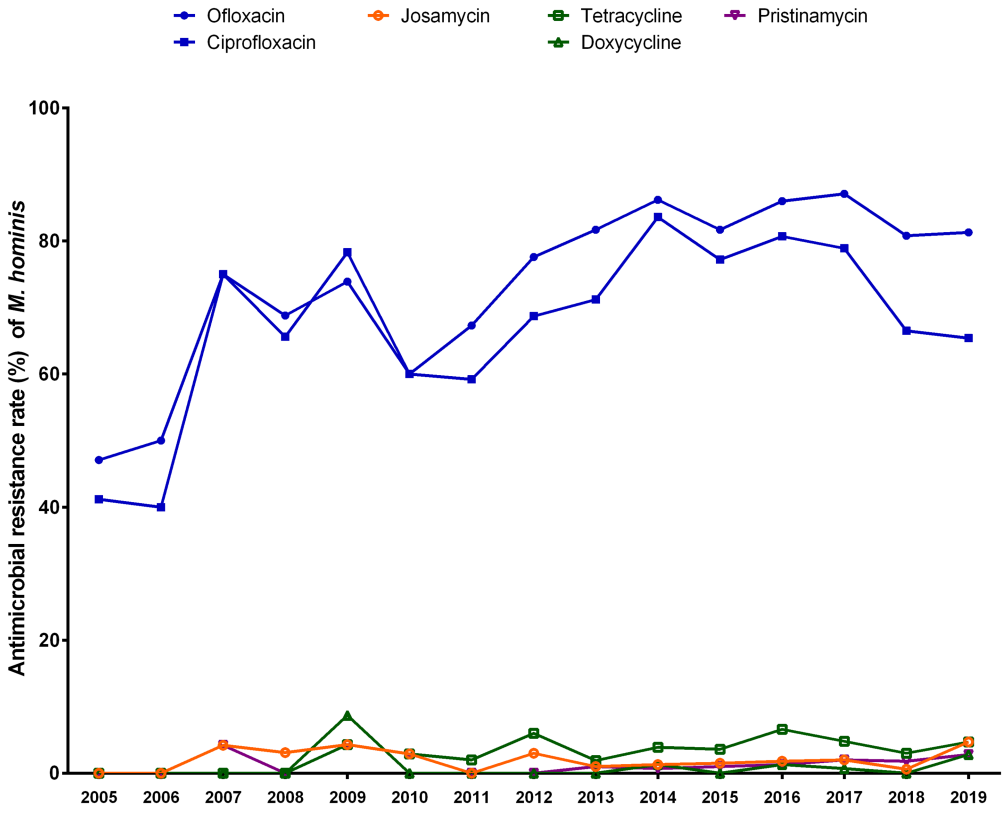

FIGURE 3
Trends in the antibiotic resistance rates of *Mycoplasma hominis* to six antibiotics from 2005 to 2019.

Antibiotic resistance is the leading cause of treatment failure in genital mycoplasmas infections, and the increasing antibiotic resistance has prompted researchers to conduct ongoing monitoring investigations. In this study, a significant variation in levels of sensitivity to various antibiotics was discovered. The majority of clinical *Ureaplasma* spp. isolates were susceptible to macrolides (erythromycin, clarithromycin, azithromycin, and josamycin), tetracyclines (tetracycline and doxycycline), and streptogramins (pristinamycin), suggesting that macrolides, tetracyclines, and streptogramins are effective antibiotics against *Ureaplasma* spp. However, the current findings revealed that *Ureaplasma* spp. were extremely resistant to fluoroquinolones, which is consistent with our and other recent studies on fluoroquinolone resistance in *Ureaplasma* spp. in China (Xie and Zhang, 2006; Song et al., 2014; Wang et al., 2016; Yang et al., 2020; Ma et al., 2021). In our recent study, the resistance rates of levofloxacin were 84.69% for *U. parvum* and 82.43% for *U. urealyticum*, and those of moxifloxacin were 51.44% for *U. parvum* and 62.16% for *U. urealyticum* (Yang et al., 2020). Notably, fluoroquinolone resistance levels differed significantly between countries. In Italy, 77.1% of *Ureaplasma* spp. were ciprofloxacin-resistant, and 26.3% of isolates were ofloxacin-resistant (Foschi et al., 2018). In the United States, however, the resistance rates of levofloxacin in *Ureaplasma* spp. were extremely low, with only 1.6% for *U. parvum* and 0% for *U. urealyticum* (Valentine-King and Brown, 2017). The primary variation is perhaps related to the strategy or inclination for using antibiotics in different regions.

M. hominis is intrinsically resistant to C14- and C15-membered macrolides (erythromycin, clarithromycin, and azithromycin), but susceptible to C16-membered macrolides (josamycin). Our results showed that the majority of clinical *M. hominis* isolates were susceptible to C16-membered macrolides (josamycin), tetracyclines (tetracycline and doxycycline), and streptogramins (pristinamycin), but most of them were resistant to fluoroquinolones (ofloxacin and ciprofloxacin). These findings were consistent with several previous studies (Wang et al., 2016; Zeng et al., 2016; Foschi et al., 2018) but differed from others (Valentine-King and Brown, 2017; Foschi et al., 2018). During the test period in China, fluoroquinolone resistance increased and reached an extraordinarily high level against both *Ureaplasma* spp. and *M. hominis*, perhaps due to the inappropriate use of fluoroquinolone agents in both poultry industry and clinical settings (Chen Z. et al., 2021; Chen H. et al., 2021).

Overall, our results showed that josamycin, tetracycline, doxycycline, and pristinamycin maintained outstanding activity against *Ureaplasma* spp. and *M. hominis*. Due to its toxicity, pristinamycin was no longer a viable alternative, so it has been unavailable for therapeutic prescription in several countries. Additionally, erythromycin, clarithromycin, and

azithromycin are all candidates for *Ureaplasma* spp. infection therapy.

This study has some important limitations. First, it was unable to discriminate between actual genital mycoplasma infection and common commensal colonization due to the lack of clinical data on the participants. Second, the Mycoplasma IST2 kit failed to separate between *Ureaplasma* spp. (*U. parvum* and *U. urealyticum*), as well as produce distinct findings for mixed cultures of *Ureaplasma* spp. and *M. hominis*, which might result in inaccurate reporting of antibiotic resistance. Third, since all clinical isolates of *Ureaplasma* spp. and *M. hominis* were generated as part of routine clinical laboratory procedures and were disposed of after being tested, the identification and antibiotic susceptibility results produced by the Mycoplasma IST2 kit cannot be compared with some other molecular-based methods or the standardized guidelines of the Clinical and Laboratory Standards Institute (CLSI) on Antimicrobial Susceptibility Testing. Regrettably, the antibiotics and breakpoints used in the Mycoplasma IST2 kit conflict with CLSI recommendations. Fourth, we were unable to perform further studies to determine the mechanisms of resistance to fluoroquinolones, macrolides, and tetracyclines in *Ureaplasma* spp. and *M. hominis*.

In conclusion, our study retrospectively analyzed the prevalence and antibiotic susceptibility of *Ureaplasma* spp. and *M. hominis* in Hangzhou, China, from 2013 to 2019. *Ureaplasma* spp. infection was relatively common, but *M. hominis* infection and *Ureaplasma* spp./*M. hominis* coinfection were exceedingly rare. Furthermore, both *Ureaplasma* spp. and *M. hominis* were more prevalent in females than in males, and their distribution was associated with age. Josamycin, doxycycline, and tetracycline are promising antibiotics with outstanding activity against *Ureaplasma* spp. and *M. hominis*.

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.

Ethics statement

This study was approved by the local Research Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, China. All isolates were generated as part of routine clinical laboratory procedures, and no identifiable patient information was collected.

Author contributions

JS, XX, and JZ designed experiments. JS and XW carried out experiments and analyzed the results. YK and HJ checked data. JS, TY, XX, and JZ 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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Acinetobacter baumannii complex-caused bloodstream infection in ICU during a 12-year period: Predicting fulminant sepsis by interpretable machine learning

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Background: *Acinetobacter* baumannii complex-caused bloodstream infection (ABCBSI) is a potentially fatal infection in intensive care units (ICUs). This study proposed an interpretable machine learning (ML) model to predict ABCBSI fulminant fatality.

Methods: A retrospective study of ICU patients with ABCBSI was performed in China from 2009 to 2020. Patients were stratified into two groups: those that suffered from fulminant sepsis and died within 48 h, and those that survived for more than 48 h. The clinical score systems and ML models with Shapley additive explanation (SHAP) were used to develop the prediction models. The ML model was internally validated with five-fold cross-validation, and its performance was assessed using seven typical evaluation indices. The top 20 features ranked by the SHAP scores were also calculated.

Results: Among 188 ICU patients with ABCBSI, 53 were assigned to the non-survival group and 135 to the survival group. The XGBoost model exhibited the greatest area under the receiver operating characteristic curve (AUC), which outperformed other models (logistic regression, AUC=0.914; support vector machine, AUC=0.895; random forest, AUC=0.972; and naive Bayesian, AUC=0.908) and clinical scores (Acute Physiology and Chronic Health Evaluation II (APACHE II), AUC=0.855; Sequential Organ Failure Assessment (SOFA), AUC=0.837). It also had a sensitivity of 0.868, a specificity of 0.970, an accuracy of 0.941, a positive predictive value of 0.920, a negative predictive value of 0.949, and an F1 score of 0.893. As well as identifying the top 12 different important predictors that contribute to early mortality, it also assessed their quantitative contribution and noteworthy thresholds.

Conclusion: Based on the XGBoost model, early mortality in ABCBSI is estimated to be more reliable than other models and clinical scores. The 12 most important features with corresponding thresholds were identified and more importantly, the SHAP method can be used to interpret this predictive model and support individual patient treatment strategies.

KEYWORDS

Acinetobacter baumannii complex-caused bloodstream infection, fulminant sepsis, machine learning model, Shapley additive explanation, treatment strategies

Introduction

Bloodstream infection (BSI) is a major cause of infectious disease morbidity and mortality, and typically refers to a patient with systemic signs and symptoms of infection who has a positive blood culture (Timsit et al., 2020). Patients in the intensive care unit (ICU) are particularly predisposed to BSI, with a prevalence of ~15.2% (Vincent et al., 2020). *Acinetobacter* baumannii complex (ABC) has a high potential for nosocomial transmission, particularly in the ICU. In 2017, carbapenem-resistant *Acinetobacter baumannii* was listed among the antibiotic-resistant "critical priority pathogens" by the World Health Organization (Tacconelli et al., 2018). ABC-caused BSI (ABCBSI) is a critical problem in the ICU as it can cause sepsis or septic shock, and prolonged hospital stays, thus increased costs and mortality rates (Guo et al., 2016; Russo et al., 2019). The 2021 Surviving Sepsis Campaign (SSC) guidelines suggested that early identification and appropriate management in the initial hours after the development of sepsis can improve outcomes (Evans et al., 2021). However, it is still unclear whether fulminant sepsis is more likely to result in higher mortality because of host- or treatment-related factors.

Prediction is common in the medical field, such as anticoagulation by risk scores, risk stratification of ICU patients, early-warning systems for sepsis, and superhuman imaging diagnostics (Chen and Asch, 2017). It is also common for clinicians to use regression analysis when testing causal hypotheses and recently, machine learning (ML) approaches have emerged from analyzing big data in medicine. Through learning the patterns of the health trajectories of large numbers of patients, the ML model can predict clinical events at an expert level, drawing from information well beyond the individual physician's practice experience (Rajkomar et al., 2019). ML has been applied in several fields of ICU, with studies using big data to predict mortality in ICU patients, readmission, and the length of ICU stay, as well as the risks of developing sepsis and acute respiratory distress syndrome (ARDS; Gutierrez, 2020). Although ML models can provide more accurate predictions, they are still difficult to translate into medical practice, especially when applied to individual patients. One reason is that the ML model makes it harder to succinctly present or explain the subtle patterns behind a particular prediction, which is often called the "black box." Thus, to better interpret changes in risk parameters on a continuous basis, we need an interpretable ML model to rationalize the quantitative relationship between clinical parameters and outcome predictions.

The rapid diagnosis and treatment of BSI patients are crucial to their prognosis since timely and effective infection treatment can significantly improve outcomes (Civitarese et al., 2017; Timsit et al., 2020). To early identify the potential risk factors which could predispose to a fulminant course of ABCBSI is essential, and it may help to provide an appropriate treatment to potentially reduce the risk of exacerbations. This study aimed to construct ML models to predict early mortality in ABCBSI and interpret the model using the Shapley additive explanation (SHAP) method so that the predictive model can not only predict the results but also provide reasonable explanations.

Materials and methods

Study population

This retrospective study was conducted in the First Affiliated Hospital, College of Medicine, Zhejiang University, from January 2009 to December 2020. All ICU adult patients (age ≥ 18 years) diagnosed with ABCBSI were considered. The exclusion criteria were: (1) positive blood cultures before ICU admission; (2) patients who were not the first infected and no patient was included twice; (3) positive blood cultures containing other pathogenic microorganisms. The study was approved by the hospital Ethics Committees (IIT20210605A) and there was no need for informed consent because of the retrospective nature of the study.

Data collection and preprocessing of data

The following data were extracted from the patients' medical records: demographic information, vital signs [temperature, mean arterial pressure (MAP), and PaO2/FiO2 (P/F) ratio], laboratory tests [white blood cells (WBCs), hemoglobin, platelets, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, creatinine, blood urea nitrogen (BUN), C-reactive protein (CRP), prothrombin time (PT), activated partial thromboplastin time (APTT), PH, bicarbonate, lactate, sodium, potassium, chloride] at the onset of ABCBSI, invasive procedures before the acquisition of BSI, antibiotic exposure, antimicrobial susceptibility, antimicrobial therapy (time of initiation, doses, routes), the Pitt bacteremia score, Acute Physiology and Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA), and outcome.

Some variables were measured more than once so their maximum, minimum, and average values were further analyzed

as independent variables. The overall missing data rate was <0.05% among all the variables and average values were input for missing variables.

Machine learning

The predictive model was based on ML algorithms with the input of variables that different ($p < 0.1$) in the univariate analysis between the non-survival and survival groups. Five ML algorithms were used: extreme gradient boosting (XGBoost), logistic regression (LR), support vector machine (SVM), random forest (RF), and naive Bayesian (NB). All analyses were performed using Python (version 3.9.10). The parameters of XGBoost can be divided into three types: general, booster, and task. General parameters define which kind of booster is used in the lifting process and the commonly used boosters are the tree model and linear model. This article uses a tree model, which is the default option. The maximum number of threads was defined as 6. The parameters for Tree Booster include the learning rate (eta = 0.01), the maximum depth of each tree (max_depth = 3), and the proportion of subsamples used to train the model in the whole sample set (subsample = 1). The main task was to solve a binary logistic regression problem (objective = binary: logistic). After building the model, the area under the receiver operating characteristics curve (AUC), sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), and F1 score were used as evaluation indicators of model performance. To select the optimal feature subset for the predictive model, 5-fold cross-validation was used for the training and validation set. Four of the five folds were used as the training set, and the remaining one was used as the validation set.

SHAP is a game-theoretic approach to explain the output of the ML model. It connects optimal credit allocation with local explanations using the classical Shapley values from game theory and their related extensions. Shapley values are a widely used approach from cooperative game theory with desirable properties. SHAP values are a unified approach for explaining the outcome of our ML model and provide consistent and locally accurate attribution values for each feature (Lundberg et al., 2018; Tseng et al., 2020).

Statistical analysis

Continuous variables are expressed as mean ± standard, and categorical variables are expressed as proportions. The variables were compared by Student's *t*-test, the Mann–Whitney test for continuous variables, and the χ^2 test or Fisher's exact test for categorical variables, respectively. A two-sided value of $p < 0.05$ was considered statistically significant. Python (version 3.9.10) was used for the statistical analysis and visualizations.

Results

Demographic and clinical characteristics

This study included 188 ICU patients with ABCBSI from 2009 to 2020 and their demographic and clinical characteristics are presented in Table 1. Overall, 28.2% (53/188) of patients with fulminant sepsis died within 48 h.

Compared to the survival group, the non-survival group was more likely to have hematological malignancy, prior exposure to carbapenems and anti-fungal agents, receive mechanical ventilation, have septic shock, immunosuppression, and higher clinical scores assessed by the Pitt bacteremia, APACHE II, and SOFA scores at the time of BSI. In addition, decreases in MAP, P/F ratio, platelets, and PH and elevated creatinine, BUN, CRP, PT, APTT, lactate, sodium, and chloride were associated with early death.

Model building and evaluation

Twenty-six features ($p < 0.1$) in the univariate analysis between the two groups were chosen as the input variables in our ML model to predict early death. The results showed that the largest AUC (0.977) to predict early mortality was constructed by XGBoost. The XGBoost model performance was superior to other models (LR, AUC = 0.914; SVM, AUC = 0.895; RF, AUC = 0.972; and NB, AUC = 0.908) and the conventional clinical scores (APACHE II, AUC = 0.855; SOFA, AUC = 0.837; Figure 1).

The XGBoost model exhibited good performance by other evaluation indices, which included sensitivity of 0.868, a specificity of 0.970, an accuracy of 0.941, a positive predictive value of 0.920, a negative predictive value of 0.949, and an F1 score of 0.893. Table 2 shows the comparison of the predictive performance of different ML models.

Explanation of risk factors

The SHAP summary was plotted for an overview of which features are most important for our XGBoost model. Figure 2A shows the top 20 risk factors in our model and the red color represents high feature value, while the blue color is the opposite. From top to bottom, the overall influence of features on the final prediction gradually decreases. For example, increases in creatinine have a positive impact and push the prediction toward mortality, whereas increases in PH have a negative impact and push the prediction toward survival. Figure 2B shows the top 20 important features evaluated by the average absolute SHAP value, the top 12 of which seem to be particularly important in our model. The level of creatinine had the strongest predictive value for all prediction horizons, followed closely by the APACHE II score, SOFA score, PH, and P/F ratio. Figures 2C,D show the individual force plots for patients who did not survive and

TABLE 1 Baseline characteristics.

Features	Survival ($n = 135$)	Nonsurvival ($n = 53$)	Value of p
Clinical parameters			
Age (year)	61.4 ± 17.6	59.5 ± 15.7	0.509
Male *n* (%)	99 (73.3%)	37 (69.8%)	0.627
Vital signs			
Temperature (°C)	38.7 ± 1.1	38.8 ± 1.0	0.294
MAP (mm Hg)	70.4 ± 12.2	59.4 ± 13.0	<0.001
P/F ratio	272.0 ± 131.2	130.5 ± 96.4	<0.001
Underlying diseases			
Hypertension	52 (38.5%)	19 (35.8%)	0.734
Diabetes mellitus	23 (17.0%)	9 (17.0%)	0.993
Solid-organ malignancy	26 (19.3%)	9 (17.0%)	0.718
CAD	21 (15.6%)	10 (18.9%)	0.582
CRF	22 (16.3%)	9 (17.0%)	0.909
Liver cirrhosis	8 (5.9%)	6 (11.3%)	0.338
COPD	25 (18.5%)	10 (18.9%)	0.956
Hematological malignancy	4 (3.0%)	9 (17.0%)	0.002
Cerebrovascular disease	14 (10.4%)	3 (5.7%)	0.465
CTD	14 (10.4%)	4 (7.5%)	0.554
Laboratory parameters			
WBCs ($\times 10^9\ L^{-1}$)	14.5 ± 10.5	11.2 ± 12.0	0.070
Hemoglobin ($g\ dL^{-1}$)	8.1 ± 2.0	8.5 ± 2.2	0.295
Platelets ($\times 10^9\ L^{-1}$)	154.1 ± 132.1	58.0 ± 70.8	<0.001
Albumin ($g\ L^{-1}$)	31.2 ± 4.7	29.0 ± 5.8	0.010
ALT ($U\ L^{-1}$)	72.9 ± 125.9	121.8 ± 254.8	0.187
AST($U\ L^{-1}$)	76.8 ± 152.6	169.0 ± 438.7	0.140
Bilirubin ($\mu mol\ L^{-1}$)	54.2 ± 97.0	71.2 ± 73.8	0.249
Creatinine ($\mu mol\ L^{-1}$)	91.1 ± 86.3	123.7 ± 92.7	0.023
BUN ($mmol\ L^{-1}$)	10.8 ± 6.4	16.5 ± 11.5	<0.001
CRP ($mg\ L^{-1}$)	116.0 ± 74.2	166.0 ± 117.0	0.005
PT (s)	14.3 ± 3.2	18.3 ± 8.0	0.001
APTT (s)	47.3 ± 22.0	59.5 ± 29.7	0.008
pH	7.4 ± 0.1	7.2 ± 0.2	<0.001
Bicarbonate ($mmol\ L^{-1}$)	24.0 ± 6.5	25.4 ± 34.6	0.762
Lactate ($mmol\ L^{-1}$)	2.9 ± 2.1	6.9 ± 4.7	<0.001
Sodium ($mmol\ L^{-1}$)	137.3 ± 6.5	141.7 ± 8.5	0.001
Potassium ($mmol\ L^{-1}$)	3.8 ± 0.6	3.8 ± 0.7	0.977
Chloride ($mmol\ L^{-1}$)	103.0 ± 6.2	106.3 ± 7.9	0.002
Invasive procedures			
Mechanical ventilation	110 (81.5%)	50 (94.3%)	0.026
Central venous catheter	102 (75.6%)	42 (79.2%)	0.591
CRRT	48 (35.6%)	23 (43.4%)	0.318
PICCO	14 (10.4%)	10 (18.9%)	0.116
Previous antibiotic used (within 1 month)			
Corticosteroid use	27 (20.0%)	16 (30.2%)	0.135
Anti-pseudomonal penicillins + beta lactamase inhibitors	81 (60.0%)	33 (62.3%)	0.775
Antipseudomonal cephalosporins	27 (20.0%)	16 (30.2%)	0.135
Aminoglycosides	8 (5.9%)	3 (5.7%)	1.000
Carbapenems	85 (63.0%)	42 (79.2%)	0.032
Quinolone	36 (26.7%)	21 (39.6%)	0.082
Tigecycline	15 (11.1%)	6 (11.3%)	0.967

(Continued)

TABLE 1 (Continued)

Features	Survival ($n = 135$)	Nonsurvival ($n = 53$)	Value of p
Anti-fungal agents	50 (37.0%)	29 (54.7%)	0.027
Carbapenem-resistant strains	119 (88.1%)	52 (98.1%)	0.063
Concurrent infection with another pathogen	55 (40.7%)	18 (34.0%)	0.391
Septic shock	51 (37.8%)	42 (79.2%)	<0.001
Immunosupression	43 (31.9%)	31 (58.5%)	0.001
Appropriate empirical therapy	34 (25.2%)	7 (13.2%)	0.074
Length of ICU stay before BSI	15.0 ± 43.0	7.7 ± 10.1	0.224
Severity of illness			
CCI	2.3 ± 2.2	2.5 ± 2.4	0.555
APACHE II score[a]	22.1 ± 8.8	34.9 ± 9.3	<0.001
SOFA score[a]	8.8 ± 4.4	15.7 ± 5.0	<0.001
Pitt bacteremia score[a]	4.5 ± 2.9	7.5 ± 2.5	<0.001

Data are n (%) or mean ± SD. MAP, mean arterial pressure; P/F, PaO_2/FiO_2; CAD, coronary artery disease; CRF, chronic renal failure; COPD, chronic obstructive pulmonary disorder; CTD, connective tissue disorder; WBCs, white blood cells; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRP, C reactive protein; CRRT, continuous renal replacement therapy; PICCO, pulse index continuous cardiac output; ICU, intensive care unit; BSI, bloodstream infection; CCI, charlson comorbidity index; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment. $p < 0.05$, which are considered statistically significant. [a]At the onset of ABCBSI.

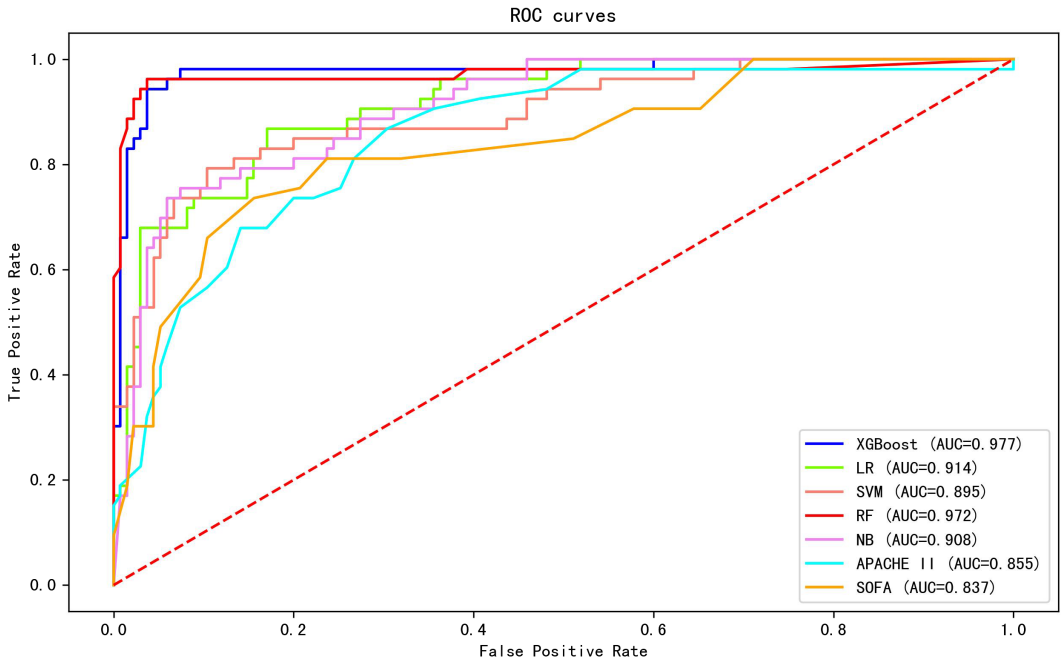

FIGURE 1
The AUC of different machine learning models and clinical scores in predicting early mortality. The results show that the XGBoost exhibited the largest AUC (0.977).

survived, respectively. The red features (on the left) indicate increased mortality risk, and the blue features indicate decreased mortality risk. For example, this patient (Figure 2C) is predicted to have a 322% risk of a poor outcome due to the elevated creatinine (114 μmol L^{-1}), sodium (157 mmol L^{-1}), APTT (30.6 s), APACHE II score (40 points), SOFA score (15 points), and lactate (3.8 mmol L^{-1}) level, and decreased PH (7.28). Creatinine is the most important risk-increasing variable and platelets ($97 \times 109\ L^{-1}$) are the most important protective variable. The patient (Figure 2D) was predicted to survive due to a lower APACHE II score (18 points), SOFA score (nine points), and normal PH (7.43), platelets ($147 \times 109\ L^{-1}$), and P/F ratio (286.7) level. The APACHE II score is the most important risk-decreasing variable.

Figure 3 shows the SHAP dependence plot of the top 12 most important variables, showing that higher creatinine, APACHE II, SOFA, lactate, and sodium and lower PH, P/F ratio, platelets,

WBCs, APTT, BUN, and albumin levels were related to higher mortality. The SHAP values for these features exceed zero, representing an increased risk of early mortality, so each feature has a cut-off point when a horizontal line is drawn.

TABLE 2 Comparison of the predictive performance of different ML.

Model	AUC	SE	SP	AC	F1 score	PPV	NPV
XGBoost	0.977	0.868	0.970	0.941	0.893	0.920	0.949
LR	0.914	0.375	0.900	0.789	0.429	0.500	0.844
SVM	0.895	0.375	0.933	0.816	0.462	0.600	0.848
RF	0.972	0.500	0.900	0.816	0.533	0.571	0.871
NB	0.908	0.625	0.933	0.789	0.556	0.500	0.893

ML, machine learning; AUC, The area under the receiver operating characteristic curve; SE, sensitivity; SP, specificity; AC, accuracy; PPV, positive predictive value; NPV, negative predictive value; XGBoost, extreme gradient boosting; LR, logistic regression; SVM, support vector machine; RF, random forest; NB, naive Bayesian.

Discussion

Acinetobacter baumannii complex is a group of nosocomial pathogens and one of the six leading multidrug-resistant pathogens causing deaths in hospitals worldwide (Murray et al., 2022). It is responsible for a variety of clinical manifestations, of which ventilator-associated pneumonia (VAP) and BSI are the most common. ICU clinicians pay the most attention to BSI caused by ABC because it can cause sepsis and septic shock which are associated with more poor outcomes. A previous systematic review and meta-analysis including 10 studies reported that the pooled mortality of patients with ABCBSI was ~56.3% (Du et al., 2019). The mortality risks for ABCBSI include old age, malignancy, chronic renal disease, chronic liver disease, neutropenia, septic shock, immunosuppressant use, total parenteral nutrition, ICU stay, previous antibiotic use, Pitt bacteremia score, APACHE II score, SOFA score, lower albumin levels,

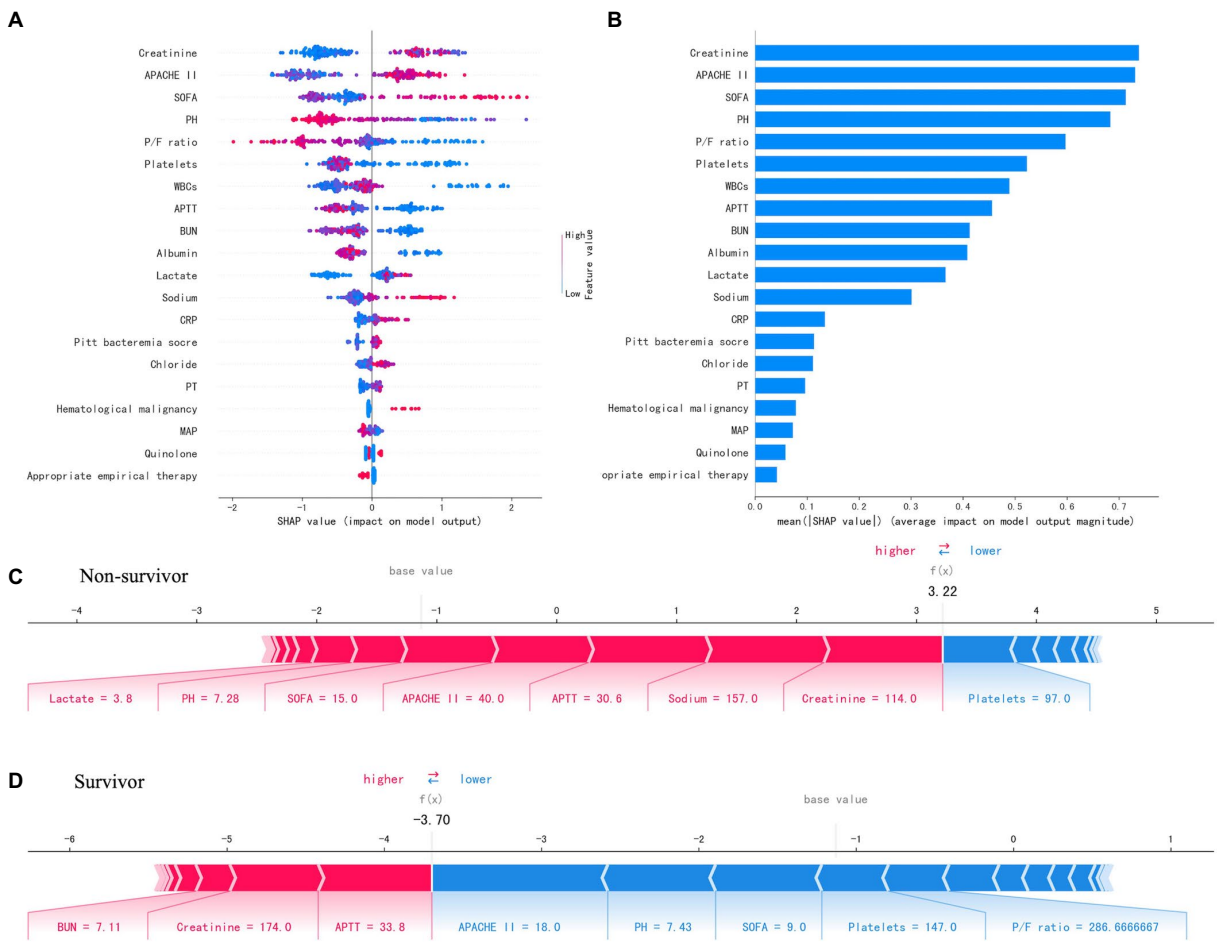

FIGURE 2
Feature analysis of the XGboost model. **(A)** A summary plot of the SHAP values for the top 20 features of our model. **(B)** The importance ranking of the top 20 variables according to the average absolute SHAP value. **(C,D)** The interpretation of model prediction results with the two samples. APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; P/F, PaO_2/FiO_2; WBCs, white blood cells; APTT, activated partial thromboplastin time; BUN, blood urea nitrogen; CRP, C-reactive protein; PT, prothrombin time; MAP, mean arterial pressure.

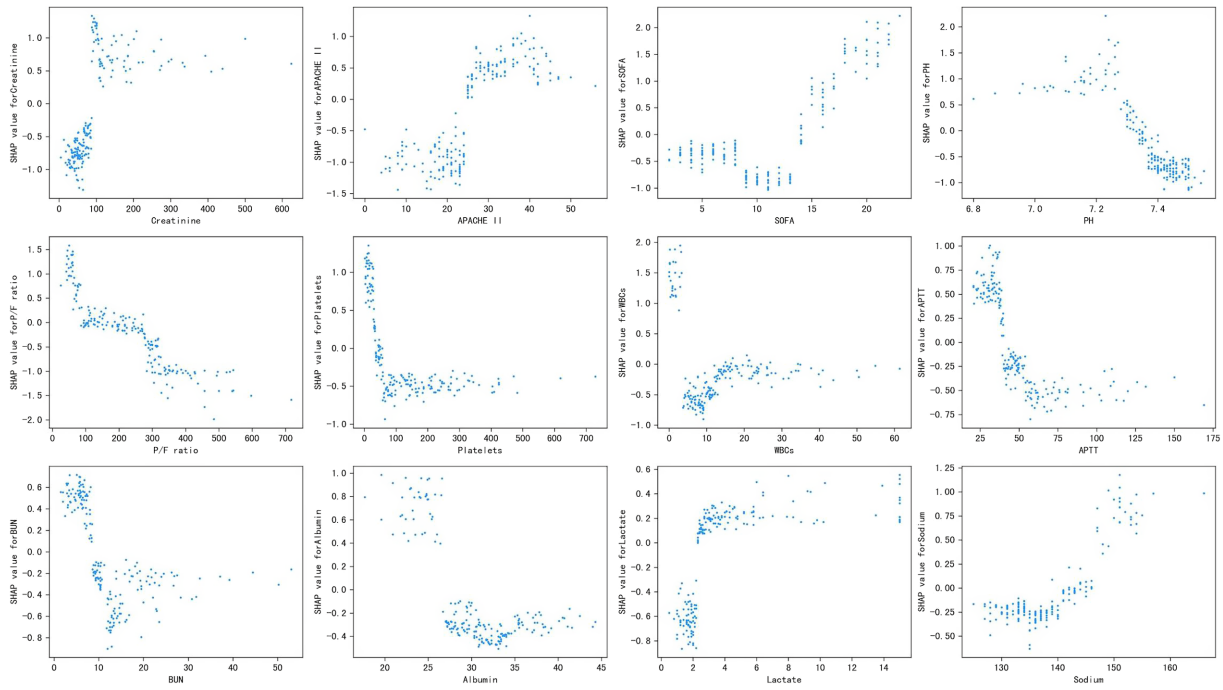

FIGURE 3
Partial SHAP dependence plot of the XGboost model. It shows how a single feature (the top 12 important variables) affects the output of the XGBoost predictive model. SHAP values for specific features exceed zero, representing an increased risk of death. APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; P/F, PaO_2/FiO_2; WBCs, white blood cells; APTT, activated partial thromboplastin time; BUN, blood urea nitrogen.

bacteremia origin, carbapenem resistance, and inappropriate initial antimicrobial therapy (Du et al., 2019; Russo et al., 2019; Zhou et al., 2019; Son et al., 2020; Gu et al., 2021; Yu et al., 2021). However, previous studies mainly focused on testing hypotheses involving causal relationships and the predictive effect of conventional regression analysis methods may be unsatisfactory because it is mainly used to solve linear problems and is difficult to fit the real distribution of data. Therefore, it is important to obtain a more accurate predictive model for mortality and the decision-making process of the model must be understood by the physician. A recent study developed an ML model to predict patient outcomes of BSI based on electronic medical records and the model AUC was 0.81 using only 25 features (Zoabi et al., 2021).

In this study, we proposed an ML model using selected features for the prediction of ABCBSI fulminant fatality. The XGBoost model performed relatively better than other models (LR, SVM, RF, and NB) as well as conventional clinical scores (APACHE II and SOFA). Among the 26 selected features in our model, the top 12 important features with absolute SHAP values were creatinine, APACHE II, SOFA, PH, P/F ratio, platelets, WBCs, APTT, BUN, albumin, lactate, and sodium, which increased or decreased the risk of early mortality of ABCBSI to varying degrees. Furthermore, the SHAP summary plot of XGBoost revealed additional important features (e.g., creatinine, APACHE II score, platelets, WBCs, APTT, albumin, lactate, etc.) that logistic regression did not include. The well-established risk factors for mortality of ABCBSI, such as creatinine, albumin, APACHE II score, and lactate have been used as prognostic markers in several studies (Du et al., 2019; Russo et al., 2019) but other factors, such as APTT and platelets, are less used as predictors of outcome in ABCBSI. Therefore, the SHAP values were used to further illustrate whether each feature contributed positively or negatively to the target outcome.

The SOFA and APACHE II scores are the most commonly used methods and authoritative critical illness evaluation systems in ICU. According to a retrospective cohort study of ICU patients with suspected infections, defining sepsis by an increase in SOFA score provided more accurate prognoses (AUC, 0.753) than either SIRS criteria (AUC, 0.589) or qSOFA (AUC, 0.607; Raith et al., 2017). The APACHE II score classifies diseases based on the severity from 0 to 71, with higher scores representing more severe illnesses and greater mortality risks. The AUC of SOFA or APACHE II score is not high and is no more than 0.85, even though they have been proven useful prognostic biomarkers for critical illnesses (Tian et al., 2022). Many studies were analyzed using multivariable logistic regression methods, with the AUC ranging from 0.76 to 0.84 (Tseng et al., 2020). Recent studies have shown that ML models tend to have better predictive power than standard scoring systems (Morgan et al., 2019; Zhang et al., 2020). In line with

these findings, our study demonstrated that the performance of the ML model was superior to the APACHE II and SOFA scores, in contrast to a systematic review that showed that logistic regression for the clinical prediction model is not inferior to the ML model (Christodoulou et al., 2019).

Our study not only generated a more accurate predictive model and identified other unrecognized key risk factors but also made it "explainable." Each component of the predictive model can be visualized and contributes differently to the final outcome. Our study benefits from using SHAP values to uncover the black box of the ML model, therefore, our predictive model can provide implications for patient management, even when applied to individual patients. Additionally, based on the SHAP dependence plot, we further demonstrated the quantitative relationship of this contribution (Figure 3). Among the 12 most important features, most had a critical threshold at which the predicted risk abruptly changed. For example, the platelets $<50 \times 109\,L^{-1}$ or lactate $>2.5\,mmol\,L^{-1}$ resulted in a significant increase in mortality risk. There were some unexpected situations, such as higher creatinine led to a higher risk of death, while higher BUN was protective. Although both serum creatinine and BUN can represent renal function, they are not completely consistent. Acute kidney injury (AKI) is defined by increased serum creatinine or decreased urine volume, which are significantly associated with mortality in sepsis (Peerapornratana et al., 2019), whereas the increase in BUN is not only affected by renal function, but also by stress-nutrition status and bleeding-volume status. A study reported a U-shape relationship between the BUN/creatinine ratio and all-cause mortality in the general population (Shen et al., 2022). Therefore, some important features may be missed due to the nonlinear relationship between features and risks in logistic regression. ML is particularly useful for handling enormous numbers of predictors, sometimes remarkably, more predictors than observations, and combining them in nonlinear and highly interactive ways (Obermeyer and Emanuel, 2016). Thus, the study offers a "warning threshold" that despite the parameters being in the normal reference range, the risk still increases in this model, and thus caution is needed.

This study has several limitations. First, it is a single-center retrospective study, so information bias and temporal bias should not be neglected. Second, the model was constructed with only a small number of patients, therefore, it needs to be externally validated in a multicenter study with a large sample size to determine its applicability. Third, in patients with other bacterial pathogens concomitantly isolated with ABC, it was not possible to judge whether the infection was caused by ABC or the concomitant pathogen(s), or both. Finally, this model was used on all patients admitted to the ICU but it needs to be tested on general wards as well and more external validation is required in the future.

Conclusion

In conclusion, an interpretable ML model with optimal performance was constructed to predict early mortality in ABCBSI. Twelve of the most important features with corresponding thresholds crucial for early mortality prediction were identified. Furthermore, clinicans should be aware of important features (such as creatinine, APACHE II score, SOFA score, PH, P/F ratio, etc) beyond their corresponding thresholds, even within the normal range. However, this study needs to be confirmed in clinical settings and externally.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. According to the national legislation and institutional requirements, patient data used in this study is confidential. To protect patient confidentiality and participant's privacy, data used for this study can be obtained in an anonymous form only according to the data privacy act. Requests to access these datasets should be directed to XZ, zxicu@zju.edu.cn.

Author contributions

JX contributed to data collection and manuscript writing. XC contributed to the data analysis. XZ contributed to the study design and revise the manuscript. All authors contributed to the article and approved the submitted version.

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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In silico characterization of bla_{NDM}-harboring plasmids in *Klebsiella pneumoniae*

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Klebsiella pneumoniae is a primary culprit of antibiotic-resistant nosocomial infections worldwide, and infections caused by NDM-producing strains are a major threat due to limited therapeutic options. The majority of bla_{NDM} cases occur on plasmids; therefore, we explored the relationships between plasmids and bla_{NDM} genes in *K. pneumoniae* by analyzing the variants of bla_{NDM}, replicon types, conjugative transfer regions of 171 bla_{NDM}-harboring plasmids from 4,451 *K. pneumoniae* plasmids. Of the nine identified bla_{NDM} variants, bla_{NDM-1} (73.68%) and bla_{NDM-5} (16.37%) were the most dominant. Over half of the bla_{NDM}-harboring plasmids of *K. pneumoniae* were classified into IncF plasmids. IncX3 single-replicon plasmids (46–57kb) carried genes encoding relaxases of the MOB_P family, T4CP genes of the VirD4/TraG subfamily, and VirB-like T4SS gene clusters, which were mainly geographically distributed in China. We found 10 bla_{NDM}-harboring IncN plasmids (38.38–63.05kb) carrying the NW-type origin of transfer (*oriT*) regions, genes coding for relaxases of MOB_F family, genes encoding T4CPs of the TrwB/TraD subfamily, and Trw-like T4SS gene clusters, which were also mainly geographically distributed in China. Moreover, we identified 21 IncC plasmids carrying bla_{NDM-1} (140.1–329.2kb), containing the A/C-type *oriT*s, genes encoding relaxases of MOB_H family, genes encoding T4CPs belonging to TrwB/TraD subfamily, and Tra_F-like T4SS gene clusters. The bla_{NDM}-harboring IncC plasmids were widely geographically distributed all over the world, mainly in the United States, China and Viet Nam. These findings enhance our understanding of the diversity of bla_{NDM}-harboring plasmids in *K. pneumoniae*.

KEYWORDS

***Klebsiella pneumoniae*, plasmid, bla_{NDM}, replicon types, conjugative transfer region**

Introduction

Klebsiella pneumoniae is a significant cause of nosocomial infections such as pneumonia, bloodstream infections, urinary tract infections, and septicemias (Pitout et al., 2015; Bengoechea and Sa Pessoa, 2019). *Klebsiella pneumoniae* represents one of the most concerning pathogens known for its high frequency and diversity of antimicrobial resistance (AMR) genes (Navon-Venezia et al., 2017; Wyres and Holt, 2018), and it has been classified as an ESKAPE organism (De Oliveira et al., 2020). The emergence and spread of carbapenem-resistant *K. pneumoniae* have become severe medical problems worldwide (Navon-Venezia et al., 2017). Resistance to carbapenems in *K. pneumoniae* involves diverse mechanisms, e.g., production of carbapenemases (e.g., KPC, NDM, and OXA-48-like), alterations in outer membrane permeability and the upregulation of efflux systems (Pitout et al., 2015).

New Delhi metallo-β-lactamase (NDM), belonging to Ambler class B β-lactamase, has the ability to hydrolyze all β-lactam antibiotics (including carbapenems) except the monobactam aztreonam (Nordmann et al., 2011). NDM-1 was first reported in a *K. pneumoniae* isolate recovered in a Swedish patient who traveled to New Delhi in 2008 (Yong et al., 2009). According to the records of the Beta-lactamase database (BLDB; Naas et al., 2017) on September 8th, 2022, more than 40 variants of NDM have been identified so far. A variety of infections caused by NDM-producing Enterobacterales strains are associated with inferior prognosis and high mortality, especially in high-risk immunocompromised patients (Guducuoglu et al., 2018). NDM-producing Enterobacterales clinical isolates, mainly *K. pneumoniae* and *Escherichia coli*, have been found worldwide, with a higher prevalence in the Indian subcontinent, the Balkans, and the Middle East (Albiger et al., 2015; Wu and Feng, 2019).

Antimicrobial resistance (AMR) in carbapenem-resistant Enterobacterales (CRE) strains is often encoded by the plasmid-borne genes (Rozwandowicz et al., 2018). Plasmids, especially conjugative plasmids, play an essential role in mediating horizontal gene transfer (HGT) and dissemination of AMR (Jiang et al., 2020). The conjugative transfer regions of conjugative plasmids typically comprise four key modules, including origin of transfer (*oriT*) region, gene encoding relaxase, gene encoding type IV coupling protein (T4CP), and gene cluster for the bacterial type IV secretion system (T4SS) apparatus (de la Cruz et al., 2010). The relaxase initiates the bacterial conjugation by recognizing and cleaving the *oriT* of the plasmid in a site-specific manner, forming a relaxosome (Llosa et al., 2002; Carballeira et al., 2014). Currently, nine types of plasmid-borne *oriT*[1] and eight main relaxase families[2] have been identified (Li et al., 2018). Conjugation requires a pilus, which is assembled by T4SS, to connect the donor and the recipient strains (de la Cruz et al., 2010). Currently, five main types of T4SS gene clusters are defined, including 18 different kinds of systems[3] (Bi et al., 2013). The T4CP connects the relaxosome to T4SS, which is required for conjugation, and currently, two main subfamilies of T4CPs[4] exist (Li et al., 2018).

Studies on the comprehensive analysis of bla_{NDM}-harboring plasmids and their conjugative transfer regions in *K. pneumoniae* are scarce. In this work, we executed *in silico* typing and comparative analysis of bla_{NDM}-harboring plasmids of *K. pneumoniae* using the bacterial plasmids available in the NCBI GenBank database. We systematically analyzed the variants of bla_{NDM}, replicon types, phylogenetic patterns, and conjugative transfer regions of the bla_{NDM}-positive plasmids of *K. pneumoniae*. This study provides deep insights into the characteristics and diversity of bla_{NDM}-harboring plasmids in *K. pneumoniae* and further emphasizes their role in dissemination of resistance genes.

Materials and methods

Plasmid sequences from the NCBI database

The GenBank Genome database (Benson et al., 2018) collect all the plasmids belonging to *K. pneumoniae*.[5] A total of 4,451 plasmids (without duplicates) of *K. pneumoniae* (Supplementary Table S1) were downloaded on April 26th, 2022. Files in FASTA DNA format of the 4,451 plasmids were downloaded in batches into our Linux-based server.

Identification of the bla_{NDM}-harboring plasmids of *Klebsiella pneumoniae*

The β-lactamase genes of the plasmids of *K. pneumoniae* were identified applying the ResFinder software, standalone version 4.1 (Bortolaia et al., 2020), with the minimum coverage of 60%, minimum identity of 90%, and species of "Klebsiella." The term "bla_{NDM}" was used to search in the "Resistance gene" list of the ResFinder results in order to judge the bla_{NDM}-harboring plasmids of *K. pneumoniae* and identify the variants of the bla_{NDM} genes. For some bla_{NDM}-harboring plasmids, the variants of bla_{NDM} were not determined by the ResFinder software; instead, they were submitted to the CARD database[6] (Alcock et al., 2020) and the Beta-lactamase database (BLDB; Naas et al., 2017) for further analysis.

1 https://bioinfo-mml.sjtu.edu.cn/oriTDB/browse_oriT_type_p.php
2 https://bioinfo-mml.sjtu.edu.cn/oriTDB/browse_relaxase.php
3 https://bioinfo-mml.sjtu.edu.cn/SecReT4/browse_type.php
4 https://bioinfo-mml.sjtu.edu.cn/oriTDB/browse_t4cp.php
5 https://www.ncbi.nlm.nih.gov/genome/browse/#!/plasmids/815/
6 https://card.mcmaster.ca

Replicon typing of the *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*

Replicon typing of the *bla*$_{NDM}$-harboring plasmids was executed *via* the PlasmidFinder software (Carattoli and Hasman, 2020). Then, selecting the database "Enterobacteriales," the FASTA-formatted DNA files were analyzed and classified in batches by using the PlasmidFinder tool version 2.0.1, with a minimum coverage cut-off of 60% and minimum identity cut-off of 95%. The database version was updated on November 29th, 2021.

Phylogenetic cladogram of the *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*

The files of the *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae*, in GenBank format, were downloaded in batches using two Bioperl modules (Bio::SeqIO and Bio::DB::GenBank). Plasmid files containing protein sequences were compiled from the plasmid files in GenBank format through the Bioperl/Bio::SeqIO module. Phylogenetic cladogram based on the presence/absence of orthologous gene families of all the *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* were constructed. First, a binary gene presence/absence matrix was built using OrthoFinder software (Emms and Kelly, 2019), and subsequently a hierarchical cluster result was generated by PAST3 (Hammer et al., 2001) and eventually displayed by iTOL (Letunic and Bork, 2016).

Geographic location and host ST types of the *bla*$_{NDM}$-harboring plasmids in *Klebsiella pneumoniae* strains

Information about geographic location of *bla*$_{NDM}$-harboring plasmids and its host strains were extracted from the files of the *bla*$_{NDM}$-harboring plasmids in GenBank format. Table containing the correspondence between strains and plasmids of *K. pneumoniae* were downloaded from the GenBank.[7] The *bla*$_{NDM}$-harboring plasmid-matched host *K. pneumoniae* strains were collected, and their DNA FASTA sequences were downloaded in batch using the Bioperl. The MLST software (Larsen et al., 2012) version 2.0.9 was downloaded from the website[8] and installed on the Linux platform. The genomes of *K. pneumoniae* strains were analyzed in batch using MLST software.

Characterization of the conjugative transfer regions of *bla*$_{NDM}$-harboring plasmids

Files in GenBank format of the *bla*$_{NDM}$-harboring plasmids in *K. pneumoniae* were analyzed in batches using oriTfinder software (local version; Li et al., 2018) to identify the presence/absence of *oriT*s, relaxase-coding genes, T4CP-coding genes, and gene clusters for T4SS. Furthermore, the types of *oriT*s, relaxases, T4CPs, and T4SSs toward the plasmids were determined based on the exhibition of the oriTDB database[9] (Li et al., 2018). In addition, the types of gene clusters for T4SS were classified based on the SecReT4 database[10] (Bi et al., 2013).

Bipartite network construction, clustering and visualization of the *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*

The bipartite network was constructed based on all the *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* using the AccNet software using default parameters (Lanza et al., 2017). The obtained network files including nodes, edges and clusters were then imported into the Cytoscape software (Shannon et al., 2003) for visualization. We displayed the relative genomic content of each plasmid by making the diameter of each node proportional to its degree.

Results

Variants of *bla*$_{NDM}$ genes in the *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*

Based on the results analyzed by ResFinder, 171 (3.84%) *bla*$_{NDM}$-harboring plasmids (Supplementary Table S2) were identified from 4,451 plasmids of *K. pneumoniae*, which were downloaded from the GenBank Genome database. Among the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae*, nine different variants of *bla*$_{NDM}$ were identified (Figure 1A). Among the nine variants of *bla*$_{NDM}$, *bla*$_{NDM-1}$ was found to be the predominant variant, accounting for 73.68% (126 *bla*$_{NDM-1}$-harboring plasmids), followed by *bla*$_{NDM-5}$, accounting for 16.37% (28 *bla*$_{NDM-5}$-harboring plasmids) (Figure 1A).

7 https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/815/
8 https://cge.food.dtu.dk/services/MLST/
9 https://bioinfo-mml.sjtu.edu.cn/oriTDB/index.php
10 https://bioinfo-mml.sjtu.edu.cn/SecReT4/

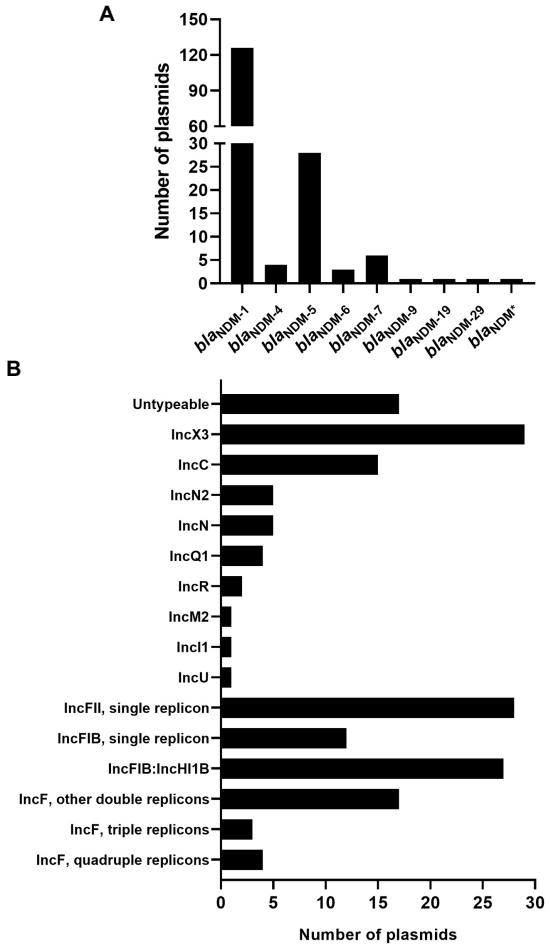

FIGURE 1
Characteristics of the 171 *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*. **(A)** Histogram of number of variants of *bla*$_{NDM}$ genes among the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae*. *bla*$_{NDM*}$ representing an unknown variant of *bla*$_{NDM}$. **(B)** An overview of the plasmid replicon types among the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* analyzed using software PlasmidFinder.

Replicon types of *bla*$_{NDM}$-harboring plasmids of *Klebsiella pneumoniae*

Replicon typing of the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* was executed using PlasmidFinder. Of the 171 plasmids, 154 were successfully identified with their replicon types, including 103 single-replicon plasmids and 51 multi-replicon plasmids (44 plasmids with two replicons, three plasmids with three replicons, and four plasmids with four replicons; Figure 1B; Supplementary Figure S1). For the 103 single-replicon plasmids harboring *bla*$_{NDM}$ in *K. pneumoniae*, the TOP5 prevalent replicons (in descending order) were IncX3 (29 plasmids), IncC (15 plasmids), IncFIB(pQil) (11 plasmids), IncFII (11 plasmids), and IncFII(Yp) (11 plasmids). Of the 44 *bla*$_{NDM}$-harboring plasmids with two replicons, 25 contained replicons IncFIB(pNDM-Mar) and IncHI1B(pNDM-MAR), which were the most prevalent two-replicon plasmids harboring *bla*$_{NDM}$ in *K. pneumoniae* (Figure 1B; Supplementary Figure S1).

In summary, 21 of the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* were found to carry the replicon of IncC, accounting for 12.28% of all the *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* in this study (Figure 1B; Supplementary Figure S1). Notably, 91 of the 171 *bla*$_{NDM}$-harboring plasmids in our study were found to be the IncF plasmids, including IncFI and IncFII plasmids, accounting for 53.22% of all the *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae* in this study (Figure 1B; Supplementary Figure S1).

Diversity of the *bla*$_{NDM}$-harboring plasmids in *Klebsiella pneumoniae*

To get the comprehensive overview of *bla*$_{NDM}$-harboring plasmids in *K. pneumoniae*, we created a phylogenetic cladogram of the 171 *bla*$_{NDM}$-harboring plasmids (Figure 2). Based on phylogenetic patterns of the 171 plasmids, combined with the replicon types, conjugative transfer regions, and genome sizes of the *bla*$_{NDM}$-harboring plasmids, most of the 171 *bla*$_{NDM}$-harboring plasmids were clustered into 10 main clades (clades I–X), representing 10 plasmid patterns carrying *bla*$_{NDM}$ genes in *K. pneumoniae* (Table 1).

Clade I: A total of 29 IncX3 plasmids were found in the clade I cluster, mainly *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ (Figure 2). For the 29 IncX3 plasmids harboring *bla*$_{NDM}$, their genome sizes varied from 45.1 to 159.3 kb (25th percentile = 46.2 kb; 75th percentile = 57.3 kb), with a median size of 53.1 kb (Supplementary Figure S2). For the conjugative transfer regions, all the plasmids belonging to clade I were found to carry genes encoding relaxases of the MOB$_P$ family characterized by the domain "Relaxase (Pfam: PF03432)," T4CPs of the VirD4/TraG subfamily characterized by the domain "T4SS-DNA_transf (Pfam: PF02534)," and VirB-like T4SS gene clusters (Figure 2; Supplementary Figure S3). Members of clade I were mainly geographically distributed in China (Figure 3; Table 1; Supplementary Table S3). No predominant ST types of isolates were found in the plasmids harboring *bla*$_{NDM}$ in *K. pneumoniae* (Table 1; Supplementary Table S3).

Clade II: Ten *bla*$_{NDM}$-positive IncN plasmids were clustered into clade II, mainly carrying *bla*$_{NDM-1}$ (Figure 2). The genome sizes of the 10 *bla*$_{NDM}$-harboring IncN plasmids varied from 38.4 to 63.1 kb (25th percentile = 47.2 kb; 75th percentile = 59.8 kb), with a median size of 52.0 kb (Supplementary Figure S1). Almost all the IncN plasmids carried the NW-type *oriT*s and genes encoding relaxases of MOB$_F$ family characterized by the domain "TrwC (PF08751)." All the 10 *bla*$_{NDM}$-positive IncN plasmids carried the genes encoding T4CPs of the TrwB/TraD subfamily characterized by the domain "TrwB_AAD_bind (PF10412)" and Trw-like T4SS gene clusters (Figure 2; Supplementary Figure S4). The members of clade II were mainly geographically distributed in China (Figure 3; Table 1; Supplementary Table S3). The 10 *bla*$_{NDM}$-positive IncN plasmids were distributed in seven ST types of

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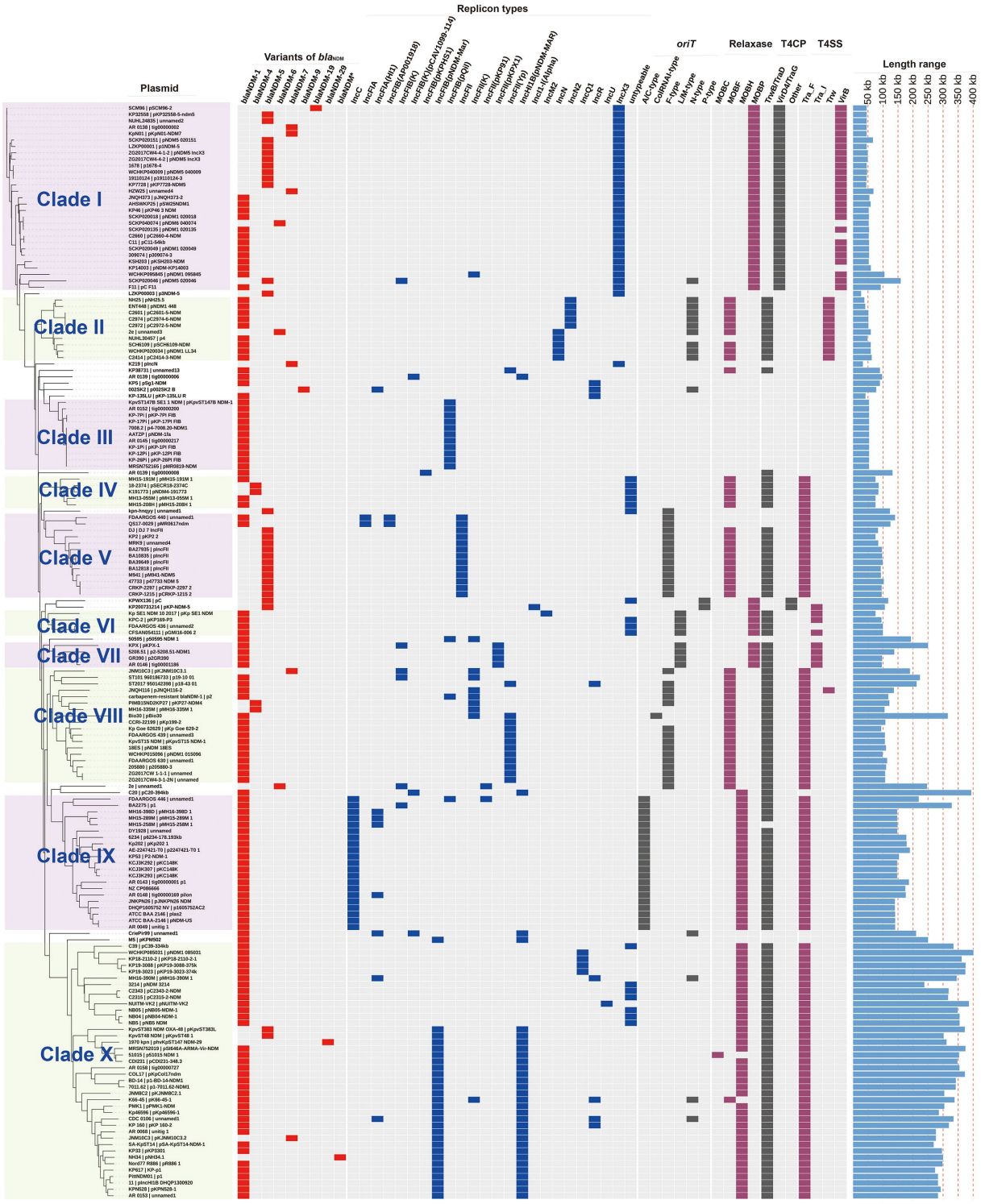

FIGURE 2
Details of variants of *bla*$_{NDM}$ genes, replicon types, conjugative transfer regions, geographical distributions, host STs, and length distribution of the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae*. The four categories of information present in this figure include the phylogenetic cladogram, variants of *bla*$_{NDM}$, replicon types, conjugative transfer regions (*oriT*, relaxase, T4CP, and T4SS), geographical distributions, ST types of host strains, and length distribution of the 171 *bla*$_{NDM}$-harboring plasmids of *K. pneumoniae*. *bla*$_{NDM*}$ representing an unknown variant of *bla*$_{NDM}$.

K. pneumoniae strains, and four plasmids were distributed in *K. pneumoniae* ST15 (Table 1; Supplementary Table S3).

Clade III: Eleven *bla*$_{NDM-1}$-positive IncF plasmids with the IncFIB(pQil) replicon were grouped into clade III, and most were

TABLE 1 Summary of the 171 bla_{NDM}-harboring plasmids of *Klebsiella pneumoniae*.

Clade	Plasmid numbers	Main replicon types	Main bla_{NDM}	Plasmid sizes (kb)	Main geographic distribution	Main ST types of hosts	Conjugative transfer region			
							oriT	Relaxase	T4CP	T4SS
I	29	IncX3	bla_{NDM-1} bla_{NDM-5}	45.1–159.3	China	–	–	MOB_P	VirD4/ TraG	VirB-like
II	10	IncN	bla_{NDM-1}	38.4–63.1	China	ST15	NW-type	MOB_F	TrwB/ TraD	Trw-like
III	11	IncFIB(pQil)	bla_{NDM-1}	54	Italy, United States	ST147	–	–	–	–
IV	5	untypeable	bla_{NDM-1} bla_{NDM-4}	75.3–86.0	Viet Nam	ST395, ST16	–	MOB_F	TrwB/ TraD	Tra_F-like
V	13	IncFII	bla_{NDM-5}	75.3–140.6	India	ST16, ST147, ST2096	F-type	MOB_F	TrwB/ TraD	Tra_F-like
VI	4	untypeable	bla_{NDM-1}	75.6–100.2	–	–	L/M-type	MOB_P	TrwB/ TraD	Tra_I-like
VII	4	IncFII(pKPX1)	bla_{NDM-1}	96.8–250.4	–	–	L/M-type	MOB_P	TrwB/ TraD	Tra_I-like
VIII	18	IncF	bla_{NDM-1}	94.4–316.2	–	–	F-type	MOB_F	TrwB/ TraD	Tra_F-like
IX	21	IncC	bla_{NDM-1}	140.1–329.2	United States, China, Viet Nam	ST11, ST1967	A/C-type	MOB_H	TrwB/ TraD	Tra_F-like
X	40	IncF	bla_{NDM-1}	238.0–401.6	China, United States	ST14, ST11, ST147	–	MOB_H	TrwB/ TraD	Tra_F-like

54-kb plasmids (Figure 2). Moreover, no conjugative transfer regions were identified in the 11 plasmids of clade III, indicating that the 11 plasmids should be non-transferable. Plasmids belonging to clade III were mainly geographically distributed in Italy and United States (Figure 3; Table 1; Supplementary Table S3). All the members of clade III were harbored by the strains of *K. pneumoniae* ST147 (Table 1; Supplementary Table S3).

Clade IV: Five bla_{NDM}-positive untypeable plasmids were clustered into clade IV, involving three bla_{NDM-1}-positive plasmids and two bla_{NDM-4}-positive plasmids (Figure 2). These five untyped plasmids, with lengths ranging from 75.3 to 86.0 kb, all carried the genes encoding relaxases of MOB_F family, genes encoding T4CPs of TrwB/TraD subfamily, and Tra_F-like T4SS gene clusters (Figure 2; Supplementary Figure S5). For the five plasmids, three were found in Viet Nam, one was found in China, and one was found in South Korea (Figure 3; Table 1; Supplementary Table S3). The STs of *K. pneumoniae* host strains containing the clade IV plasmids were distributed into ST395 and ST16 (Table 1; Supplementary Table S3).

Clade V: Thirteen plasmids with the IncFII replicon, mainly carrying bla_{NDM-5}, were classified into the clade V (Figure 2). For the 13 IncFII plasmids harboring bla_{NDM}, genome sizes varied from 75.3 to 140.6 kb (25th percentile = 88.8 kb; 75th percentile = 101.4 kb), with a median size of 96.2 kb (Supplementary Figure S1). They all carried the F-type *oriT*s and Tra_F-like T4SS gene clusters (Figure 2; Supplementary Figure S6). Most of the plasmids clustered into clade V were found to carry genes encoding relaxases of the MOB_F family and genes encoding T4CPs of the TrwB/TraD subfamily (Figure 2). The members of clade V were widely distributed in India, Southeast Asia, North America, East Asia, and Europe, with the highest prevalence in India (Figure 3; Table 1; Supplementary Table S3). The STs of *K. pneumoniae* host strains containing all Clade V plasmids were mainly distributed in ST16, ST147, and ST2096 (Table 1; Supplementary Table S3).

Clade VI: Four bla_{NDM-1}-positive plasmids, including one IncM2 plasmid and three untyped plasmids, were classified into a small cluster named clade VI in our study (Figure 2). These four plasmids, with lengths ranging from 75.6 to 100.2 kb, all carried the L/M-type *oriT*s, genes encoding relaxases of MOB_P family, genes encoding T4CPs of TrwB/TraD subfamily, and Tra_I-like T4SS gene clusters (Figure 2; Supplementary Figure S7). The four plasmids were sporadically discovered in Canada, Germany, United Kingdom, and China (Figure 3; Table 1; Supplementary Table S3). No prevalent STs of *K. pneumoniae* host strains containing all clade VI plasmids were found (Table 1; Supplementary Table S3).

Clade VII: Four bla_{NDM-1}-positive plasmids with the IncFII(pKPX1) replicon were classified into clade VII (Figure 2). The genome sizes of the four IncFII(pKPX1) plasmids varied from 96.8 to 250.4 kb. Similar to the conjugative transfer regions of plasmids belonging to clade VI, they all carried the L/M-type

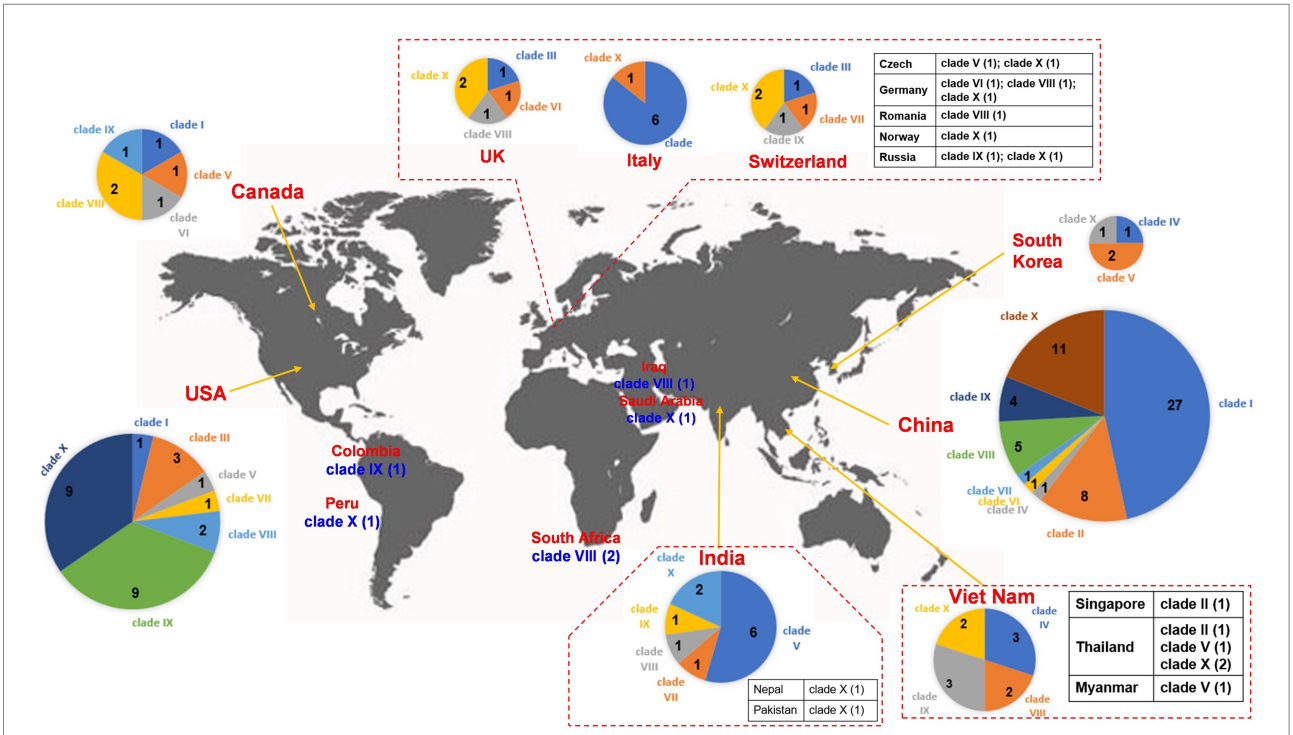

FIGURE 3
Worldwide distribution of bla_{NDM}-harboring plasmids of *K. pneumoniae*. The geographical distribution of the 10 clades (Clade I–Clade X) from the bla_{NDM}-positive plasmids of *K. pneumoniae* was calculated and displayed by pie chart. For the plasmids isolated in European countries, only those from the United Kingdom, Italy, and Switzerland were displayed by pie chart, others were displayed in the tabular form. For the plasmids isolated in Southeast Asia, only those from Viet Nam were displayed by pie chart, others were displayed in the tabular form. For the plasmids isolated in South Asia, only those from India were displayed by pie chart, others were displayed in the tabular form.

*oriT*s, genes encoding relaxases of the MOB_P family, genes encoding T4CPs of the TrwB/TraD subfamily, and Tra_I-like T4SS gene clusters (Figure 2; Supplementary Figure S8). The four plasmids were sporadically discovered in United States, India, Switzerland, and China (Figure 3; Table 1; Supplementary Table S3). No obvious common STs of strains were found (Table 1; Supplementary Table S3).

Clade VIII: Eighteen IncF plasmids, mainly carrying bla_{NDM-1}, were grouped into the clade VIII cluster (Figure 2). Most of the IncF plasmids contained the IncFII(Yp) or IncFII(K) replicon in their genomes. For the 18 bla_{NDM}-harboring plasmids of clade VIII, genome sizes varied from 94.4 to 316.2 kb (25th percentile = 106.8 kb, 75th percentile = 150.1 kb), with a median size of 110.6 kb (Supplementary Figure S1). Most of the plasmids of clade VIII were found to contain the F-type *oriT*s. They all carried the genes encoding relaxases of the MOB_F family, genes encoding T4CPs of the TrwB/TraD subfamily, and Tra_F-like T4SS gene clusters (Figure 2; Supplementary Figure S9). Notably, *K. pneumoniae* strain JNQH116 plasmid pJNQH116-2 (NZ_CP070900), belonging to the clade VIII cluster, was found to contain both Tra_F-like and Trw-like T4SS gene clusters in its genome. For clade VIII, its members were widely geographically distributed all over the world, including China, India, Southeast Asia, Middle East, North America (Canada and United States), South Africa, and some European countries (e.g., Germany, Romania, and the United Kingdom; Figure 3; Table 1; Supplementary Table S3). No prevalent STs of *K. pneumoniae* host strains containing all clade VIII plasmids were found (Table 1; Supplementary Table S3).

Clade IX: A total of 21 IncC plasmids carrying bla_{NDM-1} were grouped into the clade IX cluster of the phylogenetic cladogram (Figure 2). Their genome sizes varied from 140.1 to 329.2 kb, with the 25th percentile, median size, and 75th percentile being 144.3, 147.9, and 178.7 kb, respectively (Supplementary Figure S1). For the conjugative transfer modules, all the plasmids belonging to clade IX carried the A/C-type *oriT*s, genes encoding relaxases of the MOB_H family characterized by the domain "TraI_2 (Pfam: PF07514)," mostly genes encoding T4CPs of TrwB/TraD subfamily, and Tra_F-like T4SS gene clusters (Figure 2; Supplementary Figure S10). The IncC plasmids harboring bla_{NDM-1} were widely geographically distributed all over the world, mainly in the United States, Viet Nam, and China (Figure 3; Table 1; Supplementary Table S3). ST11 and ST1967 were the common STs strains containing bla_{NDM-1}-harboring IncC plasmids (Table 1; Supplementary Table S3).

Clade X: A total of 40 mega plasmids, where the length range varied from 238.0 to 401.6 kb (25th percentile = 293.5 kb; median = 327.3 kb; 75th percentile = 355.1 kb), mainly carrying bla_{NDM-1}, were grouped into a large cluster, named clade X in our study (Figure 2). Of the plasmids belonging to clade X, 27

(67.5%) were found to contain both replicons IncFIB (pNDM-Mar) and IncHI1B(pNDM-MAR), seven (17.5%) were unable to be typed, and four (10.0%) were IncQ1 plasmids. Moreover, all the plasmids of clade X carried genes encoding T4CPs of the TrwB/TraD subfamily and Tra_F-like T4SS gene clusters (Figure 2; Supplementary Figure S11). Most of the plasmids belonging to clade X were found to have no *oriT* and harbored the genes encoding relaxases of the MOB_H family. For the clade with the largest number, clade X, its members were widely distributed all over the world, mainly in China and the United States (Figure 3; Table 1; Supplementary Table S3). ST14, ST11, and ST147 were the common STs strains containing the plasmids of clade X (Table 1; Supplementary Table S3).

We also perform a bipartite network analysis with the 171 bla_{NDM}-harboring plasmids of *K. pneumoniae*. The bipartite network consisted of two classes of nodes: 171 plasmid units (PUs) and 2,502 homologous protein clusters (HPCs, protein families according to amino acid sequence identity, coverage, and *E*-value; Figure 4). Edges connected every PU with the HPC that it contained. The PUs of the bipartite network clearly showed distinct clustering phenomena. Overall, one homologous PU clusters (PUCs) contained the almost the same members of Clade X in the analysis above (Figure 4), which was clearly distinct from other PUs. One large region including clades III, VI – IX was also identified, which were mostly IncF and IncC plasmids. In addition, clades I, II, IV, and V were also found their corresponding PUCs in the PU-HPC bipartite network.

Discussion

Global spread of the NDM-type carbapenemases can be partly attributed to the dissemination of various bla_{NDM}-harboring plasmids (Lee et al., 2016; Dong et al., 2022). Therefore, to characterize plasmids harboring bla_{NDM} in *K. pneumoniae*, we systematically analyzed the variants of bla_{NDM}, replicon types, and conjugative transfer regions of 4,451 plasmids belonging to *K. pneumoniae* from the NCBI GenBank database. Overall, 171 bla_{NDM}-harboring plasmids of *K. pneumoniae* were identified.

In our study, nine different variants of bla_{NDM} were identified from the 171 bla_{NDM}-harboring plasmids in *K. pneumoniae*, with bla_{NDM-1} and bla_{NDM-5} being highly prevalent; bla_{NDM-1}-carrying plasmids were the most prevalent and accounted for 73.68% of the 171 bla_{NDM}-harboring plasmids. NDM-1 was first reported in 2008 on a 180-kb plasmid of *K. pneumoniae* strain isolated from a Swedish patient hospitalized in New Delhi, India (Yong et al., 2009). After the first report, NDM-1 was reported in many clinical isolates, mainly *K. pneumoniae* and *E. coli* (Kumarasamy et al., 2010). In addition, bla_{NDM-5} was another common variant in our work, accounting for 16.37% of the 171 bla_{NDM}-harboring plasmids. The variant NDM-5 was first reported on an IncF plasmid of *E. coli* EC405, isolated from a 41-year-old British patient who had a travel history to India (Hornsey et al., 2011). Notably, bla_{NDM-5} was reported to be the predominant variant in bla_{NDM}-harboring plasmids of *E. coli* (Zhang et al., 2021).

Our results showed that IncX3 single-replicon plasmids were important carriers of bla_{NDM} in *K. pneumoniae*, mainly bla_{NDM-1} and bla_{NDM-5}. IncX3 plasmid is narrow-host range plasmids in Enterobacterales (Johnson et al., 2012), which has been reported to harbor diverse carbapenemase genes in CRE worldwide (Mouftah et al., 2019). Of the 29 bla_{NDM}-harboring IncX3 plasmids grouped into clade I, most were relatively small with lengths of 46–57 kb (25th percentile = 46.2 kb; median size = 53.1 kb; 75th percentile = 57.3 kb). Based on the results analyzed by the oriTfinder software, all the 29 bla_{NDM}-harboring IncX3 plasmids of clade I contained genes encoding for relaxases belonging to the MOB_P family, with TraI protein encoded by the IncPα plasmid RP4 (Pansegrau et al., 1993) as a representative. T4CPs encoded by the 29 bla_{NDM}-harboring IncX3 plasmids of clade I belonged to the VirD4/TraG subfamily, with the TraG protein of plasmid RP4 and the VirD4 protein of Ti plasmids as representatives (Gomis-Rüth et al., 2004). The bla_{NDM}-harboring IncX3 plasmids classified into clade I contained VirB-like T4SS gene clusters, which are the best-characterized T4SS (Guglielmini et al., 2014). However, no known *oriT* site was found in most of the IncX3 plasmids harboring bla_{NDM} belonging to clade I of the phylogenetic cladogram, indicating a new type of *oriT* site, different from the nine *oriT* families collected in the oriTDB database (Li et al., 2018).

We found 10 bla_{NDM}-harboring IncN plasmids, with IncN or IncN2 replicons, clustered into clade II of the phylogenetic cladogram. They were also relatively small plasmids, with genome sizes varying from 38.38 to 63.05 kb. These bla_{NDM}-harboring IncN plasmids carried the NW-type *oriT*s, which were characterized by the conserved nick region KGTST|ATAGC ("|" refers to the *nic* site of *oriT*), with *oriT* sites of IncN plasmid R46 (Hall and Vockler, 1987) and IncW plasmid R388 (Revilla et al., 2008) as representatives. Almost all the plasmids of clade II contained genes coding for relaxases of the MOB_F family, which was characterized by the domain "TrwC (PF08751)," with R388 TrwC and F TraI as representatives (de la Cruz et al., 2010). The T4CPs encoded by the bla_{NDM}-positive IncN plasmids belonged to the TrwB/TraD subfamily, which was characterized by the domain "TrwB_AAD_bind (PF10412)," with the TrwB encoded by plasmid R388 from *E. coli* as a representative (Gomis-Rüth et al., 2004). In addition, the bla_{NDM}-positive IncN plasmids carried Trw-like T4SS gene clusters. The Trw T4SS clusters were regarded as the bacterial conjugation machines that mediate the spread of plasmids among bacterial populations (e.g., the trw locus of broad-host-range IncW plasmid R388; Seubert et al., 2003) while also mediating host-specific erythrocyte infection (e.g., the pathogenesis-related Trw system of Bartonella; Vayssier-Taussat et al., 2010).

Our work showed that 21 IncC plasmids carrying bla_{NDM-1}, with genome sizes from 140.1 kb to 329.2 kb, were clustered into the clade IX of the phylogenetic cladogram constructed by the 171 bla_{NDM}-harboring plasmids in *K. pneumoniae*. The broad-host-range IncC mega plasmids are essential contributors to the

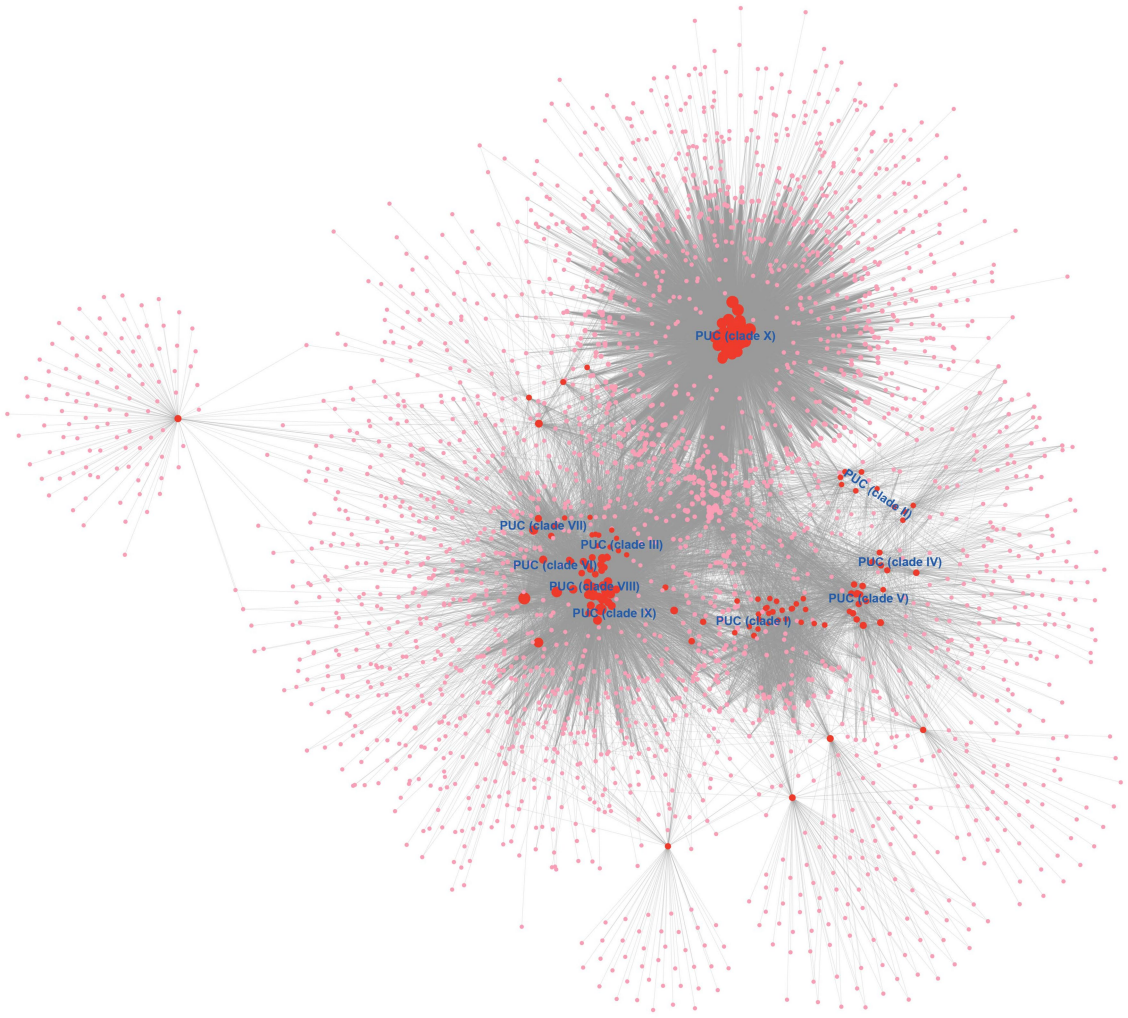

FIGURE 4
The PU-HPC bipartite network visualization of the bla_{NDM}-harboring plasmids of *K. pneumoniae*. Plasmid units (PUs, 171) and homologous protein clusters (HPCs, 2,502) are represented as colored circles, with red for PUs and pink for HPCs. The size of a circle is ranked as the degree calculated with the Cytoscape's built-in NetworkAnalyzer tool. The 10 clades (Clade I–Clade X) were labeled into the location of corresponding PUCs.

dissemination of antibiotic resistance genes, and more than 200 fully sequenced IncC plasmids have been reported (Ambrose et al., 2018). The bla_{NDM-1}-harboring IncC plasmids of clade IX contained the A/C-type *oriT*s, with the *oriT* site of IncA/C conjugative pVCR94ΔX from Vibrio cholera as the prototype (Carraro et al., 2014). Furthermore, these bla_{NDM-1}-harboring IncC plasmids carried genes encoding relaxases of the MOB_H family, characterized by the domain "TraI_2 (Pfam: PF07514)," with TraI encoded by IncHI plasmid R27, TraI encoded by IncA/C plasmid pIP1202, TraI encoded by IncJ plasmid R391, and TraI encoded by IncT plasmid Rts1 as representatives (de la Cruz et al., 2010). In addition, most of the IncC plasmids clustered into clade IX contained genes encoding T4CPs of the TrwB/TraD subfamily and Tra_F-like T4SS gene clusters.

In our work, 53.22% (91 out of 171 plasmids) of the bla_{NDM}-harboring plasmids of *K. pneumoniae* were found to be IncF plasmids, and most were multi-replicon IncF plasmids, especially IncFI-type plasmids. IncF plasmids are commonly low-copy-number plasmids, >100 kb in size (Villa et al., 2010); however, in our study, the bla_{NDM}-harboring IncF plasmids in *K. pneumoniae* were heterogeneous in size. For example, the bla_{NDM-1}-positive IncF plasmids, with the IncFIB(pQil) replicon, clustered into clade III were mostly 54-kb plasmids; the genome sizes of the IncFII plasmids grouped into clade V varied from 75.31 to 140.6 kb (25th percentile = 88.81 kb; 75th percentile = 101.4 kb); and the 27 plasmids with replicon IncFIB(pNDM-Mar) belonging to clade X were > 250 kb in size. The IncF plasmids comprise a diverse set of conjugative plasmids frequently found in Enterobacterales, which contribute to spreading AMR genes (Villa et al., 2010; Carattoli, 2011). The bla_{NDM}-harboring IncF plasmids in *K. pneumoniae* were also heterogeneous in types of conjugative transfer regions. The IncFII-type plasmids, including clades V and VIII, carried F-type *oriT*s, genes encoding relaxases of the MOB_F family, genes encoding T4CPs of the TrwB/TraD subfamily, and Tra_F-like

T4SS gene clusters belonging to the classical F-like conjugative system (de la Cruz et al., 2010). Mega plasmids with replicons IncFIB(pNDM-Mar):IncHI1B(pNDM-MAR) belonging to clade X mostly harbored the genes encoding relaxases of the MOB_H family. In our study, we found 11 bla_{NDM-1}-positive IncFIB(pQil) plasmids classified into clade III without any classical conjugative transfer regions, which were predicted as non-transferable plasmids.

Conclusion

In this study, we analyzed the variants of bla_{NDM}, replicon types, conjugative transfer regions, host STs, and geographical distributions of 171 bla_{NDM}-harboring plasmids from 4,451 *K. pneumoniae* plasmids, which were downloaded from the GenBank database. Nine variants of bla_{NDM} were found among the 171 bla_{NDM}-positive plasmids, with bla_{NDM-1} (73.68%) and bla_{NDM-5} (16.37%) as the most dominant. Over half of the bla_{NDM}-harboring plasmids of *K. pneumoniae* were classified into IncF plasmids. In addition, IncX3 single-replicon plasmids (46–57 kb), IncN plasmids (38.4–63.1 kb), IncC plasmids (140.1–329.2 kb) were also the common carriers of bla_{NDM} in *K. pneumoniae*. The bla_{NDM}-harboring IncX3 and IncN plasmids were mainly geographically distributed in China. The IncC plasmids harboring bla_{NDM-1} were widely geographically distributed all over the world, mainly in the United States, China, and Viet Nam. This study provides important insights into the diversity of bla_{NDM}-harboring plasmids in *K. pneumoniae* and further addresses their role in the acquisition and spread of resistance genes. However, the genetic diversity and characteristics of bla_{NDM}-harboring plasmids in other Gram-negative species need further study in the future.

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

XL, LuL, and XD: conceptualization. ZhuZ: methodology. LeL and LiL: software. SH and JY: validation. WL, LZ, QL, and ZhiZ: formal analysis. ZhuZ and XL: writing—original draft preparation. XL and LuL: writing—review and editing. XL: supervision. 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.2022.1008905/full#supplementary-material

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Aeromonas species isolated from aquatic organisms, insects, chicken, and humans in India show similar antimicrobial resistance profiles

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Aeromonas species are Gram-negative bacteria that infect various living organisms and are ubiquitously found in different aquatic environments. In this study, we used whole genome sequencing (WGS) to identify and compare the antimicrobial resistance (AMR) genes, integrons, transposases and plasmids found in *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii* isolated from Indian major carp (*Catla catla)*, Indian carp (*Labeo rohita)*, catfish (*Clarias batrachus*) and Nile tilapia (*Oreochromis niloticus*) sampled in India. To gain a wider comparison, we included 11 whole genome sequences of *Aeromonas* spp. from different host species in India deposited in the National Center for Biotechnology Information (NCBI). Our findings show that all 15 *Aeromonas* sequences examined had multiple AMR genes of which the Ambler classes B, C and D β-lactamase genes were the most dominant. The high similarity of AMR genes in the *Aeromonas* sequences obtained from different host species point to interspecies transmission of AMR genes. Our findings also show that all *Aeromonas* sequences examined encoded several multidrug efflux-pump proteins. As for genes linked to mobile genetic elements (MBE), only the class I integrase was detected from two fish isolates, while all transposases detected belonged to the insertion sequence (IS) family. Only seven of the 15 *Aeromonas* sequences examined had plasmids and none of the plasmids encoded AMR genes. In summary, our findings show that *Aeromonas* spp. isolated from different host species in India carry multiple AMR genes. Thus, we advocate that the control of AMR caused by *Aeromonas* spp. in India should be based on a One Health approach.

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KEYWORDS

Aeromonas, resistance, plasmids, integrase, beta lactam, antimicrobials, transposase genes

Introduction

Aeromonads are Gram-negative facultative anaerobic bacteria ubiquitously found in freshwater, estuarine, and brackish water environments (Janda and Abbott, 2010). Common disease-causing *Aeromonas* species include *Aeromonas hydrophila, Aeromonas caviae*, *Aeromonas veronii*, *Aeromonas sobria* and *Aeromonas salmonicida* (Figueras and Beaz-Hidalgo, 2015). Given their tropism for several species and ubiquitous nature in aquatic environments, *Aeromonas* spp. have the potential to transmit antimicrobial resistance (AMR) genes to multiple host species. Moreover, various *Aeromonas* spp. have been reported to carry plasmids, transposons and integrases that play a major role in acquisition and transfer of AMR genes among different bacteria species (Chang and Bolton, 1987; Sørum et al., 2003; Palu et al., 2006). Thus, a comparison of AMR genes, plasmids, and transposons found in *Aeromonas* spp. isolated from aquatic environments, insects, fish, and animals would shed insight into the role of *Aeromonas* spp. in the spread of AMR genes from the environment to different host species. Information from these studies would guide the design of effective control measures to limit AMR spread by *Aeromonas* spp. in different ecosystems.

India is the second largest consumer of antibiotics after China (Schar et al., 2020). It is also the second largest producer of farmed aquatic organisms in the world (Jayasankar, 2018). Antimicrobials may be used in aquaculture in India for the control of infectious diseases (Walia et al., 2019; Lulijwa et al., 2020) of which *Aeromonas* spp. are among the top pathogens infecting aquatic organisms (Harikrishnan and Balasundaram, 2005; Elgendy et al., 2017; Dubey et al., 2021; Saharia et al., 2021). Boeckel et al. (Van Boeckel et al., 2019) reported that India, together with China, represent the largest environmental AMR hot-spots suggesting that bacteria species like *Aeromonas* spp. ubiquitously found in the aquatic environment are likely to be among the top carriers of AMR genes. *Aeromonas* spp. have been isolated from sewage (Sudheer Khan et al., 2011; Gogry and Siddiqui, 2019), ponds (Singh et al., 2008; Zdanowicz et al., 2020), rivers (Roy et al., 2013), lakes (Joshi, 2016) and marine areas (Vivekanandhan et al., 2005). From farmed aquatic organisms they have been isolated from fresh water loach (*Lepidocephalichthys guntea*) (Roy and Barat, 2011; Roy et al., 2013), freshwater prawn (*Macrobrachium rosenbergii*) (Lijon et al., 2015), marine prawn (*Penaeus semisulcatus)* (Vivekanandhan et al., 2005), Indian white shrimp (*Penaeus indicus*) (Rahimi and Nene, 2006), and giant tiger prawn (*Penaeus monodon*) (Vaseeharan et al., 2005). In insects, they have been isolated from mosquitos (*Culex quinquefasciatus* and *Aedes aegyptii*) (Pidiyar et al., 2002) and chironomid larvae (Kuncham et al., 2017), while from birds and mammals they have been isolated from chickens (Praveen et al., 2014), pigs (Rahimi and Nene, 2006) and buffalo (Rahimi and Nene, 2006). In humans, they have been linked to keratitis, meningitis, and acute gastroenteritis (Misra et al., 1989; Seetha et al., 2004; Sinha et al., 2004; Subashkumar et al., 2006; Motukupally et al., 2014). Overall, these observations from *Aeromonas* studies performed in India are in line with findings from other countries where *Aeromonas* spp. have been isolated from various insect species such as mosquitoes, midges, and houseflies near water bodies (Smith et al., 1998; Pidiyar et al., 2002; Nayduch et al., 2005; Jazayeri et al., 2011) as well as from fish, frogs, reptiles, birds, and mammals (Parker and Shaw, 2011; Elgendy et al., 2017; Wamala et al., 2018; Abdelsalam et al., 2021). What has not been determined is whether *Aeromonas* spp. isolated from different host species carry similar AMR genes, transposons and plasmids.

From previous studies done in India, the prevalence of AMR genes in different *Aeromonas* spp. has been reported using the disc diffusion test and AMR genes by PCR (Sinha et al., 2004; Kaskhedikar and Chhabra, 2010; Roy et al., 2013). A major limiting factor with PCR as a survey tool is that it uses primers targeting only the selected AMR genes, posing the danger of omitting other vital genes contributing to AMR present in bacteria genomes. Thus, PCR lacks the ability to profile all AMR genes present in bacteria genomes. On the other hand, the disc diffusion test only gives the phenotypic characterization of AMR, but does not profile all genes responsible for the antimicrobial resistance. So, the purpose of this study was to use whole genome sequencing (WGS) to identify and compare AMR genes found in *A. hydrophila*, *A. veronii* and *A. caviae* isolated from different fish species in India. To increase our breadth of comparison, we included other publicly available whole genome sequences of *Aeromonas* spp. obtained from different host species in India deposited in the National Biotechnology Center for Information (NCBI). Thus, our work provides a comprehensive overview of AMR genes, efflux pump genes, integrases, transposases and plasmids found in different *Aeromonas* spp. isolated from different host species in India. Data generated herein is useful for creating a basis for a One Health approach in the control of AMR caused by *Aeromonas* spp.

Materials and methods

Characterization of bacteria using MALDI-TOF and PCR using 16S rRNA

Two *A. hydrophila* strains (SD/21–01 and SD/21–05) isolated from *Catla catla* and *Labeo rohita*, one *A. veronii* strain (SD/21–04) isolated from *Clarias batrachus* and one *A. caviae* strain (SD/21–11)

from *Oreochromis niloticus* from India (Table 1) were retrieved from the −80°C freezer in tryptose soy broth (TSB) and incubated at 30°C overnight. All four *Aeromonas* spp. used were isolated from disease outbreaks of fish cultured under intensive farming (Table 1; Dubey et al., 2021). Diseased fish were treated with oxytetracycline, trimethoprim and sulfonamide. Bacteria grown in TSB were also cultured on blood agar plates for individual colony purity. Purified colonies were further characterized using the Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry (MS) based on manufacturer's protocol (Singhal et al., 2015). Purified bacteria confirmed by MALDI-TOF were used for DNA extraction using the DNA extraction kit (Qiagen, Germany). Genus identification was carried out by PCR using universal 16S *rRNA* gene primers 27F and 1492R (Alcock et al., 2020). After confirmation as *Aeromonas* spp. by 16S *rRNA* gene sequencing, cultured isolates were used for genomic DNA extraction.

Testing of antimicrobial resistance using disk diffusion assay

The four *Aeromonas* spp. isolated from different fish species (Table 1) were tested for antibiotic resistance using the Kirby-Bauer disk diffusion assay (Joseph et al., 2011). Commercially available antibiotic discs (Neo-Sensitabs™, Rosco) used were ampicillin (AMP-10 µg), cefoxitin (CFO-30 µg), cephalothin (CEP-30 µg), ciprofloxacin (CIPR-5 µg), erythromycin (Ery-15 µg), gentamycin (GEN-10 µg), nitrofurantoin (NI-300 µg), penicillin (PEN-10 µg), sulfonamide (SULFA-240 µg), tetracycline (TET-30 µg), and trimethoprim (TRIM-5 µg). Overnight grown bacterial isolates were diluted to 0.5 MacFarland at a concentration of 10^8cfu/ml and 100 µl spread over the Muller Hinton agar using sterile cotton swabs (Saffari et al., 2016). Antibiotic discs were placed on the agar plate surface on a bacterial lawn followed by incubation at 30°C overnight. Antibiotic susceptibility/resistance was measured based on the manufacturer's instruction (Neo-Sensitabs™, Rosco Diagnostica, Albertslund, Denmark). All experiments were carried out based on the Clinical and Laboratory Standards Institute (CLSI) (Cockerill et al., 2012) guidelines to determine the susceptibility or resistance of bacteria to antibiotic treatment (Kahlmeter et al., 2006).

Bacterial genomic DNA extraction and QC analysis

Genomic DNA (gDNA) was extracted from the four *Aeromonas* spp. isolated from fish in India using the MagAttract® HMW DNA kit based on the manufacturer's protocols (Qiagen GmbH, Hilden, Germany) (Becker et al., 2016). A 1 ml volume containing approximately 2 ×10^9 CFU/ml freshly grown bacteria was centrifuged in 2 ml Eppendorf tubes and pellets were resuspended in 180 µl buffer ATL (tissue lysis buffer, Qiagen GmbH, Hilden, Germany). Thereafter, Proteinase K (20 mg/ml concentration) was added to each tube followed by incubation at 56°C in an Eppendorf thermomixer for 30 min. After incubation, 4 µl RNase was added to the suspension followed by pulse vortexing. This was followed by adding 15 µl of MagAttract Suspension G and 280 µl Buffer MB to each vial followed by pulse vortexing (Tarumoto et al., 2017). The suspension from each tube was transferred onto the MagAttract holder followed by mixing for 1 min on an Eppendorf thermomixer. Magnetic beads

TABLE 1 Genebank accession numbers of *Aeromonas* spp. used in the study.

Strain	Year	Bacteria species	Host species	Clinical history	Accession no	Source/References
SD/21–04 (Ah2)	2009	*A. veronii*	Walking catfish (*Clarias batrachus*)	Diseased fish	JAJVCV000000000	This study
SD/21–01 (Ah1536)	2009	*A. hydrophila*	Indian carp (*Catla catla*)	Diseased fish	JAJVCT000000000	This study
SD/21–05 (Ah4)	2009	*A. hydrophila*	Rohu *(Labeo rohita)*	Diseased fish	JAJVCU000000000	This study
SD/21–11 (Ah27)	2009	*A. caviae*	Nile tilapia (*Oreochromis niloticus*)	Diseased fish	JAJVCW000000000	This study
XhG1.2	2017	*A. veronii*	Green swordtail (*Xiphophorus hellerii*)	Diseased fish	JACGXR000000000.1	Das et al. (2021)
A8-AHP	2016	*A. veronii*	Rohu *(Labeo rohita)*	Diseased fish	CP046407.1	Tyagi et al. (2022)
Phln2	2010	A. veronii	Fish intestine	Unknown	ANNT00000000.1	
F2S2–1	2015	*A. dhakensis*	Indian oil sardine (*Sardinella longiceps*)	Not specified	LZFM00000000.1	Nadiga et al. (2016)
Y557	2015	*A. salmonicida*	Bighead carp (*Aristichthys nobilis*)	Market foods	JZTH00000000.1	Vincent et al. (2016)
Y567	2015	*A. salmonicida*	Buffer catfish (*Ompok bimaculatus*)	Market foods	JZTG00000000.1	Vincent et al. (2016)
A527	2007	*A. salmonicida*	Giant river prawn (*Macrobrachium rosenbergii*)	Market foods	CP022550.1	Vincent et al. (2017)
CMF	2019	*A. veronii*	Insect gut *(Chrysomya megacephala)*	Unknown	WVRP00000000.1	
FC951	2017	*A. veronii*	Human (*Homo sapiens*)	Asymptomatic patients	CP032839.1	Ragupathi et al. (2020)
VBF557	2015	*A. veronii*	Human (*Homo sapiens*)	Unknown	LXJN00000000.1	
Y47	2015	*A. salmonicida*	Chicken (*Gallus domesticus*)	Market foods	JZTF00000000.1	Vincent et al. (2016)

containing gDNA were separated on the MagAttract magnetic rack for around 1 min, and supernatants were removed without disturbing the beads. Magnetic beads were washed twice using MW1 and PE buffer (Becker et al., 2016; Tarumoto et al., 2017). The remaining suspension from each vial was removed by rinsing the beads with 1 ml RNase-free water twice (Qiagen GmbH, Hilden, Germany) (Becker et al., 2016). The harvested gDNA was eluted in 100 µl buffer EB. The purity of gDNA was assessed using the NanoDrop (Thermo Fisher, Arbor, Michigan United States) and gel electrophoresis using 1% agarose. Quantification of gDNA was done using the Qubit double-stranded DNA high-CHS kit based on the manufacturer's instructions (Life Technologies Inc., Carlsbad, CA, United States) (Guan et al., 2020).

Library preparation, sequencing and bioinformatic analysis

Aeromonas spp. sequence libraries were prepared using the paired-end genome libraries using the Nextera DNA Flex Tagmentation (Illumina Inc. San Diego, CA, United States) (Gaio et al., 2021). Illumina libraries were quantified using the Qubit® DNA HS Assay Kit in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) while the size of library fragments was checked using an Agilent 2,100 Bioanalyzer System using the Agilent HS DNA Kit (Agilent Technologies, CA, United States). Illumina MiSeq (Illumina Inc., United States) were sequenced using V3 reagent kits using paired-end read length of 2×300 bp (Kaspersen et al., 2020). Four bacterial raw DNA reads from this study and 11 sequence reads archives (SRAs) were retrieved from NCBI (Table 1) and were analyzed using the online Galaxy platform (https://usegalaxy.no/) version 21.05. Quality of both forward and reverse raw reads were analyzed using the FastQC Version 0.11.9 software (Bioinformatics, 2011), while the Trimmometric version 0.38.1 was used to remove the adapters and low-quality reads from paired-end sequences (Bolger et al., 2014). The resulting paired-end sequence reads were *de novo* assembled into contigs using SPAdes v. 3.12.0 (Coil et al., 2015) with 33 to 91 k-mers (Bankevich et al., 2012), while genome annotation was conducted using the prokaryotic genome annotation pipeline (PGAP) (Tatusova et al., 2016) from the NCBI and Prokka (Seemann, 2014).

Prediction of antimicrobial resistance genes

In addition to genome sequences of the four isolates from fish in India, we retrieved 11 whole genome sequences (WGS) of *Aeromonas* spp. from different host species in India from the NCBI database for comparison with our isolates (Table 1). Among the retrieved genomes from NCBI, *A. veronii* strain A8-AHP was isolated from the kidney tissue of diseased *Labeo rohita* and was shown to have reduced susceptibility for ampicillin and imipenem on the disk diffusion test (Tyagi et al., 2022), while *A. veronii* strain XhG1.2 was isolated from gills and intestine of diseased green swordtail fish and no antibiotic resistance test was reported (Das et al., 2020). Other *A. veronii* isolates include strain FC951 isolated from healthy humans, VBF557 from humans with unknown clinical history, CMF from insect gut (*Chrysomya megacephala*) and PhIn2 from fish intestines with unknown clinical history (Table 1). Similarly, *A. dhakensis* strain F2S2–1 was isolated from the skin surface of an Indian oil sardine (Nadiga et al., 2016). The *A. salmonicida* strains Y47, Y567, A527 and Y577 were isolated from a chicken, butter catfish (*Ompok bimaculatus*), prawn (*Macrobrachium rosenbergii*), and bighead carp (*Aristichthys nobilis*), respectively, sold as food at a market in Mumbai in India (Nagar et al., 2011; Vincent et al., 2017). There was no information available regarding the antibiotic treatment of the host species for the genomes retrieved from the NCBI database and no record of disc diffusion test for all isolates, except *A. veronii* strain A8-AHP. Altogether, a total of 15 sequences were used for WGS comparison of AMR genes, plasmids and transposases profiles. Antibiotic resistance genes were identified using staramr version 0.7.2 (Tran et al., 2021) and ABRicate version 1.0.1 (Seemann, 2016) in the Comprehensive Antimicrobial Resistance Database (CARD) (Alcock et al., 2020). The threshold for AMR gene identification using the CARD was set at 80%. Plasmidfinder v 2.0 (Ullah et al., 2020) was used to identify plasmids in the bacterial genomes.

Pangenome analysis

The pangenome of the 15 *Aeromonas* isolates from India was constructed using Roary version 3.13.0 using general feature files 3 (.gff) generated from Prokka Version 1.14.5. The minimum percent identity cut-off limit was set at 95% (Seemann, 2014; Page et al., 2015). The distribution of core genes (genes present in all genomes), shell genes (genes not shared by all genomes but present in more than one isolate), and cloud genes (genes only found in one isolate) were determined using the online usegalaxy. no platform with minimum gene identity cut-off of 99% (Page et al., 2015). The pangenome of all 15 *Aeromonas* genomes was generated using the online genome viewer Phandango (Hadfield et al., 2018), while accompanying phylogenetic trees were created using Gene_presence_absence and Newick files obtained from Roary and *Aeromonas* genomes were grouped in similarity clusters.

Phylogenetic analysis of antimicrobial resistance genes

Phylogenetic analysis of the Ambler classes B, C and D β-lactamase genes was carried out using the Molecular Evolutionary Genetic Analysis version 7 (MEGA-7) bioinformatics software (Kumar et al., 2016). The AMR genes used for phylogenetic analyses were retrieved after screening of AMR genes using ABRicate version 1.0.1 for all 15 *Aeromonas* spp.

genomes. Phylogenetic trees were generated using the Neighbor-joining and BioNJ algorithm to a pairwise matrix estimated using JTT model and expressed as number of base substitution per site (Jones et al., 1992). The outlier groups for the Ambler classes B, and C β lactamase genes used were *Shigella sonnei* tetracycline gene *tet(A)* ANN06707.1 while *Vibrio fluvialis* sulfonamide gene *sul1* AEJ33969.1 was used as out group for the class D β-lactamase and the *CRP* gene, respectively.

Results

Phenotype characterization of antimicrobial resistance using the disc diffusion test

All four *Aeromonas* spp. isolated from fish in India showed multidrug resistance (MDR) to three or more antibiotics on the disk diffusion test (Table 2). *A. hydrophila* strain SD/21–01 from Indian carp (*C. catla*) was resistant to AMP-10, CEP-30, PEN-10, ERY-15, SULFA-240, while the *A. hydrophila* strain SD/21–05 (*L. rohita*) was also resistant to AMP-10, ERY-15, PEN-10, SULFA-240 and TRIM-5. The *A. veronii* isolate from catfish (SD/21–04) was resistant to AMP-10, CFO-30, CEP-30, ERY-15, GEN-10, PEN-10, and TET-30 while *A. caviae* from Nile tilapia (SD/21–11) was resistant to AMP-10, PEN-10, ERY-15, GEN-10 and SULFA-240. All four isolates were susceptible to CIPR, and NI300. In addition, *A. hydrophila* from India carp (SD/21–05) and catfish (SD/21–01) together with *A. caviae* from Nile tilapia (SD/21–11) showed susceptibility to TET-30 while *A. veronii* from catfish (SD/21–04) was susceptible to SULFA-240.

Genome comparison

Draft genomes of all the four *Aeromonas* isolates from India sequenced using the MiSeq 300 generated varied between 44.5–52.0 million DNA reads with a phred quality score > 36 for all four isolates (Table 3). After quality filter (Q > 30), approximately 42.6–44.3 million reads were *de novo* assembled using SPAdes v. 3.12.0. Raw data generated after sequencing have been deposited in NCBI under the sequence read archive (SRA) accession numbers from SRR17405115 to SRR17405118. Genome assembly and annotation features of the four fish isolates together with 11 genomes from other species are shown in Table 3. Final genome assembly of the four Indian fish isolates SD/21–01, SD/21–05, SD/21–04 and SD/21–11 consisted of 4,701,638 bp, 4,940,355 bp, 4,570,779 bp, 4,231,844 bp, with N50 value 766,346 bp, 239,795 bp, 184,893 bp, 101,699 bp, respectively. Total number of contigs for *A. hydrophila* (SD/21–01 and SD/21–05), *A. veronii* (SD/21–04) and *A. caviae* (SD/21–14) were 30, 78, 69 and 99, respectively. All four fish genomes have been deposited at DDBJ/ENA/GenBank with accession JAJVCT000000000 to JAJVCW000000000 (Table 1). The size of all 15 genomes is shown in Table 3. Equally, a comparison of other parameters such as contigs, G + C content %, genes (total), genes (RNA), protein coding genes (CDS), and Pseudo Genes is shown in Table 3.

Pangenome analysis

The total number of genes detected from the 15 *Aeromonas* genomes (Table 1) based on pangenome analysis was 20,415 genes of which 621 genes were core-, 7,139 shell- and 12,655 cloud genes (Figure 1). Four groups were generated based on *Aeromonas* species classification. Group 1 consisted of seven *A. veronii* genomes obtained from catfish (SD/21–04), human (FC951 and VBF557), fish (Ph1n2), Indian carp (A8-AHP), swordtail (XhG1.2) and insect (CMF). The total number of genes from group-1 was 8,911 genes that comprised of 2,388 core-, 1898 shell- and 4,625 cloud genes. Group-2 only comprised of genes from *A. caviae* isolated from Nile tilapia (SD/21–11). Group-3 consisted of genes from four *A. salmonicida* genomes from prawn (A527), butter catfish (Y567), chicken (Y47) and bighead carp (Y557) that had a total of 6,048

TABLE 2 Antibiotic susceptibility of *Aeromonas* spp. based on disk diffusion test.

Antibiotics (μg)	SD/21–04	SD/21–01	SD/21–05	SD/21–11
Ampicillin (AMP-10)	R	R	R	R
Cefoxitin (CFO-30)	R	R	S	S
Cephalothin (CEP-30)	R	R	S	S
Ciprofloxacin (CIPR-5)	S	I	S	I
Erythromycin (ERY-15)	R	R	R	R
Gentamycin (GEN-10)	R	R	I	R
Nitrofurantoin (NI-300)	S	S	S	S
Penicillin (PEN-10)	R	R	R	R
Sulfonamide (SULFA-240)	R	R	R	R
Tetracycline (TET-30)	R	I	I	S
Trimethoprim (TRIM-5)	I	I	R	I

Determination of susceptibility or resistance to antibiotic treatment was based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (Kahlmeter et al., 2006; Cockerill et al., 2012).

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TABLE 3 Whole genome sequence data of *Aeromonas* spp. used in the study.

Genome feature	SD/21–04	SD/21–01	SD/21–05	SD/21–11	XhG1.2	A8-AHP	PhIn2	F2S2–1	Y557	Y 567	A527	CMF	FC951	VBF557	Y47
Source	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Prawn	Insect gut	Human	Human	Chicken
Country of origin	India	India	India	India	India	India	India	India	India	India	India	India	India	India	India
Contig	69	30	78	99	35	1	1899	64	104	47	1	200	1	526	118
Genome size (bp)	4,570,779	4,701,638	4,940,355	4,231,844	4,573,855	4,744,657	4,300,552	4,750,839	4,736,406	4,554,843	4,806,250	4,562,071	4,666,657	4,696,503	4,710,230
Largest contig	602,701	1,325,364	610,305	352,808	-	4,744,657	23,680	-	-	-	4,806,250	-	4,666,657	-	-
N50	184,893	766,346	239,795	101,699	305,294		3,789	260,482	101,766	217,341		40,276	-	19,666	117,778
G + C content %	58.60	61.26	60.92	62.02	58.7	58.36	58.68	60.30	62.05	59.67	61.87	56.7	60.50	61.53	62.5
Genes (total)	4,310.0	4,361	4,613	3,993	4,165	4,419	3,993	4,225	4,389	4,215	4,470	4,282	4,575	4,460.0	4,400.0
CDSs (with protein)	4,152	4,203	4,425	3,822	4,038	4,205	3,822	4,075	4,154	4,007	4,138	4,143	4,056	3,325	4,165
Genes (RNA)	109	109	108	101	78	145	101	115	146	147	162	96	147	93	154
rRNAs	4	5	4	5	4	31	5	21	31	33	31	11	21	15	35
tRNAs	100	97	96	90	70	108	90	88	115	114	125	81	112	74	119
Pseudo Genes (total)	49	49	80	70	53	69	70	35	88	57	170	43	372	1,042	78

genes comprising of 3,320 core and 2,728 shell genes. Group-4 consisted of genes from two *A. hydrophila* genomes isolated from Indian carp (SD/21–01 and SD/21–05) and one *A. dhakensis* genome from sardine (F2S2–1). The total number of genes in group-4 was 6,321 genes of which 2,786 were core- and 3,535 shell genes.

Antibiotic resistance genes

All 15 *Aeromonas* genomes analyzed had three or more AMR genes of which the Ambler classes B, C and D β-lactam genes accounted for the majority (Table 4).

Ambler class B metallo-β-lactam resistance genes

Among the Ambler class B metallo-β-lactamase (MBL) resistance genes, only five AMR genes were detected from 14 of the 15 *Aeromonas* genomes examined (Table 4) comprising of (i) carbapenem gene *ImiH* from *A. hydrophila* isolated from Indian carp (SD/21–01), (ii) carbapenem gene *cphA3* from *A. veronii* isolated from humans (FC951 and VBF-557), Swordtail fish (XhG1.2), Indian carp (A8-AHP), and *A. dhakensis* from sardine (F2S2–1), (iii) carbapenem gene *cphA4* from *A. veronii* from catfish (SD/21–04), insect gut (CMF) and fish (PhIp2), (vi) carbapenem gene *cph5* detected from *A. salmonicida* isolated from chicken (Y47), prawn (A527), butterfish (Y567) bighead fish (Y557), and (v) carbapenem gene *cphA8* from *A. hydrophila* isolated from Indian carp (SD/21–05). Phylogenetic analysis showed a close similarity for all Ambler class B MBL genes genes isolated from different *Aeromonas* spp. in spite of the bacteria isolates coming from different host species (Figure 2).

Class C β-lactamase resistance genes

Of the 15 aeromonas genomes examined, only nine had class C β-lactam resistance genes (Table 4) consisting of (i) β-lactamase gene bla_{AQU-2} from *A. hydrophila* isolated from Indian carp (SD/21–1) and *A. dhakensis* (F2S2–1) from sardine, (ii) cephalosporin gene *cepS* from *A. hydrophila* (SD/21–05) isolated from Indian carp and human (VBF557), (iii) bla_{MOX-7} from *A. caviae* isolated from Nile tilapia (SD/21–11), and (iv) bla_{FOX-7} from *A. veronii* isolated from Indian carp (A8-APH). Resistance genes detected from *A. salmonicida* isolates included (v) bla_{FOX-2} from butter catfish (Y567) and prawn (A527) (vii), bla_{FOX-4} from bighead fish (Y557) and bla_{FOX-5} from *A. salmonicida* from chicken (Y47). The phylogenetic tree divided the Class C β-lactamase resistance genes in two groups of which group 1 comprised of the bla_{AQU-2}, *cepS*, bla_{MOX-7} and bla_{FOX-7} genes while the bla_{Fox} genes from *A. salmonicida* were clustered together in group 2 (Figure 3). Phylogenetic analysis showed that *A. hydrophila* strain SD/21–01 from Indian carp and *A. dhakensis* strain F2S2–1 from sardine that had the β-lactamase gene bla_{AQU-2} were paired together, while *A. hydrophila* strain SD/21–05 from Indian carp and strain VBF557 from human that also had the cephalosporin gene *cepS*

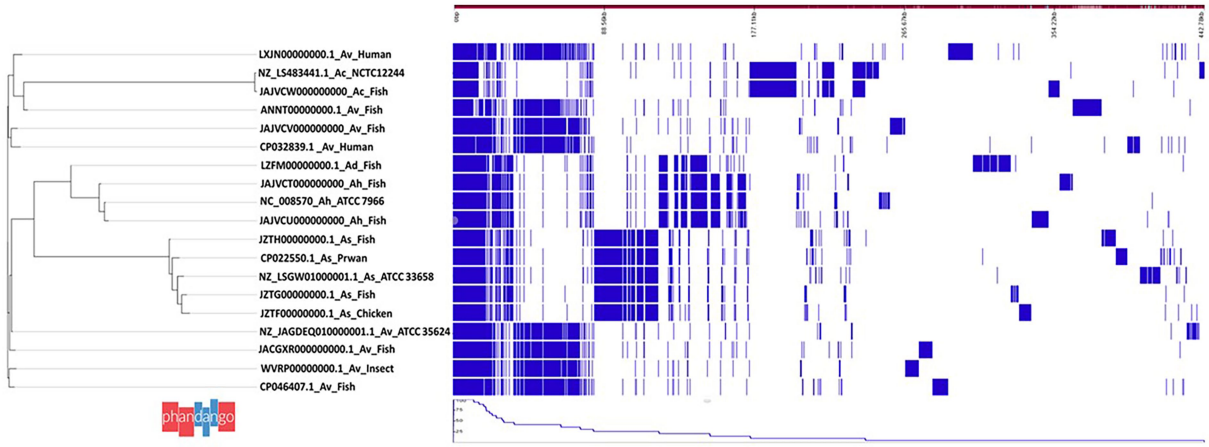

FIGURE 1
Pangenome analysis of 15 *Aeromonas* spp. isolated from different host species in India. Note that the 15 *Aeromonas* spp. are in four groups based on species. (i) Group comprises of *Aeromonas veronii* isolates and reference strain NZ_LS4883441.1 NCTC12244, (ii) Group 2 consists of a *A. hydrophila* and *A. veronii* isolate and reference strain NC_008570 Ah_ATCC7966, (iii) Group 3 consists of *A. salmonicida* isolates and reference strain NZ_LSGW01000001.1_As_ATCC33658, and (iv) Group 4 consists of *A. hydrophila* and *A. dhakensis* isolates together with the NZ_JAGDE01000001.1 Av_ATCC35624 reference strain.

were also put next to each other in group I. Equally, the $bla_{\text{FOX-2}}$ gene from *A. salmonicida* strains Y567 from butter catfish and A527 from prawn were placed next to each other in group II. Altogether, these findings show that genes identified to be similar using the CARD (Alcock et al., 2020; Table 4) also had a high similarity in the phylogenetic tree (Figure 4). Overall, these findings point to high similarity among C β-lactamase resistance genes in spite of the bacteria isolates coming from different host species (Figure 3).

Classes D β-lactamase resistance genes

Only resistance genes belonging to the bla_{OXA} group were detected in class D β-lactamase. The first group consisted of $bla_{\text{OXA-12}}$ from *A. veronii* isolated from catfish (*C. catla*) (SD/21–04) and Swordtail fish (XhG1.2) together with *A. veronii* from humans (FC951 and VBF-557), insect (CMF), Indian carp (A8-AHP), and fish (Phln2) (Table 4). The second group comprised of $bla_{\text{OXA-724}}$ from *A. hydrophila* isolated from Indian carp (SD/21–01 and SD/21–05) and *A. dhakensis* from sardine (F2S2–1). The third group consists of $bla_{\text{OXA-427}}$ from *A. salmonicida* isolates from bighead fish (Y557), prawn (A527), chicken (Y47), and butter catfish (Y567). The final group consisted of $bla_{\text{OXA-780}}$ from *A. caviae* isolated from Nile tilapia (SD/21–11). The phylogenetic tree showed that all seven isolates having the $bla_{\text{OXA-12}}$ had 100% similarity comprising of *A. veronii* from catfish (*C. catla*) (SD/21–04), Indian carp (A8-APH), insect (CMF), human (FC951), swordtail (XhG1.2), fish intestine (Ph1n2) and human (VBF557) clustered together in group 1 (Figure 4). Equally, isolates that had the $bla_{\text{OXA-724}}$ gene inclusive of *A. hydrophila* isolated from Indian carp (SD21/05 and SD21/01) and *A. veronii* from sardine (F2S2–1) had a 100% similarity and were clustered in group 2 while the $bla_{\text{OXA-427}}$ gene detected in four *A. salmonicida* isolates from bighead (Y557), butter catfish (Y567) and prawn (Y47) was associated with group 3 with 100% similarity (Figure 4). Thus, these findings show that the similarity in AMR genes identified based on the CARD (Alcock et al., 2020; Table 4) corresponded with the similarity seen in the phylogenetic tree (Figure 4). Altogether, these findings show high similarity of class D β-lactam resistance genes irrespective of the bacteria being isolated from different host species.

Other antibiotic resistance genes

Only *A. hydrophila* isolated from the Indian carp (SD/21–05) possessed the trimethoprim resistance gene *dfrA12* (Table 4) being in agreement with the disk diffusion test results of resistance to trimethoprim (Table 2). The sulfanomide resistance gene *sul1* was only detected from *A. hydrophila* and *A. caviae* isolated from Indian carp (SD/21–05) and Nile tilapia (SD/21–11) (Table 4) that also showed resistance to sulfonamide in the disk diffusion test (Table 2). Aminoglycoside resistance genes comprised of *aadA2* from *A. hydrophila* isolated from Indian carp (SD/21–05), *APH(3′)-Ia* from *A. veronii* isolated from Indian carp (A8-AHP), and *ANT(3″)-IIa* from *A. caviae* isolated from Nile tilapia (SD/21–11). The tetE gene was only detected from *A. veronii* (SD/21–04) isolated from catfish that also showed resistance to tetracycline in the disk diffusion test (Table 2) and *A. salmonicida* from chicken (Y47), while $bla_{\text{TEM-150}}$ was only detected from *A. veronii* isolated Indian carp (PhIn2). Other genes detected include the chloramphenicol gene *cmlA1* and colistin gene *mrc-3* from *A. veronii* isolated from Nile tilapia (SD/21–11), humans (FC951), and Swordtail fish (XhG1.2), respectively. Correlation of the phenotypic profile determined by the disk diffusion test with the genotypic profile based on genes identified using the CARD (Alcock et al., 2020) showed 93% specificity and 88% sensitivity with an overall kappa score (K) of 0.88 determined using the Cohen's kappa test (Cohen, 1968).

TABLE 4 Antimicrobial resistance genes detected in the *Aeromonas* genomes.

AMR gene description	Gene	SD/21-04	SD/21-01	SD/21-05	SD/21-11	XhG1.2	A8-AHP	Phln2	F2S2-1	Y557	Y567	A527	CMF	FC951	VBF557	Y47
Class A β-lactamase	*TEM-150*															
Ambler Class B MBL	*ImiH*															
(Carbapenem)																
	cphA4															
	cphA3															
	cphA5															
	cphA8															
Class C beta-lactamase	*Aqu-2*															
(Cephalosporin Cephamycin																
Penam)																
	*CepS_*															
	bla$_{MOX-7}$															
	bla$_{FOX-7}$															
	bla$_{FOX-4}$															
	bla$_{FOX-2}$															
	bla$_{FOX-5}$															
Class D β-lactamase	*bla*$_{OXA-12}$															
(Cephalosporin, Penam)																
	bla$_{OXA-427}$															
	bla$_{OXA-724}$															
	bla$_{OXA-780}$															
Trimethoprim-resistant	*dfrA12*															
Aminoglycoside	*aadA2*															
	APH(3')-Ia															
Sulfonamide resistant	*sul1*															
Aaminoglycoside	*ANT(3")-IIa*															
Total resistance genes	3	3	6	3	3	3	3	3	3	3	3	3	3	3	3	

All antimicrobial resistance genes detected and identified using staramr version 0.7.2 (Tran et al., 2021) and ABRicate version 1.0.1 (Seemann, 2016) in the Comprehensive Antimicrobial Resistance Database (CARD) (Alcock et al., 2020).
Blue = presence of resistance genes (detected), White/blank = absence of gene (not detected).

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Drug resistance efflux pump genes

All 15 *Aeromonas* genomes had multiple multidrug efflux pump genes (Table 5). Dominant genes included the multidrug resistance protein (*mdtH*) and the zinc/cadmium/mercury/lead-transporting ATPase *zntA* gene detected in all 15 *Aeromonas* genomes. Other dominant genes included *mdtL*, which was detected in 11 of the 15 *Aeromonas* genomes except for *A. veronii* isolated from catfish (SD/21–04), green swordtail fish (XhG1.2), insect (CMF), and *A. salmonicida* from prawn (A527) (Table 5). The multidrug efflux MFS transporter (*emrD*), β-lactam sensor histidine kinase (*blrB*), and bleomycin resistance family protein (brp) were detected in *A. hydrophila* isolated from Indian carp (SD/21–01 and SD/21–05) and

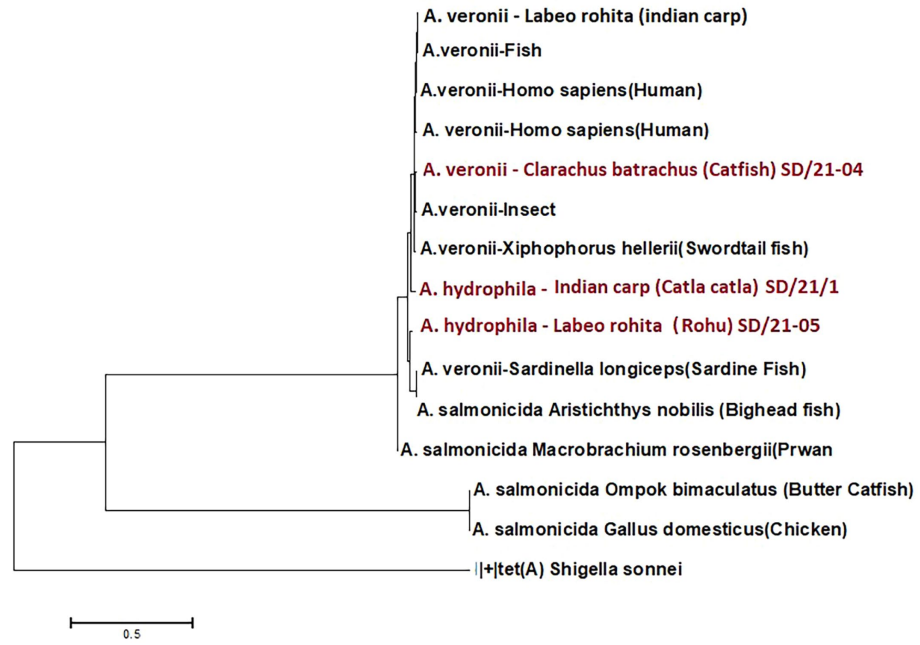

FIGURE 2
Phylogenetic analysis of Ambler class B metallo-β-lactam (MBL) resistance genes from 13 *Aeromonas* spp. isolated from different host species in India.

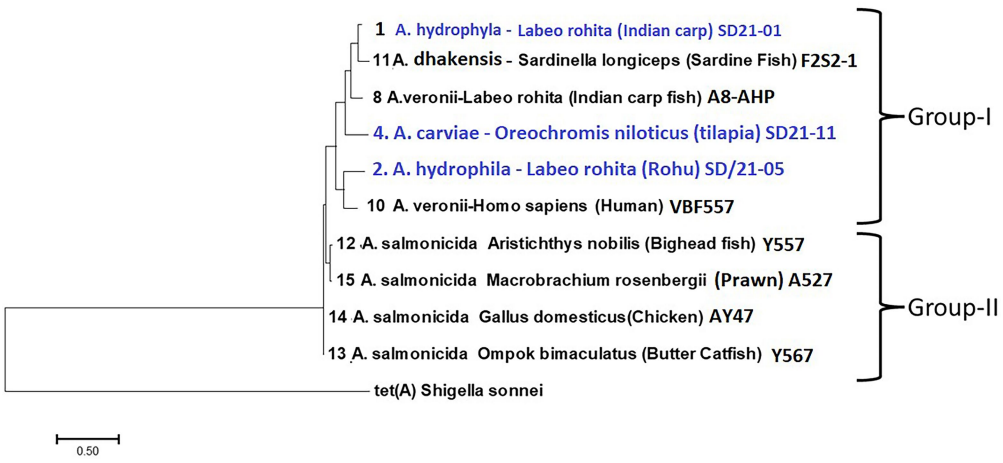

FIGURE 3
Phylogenetic analysis of Ambler class C β-lactam resistance genes from 10 *Aeromonas* spp. isolated from different host species in India. The isolates were put in two groups. (i) Group 1 consists of bla_{AQU-2}, *cepS*, bla_{MOX-7}, and bla_{FOX-7} genes from *A. hydrophila*, *A. veronii*, and *A. caviae* isolated from different host species. Note that Strains SD/21–01 and F2S2/1 having the bla_{AQU-2} gene were put together while strains SD/21–05 and VBF557 having the *cepS* gene were also placed together. (ii) Group 2 consists of bla_{FOX-2}, bla_{FOX-4} and bla_{FOX-5} genes from *A. salmonicida* isolates of which strains Y567 and Y527 having the bla_{FOX-2} gene were put together.

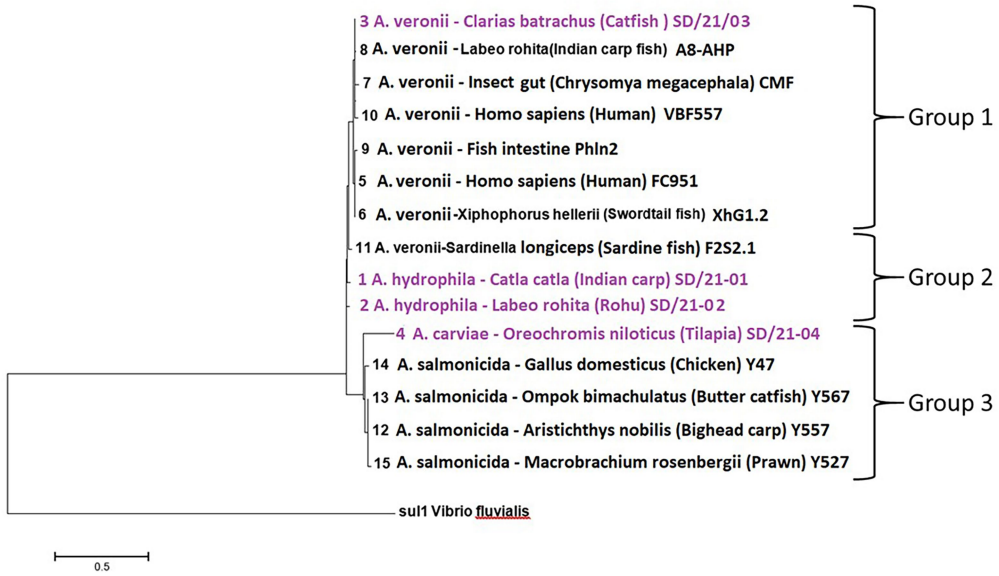

FIGURE 4
Phylogenetic analysis of D class β-lactam resistance genes from 15 *Aeromonas* spp. from different host species in India. (i) Group 1 consists of the bla_{OXA-12} gene from *A. veronii* isolates, (ii) Group 1 had the $bla_{OXA-724}$ gene from *A. veronii*, and *A. hydrophila* isolates while (iii) Group 3 has the $bla_{OXA-427}$ gene from *A. caviae* and *A. salmonicida* isolates.

A. veronii isolated from catfish (SD/21–04), human (FC951), and insect (CMF) but not from the *A. salmonicida* and *A. dhakensis* (F2S2–1). On the contrary, the emrB/QacA family drug resistance (*emrB*), bicyclomycin resistance protein (*BCM*), Putative chloramphenical resistance permease protein (*rarD*) and fluoroquinolone (*qnr*) were dominant in the *A. salmonicida* and *A. dhakensis* isolates but absent in *A. hydrophila* and less dominant in *A. veronii* isolates. Finally, the resistance nodulation cell division (RND) multidrug efflux pump *crp* was detected from five *A. veronii* isolates from insect (CMF), Indian carp (A8-AHP), fish (PhIn2), and humans (VBF557) as well as *A. dhakensis* from sardine (F2S2–1). In addition, *crp* was also detected from four *A. salmonicida* isolates from bighead fish (Y557), prawn (A527), chicken (Y47), and butter catfish (Y567). Phylogenetic analysis showed a similarity of 100% *crp* from *A. veronii* isolates from insect, Indian carp, and human isolates (Figure 5). The homology among *crp* from the nine host species varied between 99.1 and 100.0%. Other resistance drug efflux genes detected are shown in Table 5.

Resistance genes detected together with integrase and efflux pumps

The circular map for *A. hydrophila* strain SD/21–05 (Figure 6A) genome showed presence of all six resistance genes ($bla_{OXA-724}$, *cepS*, *cphA8*, *dfrA12*, *aadA2*, and *sul1*) detected using the CARD (Alcock et al., 2020; Table 4). It is noteworthy that the integrase *intl1* gene was located next to the trimethoprim (*dfrA12*), aminoglycoside (*aadA2*), and sulfanomide (*sul1*) genes together with the major facilitator superfamily (MFS) efflux pump *QacEdelta-1* (Figure 6A). The circular map of the *A. veronii* strain SD/21–04 (Figure 6B) genome shows that the *tetR* gene was located next to the Tet(E) efflux pump together with an unknown hypothetical protein while other genes detected included the cephalosporin/penam *cphA4* and bla_{OXA-12} genes (Figure 6B). As for *A. caviae* strain SD/21–11, our findings show that all four AMR genes bla_{OXA-12}, $bla_{OXA-780}$, *suI2* and *ANT(3)-IIa* detected using the CARD (Alcock et al., 2020) were found in its genome of which the *intI1* integrase was located next to the sulfonamide *suI2* and aminoglycoside *ANT(3)-IIa* genes together with the chloramphenicol *cmlAI* and MFS *QacEdelta-1* efflux pumps (Figure 6C). Finally, the circular map for *A. hydrophila* strain SD/21–01 showed presence of *lmiH*, bla_{AQU-2}, $bla_{OXA-724}$ and *tet(R)* genes in its genome of which the tetracycline gene *tet(R)* was located next to the multidrug complex MexAB-OprM and the RND smeD efflux pumps.

Plasmids found in the *Aeromonas* spp.

Of the four *Aeromonas* spp. sequenced in the present study, three had plasmids (Supplementary Table S1). *A. hydrophila* from Indian carp (SD/21–05) had two plasmids of which pSD2105-1 had a size of 5,278 bp while pSD2105-2 was 3,599 bp (Figure 6A). Genes found in pSD2105-1 included *D-met*, *mebB*, *mebD*, and *MobDI*, whereas pSD2105-2 had *mobC*, *mbeB*, *mbeD* and *Bor* genes (Supplementary Table S1; Figure 6A). Equally, *A. veronii* from catfish (SD/21–04) had two plasmids

TABLE 5 Multidrug efflux pump genes detected in *Aeromonas* genomes.

Gene description	Gene	SD/21–04	SD/21–01	SD/21–05	SD/21–11	XhG1.2	A8-AHP	Phln2	F2S2–1	Y557	Y567	A527	CMF	FC951	VBF557	Y47
Multidrug resistance protein (fluoroquinolones, ceftriaxone)	*mdtL*		■	■	■		■	■	■	■	■			■	■	■
Multidrug resistance protein (fluoroquinolone)	*mdtH*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Multidrug resistance protein (Aminocoumarin)	*mdtB*	■	■	■	■	■		■		■		■				
multidrug efflux MFS transporter (Phenicol)	*emrD*	■	■	■	■	■	■						■	■		
EmrB/QacA family drug resistance	*emrB*								■	■	■	■			■	■
multidrug efflux RND transporter (tetracycline, chloramphenicol)	*MDR*	■	■	■	■	■	■		■			■	■	■		
chloramphenical resistance permease	*rarD*								■	■	■				■	■
fluoroquinolone resistance protein	*FQR*								■	■	■	■			■	■
Quaternary ammonium efflux SMR transporter	*QacEdelta1*				■	■	■					■	■			■
Zinc/cadmium/mercury/lead-transporting ATPase	*zntA*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Beta-lactam sensor histidine kinase	*blrB*	■	■	■	■	■	■						■			
organic hydroperoxide resistance protein	*ohrP*		■	■							■					■
bleomycin resistance family protein	*ble*	■	■	■		■	■						■	■		■
fosfomycin resistance glutathione transferase	*FosA*				■			■	■		■	■		■		■
Bicyclomycin resistance protein	*BCM*								■	■	■					■
cAMP-activated global transcriptional regulator	*crp*						■	■	■	■	■	■			■	■

All multidrug efflux pump genes were detected and identified using staramr version 0.7.2 (Tran et al., 2021) and ABRicate version 1.0.1 (Seemann, 2016) in the Comprehensive Antimicrobial Resistance Database (CARD) (Alcock et al., 2020).
Blue = presence of genes (detected), White/blank = absence of gene (not detected).

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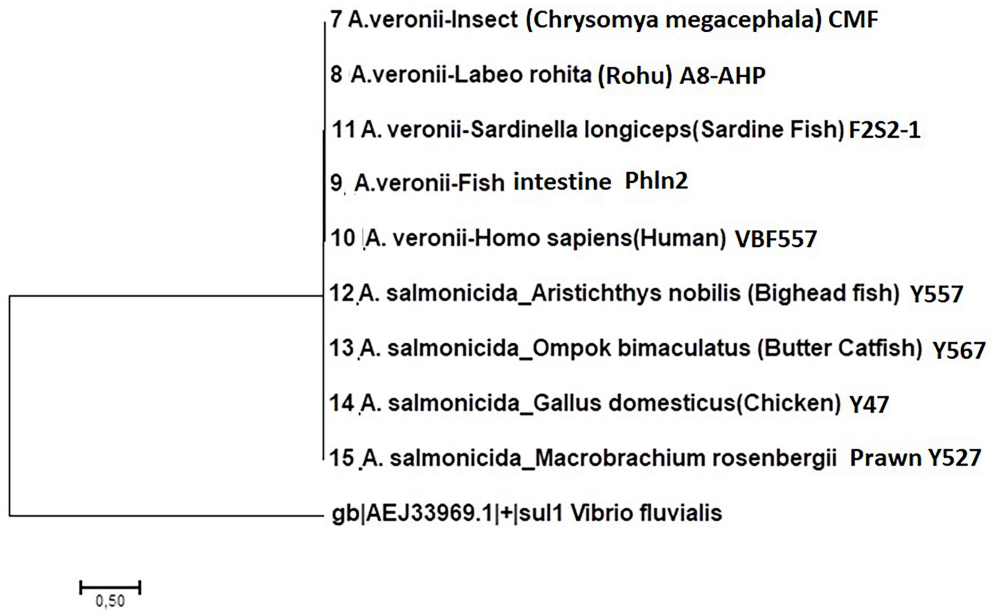

FIGURE 5
Phylogenetic analysis of *crp* resistance genes detected from sequences of nine *Aeromonas* spp. isolated from different host species in India.

with sizes of 7,480 bp (pSD2104-1) and 1740 bp (pSD2104-2) (Figure 6B). Genes detected in pSD2104-1 were *parB*, *repB*, *relB*, *relE*, *mqsA*, and *mqrR*, whereas pSD2104-2 had *hyp* and *repB* (Supplementary Table S1). *A. caviae* from Nile tilapia (SD/21–11) had only one plasmid (pSD21-11) with a size of 9,364 bp that had *repB*, *parB*, *copG*, *relE* and *sel1* genes (Figure 6C). Suffice to point out that only pSD211-11 had a "site-specific integrase." Only *A. hydrophila* from catfish (SD/21–01) had no plasmid (Figure 6D) out of the four *Aeromonas* spp. sequenced in the present study. Of the 11 *Aeromonas* genomes retrieved from NCBI, only three had plasmids (Supplementary Table S1). *A. veronii* from human (FC9l51) had one plasmid (196,528 bp) and no AMR genes detected. Similarly, *A. salmonicida* from big head carp (Y577) had one plasmid (5,402 bp) and no AMR genes. *A. veronii* from Indian carp (A8-APH) and *A. salmonicida* from chicken (Y47) had three plasmids that had no AMR genes (Supplementary Table S1).

Transposons detected in the genomes

We found several transposases and integrases in the *Aeromonas* genomes with each isolate having more than six transposases (Table 6). The most dominant transposases were part of insertion sequence (IS) elements; IS481, IS1595, IS110, IS3, IS5 and IS4 that were found in several isolates. Some of the transposases were associated with resistance genes and efflux pumps as shown in Figure 6D that IS5/IS1182 was located close to the tet(E) efflux pump and *tetR* gene in *A. veronii* strain SD21/04. The class I integrase *Int1* was only detected from two fish isolates (SD21-05 and SD21-11).

Discussion

In this study, we have shown that all 15 *Aeromonas* genomes examined had multiple AMR genes suggesting that *Aeromonas* spp. infecting different host species in India could be carriers of multidrug resistance (MDR) genes. We have also shown that WGS is a reliable tool able to profile all AMR genes, efflux pump proteins, integrases, transposes and plasmids present in bacteria genomes, unlike PCR that use primers targeting selected genes posing the danger of missing some of the vital AMR genes encoded in bacteria genomes. In addition, we have shown that pangenome analysis is a reliable tool able to classify members of the genus *Aeromonas* into species by separating the shell genes that are species specific from the core genes shared by all aeromonads. Although the pangenome classified the 15 genomes into four groups based on species, the high similarity of AMR genes determined by phylogenetic analyses is suggestive that there is interspecies transmission of AMR genes among bacteria species isolated from different hosts. This is also indicative that these genes could be part of the conserved genome across the aeromonads being in line with previous observations that Aeromonads are intrinsically resistant to β-lactams (Baron et al., 2017; Kabwe et al., 2020; Sakulworakan et al., 2021).

In general, the ambler classes B, C and D genes accounted for the largest proportion of AMR genes detected from the 15 *Aeromonas* genomes examined. The *Aeromonas* genus contains

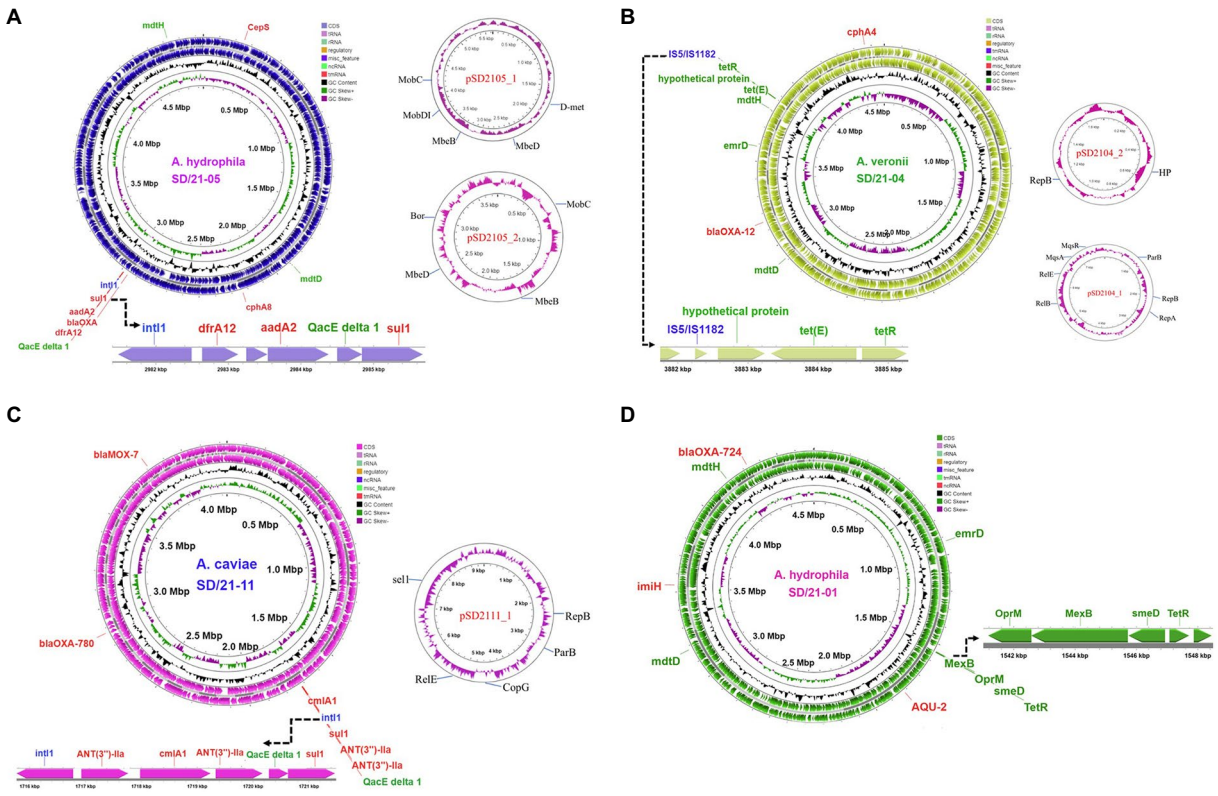

FIGURE 6
Circular maps of whole genome sequences of *Aeromonas* spp. isolated from four fish species in India. **(A)** Circular map of whole genome sequence of *Aeromonas hydrophila* strain SD21/21–05 (blue) isolated from Indian carp (*Labeo rohita*) showing the loci for the resistance genes (red), efflux pumps (green) and integrase (blue). The extended linear map shows the integrase *intI1* linked to *dfrA12*, *aadA2*, *QacEdelta 1* and *sul1*. Circular maps of the two plasmids detected are shown in pink. **(B)** Circular map of whole genome of *A. veronii* strain SD/21–04 isolated from *Clarias batrachus* showing the loci for resistance genes (red), efflux pumps (green), and transposases (blue). The linear map shows the transposases linked to the hypothetical protein, tet(E) efflux pump and *TetR* is the repressor of the tetracycline resistance element. Circular maps of the two plasmids are shown in pink. **(C)** Circular map of the *A. caviae* strain SD/21–11 isolated from *Oreochromis niloticus* showing positions of the resistance genes (red), integrase (blue) and efflux pumps (green) with the linear map showing the integrase *intI1* linked to the *ANT(3″)-IIa*, *cmlA1*, *ANT(3″)-IIa*, *QacEdelta 1* and *sul1* genes. Circular map of the single plasmid is shown in red. **(D)** Circular map of *A. hydrophila* strain SD/21–01 isolated from *Catla catla* showing resistance genes (red) and efflux pump proteins (green). The extended linear map shows the multidrug complex OprM-MexB and RND pumps linked to the *tetR* gene.

several aquatic bacteria species both commensals and fish pathogens that host chromosomally located *amp* resistance genes that can be functional (De Luca et al., 2010). Among the class B MBL genes, the high similarity of carbapenem genes *cphA3* and *cphA4* genes from *A. veronii*, *A. hydrophila* and *A. caviae* isolated from humans, Indian carp, sardine, insect, and tilapia demonstrate the ability of different *Aeromonas* sp. isolated from different host species to harbor similar AMR genes. On the other hand, the high similarity of carbapenem gene *cphA5* detected in the *A. salmonicida* genomes from chicken, and butter catfish is suggestive that one *Aeromonas* sp. carrying a similar gene can be a source of AMR transmission to different host species. Wang Y. et al. (2021) and Kabwe et al. (2020) have shown that aeromonads have several MBL genes that include *cphA*, *imiH*, and *ceph-A3* encoded in their chromosomes suggesting that their presence in the *Aeromonas* spp. examined in this study could be that they intrinsically are encoded in the genomes. Despite so, suffice to point out that the MBL genes detected in this study have been reported from different bacteria species isolated from humans, animals, fish, chickens, mussel and the environments in different countries (Maravić et al., 2013; Bottoni et al., 2015; Hilt et al., 2020; Ramsamy et al., 2020; Bertran et al., 2021; Wang Y. et al., 2021). Thus, it is likely that these AMR genes exist in other bacteria species in different aquatic environments and a wide range of host species in India.

As pointed out by Chen et al. (2019) that the diversity of bla_{OXA} genes has been expanding to include new variants of bla_{OXA-12} such as $bla_{OXA-427}$, $bla_{OXA-724}$ and $bla_{OXA-780}$, all detected in this study. We found bla_{OXA-12} in sequences of four *A. veronii* isolates obtained from humans, catfish, carp, and insect. Previously, it was detected in *Aeromonas* spp. such as *A. hydrophila*, *A. allosaccharophila*, *A. veronii*, and *A. rivipollensis* isolated from humans, chicken, pork, and wild nutria (*Myocastor coypus*) (Park et al., 2018; Shen et al., 2018; Xiao et al., 2020), respectively. We also found $bla_{OXA-427}$ in *A. salmonicida* isolated from bighead fish, butter catfish and prawn, which is an emerging class D

TABLE 6 Transposases and integrases detected from the *Aeromonas* genomes.

Blue = presence of genes (detected), white/blank = absence of gene (not detected).

carbapenemase that confer resistance against a wide range of β-lactams including broad-spectrum penicillins, cephalosporins, and carbapenems (Bogaerts et al., 2015, 2017). Although outbreaks in humans have only been reported from hospitals in Belgium where $bla_{OXA-427}$ was isolated from nosocomial *Klebsiella pneumoniae* and *Enterobacter cloacae* infections (Desmet et al., 2018), its presence among *Aeromonas* spp. shows global distribution involving humans, animals and fish. For example, it has been detected from *A. caviae* and *A. hydrophila* isolated from humans in China (Tang et al., 2020; Lin et al., 2021), *Aeromonas* spp. from reservoir water in Singapore (Zhong et al., 2021), *A. media* in Nebraska watershed (Donner et al., 2022), *A. salmonicida* from Atlantic salmon (*Salmo salar* L) in Chile (Vásquez-Ponce et al., 2022), and pork processing plant in Spain (Cobo-Díaz et al., 2021). Similarly, $bla_{OXA-724}$ has been detected from *A. dhakensis* isolated from humans in Spain (Bertran et al., 2021), *A. hydrophila* from pigs in South Africa (Ramsamy et al., 2021) as well as *A. jandaei* and *A. hydrophila* from chicken and

catfish in the United States (Wang Y. et al., 2021), while in the present study it was found in *A. hydrophila* from carp and *A. veronii* from sardine from India. bla_{OXA-72} has been detected from *Acinetobacter baumannii* in humans where it has been associated with pneumonia, septic shock, and respiratory failure (Jia et al., 2019). Given that various bla_{OXA} genes have been shown to be intrinsically encoded in the chromosomes of various *Aeromonas* spp. (Kabwe et al., 2020; Wang Y. et al., 2021), these findings show that *Aeromonas* spp. found in different host species and aquatic environment could play a vital role in the global spread of emerging β-lactam resistance genes such as $bla_{OXA-427}$ and $bla_{OXA-724}$.

In this study, we detected class C β-lactamase genes from 10 of the 15 *Aeromonas* genomes examined unlike class D genes that were detected in all genomes. As shown in our findings, class C genes comprised of the cephalosporin/penam *cepS*, bla_{MOX} and bla_{FOX} genes as well as the bla_{AQU-2} β-lactamase gene. Among these, *cepS* has previously been detected from various *Aeromonas* spp. isolated from humans, pigs, catfish, chicken, frogs, mullet and the environment while bla_{AQU-2} has been reported from *Aeromonas* spp. isolated from humans and chicken in different countries (Walsh et al., 1997; Ramadan et al., 2018; Seo and Lee, 2018; Wang et al., 2019; Bertran et al., 2021; Kimera et al., 2021; Wang Y. et al., 2021). Similarly, bla_{FOX-2}, bla_{FOX-4}, bla_{FOX-5} and bla_{MOX-7}, have been detected from different bacteria species including *Aeromonas* spp. isolated from humans, wastewater, fish tanks, mussel, and wild animals in different countries (Maravić et al., 2013; Bertran et al., 2021). Altogether, these studies show that the class C β-lactamase genes are prevalent in a wide range of host species in various countries indicating they could be present in several other species not included in this study found in India. Suffice to point out that *cepS*, bla_{AQU-2}, and $bla_{FOX/MOX}$ have been detected in the chromosomes of various *Aeromonas* spp. (Kabwe et al., 2020; Wang Y. et al., 2021), suggesting that the class C β-lactamase genes detected in this study could have been intrinsically encoded in the genomes of the *Aeromonas* spp. examined. This is also supported by the high prevalence of the *crp* gene detected in nine of the 15 genomes examined in this study, which is a RND efflux pump associated with resistance against penam, cephalosporin, macrolide, trimethoprim and fluoroquinolone (Nishino et al., 2008). This finding points to its wide prevalence among *Aeromonas* spp. infecting humans, fish, insect and animals in India. Previously, *crp* has been found in *Cronobacter* spp. isolated from infant food (Carvalho et al., 2020), *C. sakazakii* from powdered milk (Holý et al., 2020), *Enterobacter hormaechi* from yoghurt (Tóth et al., 2020), *Salmonella enterica* from ducks, (Yu et al., 2022), and *Vibrio* spp. from human and environmental samples (Pérez-Duque et al., 2021; Nguyen et al., 2022).

Several studies have shown that environmental aeromonads contain chromosomally encoded β-lactamases that cause resistance to drugs including ampicillins, cephalosporin and penicillin (Richardson et al., 1982; Zemelman et al., 1984; Motyl et al., 1985; Shannon et al., 1986; Chang and Bolton, 1987; Fosse et al., 2003; Girlich et al., 2011). Thus, it is likely that the resistance observed against ampicillin, penicillin and cephalosporin in our phenotypic analysis was encoded in genomes of *Aeromonas* spp. examined. So, it can be speculated that Aeromonads could be an important source for the spread of novel β-lactamases to human clinically important bacteria in line with Fosse et al. (2003) and Girlich et al. (2011), who pointed out that the resistance originating from aeromonads poses a significant public health risk to humans.

The resistance against gentamycin in the genus *Aeromonas* has been linked to variable results as shown that gentamycin sensitive *Aeromonas* spp. have previously been isolated from rainbow trout (*Oncorhynchus mykiss*) (Akinbowale et al., 2007), carp (Öztürk et al., 2007) and Nile crocodile (*Crocodylus niloticus*) (Turutoglu et al., 2005) while gentamycin resistant *Aeromonas* spp. have been isolated from catfish (Chinedu et al., 2020) and European rivers (Goñi-Urriza et al., 2000). Hence, it is unknown whether the *aadA2* and *ANT(3″)-IIa* aminoglycoside resistance observed in our fish isolates was intrinsically or extrinsically acquired. Detection of the *ANT(3″)-IIa* aminoglycoside gene linked to *intl1* the integrase together with the chloramphenicol *cmlAI* and MFS *QacEdelta-1* efflux pumps in *A. caviae* strain SD/21–11 in this study is suggestive that there might be some transfer or acquisition of gentamycin genes into *Aeromonas* genomes. This is supported with observations seen in *A. hydrophila* strain SD/21–05 that also had the *intl* integrase linked to the aminoglycoside (*aadA2*), sulfonamide (*sul1*) and trimethoprim (*dfr12*) genes together with the MFS *QacEdelta-1* efflux pump pointing to transfer or acquisition of chloramphenicol, trimethoprim and gentamycin resistance genes into *Aeromonas* genomes. Even though several studies (Koksal et al., 2007; Awan et al., 2009; Saengsitthisak et al., 2020; Dhanapala et al., 2021) have reported erythromycin resistance in *Aeromonas* spp. suggesting that it could be chromosomally integrated, isolates of *A. sobria* from prawn (*Penaus monodon*) (Vaseeharan et al., 2005), *A. veronii* from sea bass (*Lateolabrax maculatus*) (Wang B. et al., 2021) and *A. hydrophila* from humans (Von Graevenitz and Mensch, 1968) were shown to be sensitive to erythromycin. Although our fish isolates showed resistance to erythromycin, it is unknown whether the resistance was intrinsic or extrinsically acquired. However, it is likely that the resistance seen against tetracycline, sulfonamide and trimethoprim could have been acquired from treatment of diseased fish using these antibiotics as reported from clinical reports. Moreover, these antibiotics are widely used in aquaculture in India and resistance based on disc diffusion test has been reported previously (Abraham et al., 2017; Roy et al., 2021; Sivaraman et al., 2021; Patil et al., 2022).

Our findings show that all 15 *Aeromonas* genomes had multidrug efflux pump proteins. The *mdtL* protein which is one of the first line of defence against antimicrobials involved in decreasing intracellular drugs levels (Rahman et al., 2017) was detected in most isolates. *mdtL* has been shown to increase resistance against fosfomycin and chloramphenicol (Kvist et al., 2008). Among the major facilitator superfamily (MFS), *emrB* and *emrD* involved in resistance against several drugs like norfloxacin, tetracycline, chloramphenicol, novobiocin, fluoroquinolone and nalidixic acid (Jahan et al., 2021) were detected in several isolates.

As for the RND proteins, we detected the *tet(E)* gene known to encode the tetracycline efflux pumps (Møller et al., 2016). Other multidrug efflux pump proteins detected include *rarD*, *qnr*, *mdtH*, *mdtD, pbp1A* and *qacEdelta1* involved in resistance against chloramphenicol, fluoroquinolone, novobiocin amoxicillin, and several other drugs (Kazama et al., 1999; Nagakubo et al., 2002; Stanhope et al., 2008; Ovchinnikov et al., 2015; Zago et al., 2020). In the present study, the MFS *QacEdelta-1* efflux pump gene was linked to the trimethoprim (*dfrA12*), aminoglycoside (*aad2*) and sulfonamide (*sul2*) resistance genes in *A. hydrophila* strain SD/21–05 while the tet(E) pump was linked to the *tetR* tetracycline gene in *A. veronii* strain SD/21–04. In *A. caviae* strain SD/21–11, the chloramphenicol *cmlA1* and MFS *QacEdelta-1* efflux pumps gene were linked to the *ANT(3″)-IIa* aminoglycoside gene and *sul1* sulfonamide genes whereas in *A. hydrophila* strain SD/21–01 the multidrug complex OprM-MexB and RND smeD efflux pumps were linked to the tetracycline *tetR* gene. We also detected *fosC2*, *blrB*, *BRP*, and *BMC* involved in resistance against fosfomycin, β-lactams, glycopeptide, and bicyclomycin (Galm et al., 2005; Nikolaidis et al., 2014; Jahan et al., 2021). These findings concur with previous studies (Li and Nikaido, 2004, 2009) showing that AMR genes expressed by *Aeromonas* spp. are often linked to multidrug resistance proteins.

The most important mobile genetic elements (MGEs) known to play a key role in the spread of AMR genes include class 1 integrons (*intI*), transposons and plasmids (Liebert et al., 1999; Carattoli, 2001; Stalder et al., 2012). In this study, *intI* was only detected from two out of the four isolates sequenced in this study. In *A. hydrophila* strain SD/21–05, it was located next to the trimethoprim *dfrA12*, aminoglycoside *aadA2* and sulphonamide *sul1* genes, whereas in *A. caviae* strain SD/21–11 it was linked to aminoglycoside *ANT(3″)-IIa*, chloramphenicol *cmlA1* and sulphonamide *sul1* genes. These findings are in line with Ranjbar et al. (2019), Pérez-Valdespino et al. (2009), and Schmidt et al. (2001) who found *sul1, dfrA12*, *aadA2*, *aadA1*, bla_{OXA}, *cmlA4* and *ANT(3″)* gene cassettes in sequences linked to *intI* obtained from different *Aeromonas* spp. The IS classes of transposases reported in this study corroborates with several studies that found IS66, IS30, IS3, IS4, IS5, IS66, IS630, ISA110, ISA1182 transposases in *Aeromonas* spp. isolated from aquatic environments and different host species (Najimi et al., 2009; Studer et al., 2013; Adamczuk and Dziewit, 2017; Vincent et al., 2017; Jin et al., 2020; Ragupathi et al., 2020). In the present study, some transposases were linked to multidrug efflux pumps and AMR genes as shown that the IS5/IS1182 transposase was linked to the Tet(E) efflux pump and tetracycline *tetR* gene in *A. veronii* strain SD/21–04. These transposases have also been found in plasmids linked to AMR (Najimi et al., 2009). For example, IS630 and IS600 were found in the pFBAOT6 plasmid linked to tetracycline resistance in *A. caviae* (Rhodes et al., 2004) while IS4 was found in the Inc-Q3 plasmid showing resistance against quinoline in *Aeromonas* spp. (Piotrowska et al., 2020). Finally, the detection of genes like *D-met*, *mbeD*, *mobDI*, *parB*, *repB*, and *relE* (Carattoli, 2001) in the plasmids of *Aeromonas* spp. shows that the identified plasmids in this study had the potential to transfer AMR genes to other bacteria.

Conclusion

In this study, we have shown that *Aeromonas* spp. isolated from fish, prawn, insect, chicken and humans in India carry various AMR genes. The sequenced isolates of aeromonads from aquaculture reveal well-known AMR genes and class 1 integrons documented from similar studies from aquaculture worldwide, while aeromonads from other environmental sources do not contain commonly transferable AMR genes. These findings also showed high similarity of AMR genes found in different *Aeromonas* spp. despite the bacteria being isolated from different host species. Thus, we advocate that the control of AMR caused by *Aeromonas* spp. in India should be done using a One Health approach.

Data availability statement

The data presented in the study are deposited in the NCBI repository, available at: https://www.ncbi.nlm.nih.gov/nuccore/JAJVCT000000000, https://www.ncbi.nlm.nih.gov/nuccore/JAJVCU000000000, https://www.ncbi.nlm.nih.gov/nuccore/JAJVCV000000000, and https://www.ncbi.nlm.nih.gov/nuccore/JAJVCW000000000.1.

Author contributions

SD, HS, and HM: conceptualization, methodology, data curation, formal analysis, manuscript preparation, and resources. EA-W, JK, BP, InK, IdK, and ØE: data analysis and preparation of 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.2022.1008870/full#supplementary-material

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Characterization of virulence and antimicrobial resistance genes of *Aeromonas media* strain SD/21–15 from marine sediments in comparison with other *Aeromonas* spp.

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Aeromonas media is a Gram-negative bacterium ubiquitously found in aquatic environments. It is a foodborne pathogen associated with diarrhea in humans and skin ulceration in fish. In this study, we used whole genome sequencing to profile all antimicrobial resistance (AMR) and virulence genes found in *A. media* strain SD/21–15 isolated from marine sediments in Denmark. To gain a better understanding of virulence and AMR genes found in several *A. media* strains, we included 24 whole genomes retrieved from the public databanks whose isolates originate from different host species and environmental samples from Asia, Europe, and North America. We also compared the virulence genes of strain SD/21–15 with *A. hydrophila*, *A. veronii*, and *A. salmonicida* reference strains. We detected *Msh* pili, tap IV pili, and lateral flagella genes responsible for expression of motility and adherence proteins in all isolates. We also found *hylA*, *hylIII*, and *TSH* hemolysin genes in all isolates responsible for virulence in all isolates while the *aerA* gene was not detected in all *A. media* isolates but was present in *A. hydrophila, A. veronii*, and *A. salmonicida* reference strains. In addition, we detected *LuxS* and *mshA-Q* responsible for quorum sensing and biofilm formation as well as the ferric uptake regulator (*Fur*), heme and siderophore genes responsible for iron acquisition in all *A. media* isolates. As for the secretory systems, we found all genes that form the T2SS in all isolates while only the *vgrG1*, *vrgG3*, *hcp*, and *ats* genes that form parts of the T6SS were detected in some isolates. Presence of bla_{MOX-9} and $bla_{OXA-427}$ β-lactamases as well as *crp* and *mcr* genes in all isolates is suggestive that these genes were intrinsically encoded in the genomes of all *A. media* isolates. Finally, the presence of various transposases, integrases, recombinases, virulence, and AMR genes in the plasmids examined in this study is suggestive that *A. media* has the potential to transfer virulence and

AMR genes to other bacteria. Overall, we anticipate these data will pave way for further studies on virulence mechanisms and the role of *A. media* in the spread of AMR genes.

KEYWORDS

***Aeromonas media*, antimicrobial resistance, virulence, plasmid, intrinsic–extrinsic, whole genome sequencing**

Introduction

Aeromonas media was first reported as a new species by Allen et al. (1983) who isolated the bacterium from River Avon in Hampshire, England. Since then, it has been reported from sewage, sludge, lakes, rivers, and drinking water (Singh, 2000; Picao et al., 2008; Picão et al., 2008; Figueira et al., 2011; Pablos et al., 2011). In humans, *A. media* has mostly been isolated from diarrhea patients (Singh, 2000) while in fish it has been linked to skin ulcerations (Lü et al., 2016). In fish, it has been isolated from Koi carp (Cyprinus carpio; Lü et al., 2016), catfish (*Clarias batrachus*; Singh, 2000), bluntnose bream (*Megalobrama amblycephala*), eel (*Anguilla anguilla*; Yi et al., 2013), southern black bream (*Acanthopagrus butcheri*; Zhou et al., 2013), and crucian carp (*Carassius carassius*; Hu et al., 2012). In shellfish, it has been isolated from oysters (*Crassostrea rhizophorea*; Evangelista-Barreto et al., 2006), snails (Roger et al., 2012; Talagrand-Reboul et al., 2017), Yesso scallop (*Patinopecten yessoensis*; De Silva et al., 2019), shrimps (*Litopenaeus vannamei*; De Silva et al., 2018), cockles (*Tegillarca granosa*; Dahanayake et al., 2020), and clam (*Ruditapes philippinarum*; Dahanayake et al., 2019). In Norwegian markets, it has been isolated from retail foods such as sushi, oysters, and scallops (Hoel et al., 2017; Lee et al., 2021), while in Korean markets it has also been isolated from frozen shrimps, clams, and Yesso scallop (De Silva et al., 2018, 2019; Dahanayake et al., 2019, 2020). It has also been isolated from chilled chicken in China (Wang et al., 2017; Shao et al., 2022), turkey in Germany (Shen et al., 2018), and pork and pig slaughter house in Portugal (Fontes et al., 2011). These studies show that *A. media* can be transmitted to humans through food, drinking water and the environment.

Although *A. media* has been linked to diarrhea in humans and skin ulcerations in fish (Lü et al., 2016), there is limited information describing the profile of virulence factors found in *A. media*. It is unknown whether *A. media* shares a similar composition of virulence genes with other *Aeromonas* spp. like *A. hydrophila*, *A. veronii*, and *A. salmonicida*. As pointed out by Guerra et al. (2007) and Bhowmick and Bhattacharjee (2018) *Aeromonas* virulence is multifactorial involving various factors like endotoxins, enterotoxins, cytotoxins, hemolysins, proteases, and adhesins. However, *A. hydrophila*, *A. veronii*, *A. caviae*, and *A. sobria* are considered as major pathogens in the genus *Aeromonas* because they account for the largest proportion of the aeromonads isolated from clinical cases unlike *A. media*, which is considered a minor pathogen because of fewer cases isolated from human and animal diseases. Thus, there have been more virulence factor studies done for the major *Aeromonas* pathogens than for minor pathogenic species like *A. media* (Piotrowska and Popowska, 2015; Rasmussen-Ivey et al., 2016; Romero et al., 2016; Gauthier et al., 2017; Talagrand-Reboul et al., 2017). However, the increasing number of cases linked to human and animal infections reported in recent years coupled with increasing isolations from retail ready-to-eat foods (Fontes et al., 2011; Wang et al., 2017; Shen et al., 2018; De Silva et al., 2019; Shao et al., 2022) indicates that *A. media* is emerging as an important environmental and foodborne pathogen with significant public health implications. Thus, there is need to elucidate the virulence factors of *A. media* isolated from different aquatic environments and host species with the view of developing effective control measures.

Antimicrobial resistance (AMR) has emerged to be an important global public health threat classified as among the top 10 global priorities by World Health Organization (2021). Multidrug resistant *Aeromonas* spp. have been isolated from different aquatic environments, animals, and retail foods (Stratev and Odeyemi, 2016; Teodoro et al., 2022). Also, *Aeromonas* spp. have been shown to carry plasmids encoding AMR and virulence genes (Tomás, 2012). Although previous studies reported the presence of AMR and virulence genes from *Aeromonas* spp. that included *A. media* isolated from ready-to-eat foods in Norway, the major limitation with these studies was that they used primers that targeted only a few selected genes, which did not give a global overview of all AMR genes present in bacteria genomes. Thus, in the present study, we used whole genome sequencing (WGS) to characterize all virulence and AMR genes present in *A. media* isolated from marine sediments collected from the Øresund Bay in Denmark. To gain a wide overview of the virulence and AMR genes found in *A. media* strains isolated from different geographical areas, we compared our isolate (strain SD/21–15) with genomes of 24 other isolates from Europe, North America, and South America retrieved from the National Center for Biotechnology Information (NCBI). We also compared our isolate with whole genome sequences of *A. hydrophila*, *A. veronii*, and *A. salmonicida* reference strains to determine the difference in the composition of virulence genes between *A. media* strain SD/21–15 and other *Aeromonas* spp. Our findings show that WGS is a reliable tool able to profile all AMR and virulence genes found in

bacteria genomes unlike PCR based assays that only identify a few selected genes based on the primers used in the assay. Thus, we found a high similarity in the profile of AMR and virulence genes found in strain SD/21–15 with other *A. media* strains isolated from different host species and geographical areas in the world. Our findings show that *A. media* harbors several intrinsic AMR genes that could be transmissible to other bacteria species and it also harbors several virulence genes that could be responsible for its pathogenicity in different host species. We anticipate that data generated in this study will shed new insights on the role of *A. media* in the spread AMR genes and that it will pave way for studies aimed at elucidating the virulence mechanisms of *A. media* in different susceptible hosts.

Materials and methods

Characterization of bacteria using MALDI-TOF and sequences of The 16S rRNA gene

The *A. media* isolate designated as strain SD/21–15, originally isolated from marine sediments collected from Øresund in Denmark in 1992 (Andersen and Sandaa, 1994), was retrieved from the −80°C freezer and cultured in tryptose soy broth (TSB) followed by incubation at 10°C for 5–7 days. The isolate was previous classified as *Aeromonas* spp. (Andersen and Sandaa, 1994). The bacteria initially grown in TSB was later cultured on blood agar plates by incubation at 10°C for 5–7 days for individual colony purity followed by characterization using the Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry (MS; Singhal et al., 2015). The purified bacteria confirmed by MALDI-TOF were used for DNA extraction using the DNA extraction kit based on the manufacturer's protocol (Qiagen, Germany). Species identification and confirmation was carried out by PCR amplification of the 16S rRNA gene using the universal primers 27F and 1492R (Kuncham et al., 2017).

Testing of antimicrobial resistance using disk diffusion assay

The antibiotic disk experiment was carried out based on the Clinical and Laboratory Standards Institute (CLSI; Cockerill et al., 2012) guidelines to determine the susceptibility or resistance of bacteria to antibiotic treatment (Kahlmeter et al., 2006). The *A. media* isolate from Øresund in Denmark (Andersen and Sandaa, 1994) was tested for antibiotic resistance using the Kirby-Bauer disk diffusion assay (Joseph et al., 2011) using commercially available antibiotic discs (Neo-SensitabsTM, Rosco). Antibiotics used in the disk diffusion test were Ciprofloxacin (CIPR—5 μg), Erythromycin (Ery—15 μg), Gentamycin (GEN—10 μg), Ampicillin (AMP—10 μg), Cefoxitin (CFO—30 μg), Cephalothin (CEP—30 μg), Nitrofurantoin (NI—300 μg), Penicillin (PEN—10 μg), Tetracycline (TET—30 μg), Trimethoprim (TRIM—5 μg), Colistin (CO—150 μg), Sulfonamide (SULFA—240 μg), Amoxicillin (AMOXY—30 μg), Rifampicin (RIF—5 μg). The bacteria cultured overnight was diluted to 0.5 McFarland at a concentration of 10^8 CFU/ml and was spread on the surface of the Muller Hinton agar using sterile cotton swabs (Saffari et al., 2016). The antibiotics discs were put on the plate containing the bacterial lawn. This was followed by incubation at 10°C for 5–7 days. Afterward, antibiotic susceptibility and resistance was measured based on the manufacturer's instruction (Neo-SensitabsTM, Rosco).

Bacterial genomic DNA extraction and quality control analysis

Genomic DNA (gDNA) was extracted from *A. media* strain SD/21–15 isolate using the MagAttract® HMW DNA kit based on manufacturer's protocols (Qiagen, Germany) (Becker et al., 2016). A concentration of 2×10^9 CFU/ml freshly grown *A. media* strain SD/21–15 was centrifuged in 2 ml Eppendorf tubes, and pellets were resuspended in 180 μl ATL buffer followed by adding 20 μl Proteinase K to each tube. This was followed by incubation at 56°C in an Eppendorf thermomixer for 30 min. Afterward, 4 μl RNase was added to each tube followed by pulse vortexing and adding 15 μl of MagAttract Suspension G and 280 μl Buffer MB to each vial (Tarumoto et al., 2017). Next, the suspension from each tube was transferred onto a MagAttract holder followed by mixing for 60 s on an Eppendorf thermomixer. Magnetic beads containing gDNA were separated on the MagAttract magnetic rack for 60 s. Supernatants were removed without disturbing the beads and were washed twice using MW1 and PE buffer (Becker et al., 2016; Tarumoto et al., 2017). Thereafter, the remaining suspension from each vial was removed by rinsing the beads with 1 ml distilled water twice. The gDNA was harvested by eluting in 100 μl buffer EB while the purity of the gDNA was assessed using the NanoDrop (Thermo fisher, United States) followed by gel electrophoresis using 1% agarose. The harvested gDNA was quantified using the Qubit double-stranded DNA (dsDNA) high-CHS kit based on the manufacturer's instructions (Life Technologies Inc., Carlsbad, CA, United States; Guan et al., 2020).

Library preparation and sequencing

The sequence library for *A. media* strain SD/21–15 was prepared using the paired end DNA libraries using the Nextera DNA Flex Tagmentation (Illumina Inc. San Diego, CA, United States; Gaio et al., 2021) while the Illumina library was quantified using the Qubit® DNA HS Assay Kit in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA,

United States). Agilent HS DNA Kit (Agilent Technologies, CA, United States) based on the Agilent 2,100 Bioanalyzer System was used to check the size of library fragments. Illumina MiSeq (Illumina Inc., United States) was used for sequencing using V3 reagent kits using paired-end read length of 2 × 300 bp as previously described (Kaspersen et al., 2020). Bioinformatic analysis was done using the online Galaxy platform[1] version 21.05. Quality of both forward and reverse raw reads was analyzed using the FastQC Version 0.11.9 software (Bioinformatics, 2011). Adapters and low-quality reads from paired end sequences were removed using Trimmometric version 0.38.1 (Bolger et al., 2014). Afterward, the resulting paired-end sequence reads were *de novo* assembled into contigs using A5-miseq assembler (Coil et al., 2015). Quality read sequence contigs with 33–91 k-mers were assembled using SPAdes v. 3.12.0 (Bankevich et al., 2012). Genome annotation was made using the prokaryotic genome annotation pipeline (PGAP; Tatusova et al., 2016) from NCBI while annotation was done using Prokka (Seemann, 2014).

Prediction of average nucleotide identity and virulence genes

In addition to the *A. media* strain SD/21–15 whole genome sequence (WGS), we retrieved 24 WGSs of *A. media* isolates from the NCBI database obtained from different host species and environmental samples from Asia, Europe, and North America (Table 1). It is noteworthy that although *A. media* strain SD/21–15 was isolated in 1992 when it was classified as *Aeromonas* spp. (Andersen and Sandaa, 1994), the 24 genomes retrieved from the NCBI database covered the period 2013–2022 because there were no whole genome sequences of *A. media* prior to 2013 found in the NCBI database. The Galaxy platform using abricate v. 1.0.1 was used to identify genes of the virulence factors of pathogenic bacteria (VFDB; Seemann, 2014, 2020; Chen et al., 2005) of which the threshold for virulence-gene identification using the VFDB was set at 80%. On the other hand, the Average Nucleotide Identity (ANI) of all 25 *A. media* genomes was analyzed using the online Galaxy Europe[2] using FastANI v. 1.3. *Aeromonas media* strain MC64 from the Chinese hospital (CP047962.1) was used as a reference to calculate the ANI of all the 25 *A. media* genomes (Table 1). The threshold for FastANI was set at 90% based on pairwise sequence mapping (Jain et al., 2018) while heatmap based on calculated ANI for all 25 *A. media* genomes were generated using the package heatmap in the R studio v 4.0.4 statistical software with online Orion NMBU software.[3]

Prediction of antimicrobial resistance genes and mobile genetic elements

A total of 25 *A. media* whole genome sequences were used for identification of AMR genes, plasmids and transposons. Staramr version 0.7.2 (Tran et al., 2021) and ABRicate version 1.0.1 (Seemann, 2014, 2020) were used for identification of antibiotic resistance genes in the Comprehensive Antimicrobial Resistance Database (CARD) software (Alcock et al., 2020) of which the CARD identification threshold for AMR-genes was set at 80%. Identification of plasmids in bacterial genomes was done using Plasmidfinder v 2.0 (Ullah et al., 2020) with the threshold for plasmid identification set at 80%. Proksee software[4] was used to generate circular maps of all 25 *A. media* genomes and plasmids online.

Results

Whole genome sequencing and phylogenetic analysis

The *A. media* genomes retrieved from the NCBI databank were from Asia, Europe, and North America with the majority coming from Asia (Table 1). Thus, we did not find whole genome sequences of *A. media* isolates from Africa, Central, and South America in the public database. The genome size varied between 4.5 and 5.2 Mb while the GC content varied between 59 and 62.5% for all isolates (Table 1). The number of genes detected varied between 3,934 and 5,101 while the number of proteins varied between 3,535 and 4,704. Apart from the archived strain SD/21–15 obtained from marine sediments in Øresund in Denmark in 1992, all *A. media* strains used were isolated from the period 2013 to 2022 indicating that they were isolated in the last decade. Other details that include strain names, country of origin, and accession numbers are shown in Table 1. The circular map showing all *A. media* genomes used shows that strain SD/21–15 had a complete genome comparable with other *A. media* isolates obtained from different host species and the environment (Figure 1). Equally, phylogenetic analysis showed high similarity (>94%) of strain SD/21–15 with other *A. media* isolates from different host species and environments.

Average nucleotide identity and heatmap

The ANI phylogenetic analysis showed high similarity (>94%) of all *A. media* isolates despite emanating from different host species and geographical areas (Figure 2). The ANI of *A. media*

1 https://usegalaxy.no/
2 https://usegalaxy.eu/
3 RStudio nmbu.no
4 https://proksee.ca/

TABLE 1 Genome data of *Aeromonas media* strains used in the study.

No	Strain name	Country	Sources	Year	Level	Size (Mb)	GC%	Scafold	Genes	Proteins	Accession No.
1	TR3_1	China	Waste water	2021	Complete genome	4,531	61	1	4,193	3,954	CP075564.1
2	SD/21–15	Denmark	Marine sediment	2022	Contig	4,889	59	214	4,663	4,444	JAJVCY000000000
3	ARB13	Japan	River water	2014	Contig	4,612	61	180	4,330	4,124	JRBF00000000.1
4	ARB20	Japan	River water	2013	Contig	4,6	60.5	185	4,337	4,126	JRBG00000000.1
5	CECT 4232	USA		2013	Contig	4,5	61	329	4,299	4,043	CDBZ00000000.1
6	NXB	China	Chicken meat	2017	Scaffold	4,5	61	131	4,199	4,001	NXBV00000000.1
7	BAQ071013-132	USA	Perch	2019	Scaffold	4,7	61	165	4,394	4,174	NKWW00000000.1
8	BAQ071013-115	USA	Perch	2019	Scaffold	4,6	62	89	4,252	4,080	NKWY00000000.1
9	MC64	China	Hospital	2017	Complete genome	5	60	1	4,680	4,239	CP047962.1
10	T0.1–19	China	Sludge	2016	Complete genome	4,9	60	1	4,612	4,248	CP038441.1
11	R1-18	China	Sludge	2016	Complete genome	4,7	61	1	4,450	4,006	CP038443.1
12	T5-8	China	Sludge	2016	Complete genome	4,8	60.5	1	4,502	4,131	CP038444.1
13	R25-3	China	Sludge	2016	Complete genome	4,9	60.6	1	4,547	4,233	CP038445.1
14	R50-22	China	Sludge	2016	Complete genome	5,1	60	1	4,767	4,430	CP038448.1
15	R1-26	China	Biofilm reactor	2018	Complete genome	4,7	60.5	1	4,361	4,069	CP043579.1
16	WP7-W18-ESBL-02	Japan	Waste water	2020	Complete genome	4,8	61	1	4,424	4,165	AP022188.1
17	E31	China	water	2021	Complete genome	5,3	60	1	4,953	4,526	CP067417.1
18	CN17A0010	China	Human stool	2021	Contig	4,6	62	18	4,220	4,025	JAEHIH000000000.1
19	Colony414	Thailand	food	2021	Complete genome	4,7	62.5	1	3,934	3,535	CP070623.1
20	D180	Spain	Fish	2021	Contig	4,5	61.5	52	4,179	3,936	JAGDES000000000.1
21	ATCC 33907	Spain	River water	2021	Contig	4,5	61	199	4,227	4,001	JAGDEO000000000.1
22	Z1-6	China	Human	2018	Scaffold	4,5	61	131	4,209	4,008	UETL00000000.1
23	KLG6	UK	River	2019	Contig	4,5	61	454	4,430	3,961	CAAKNK000000000.1
24	INSAq193	Portugal	Fish	2022	Scaffold	5,2	60	532	5,101	4,704	JAKCNH000000000.1
25	WS	China	Water sample	2014	Complete genome	4,8	60.5	1	4,452	4,089	CP007567.1

strain SD/21–15 was >97% similar with the Chinese hospital strain MC64 (CP047962.1) used as a reference. The ANI phylogenetic tree clustered all 25 isolates into two groups, of which group-I comprised of 17 isolates with >97% similarities that included isolates from Denmark (SD/21–15), Japan (ARB13, ARB20, and WP7-W18-ESBL-02), China (T0.1–19, R1-26, and E31), United States (CECT 4232), Spain (ATCC 33907), and United Kingdom (KLG6; Figure 2). On the other hand, group-II comprised seven isolates with >93% similarities consisting of isolates from United States (BAQ071013-132 and BAQ071013-115), Spain (D180), Portugal (INSAq193), Thailand (Colony414), and China (CN17A0010 and Z1-6).

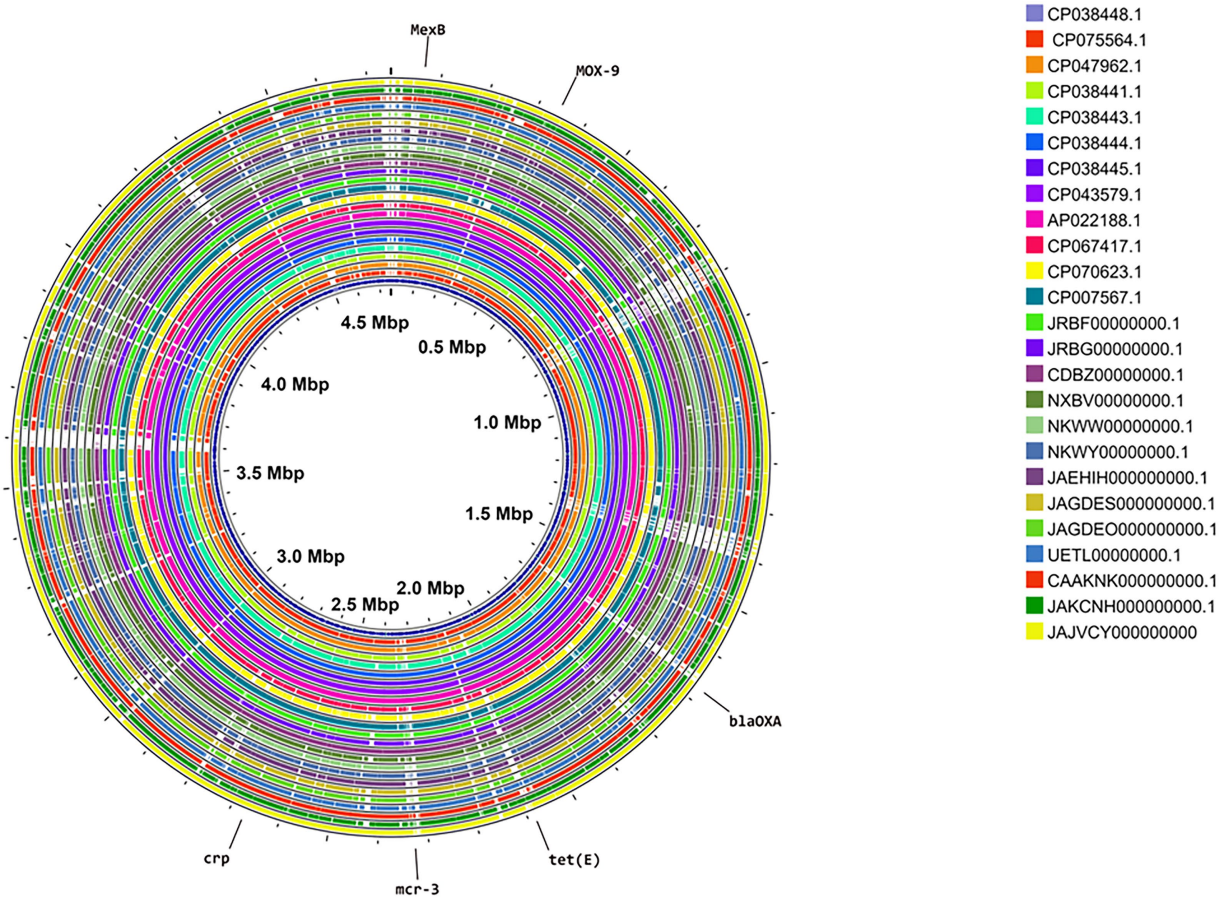

FIGURE 1
Circular map showing a comparison of the genome of *Aeromonas media* strain SD/21–15 together with genomes retrieved from the National Center for Biotechnology Information (NCBI) public databank obtained from different host species and environmental samples from different geographical areas in the world (See Table 1). Note strain SD/21–15 (JAJVCY000000000; outermost) shows a complete circular map similar with other 25 *A. media* strains. Figure 1 was created in Proksee (https://proksee.ca/).

Virulence factors

The virulence factors examined comprised of six elements, namely; (i) adherence and motility, (ii) immune evasion, (iii) secretions system, (iv) toxins, (v) iron acquisition, and (vi) biofilm formation together with quorum sensing (Figure 3; Table 2).

Adherence, motility proteins, and immune evasion genes

The adherence and motility genes detected were classified into four groups namely the (i) Msh pilus, (ii) Tap type IV pili, (iii) polar flagellar, and (iv) lateral flagella (Table 2). The Msh pilus, polar flagellar, and Tap type IV pili genes were detected in all 25 *A. media* strains including strain SD/21–15 while the lateral flagella genes were only found in strains BAQ071013-132 and BAQ071013-1115 isolated from perch in the United States as well as strain D180 isolated from fish in Spain (Table 2). Comparison of strain SD/21–15 with other *Aeromonas* spp. showed that it had all genes that form the Flp type IV and polar flagella proteins similar with the *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449) reference strains (Supplementary Table S1). However, it only had 15 genes that form the Tap type IV pili unlike *A. veronii* (B565), *A. hydrophila* (ATCC 7966), and *A. salmonicida* (A449) reference strains that had 20, 22, and 23 proteins, respectively. Our findings also show that *A. media* SD/21–15 strain did not have type I fimbriae genes found in *A. veronii* (B565), *A. hydrophila* (ATCC 7966), and *A. salmonicida* (A449) reference strains. In addition, genes that form the lateral flagella were only detected in *A. salmonicida* (A449) but not in *A. media* strain SD21/01–15 and the other *Aeromonas* reference strains (Supplementary Table S1). On the contrary, the polar flagella genes were detected in all four *Aeromonas* spp. examined although *A. hydrophila* (ATCC 7966) and *A. media* strain SD/21–15 had more genes that form the polar flagella than *A. veronii* (B565) and *A. salmonicida* (A449) reference strains (Supplementary Table S1).

Capsule and immune evasion genes

Our findings show that only eight of the 25 isolates examined had capsules and those included the strains

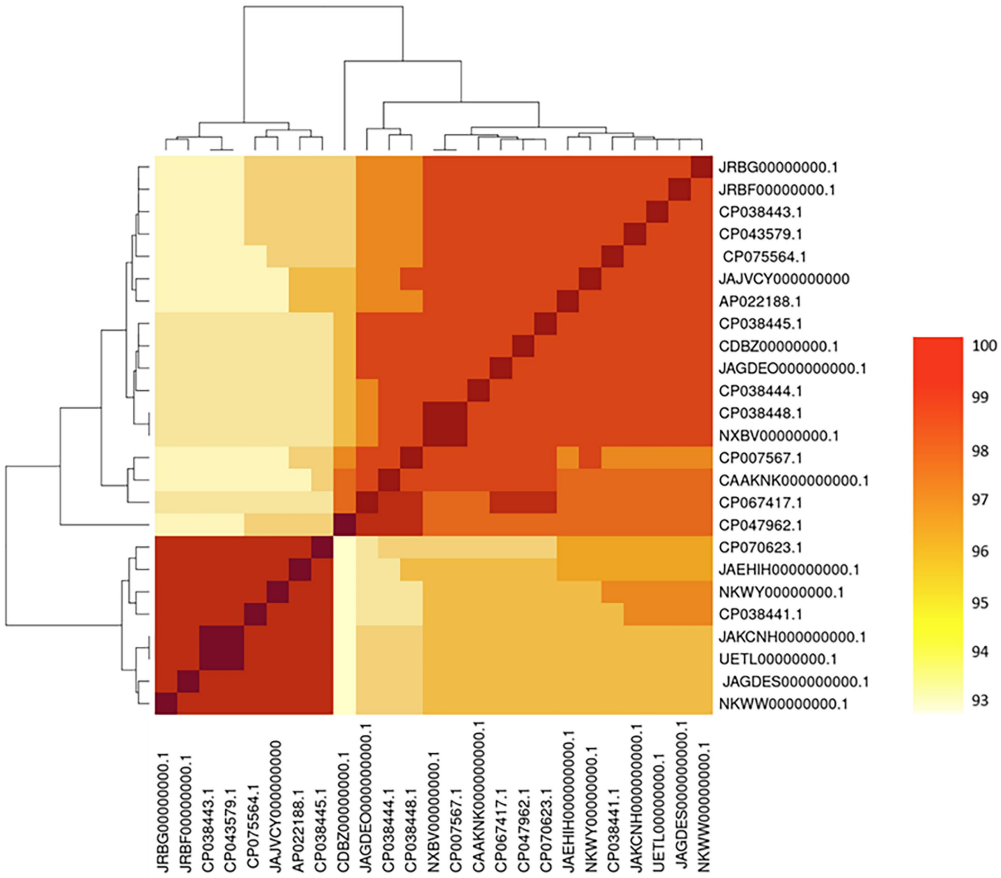

FIGURE 2
Heatmap/cladogram combined with phylogenetic comparison of *Aeromonas media* strain SD/21–15 isolated from an aquatic environment in Denmark with other strains retrieved from the National Center for Biotechnology Information (NCBI) public databank isolated from different host species and environments in the world. Note the high similarity among all *25 A. media* strains with homology varying between 93 and 100%.

SD/21–15, ARB13, CN17A0010, D180, ATCC 33907, Z1-6, KLG6, and INSAq193 (Table 2). A comparison of strain SD/21–15 with other *Aeromonas* spp. showed that only strain SD/21–15 had a capsule, and no capsule genes were detected in the genomes of *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449; Supplementary Table S1). Other immune evasion genes detected in *A. media* strain SD/21–15 include the nitrate reductase (*narH*), antiphagocytosis capsule (*wzb*), serum resistance LPS (*rfb*), and stress adaptation catalase peroxidase (*karG*) genes (Supplementary Table S1).

Secretion system

Although we investigated the presence of all secretory systems, our findings show that only the type II secretory system (T2SS) was detected in all 25 *A. media* (Table 2). Comparative analysis showed that strain SD/21–15 had 14 of the 15 T2SS genes ranging from *exeA* to *exeM* with the exception of *exeN* while *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449) had all 15 genes from *exeA* to *exeN* (Supplementary Table S1). On the contrary, the type III secretory system (T3SS) was not detected in all 25 *A. media* genomes, and it was not detected in *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449) reference strains. As for the type VI secretory system (T6SS), only three isolates had the genes *vrG1*, *vgrG3*, *hcp*, and *ats* genes in their genomes while most strains only had two of these genes detected (Table 2). Comparison of strain SD/21–15 with other *Aeromonas* spp. showed that only *A. hydrophila* (ATCC 7966) had all 25 genes that form the T6SS while *A. salmonicida* (A449) had 14 and *A. veronii* (B565) had none (Supplementary Table S1).

Hemolysin and other toxin genes

All *A. media* isolates had hemolysin genes namely hemolysin HlyA (*hlyA*), hemolysin III (*hlyIII*), and thermostable hemolysin (*TSH*) genes (Table 2). On the contrary, the aerolysin gene was not detected in all 25 *A. media* strains while the *RTX* toxin genes were only detected from Strain NXB, T0.1–19, and Z1-6 from chicken meat, sludge, and humans in China (Tables 1, 2), respectively. Comparison of strain SD/21–15 with other *Aeromonas* spp. showed that the aerolysin AerA/cytotoxic enterotoxin *aerA/act* gene was present in *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449) reference strains but not in strain SD/21–15. Other toxin genes, such as the heat stable cytotoxic

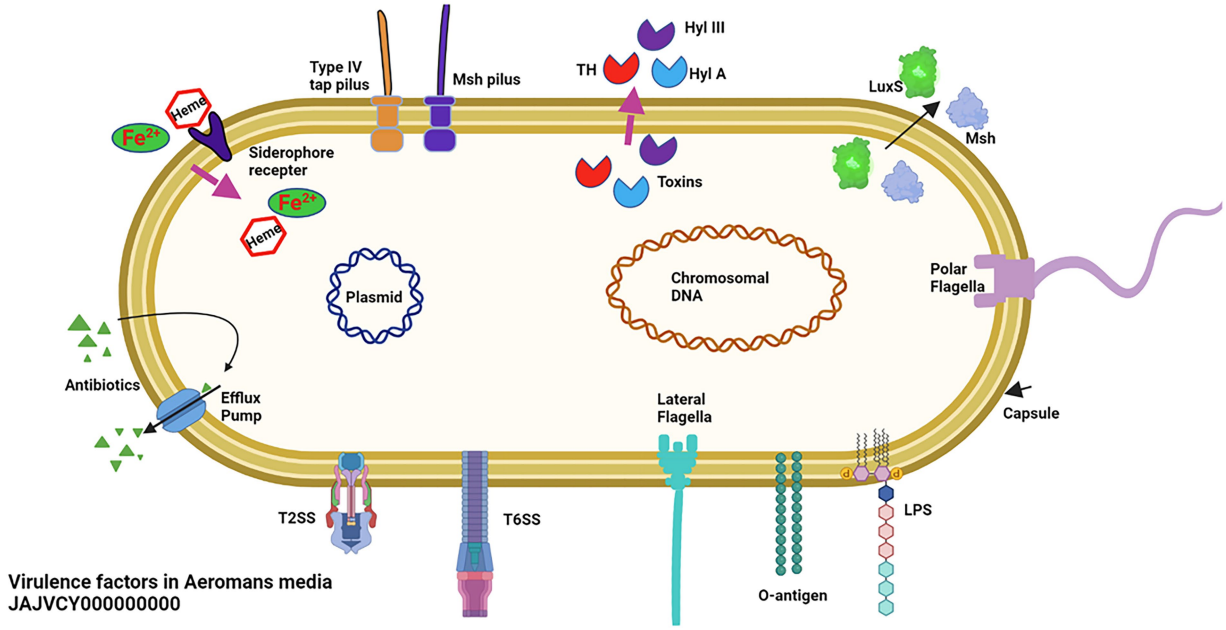

FIGURE 3
Schematic diagram of virulence genes on *Aeromonas media* included investigated showing (i) adherence proteins consisting of the *Msh* and type tap IV pili as well as the polar and lateral flagella, (ii) iron acquisition components comprising of the ferric uptake regulator (*Fur*), heme, and siderophore proteins, (iii) secretion system consisting of T2SS and T6SS, (iv) hemolysins consisting of hemolysin A (*hylA*), hemolysin III (*hylIII*), and thermostable heat (TH) protein, (v) biofilm and quorum sensing components consisting of *S*-ribosylhomocysteinase (*LuxS*) and MQS, (iv) Immune proteins consisting of capsule, lipopolysaccharide (LPS), and somatic O-antigen. In addition, schematic diagram shows *A. media* components associated with antimicrobial resistance (AMR) components consisting of (vii) efflux pumps and (viii) plasmid. Figure 3 was created in BioRender.com (https://biorender.com/).

enterotoxin (*ast*) and the repeat toxins (*RTX*; *rtxA*, *rtxB*, *rtxC*, *rtxD*, *rtxE*, and *rtxH*) genes only found in *A. hydrophila* (ATCC 7966) were not detected in strain SD/21–15. It also lacked the extracellular hemolysin (*ahh1*) gene found in *A. hydrophila* (ATCC 7966) and *A. salmonicida* (A449). On the other hand, *hlyA*, *hlyIII*, and TH toxin genes found in strain SD/21–15 were also present in *A. hydrophila* (ATCC 7966), *A. veronii* (B565), and *A. salmonicida* (A449) reference strains.

Iron acquisition, biofilm formation, and quorum sensing genes

The biofilm and quorum sensing *luxS* and *mshA-Q* genes were present in the genomes of all *A. media* isolates examined (Table 2). Similarly, the iron acquisition genes consisting of the gene of ferric uptake regulator (*fur*), siderophore synthesis, and heme uptake genes were present in all *A. media* isolates (Table 2).

Antimicrobial resistance

Phenotype characterization using the disk diffusion test

The *A. media* strain SD/21–15 showed multidrug resistance (MDR) to more than five antibiotics that included AMP-10 and PEN-10. They also showed resistance to CFO-30, CEP-30, TET-30, and AMOXY-30. It showed intermediate resistance for CIPR-5, ERY-15, and RIF-5 but was susceptible to Gentamycin GEN-10, NI-300, SULFA-, and TRIM-5 (Table 3) on the disk diffusion test.

Antimicrobial resistance genes

Whole genome sequence analysis showed that all 25 *A. media* isolates had multiple AMR genes encoded in their genomes (Table 4). Only $bla_{\text{KPC-1}}$ and $bla_{\text{TEM-1}}$ were detected among class A β-lactamases, of which $bla_{\text{KPC-1}}$ was found in strains MC64 and E31 that were isolated from a hospital and water in China while $bla_{\text{TEM-1}}$ was found in strains MC64, E31 and INSAq193 isolated from hospital, water and fish from China and Portugal, respectively. The only gene identified in the class B metallo-β-lactamases (MBL) was *cphA7* found in strains R1-18 and R1-26 isolated from sludge and biofilm reactors in China, respectively. The class C β-lactamase group was dominated by $bla_{\text{MOX-9}}$ found in all 25 *A. media* isolates while $bla_{\text{CMY-8b}}$ was only detected in strain SD/21–15. Equally, class D was dominated by $bla_{\text{OXA-427}}$ found in all 25 *A. media* strains while $bla_{\text{OXA-1}}$ was only found in strain R50-22 and $bla_{\text{OXA-10}}$ in strain WP7-W18-ESBL-02. Outside the β-lactamase, the dominant AMR genes detected were *CRP* and *MCR* that were present in all 25 *A. media* isolates followed by *MCR-3* and *MCR-3.6* that were detected in eight, and *sul1* from five isolates. Other AMR genes detected from different *A. media* strains are shown in Table 4.

TABLE 2 Comparison of virulence genes in *Aeromonas media* sequences.

Strain name	Adherence and motility				Immune evasion	Secretion system						Toxins					Iron acquisition			Biofilm and quorum sensing	
	Msh pilus	Polar flagella	Tap type IV pili	Lateral flagella	Capsule	T2SS	T3SS	T6SS				*hylHlA*	*hylIII*	*TSH*	AerA	RTX toxin	Ferric uptake	Siderophore synthesis	Heme uptake	LuxS	mshA-Q
								vgrG1	vgrG3	hcp	ats										
TR3_1	■	■	■			■		■	■	■		■	■	■			■	■	■	■	■
SD/21–15	■	■	■		■	■		■		■		■	■	■			■	■	■	■	■
ARB13	■	■	■		■	■			■	■		■	■	■			■	■	■	■	■
ARB20	■	■	■			■			■	■		■	■	■			■	■	■	■	■
CECT 4232	■	■	■			■		■		■		■	■	■			■	■	■	■	■
NXB	■	■	■			■						■	■	■		■	■	■	■	■	■
BAQ071013-132	■	■	■	■		■		■	■	■	■	■	■	■			■	■	■	■	■
BAQ071013-115	■	■	■	■		■		■	■	■	■	■	■	■			■	■	■	■	■
MC64	■	■	■			■		■		■		■	■	■			■	■	■	■	■
T0.1–19	■	■	■			■			■		■	■	■	■		■	■	■	■	■	■
R1-18	■	■	■			■						■	■	■			■	■	■	■	■
T5-8	■	■	■			■		■		■		■	■	■			■	■	■	■	■
R25-3	■	■	■			■		■		■		■	■	■			■	■	■	■	■
R50-22	■	■	■			■		■		■	■	■	■	■			■	■	■	■	■
R1-26	■	■	■			■		■		■		■	■	■			■	■	■	■	■
WP7-W18-ESBL-02	■	■	■			■						■	■	■			■	■	■	■	■
E31	■	■	■			■		■		■		■	■	■			■	■	■	■	■
CN17A0010	■	■	■		■	■						■	■	■			■	■	■	■	■
Colony414	■	■	■			■						■	■	■			■	■	■	■	■
D180	■	■	■	■	■	■		■	■	■	■	■	■	■			■	■	■	■	■
ATCC 33907	■	■	■		■	■		■		■		■	■	■			■	■	■	■	■
Z1-6	■	■	■		■	■						■	■	■		■	■	■	■	■	■
KLG6	■	■	■		■	■						■	■	■			■	■	■	■	■
INSAq193	■	■	■		■	■				■	■	■	■	■			■	■	■	■	■
WS	■	■	■			■		■		■		■	■	■			■	■	■	■	■

Blue, presence of gene, white/blank, absence of the gene.

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TABLE 3 Antimicrobial resistance of *Aeromonas media* strain SD/21–15 based on disc diffusion test.

Antibiotics	Susceptibility/Resistance	
Ampicillin (AMP-10)	Resistant	R
Cefoxitin (CFO30)	Resistant	R
Cephalothin (CEP 30)	Resistant	R
Ciprofloxin (CIPR5)	19 (Susceptible)	S
Erythromycin (ERY15)	11 (Susceptible)	S
Gentamycin (GEN10)	24 (Susceptible)	S
Nitrofurantoin (NI300)	20(Susceptible)	S
Penicillin (PEN10)	Resistant	R
Colistin (CO150)	28 (Susceptible)	S
Sulphonomide (SULFA)	17 (Susceptible)	S
Tetracycline (TET30)	Resistant	R
Trimethoprim (TRIM5)	20 (Susceptible)	S
Amoxicillin (AMOXY)	Resistant	R
Rifampicin (RIF5)	15 (Susceptible)	S

Multidrug resistance proteins

Our findings show that different genes encoding multidrug resistance proteins were detected from each of the 25 *A. media* isolates. Among these *mexB* was detected in 24 of the total 25 *A. media* isolates while *tetE* was detected in 11 and *Mcr3* in seven of the 25 isolates (Table 4). Other multidrug efflux pump proteins detected included *vatF*, *catB*, *mphA*, *mphE*, *msrE*, *arr-3*, and *ugd tet*(*E*) (Table 4).

Mobile genetic elements

Components of the mobile genetic elements (MBE) identified consisted of the transposases, integrases, recombinases, and plasmids (Tables 5, 6; Figure 5). Our findings show that strain D/21–15 had more transposons detected than the other strains (Table 5). Although the Tn3 family of transposons was detected in several strains, the insertion sequence (IS) class of transposons was the most dominant in all *A. media* isolates. Although integrase was detected in all 25 isolates only six isolates had all four components of the recombinases comprising of the recombinase family protein, tyrosine recombinase XerC, recombinase RecA, and site-specific tyrosine recombinase XerD while most strains only had the tyrosine recombinase XerC and site specific tyrosine recombinase XerD (Table 5). Finally, only 10 isolates had plasmids of which strains MC64, R25-3, R50-22, and E31 had two plasmids each while strains TR3_1, SD/21–15, T0.1–19, T5-8, INSAq193, and WS had only one plasmid each (Table 6). We generated circular maps from six out of the 10 plasmids detected to determine whether they encoded AMR genes, transposons, and integrases. The circular map of strain SD/21–15 plasmid has no AMR genes, transposases, or integrase encoded in its genome (Figure 4A). However, the circular maps of strains TR3_1, R50-22, MC64, PE31A, and T5-1 plasmids encoded AMR genes, transposases, virulence factors, efflux pumps, recombinases, and other genes (Figures 4B–F).

Discussion

In this study, we have shown that strain SD/21–15 has several virulence and AMR genes similar to those found in other *Aeromonas* spp. Although we did not find whole genome sequences of *A. media* from Africa, Central, and South America in the public databanks, the 25 strains used in this study show a wide geographical distribution covering North America, Europe, and Asia. The absence of whole genome sequences of *A. media* from Africa and South America in the NCBI database is unknown whether this is due to lack of studies or resources for WGS of *A. media* in these continents. In terms of host distribution, the 25 isolates used covered a wide range of hosts from humans, fish, and chickens while environmental samples were from rivers, sludge, water treatment facility, hospital, biofilm reactors, and marine environments. As for the time span covered, the isolates used covered the period 2013–2022, with the exception of strain SD/21–15 isolated in 1992, because we did not find whole genome sequences of *A. media* deposited in the NCBI database prior to 2013. Note that strain SD/21–15 was initially classified as *Aeromonas* spp. using morphological, motility, and biochemical tests in 1992 (Andersen and Sandaa, 1994) but the WGS carried out in the present study classified the isolate as *A. media*. Thus, it is likely that several other isolates previously classified as *Aeromonas* spp. using morphological and biochemical tests could classified as *A. media* using WGS. Nonetheless, our comparison of AMR and virulence genes for *A. media* in the present study is based on a collection of genomes from a broad geographical distribution and wide host species using recent data. The similarity of AMR genes detected in strain SD/21–15 from marine sediments isolated in 1992 with recent isolates covering the period 2013 to 2022 is suggestive that *A. media* could be a hidden environmental risk carrying several intrinsic AMR genes as a source of transmission to other bacteria. The diverse host range and environmental source is suggestive that *A. media*, like other aeromonads, bridges the gap between the environment, aquaculture, animals and humans in the transmission of AMR genes.

The adherence of bacteria to host cells using pili and flagella is a crucial pathogenicity step in early stages of bacterial infection. The presence of genes that form *Msh* pili, tap type IV pili, and polar flagella proteins in all 25 isolates is suggestive that these proteins could be important for the adherence of *A. media* to host cells. This finding shows that *A. media* shares similar adherence proteins with other *Aeromonas* spp. where these proteins are used for intestinal adherence, colonization and biofilm formation (Canals et al., 2006; Hadi et al., 2012). However, only three isolates had the lateral flagella genes suggesting that this protein might not be obligatory for the adherence and biofilm formation in *A. media*. Other genes detected include *luxS* needed for biofilm formation and quorum sensing (Kozlova et al., 2008) and *mshQ* required for mannose-sensitive hemagglutinin pilus biosynthesis (Qin et al., 2014). Thus, detection of *luxS* and *mshQ* in all 25 isolates is

TABLE 4 Antimicrobial resistance and efflux pump proteins detected in the *Aeromonas media* strains.

No	Strain name	Beta lactamase					Other AMR genes								Efflux pump proteins	
		Class A		Class B MBL	Class C	Class D										
1	TR3_1				MOX-9 (98.35)	OXA-427 (98.36)	CRP (78.41)	MCR-7.1 (72.31)					QnrS2 (100)		tet E (99.92)	MexB (73.00)
2	SD/21–15				MOX-9 (98.35)	CMY-8b OXA-427 (98.74)	CRP (78.41)	MCR-7.2 (73.57)							tet E (99.95)	MexB (72.73)
3	ARB13				Mox-9 (98.09)	OXA-427 98.74	CRP (78.41)	MCR-7.1 (72.53)						ugd (70.40)		MexB (72.67)
4	ARB20				MOX-9 (89.09)	OXA-427 (98.74)	CRP (78.41)	MCR-7.1 (72.53)						ugd (70.40)		MexB (72.67)
5	CECT 4232				MOX-9 (99.91)	OXA-427 (97.99)	CRP (78.41)	MCR-7.1 (73.19)	MCR-3 (83.77)	MCR-3.6 (99.88)		vatF (71.04)				MexB (72.47)
6	NXB				MOX-9 (85.62)	OXA-427 (89.31)	CRP (78.73)	MCR-7.1 (73.29)	MCR-3 (84.26)	MCR-3.6 (96.55)					tet E (95.28)	MexB (72.73)
7	BAQ071013-132				mox-9 (85.96)	OXA-427 (89.43)	crp (78.56)	MCR-7.1 (73.22)								MexB (72.40)
8	BAQ071013-115				MOX-9 (88.29)	OXA-427 (89.31)	CRP (77.94)	MCR-7.1 (73.54)				vatF (71.92)				MexB (72.82)
9	MC64 (Plasmid)	KPC-1 (100)	TEM-1		MOX-9 (98.44)	OXA-427 (99.12)	CRP (78.41)	MCR-7.1 (73.38)				AAC(3)-Iid (99.88)	mphA (100)	ugd (71.24)		MexB (72.70)
10	T0.1–19				MOX-9 (85.60)	OXA-427 (88.68)	CRP (78.73)	MCR-7.1 (73.43)								MexB (72.41)
11	R1-18			cphA7 (94.12)	MOX-9 (99.91)	OXA-427 (98.99)	CRP (78.41)	MCR –7.1 (72.69)			sul1 (100)	ANT (3)-II a(99.07)	aadA16 (99.29)	catB (100)	dfrB4 (100)	tet E (95.19)	MexB (72.48)
12	T5-8				Mox-9 (99.90)	OXA-427 (97.99)	CRP (78.73)	MCR-7.1 (73.42)	MCR-3 (83.77)	MCR-3.6 (99.88)		vatF (71.04)				tet E (99.92)	MexB (72.73)
13	R25-3				Mox-9 (99.90)	OXA-427 (97.99)	CRP (78.41)	MCR-7.1 (73.19)	MCR-3 (83.77)	MCR-3.6 (99.88)		vatF (71.04)				tet E (99.92)	MexB (72.73)
14	R50-22 (Plasmid)				MOX-9 (99.90)	OXA-427 (97.99)	OXA-1 (100) CRP (78.41)	MCR-7. nnnnn(73.19)	MCR-3 (99.89)	MRC 3.6 (99.88)	sul1 (100)	AAC (6)-Ib-cr (100)	arr-3 (100)	catB (100)	mphE (100) msrE (100)	tet E(99.92)	MexD (80.230)

(Continued)

10.3389/fmicb.2022.1022639

TABLE 4 (Continued)

No	Strain name	Beta lactamase						Other AMR genes								Efflux pump proteins	
		Class A		Class B MBL	Class C	Class D											
15	R1-26			cphA7 (94.12)	MOX-9 (99.91)	OXA-427 (98.99)		CRP (78.41)	MCR-7.1 (72.97)			sul1 (100)	ANT(3)-Iia	tet A (100)	catB3 (100)	dfrB4 (100)		MexB (72.35)
16	WP7-W18-ESBL-02				MOX-9 (98.09)	OXA-427 (98.99)	OXA-10	CRP (78.57)	MCR-7.1 (72.96)			sul1 (100)	ANT (3)-IIa	tet A (100)	catB3 (100)	dfrB4 (100)		MexB (72.92)
17	E31	KPC-1 (99.89)	TEM-1 (99.89)		MOX-9 (98.44)	OXA-427 (98.99)		CRP (78.41)	MCR-7.1 (73.38)				AAC(3)-Iid (99.88)		mphA (100)		tet E(96.95)	MexB (72.62)
18	CN17A0010				MOX-9 (88.29)	OXA-427 (89.56)		CRP (97.95)	MCR 7.1 (73.51)				vatF (71.07)					MexB (72.75)
19	Colony414				MOX-9 (89.21)	OXA-427 (89.38)		CRP (77.94)	MCR-7.1 (73.02)									MexB (72.36)
20	D180				MOX-9 (86.22)	OXA-427 (89.06)		CRP (78.41)	MCR-7.1 (73.48)									MexB (72.31)
21	ATCC 33907				MOX-9 (99.74)	OXA-427 (97.99)		CRP (78.41)	MCR-7.1 (73.19)	MCR3 (83.77) MCr	MCR-3.6 (99.88)							MexB (72.47)
22	Z1-6				MOX-9 (99.74)	OXA-427 (89.31)		CRP (78.73)	MCR-7.1 (73.29)	MCR-3 (84.26)	MCR3.6 (96.55)						tet (E) (95.28)	MexB (72.73)
23	KLG6				MOX-9 (98.44)	OXA-427 (98.99)		CRP (78.41)	MCR-7.1 (73.56)					tet A (100)				MexB (72.92)
24	INSAq193		TEM-1		MOX-9 (85.87)	OXA-427 (89.12)		CRP (78.57)	MCR 7.1 (71)		acrD (98.81)	sul2 (99.63)	APH (3)-Ib	QnrB5 (99.12)		dfrA5 (98.73)	tet E (99.92)	
25	WS				MOX-9 (98.44)	OXA-427 (98.99)		CRP (78.09)	MCR-7.1 (73.45)								tet E (95.19)	MexB (72.70)

10.3389/fmicb.2022.1022639

TABLE 5 Transposes, integrases and recombinases detected in the *Aeromonas media* genomes.

Transposes/ Integrases gene description		JAJVCY000000001	CP047962.1	CP075564.2	JRBF00000000.1	JRBG00000000.1	CDBZ00000000.1	NXBV00000000.1	NKWW00000000.1	NKWY00000000.1	CP038441.1	CP038443.1	CP038444.1	CP038445.1	CP038448.1	CP043579.1	AP022188.1	CP067417.1	JAEHIH000000000.1	CP070623.1	JAGDES000000000.1	JAGDEO000000000.1	UETL00000000.1	CAAKNK000000000.1	JAKCNH000000000.1	CP007567.1
Transposes	DDE-type integrase/transposase/recombinase	■		■														■								
	IS5/IS1182 family transposase	■		■							■	■	■	■				■								
	IS1595 family transposase	■	■	■	■	■	■	■	■	■		■	■	■	■	■		■	■		■	■	■	■	■	
	IS110 family transposase	■	■		■	■	■	■			■	■	■	■	■		■	■	■			■	■	■	■	
	IS3 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	
	IS5 family transposase	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■				■	■	■	■	■
	IS66 family transposase	■	■		■	■	■				■	■	■	■	■	■		■		■	■	■		■	■	■
	IS630 family transposase	■	■	■	■	■	■		■		■	■		■	■	■	■	■			■	■		■	■	■
	IS4 family transposase	■	■		■	■	■	■	■		■	■	■	■	■	■	■	■		■	■	■	■	■	■	■
	IS21 family transposase	■	■	■	■	■	■	■			■	■		■	■	■		■				■	■	■	■	
	IS30 family transposase	■	■	■	■	■	■	■			■	■	■	■	■	■	■	■				■	■	■		
	IS200/IS605 family transposase	■	■		■	■	■	■					■		■	■		■				■	■	■	■	
	IS256 family transposase	■	■	■	■		■					■				■		■				■		■		■
	Tn3 family transposase	■			■		■	■	■	■		■				■			■	■	■	■	■	■	■	
	IS1634 family transposase	■	■				■						■	■				■				■			■	
	Site-specific integrase	■	■	■							■	■	■	■	■			■		■						■
Integrase	Integrase	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	Tyrosine-type recombinase/integrase	■	■	■														■		■						
	Recombinase family protein	■	■								■	■	■					■								
Recombinase	Tyrosine recombinase XerC	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■
	Recombinase RecA	■	■	■							■	■	■	■	■			■		■						
	Site-specific tyrosine recombinase XerD	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	

Blue=presence of the gene, while/blank=absence of the gene.

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TABLE 6 Plasmids detected in the *Aeromonas media* whole genome sequences.

No	Strain name	Accession number	Plasmid-1	Plasmid-2
1	TR3_1	CP075564.1	CP075565.1 (qnrS) (9,182 bp)	
2	SD/21–15	JAJVCY000000000	Contig 81, 9,295 bp	
3	ARB13	JRBF00000000.1		
4	ARB20	JRBG00000000.1		
5	CECT 4232	CDBZ00000000.1		
6	NXB	NXBV00000000.1		
7	BAQ071013-132	NKWW00000000.1		
8	BAQ071013-115	NKWY00000000.1		
9	MC64 (Plasmid)	CP047962.1	CP047963.1 (283,486 bp)	CP047964.1 (24,044 bp)
10	T0.1–19	CP038441.1	CP038442 (2,785 bp)	
11	R1-18	CP038443.1		
12	T5-8	CP038444.1	CP061478.1 (100,709 bp)	
13	R25-3	CP038445.1	CP038446.1 (190,780 bp)	CP038447.1 (4,795 bp)
14	R50-22 (Plasmid)	CP038448.1	CP038449.1 (198,927 bp)	CP038450.1 (199,818 bp)
15	R1-26	CP043579.1		
16	WP7-W18-ESBL-02	AP022188.1		
17	E31	CP067417.1	CP067418.1 (373,184 bp) KPC,	CP067419.1 (15,349 bp)
18	CN17A0010	JAEHIH000000000.1		
19	Colony414	CP070623.1		
20	D180	JAGDES000000000.1		
21	ATCC 33907	JAGDEO000000000.1		
22	Z1-6	UETL00000000.1		
23	KLG6	CAAKNK000000000.1		
24	INSAq193	JAKCNH000000000.1	JAKCNH010000351.1	
25	WS	CP007567.1	CP007568.1 (11,276 bp)	

suggestive that these proteins could be vital for biofilm and quorum sensing in *A. media*.

Previous studies reported presence of four (T2SS, T3SS, T4SS, and T6SS) secretory systems in *Aeromonas* spp. out of the six characterized in Gram negative bacteria (Beaz-Hidalgo and Figueras, 2013). However, only the T2SS and T6SS were detected in the *A. media* isolates examined in this study. Detection of all T2SS genes in all isolates may indicate that it might be required for *A. media* virulence. In other *Aeromonas* spp., T2SS has been linked with the presence of various proteins such as amylases, DNases, proteases, and the aerolysin-related cytotoxic enterotoxin *Act* shown to cause diarrhea (Xu et al., 1998; Sandkvist, 2001; Sha et al., 2002; Galindo et al., 2004). On the other hand, the T6SS uses the glycine repeat G (*VrgG*) and hemolysin-coregulated (*Hcp*) genes as part of the pore-forming protein to inject toxins into host cells (Bingle et al., 2008). Our findings show that only 3/25 isolates had the genes for all four proteins (VgR1, VgrG1, VgrG3, and Hcp) characterized to be crucial for T6SS virulence in *Aeromonas* spp. (Suarez et al., 2008, 2010). Interestingly, Rasmussen-Ivey et al. (2016) pointed out that T6SS is not obligatory for *Aeromonas* virulence as shown that not all hypervirulent *A. hydrophila* strains causing diseases in fish possess the T6SS. Similarly, it is likely that T6SS is not obligatory for the virulence of *A. media* given that most isolates used in this study did not have all T6SS genes. However, there is need for *in vivo* studies to validate these observations.

We detected three hemolysin genes namely *hlyA*, *hlyIII*, and *TSH* in all 25 isolates suggesting that these genes might be important for *A. media* virulence. Previous studies have shown that *hlyA*, *hlyIII*, and *TH* are pore forming cytotoxic enterotoxins found in different bacteria species including *Aeromonas* spp. that cause membrane damage and fluid accumulation in host cells leading to diarrhea (Agger et al., 1985; Kozaki et al., 1987; Honda et al., 1992; Baida and Kuzmin, 1996; Stanley et al., 1998; Chopra and Houston, 1999; Abrami et al., 2000; DelVecchio et al., 2002; Wang et al., 2003; Maté et al., 2014; Abdel-Fattah et al., 2017). Thus, it is likely that the diarrhea reported in humans infected by *A. media* might be caused by the hemolysin genes. However, we did not find the *aerA* gene in all 25 *A. media* isolates and yet it was present in other *Aeromonas* spp. examined (Hirono and Aoki, 1991; Wang et al., 2003). Wong et al. (1998) and Heuzenroeder et al. (1999) showed that a combination of the *hlyA*(+)*aerA*(+) double mutant significantly reduced the virulence of *A. hydrophila* in mice. They observed that cytotoxicity to buffalo green monkey kidney cells and hemolysis on horse blood agar were eliminated only in the double and not in the single mutants of *A. hydrophila*, *A. veronii*, and *A. caviae*. They also showed that only the double

FIGURE 4
Circular maps of plasmids (i) pSD/21–15 isolated from Denmark **(A)**, (ii) pTR3_1 waste water treatment from United States **(B)**, (iii) pR50_22 from a sludge in China **(C)**, (iv) pMC64 from a hospital in China **(D)**, (v) pE31 from water in China **(E)**, and (vi) pT5-1 from a sludge in China **(F)**. Note that the location of virulence factors, antimicrobial resistance (AMR) genes, transposases, efflux pumps, secretion system, and other genes are shown in the circular map for each the plasmid.

mutant eliminated the β-hemolysis on horse blood agar and cytotoxic activities on buffalo green monkey and Vero cells. Inactivation of the double mutant completely attenuated the virulence of *A. hydrophila* in mice (Heuzenroeder et al., 1999). In this study, all *A. media* isolates only had *hlyA* but not *aerA*. So, it is unknown whether the absence of *aerA* renders *A. media* isolates less pathogenic than other *Aeromonas* spp. that have the *hlyA*(+)-*aerA*(+) combination.

Iron is a vital cofactor used for various metabolic processes for the survival of bacteria in infected hosts (Ratledge and Dover, 2000; Wandersman and Delepelaire, 2004; Maltz et al., 2015). Thus, different bacteria species have devised various mechanisms for getting iron from their hosts (Byers, 1987; Calderwood and Mekalanos, 1987; Litwin and Calderwood, 1993; Morton et al., 2007, 2009). So, the uptake of iron from host cells is considered a virulence factor because of the damage impacted on the host due to iron deprivation. Common molecules used by Gram negative bacteria for iron uptake include the ferric uptake regulator (*fur*), siderophores, and heme (Barghouthi et al., 1989a,b; Litwin and Calderwood, 1993; Morton et al., 2007). Ebanks et al. (2013) showed that *fur* knockout

mutants reduced the pathogenicity of *A. salmonicida* while Najimi et al. (2008a) showed that mutation in the hemin-binding protein caused a drastic reduction in the pathogenicity of *A. salmonicida* due to reduced heme uptake as a source of iron. In another study, Najimi et al. (2008b) showed that mutations in genes used for catecholate siderophore production reduced the pathogenicity of *A. salmonicida*. Thus, the detection of the genes encoding *fur*, siderophore, and heme in all 25 isolates is suggestive that these genes could be crucial for iron acquisition in *A. media* being similar to observations seen in other *Aeromonas* spp. (Byers, 1987; Najimi et al., 2008b; Ebanks et al., 2013).

A recent study by Ebmeyer et al. (2019) reported *Aeromonas* spp. as the origin of several clinically significant β-lactamases such as the CMY-1/MOX-family that include bla_{AmpC}, bla_{MOX-1}, bla_{MOX-2}, and bla_{MOX-9}. Thus, the detection of bla_{MOX-9} in all 25 isolates from different host species and geographical areas in the world corroborates with Ebmeyer (2021) who reported *A. media* as the origin of bla_{MOX-9}. In 2017, Bogaerts et al. (2017) reported that $bla_{OXA-427}$ from Enterobacteriaceae was closely related to isolates from *A. media*, *A. hydrophila*, and *A. sobria* as a novel emerging carbapenem-hydrolysing class D β-lactamase (CHDL) from patients in a Belgian hospital. They showed that $bla_{OXA-427}$ hydrolyzed imipenem and conferred resistance to extended-spectrum cephalosporins, penicillin and carbapenems when expressed in *Escherichia coli*. Its presence in all 25 isolates emanating from North America, Europe, and Asia is suggestive that $bla_{OXA-427}$ could be highly prevalent in *A. media* strains across the world posing the danger of being the source of $bla_{OXA-427}$ transmission to humans and animals. Its higher presence among *Aeromonas* spp. than other bacterial species, support observations made by Bogaerts et al. (2017) who pointed out that CHDLs are restricted to a few bacterial genera. Detection of *CRP* and *MCR* genes in all 25 *A. media* shows that the presence of these genes in *A. media* extends across several continents while the presence of bla_{KPC-1} in *A. media* isolates is a significant finding given that infections caused by bla_{KPC-1} producing bacteria are extremely difficult to treat because of their multidrug resistance linked to high mortalities in humans (Sacha et al., 2009). The presence of *MexB* in all isolates is suggestive that this efflux pump could be important for transportation of genes like bla_{MOX-9}, $bla_{OXA-427}$, *crp*, and *mcr* genes found in all *A. media* isolates.

Although the plasmid of strain SD/21–15 had no AMR-genes in its genome, other *A. media* isolates had plasmids having various AMR genes that included bla_{KPC-1}, $bla_{OXA-427}$, *sul1*, bla_{OXA-1}, and *qnr* genes. In additions, the detected plasmids had several transposases, such as Tn3, ISAs1, IS1595, and IS4 known to carry various AMR-gene cassettes (Dziewit et al., 2012; Baquero et al., 2013; Carvalho et al., 2021). The plasmids also encoded various efflux pump proteins, such as *tet(E)*, merD, mexC, OprJ, mph(E), (Chopra, 2002), and mph(A) known to play significant roles in drug trafficking across cell membranes (Dayao et al., 2016; Kim et al., 2017; Yang et al., 2021). The presence of type II toxin-antitoxin RelEParE and T2SS is indicative that the plasmids also carry virulence genes. The presence of proteins such as the conjugal transfer protein TraF points to the presence of proteins that facilitate gene transfer between bacteria species. Other researchers (Majumdar et al., 2006; Preena et al., 2021) have noted that plasmids of aeromonads can be cured after sub-culturing, and depending on the history of the isolates after primary isolation, we may not have sequenced all plasmids of the original isolates in this study. Altogether, these observations show that *A. media* strains isolated from different geographical and host species in the world carry various multidrug efflux pump proteins, transposons, AMR, and virulence genes. However, this is need for *in vivo* studies using approaches such as mutagenesis, cloning, and purification of virulent genes identified in this study in order to determine their virulence mechanisms in different host species. Such studies would shed more insight on genes that are crucial for the pathogenicity of *A. media*.

Conclusion

In this study, we have shown that *A. media* strain SD/21–15 isolated from marine sediments in Denmark shares several virulence genes such as adherence proteins, hemolysins, secretion system, iron acquisition, biofilm formation and quorum sensing genes with other *A. media* strains isolated from different host species and geographical areas in the world. We have also shown that strain SD/21–15 shares several genes like hemolysins, adherence proteins, and T2SS with other *Aeromonas* spp. although it lacks the cytotoxic *aerA* gene. The presence of bla_{MOX-9}, $bla_{OXA-427}$, *crp,* and *mcr* genes in all 25 isolates is indicative that these AMR genes are highly prevalent in *A. media* isolates found in different ecosystems. The presence of transposases, integrase, recombinases, virulence, and AMR genes in the plasmids is indicative that the *A. media* strains examined in this study had the potential to transmit virulence and AMR genes to other bacteria. In summary, our findings shed new insights on virulence genes and the role of *A. media* in the spread of AMR genes.

Data availability statement

The datasets presented in this study can be found in online repositories. The link to the repository can be found below: https://www.ncbi.nlm.nih.gov/nuccore/JAJVCY000000000.1.

Author contributions

SD, HS, and HM: conceptualization, methodology, supervision, data curation, bioinformatics analysis, and mobilizing resources. SD, EA-W, BP, ØE, HS, and HM: manuscript preparation, editing, and submission. 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.2022.1022639/full#supplementary-material

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Conjugation of plasmid harboring *bla*$_{NDM-1}$ in a clinical *Providencia rettgeri* strain through the formation of a fusion plasmid

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Providencia rettgeri has recently gained increased importance owing to the New Delhi metallo-β-lactamase (NDM) and other β-lactamases produced by its clinical isolates. These enzymes reduce the efficiency of antimicrobial therapy. Herein, we reported the findings of whole-genome sequence analysis and a comprehensive pan-genome analysis performed on a multidrug-resistant *P. rettgeri* 18004577 clinical strain recovered from the urine of a hospitalized patient in Shandong, China, in 2018. *Providencia rettgeri* 18004577 was found to have a genome assembly size of 4.6Mb with a G+C content of 41%; a circular plasmid p18004577_NDM of 273.3Kb, harboring an accessory multidrug-resistant region; and a circular, stable IncT plasmid p18004577_Rts of 146.2Kb. Additionally, various resistance genes were identified in its genome, including *bla*$_{NDM-1}$, *bla*$_{OXA-10}$, *bla*$_{PER-4}$, *aph*(3′)-*VI*, *ant*(2″)-*Ia*, *ant*(3′)-*Ia*, *sul1*, *catB8*, *catA1*, *mph*(*E*), and *tet*. Conjugation experiments and whole-genome sequencing revealed that the *bla*$_{NDM-1}$ gene could be transferred to the transconjugant *via* the formation of pJ18004577_NDM, a novel hybrid plasmid. Based on the genetic comparison, the main possible formation process for pJ18004577_*NDM* was the insertion of the [*ΔISKox2-IS26-ΔISKox2*]-*aph*(3′)-*VI*-*bla*$_{NDM-1}$ translocatable unit module from p18004577_*NDM* into plasmid p18004577_Rts in the Russian doll insertion structure (*ΔISKox2-IS26-ΔISKox2*), which played a role similar to that of *IS26* using the "copy-in" route in the mobilization of [*aph*(3′)-*VI*]-*bla*$_{NDM-1}$. The array, multiplicity, and diversity of the resistance and virulence genes in this strain necessitate stringent infection control, antibiotic stewardship, and periodic resistance surveillance/monitoring policies to preempt further horizontal and vertical spread of the resistance genes. Roary analysis based on 30 *P. rettgeri strains* pan genome identified 415 core, 756 soft core, 5,744 shell, and 12,967 cloud genes, highlighting the "close" nature of *P. rettgeri* pan-genome. After a comprehensive pan-genome analysis, representative biological information was revealed that included phylogenetic distances, presence or absence

of genes across the *P. rettgeri* bacteria clade, and functional distribution of proteins. Moreover, pan-genome analysis has been shown to be an effective approach to better understand *P. rettgeri* bacteria because it helps develop various tailored therapeutic strategies based on their biological similarities and differences.

KEYWORDS

Providencia rettgeri*, bla_{NDM-1}, bla_{OXA-10}, bla_{PER-4}, class-1 integrons (*IntI*), *IS26*, *ISKox2

1. Introduction

Providencia rettgeri is an opportunistic human pathogen that belongs to the genus *Providencia*, family Morganellaceae, and order Enterobacterales. It is mainly associated with hospital-acquired infections, including catheter-related urinary tract infections, bacteremia, meningitis, diarrhea, endocarditis, and wound and eye infections (Abdallah and Balshi, 2018). *Providencia rettgeri* exhibits intrinsic resistance to many antimicrobials, including ampicillin, first-generation cephalosporins, polymyxins, and tigecycline (Shin et al., 2018), which makes the treatment of infections caused by this pathogen challenging.

Since the first carbapenem-resistant *Providencia* strain was reported in Japan in 2003 (Shibata et al., 2003), *P. rettgeri* became extremely popular as a carbapenemase producer; the production of metallo-β-lactamase (MBL) carbapenemases, *NDM-1* being the most common, by *P. rettgeri* has gained extensive attention. This indicated that the clinically available β-lactamase inhibitors, including avibactam, relebactam, and vaborbactam, were not effective against infections caused by carbapenem-resistant *Providencia* strains. Moreover, the emergence of multidrug-resistant *P. rettgeri* strains poses a serious threat to public health.

Horizontal gene transfer remains the most effective means of bacterial evolution, allowing bacteria to rapidly acquire new functional genes, including virulence and antibiotic resistance (AR) genes, with the help of mobile genetic elements (MGEs). These MGEs include a series of insertion sequences (ISs), plasmids, prophages, and viruses (Zhong et al., 2019). In recent years, the *IS6/IS26* family of ISs was reported to form cointegrates *via* both, the copy-in and targeted conservative mechanisms (Harmer and Hall, 2020). However, it remains unknown whether other ISs can perform the same reaction. In particular, the role of MGEs in the dissemination of bla_{NDM-1} among *P. rettgeri* strains is not well established.

In this study, we detected a bla_{NDM-1}-producing *P. rettgeri* isolate in a patient with urinary tract infection at a teaching hospital in Shandong, China. Comparative genomic analyses of the plasmids harboring bla_{NDM-1}, bla_{OXA-10}, and bla_{PER-4} were conducted to elucidate the genetic environment and recombination during conjugation. Two copies of the same IS could form a composite transposon capable of mobilizing the intervening components at multiple nested genetic levels, analogous to a Russian doll set, drives rapid dissemination of the carbapenem resistance genes (Feng et al., 2015; Sheppard et al., 2016). To the best of our knowledge, this is the first report that describes a nested insertion structure *ΔISKox2-IS26-ΔISKox2*, playing a role similar to that of the *IS26* isoform in horizontal gene transfer progression.

The pan-genome has become crucial for understanding species diversity and evolution. The pan-genome refers to a complete set of genes present in a collection of organisms. Pan-genome is divided into core and accessory genomes (Tettelin et al., 2005). The core genome is likely essential for the growth or survival of the clade whereas the accessory genome is considered to be composed of major genes to understand variations in the clade's genomes and their specific lifestyles and evolutionary trajectories (Kim et al., 2020).

In this study, a comprehensive analysis of *P. rettgeri* strain genomes, by means of pan-genome analysis, both at the phylogenetical and functional level, may provide useful insights into the different properties of *P. rettgeri* strains.

2. Materials and methods

2.1. Clinical case, clinical strain, and susceptibility assays

Carbapenem-resistant *P. rettgeri* strain 18004577 was recovered from the urine sample of an 18-year-old male patient with chest and abdominal trauma who was admitted to Shandong Provincial Hospital, China, in 2018. The patient was diagnosed with multiple fractures and lung and kidney contusions after falling from a six-story building. Urinary tract infection occurred during hospitalization. Finally, the patient recovered and was discharged. The patient's medication history and hospital course details were retrieved from the hospital record information system. The case history collection and reporting protocols used in the present study were approved by the Ethics Committee of Shandong Provincial Hospital.

Species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (BioMérieux, France). Phenotypic detection of carbapenemases

was conducted using the carbapenem inactivation method (CIM) and the EDTA-modified CIM (eCIM) test, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Antibiotic susceptibility testing for aztreonam, cefepime, ceftriaxone, ceftazidime, ertapenem, imipenem, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, ciprofloxacin, levofloxacin, gentamicin, amikacin, ampicillin, ampicillin–sulbactam, cefazolin, cefotetan, tobramycin, and nitrofurantoin was conducted using the VITEK-2 compact system (BioMérieux, France); the test results were interpreted using CLSI breakpoints (CLSI, 2020).

2.2. Conjugation assay and confirmation of the locations of the resistance genes

Conjugation experiments were conducted as described previously, with sodium azide-resistant *E.coli* J53AziR as the recipient strain and *P. rettgeri* 18004577 as the donor strain. Transconjugants harboring carbapenemase resistance genes were selected on Mueller–Hinton agar plates containing 6 μg/ml ceftazidime and 100 mg/ml sodium azide. Antibiotic susceptibility tests and PCR were performed to confirm carbapenemase gene transfer (Poirel et al., 2011; Xiang et al., 2015).

S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) was performed to determine the size and number of plasmids carried by *P. rettgeri* 18004577 (clinical strain) and J-18004577, a transconjugant. The genomic DNA of the clinical strain and transconjugant embedded in the gel plugs was digested with QuickCut S1 nuclease (Takara, Shiga, Japan) for 1 h or with QuickCut sfi nuclease (Takara, Shiga, Japan) for 2.5 h and then separated using S1-PFGE for 17 h. The pulse time was switched from 2.16 to 63.8 s. *Salmonella* strain H9812 was digested with QuickCut XbaI (Takara, Shiga, Japan) and used as the reference marker. Southern blotting was conducted to confirm the locations of blaNDM−1, blaOXA-10, and blaPER-4 using specific probes labeled with digoxigenin (Roche, Basel, Switzerland; Supplementary Figure 1A) A 621-bp probe for blaNDM-1 was synthesized using PCR amplification with the primers 5′-CGGAATGGCTCATCACGATCCAC-3′ (forward) and 5′-GGTTTGGCGATCTGGTTTTC-3′ (reverse). A 504-bp probe for blaOXA-10 was synthesized using the primers 5′-TCTGCCGAAGCCGTCAATGGT-3′ (forward) and 5′-ATATTCAGGTGCCGCCTCCGTTA-3′ (reverse). Finally, a 504-bp probe for blaPER-4 was synthesized using the primers 5`-GCAATACTCGGTCTCGCACAC′-3′ (forward) and 5′-TGATACGCAGTCTGAGCAACCT-3′ (reverse).

2.3. Whole genome sequencing and annotation

DNA was extracted from the clinical isolates and transformants using a genomic DNA commercial kit (Qiagen, Hilden, Germany). Genomic DNA was sequenced using the Illumina HiSeq platform (Novogene Co., Ltd., Beijing, China) and PacBio RSII sequencer (Biozeron Biological Technology Co., Ltd., Shanghai, China). Paired-end short Illumina reads were used to correct long PacBio reads using proofread for large-scale high-accuracy PacBio correction, and the corrected PacBio reads were then assembled *de novo* utilizing using the functions available at https://github.com/ruanjue/smartdenovo.

Sequence annotation was conducted using RAST 2.0[1] combined with BLASTP/BLASTN searches against UniProtKB/SwissProt and RefSeq databases. Annotation of the resistance genes and mobile elements was conducted using online databases, including CARD[2] and ISfinder.[3] PHAge Search Tool Enhanced Release was used to identify prophages (Arndt et al., 2016). The virulence genes were identified by alignment of the gene sequences against the sequences available in the Virulence Factors Database (Liu et al., 2019).

2.4. Comparison analysis of plasmid sequences

The sequences of five plasmids were retrieved from the NCBI database for comparative analysis: pBML2531 (GenBank accession no. AP022376.1), pNDM15-1091 (GenBank accession no.CP012903.1), pRts1 (GenBank accession no. MN626604.1), pT-OXA-181 (GenBank accession no. NC_020123.1), and Rts1 (GenBank accession no. NC_003905.1).

A BLAST Ring Image Generator[4] was used to generate and visualize the comparisons of the plasmids and their genetic structures. More detailed genome alignment between closely related plasmids was conducted using local BLAST, and the findings were visualized using Easyfig.[5]

2.5. Plasmids stability testing

Plasmid stability testing was performed using Luria–Bertani broth as previously described, but with some modifications (Nang et al., 2018). Briefly, the *P. rettgeri* 18004577 clinical strain was cultured at 37°C in a shaking bath (150 rpm/min) and serially passaged for 7 days with a 1:1,000 dilution of antibiotic-free Luria–Bertani broth. Then, 100 μl of the seventh-day culture was plated onto antibiotic-free Mueller Hinton agar. Subsequently, 50 colonies were randomly selected and subjected to PCR for amplification of the *repA* gene of the plasmid p18004577_Rts. The

1 https://rast.nmpdr.org/
2 http://arpcard.mcmaster.ca
3 https://www-is.biotoul.fr/
4 https://sourceforge.net/projects/brig/
5 http://mjsull.github.io/Easyfig/files.html

TABLE 1 Antibiotic susceptibility testing of *Providencia rettgeri* 18004577 and the transconjugant J-18004577.

Strains		MIC (μg/ml)														
		AMP	SAM	TZP	CZO	CTT	CRO	CAZ	FEP	ATM	ETP	IMP	AMK	CEN	TOB	CIP	LVX	FIT	SXT
P. rettgri	1.8E+07	≥32 (R)	≥32 (R)	64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥8 (R)	≥16 (R)	4 (S)	8 (I)	8 (I)	≥4 (R)	4 (I)	128 (R)	≤20 (S)
Transconjugant	J-18004577	≥32 (R)	≥32 (R)	≥128 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≤1 (S)	≥8 (R)	≥16 (R)	≤2 (S)	≤1 (S)	≤1 (S)	≤0.25 (S)	≤0.25 (S)	≤16 (S)	≤20 (S)

MIC, Minimal inhibitory concentrations; AMP, ampicillin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; CZO, cefazolin; CTT, cefotetan; CRO, cefatriaxone; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IMP, imipenem; AMK, amikacin; CEN, gentamicin; TOB, tobramycin; CIP, ciprfloxacin; LVX, levofloxacin; FIT, nitrofurantion; and SXT, trimethoprim/sulfamethoxazole.

plasmids were considered stable if more than 85% of the colonies harbored the repA gene.

2.6. Pan-genome analysis of the reported *Providencia rettgeri* strains

Combining with sequencing data of 29 published *Providencia rettgeri* genomes from the National Center for Biotechnology Information (NCBI) database, pan-genome analysis was carried out (Supplementary Table 1). The criteria for data-selection was that *Providencia rettgeri* strain with select columns "level" had "complete" genome files available on NCBI in the beginning of our project. We first determined the phylogenetic relationship between the 30 *P. rettgeri* strains using OrthoFinder (Emms and Kelly, 2015), by using the protein sequences of the strains obtained from the Prokaryotic Genome Annotation System (Prokka; Seemann, 2014). Further, the multiple sequence alignment analysis of the resulting single-copy direct-line homologous protein long sequence was carried out using the MAFFT software (Katoh, 2005). Then, we constructed the maximum-likelihood phylogenetic tree of 30 genomes using the RaxML-NG software, with a 1,000-bootstrap test (Kozlov et al., 2019). The calculation of the best amino-acid substitution model was performed using ModelTest-NG software before the phylogenetic tree construction (Darriba et al., 2020). After obtaining the best tree, we visualized the evolutionary tree using the R software with ggtree package (Yu, 2020). Average nucleotide identity (ANI) was performed to explore the taxonomic boundary of the genomes using FastANI (Jain et al., 2018).

After the determination of potential confounding strains, pan-genome analysis was conducted with Roary (Page et al., 2015) using the GFF3 files generated by Prokka (Seemann, 2014). To explore the molecular and biological functions of core genes from the 30 genomes, GO functional enrichment analysis was carried using the R language clusterProfiler package (Wu et al., 2021).

3. Results

3.1. Overview of the *Providencia rettgeri* clinical isolate

Carbapenem-resistant *P. rettgeri* 18004577 was identified as an MBL-producing strain using eCIM. The antimicrobial susceptibility testing results are presented in Table 1. The clinical strain was resistant to almost all of the 18 antibiotics, except for amikacin and trimethoprim/sulfamethoxazole. The transconjugant J-18004577 remained susceptible to amikacin, trimethoprim/sulfamethoxazole, aztreonam, and nitrofurantoin.

According to the whole-genome sequencing analysis, the complete genome of strain 18004577 contained a circular chromosome of 4.6 Mb with a G + C content of 41%, a circular plasmid p18004577_NDM of 273.2 Kb with a G + C content of

47.6%, and a circular plasmid p18004577_Rts of 146.2 Kb with a G + C content of 45.5%.

A total of 10 prophage regions were identified, of which eight were located on the genome and two were located on the plasmid p18004577_NDM (Supplementary Table 2). A CRISPR region was identified in the genome of 18004577 at nucleotide positions 565, 107–565, 213-bp, with one 29-bp long spacer sequence.

3.2. Distribution of virulence genes

The distribution of virulence genes was investigated to identify the key pathogenicity genes of *P. rettgeri* 18004577. The strain harbored several virulence genes, such as *flhA*, *fliC*, *clpB*, *hcp*, *fcl*, and *gmd*, associated with flagellar biosynthesis, type VI secretion system, and O-antigen (Supplementary Table 3).

3.3. Characteristics of plasmids carrying bla_{NDM-1} in the clinical strain and transconjugant

According to the results of S1-PFGE, the clinical strain *P. rettgeri* 18004577 harbored two plasmids of approximately 300 and 100 Kb (Supplementary Figure 1B). Based on the whole-genome analysis, the larger plasmid named p18004577_NDM (GenBank accession no. CP098041.1) was found to harbor multiple resistance genes, including bla_{NDM-1}, bla_{OXA-10}, bla_{PER-4}, *aph*(3′)-*VI* (kanamycin resistance), *ant*(2″)-*Ia* (gentamicin and tobramycin resistance), *ant*(3′)-*IIa* (streptomycin resistance), *sul1* (sulfamethoxazole resistance), *catB8* and *catA1* (chloramphenicol resistance), *mph*(*E*; erythromycin resistance), and *tet* (tetracycline resistance; Supplementary Table 4).

The New Delhi MBL (NDM)-harboring plasmid of the clinical strain was transferred into *E. coli* J53Azi^R *via* conjugation. The presence of NDM-1 in the transconjugant was confirmed using PCR. Two resistance genes were identified in the transconjugant J-18004577––bla_{NDM-1} and *aph*(3′)-*VI*––which were obtained from the clinical isolate *P. rettgeri* 18004577 by conjugation assay (Figure 1). Southern blotting confirmed that bla_{NDM-1}, bla_{OXA-10}, and bla_{PER-4} were located on the larger plasmid in the clinical strain, and only bla_{NDM-1} was present on the hybrid plasmid, with a size of approximately 220 Kb of the transconjugant (Supplementary Figures 1C,D).

p18004577_Rts was found to be a type IncT plasmid with a 146,248-bp size and an average GC content of 45.5%; it had at least 202 predicted coding sequences. No resistance gene was predicted, and it included 15 tra-type genes, except for *trb*, in the transfer region. BLAST analysis showed that p18004577_Rts shared 99% nucleotide identity with 98% cover with plasmid Rts1 from *Proteus vulgaris* (NC_003905.1; Figure 2).

p18004577_Rts (GenBank accession no. CP098042.1) harbored by *P. rettgeri* 18004577 contained a replication initiation protein encoded by *repA* (at 155–1177 bp) and a short segment containing the replication origin ori (at 51–238 bp) that provides two elements for its autonomous replication (Murata et al., 2002), a process which is similar to the one found in plasmid Rts1. The replication of this mini-Rts1 plasmid was stable at 37°C (Itoh et al., 1982) The stability of p18004577_Rts was confirmed using PCR amplification performed after 7 days of culture; 48 of 50 colonies were found to carry *repA*, indicating the high stability of p18004577_Rts.

pJ18004577_NDM (GenBank accession no. CP114206) was a hybrid plasmid generated from p18004577_NDM to p18004577_Rts, with a length of 154,303 bp, a GC content of 45.5%, and 209 predicted coding sequences. The features of the variable region (at 8,779–24,607 bp) of pJ18004577_NDM shared high homology with the bla_{NDM-1}-containing region of p18004577_NDM, and the

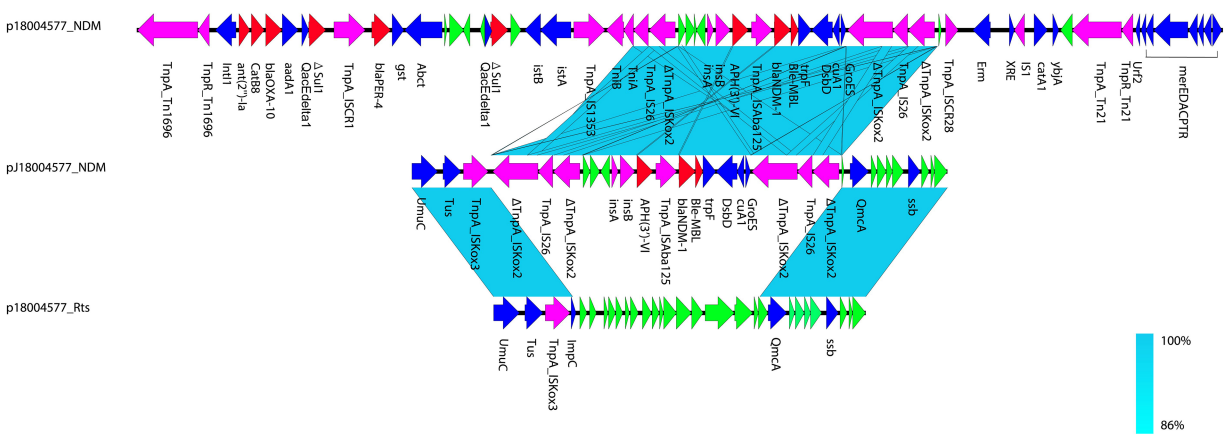

FIGURE 1
Comparison of p18004577_NDM, pJ18004577_NDM, and p18004577_Rts. Colors of the arrows indicate the functions of the predicted ORFs: accessory genes (blue), antimicrobial resistance genes (red), transposon-related genes and insertion sequences (purple), and hypothetical proteins (green). Regions with homology are shaded light blue.

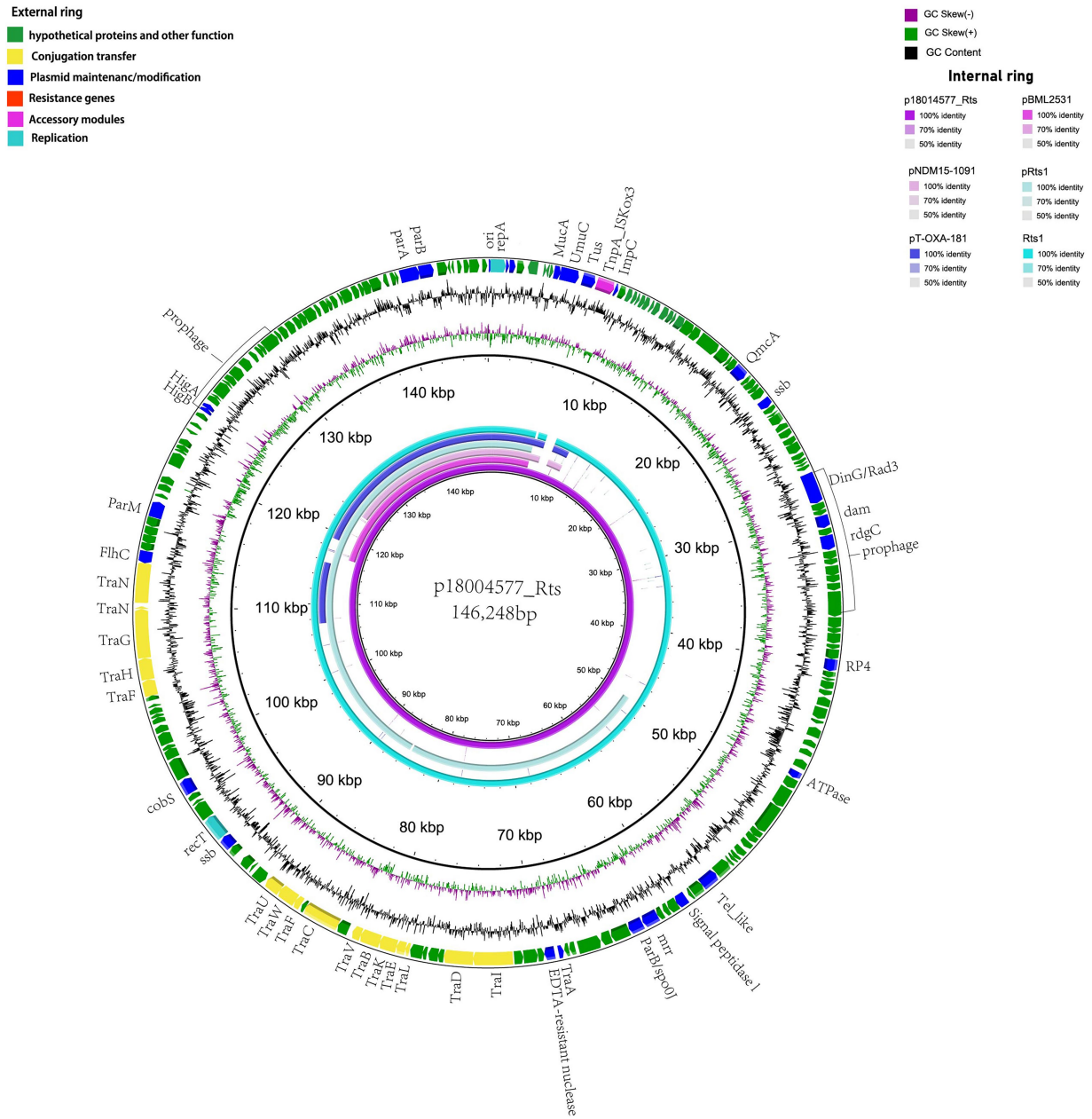

FIGURE 2
Structural comparisons of p18004577_Rts with the similar plasmids. The outer ring represents the CDSs of the reference sequence p18004577_Rts. The internal six rings show the comparative analysis of plasmids pBML2531 (GenBank accession no. AP022376.1), pNDM15-1091 (GenBank accession no.CP012903.1), pRts1 (GenBank accession no. MN626604.1), pT-OXA-181 (GenBank accession no. NC_020123.1), and Rts1 (GenBank accession no. NC_003905.1) with p18004577_Rts.

backbone region of pJ18004577_NDM was almost identical to that of p18004577_Rts (Figure 3).

3.4. Comparative analysis of the genetic environment of blaNDM-1

According to the whole-genome analysis, p18004577_NDM harboring bla_{NDM-1} from clinical strain *P. rettgeri* 18004577 was a 273,271-bp long untypeable plasmid, based on replication module analysis. p18004577_NDM contained an accessory multidrug-resistant region generated by *ΔTn1696*, class 1 integrons (*IntI*) harboring bla_{OXA-10}, *ant* (*2″*)-*Ia*, *catB8*, bla_{PER-4} and *sul*, *ΔIS1326*, *ΔTn125* harboring bla_{NDM-1}, *ΔISKoX2*, *ΔTn21*, *mer* operon, and *ΔTn2501*, which conferred resistance to aminoglycosides, quinolones, and β-lactams. In addition, two prophage regions (at 218,975–233,789 and 288,401–260,814 bp) were predicted, the physiological functions of which are unknown (Figure 4).

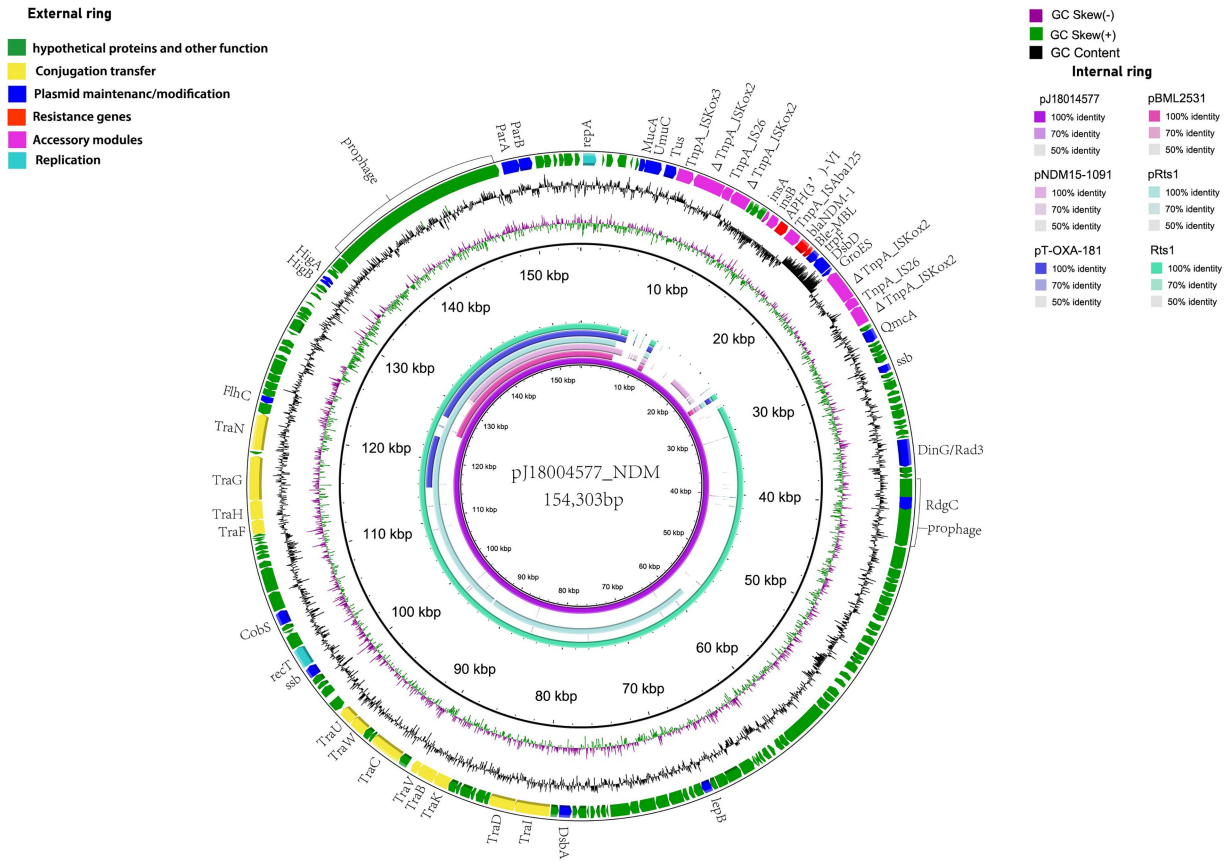

FIGURE 3
Comparisons of genetic context of pJ18004577_NDM with similar plasmids. Red and purple arrows represent antibiotic resistance genes and accessory modules, respectively. The features of the variable region (at 8,779–24,607bp) shared high homology with the bla_{NDM-1}-containing region of p18004577_NDM, and the backbone region was almost identical to that of p18004577_Rts.

In p18004577_NDM, a variant of *Tn1696* was observed upstream of the class I integron, which was found to be similar to pSx1 (accession no. CP013115), pT_OXA_181 (accession no. JQ996150), and *Tn1696* (accession no. U12338; Figure 5). The class I integron (at 27,283–48,006 bp) was flanked by an *IS26* sequence that interrupted the transposase *TniA* at a downstream position, and the cassette content of the class I integron comprised multiple resistance genes, such as bla_{OXA-10}, *ant* (*2′*)-*Ia*, *catB8*, bla_{PER-4}, two *Δsul1*, two *qacEdelta1*, and one *IS1326* element truncated by *IS1353*. The bla_{NDM-1} containing region (at 53,799–58,491 bp) was truncated by *Tn125* that was flanked by an *IS14* element at an upstream location and by *ISKoX2* inserted by *IS26* at a downstream location (Figures 1, 4).

The plasmid pJ18004577_NDM showed partial homology with several other plasmids, such as pBML2531, pNDM15-1,091, pRts1, pT-OXA-181, and Rts1 (Figure 3). The features of the variable region (at 8,779–24,607 bp) of pJ18004577_NDM shared high homology with the bla_{NDM-1}-containing region of p18004577_NDM, and the backbone region of pJ18004577_NDM was highly similar to that of p18004577_Rts.

Based on the results of genetic comparison, the main possible formation process of pJ18004577_NDM was the insertion of the [*ΔISKox2-IS26-ΔISKox2*]-[*aph*(*3′*)-*VI*]-bla_{NDM-1} module from p18004577_NDM (at 48,007–63,004 bp) into plasmid p18004577_Rts (at 8,779–17,992 bp; Figure 1). The insertion module in pJ18004577_NDM seemed to be surrounded by an 8-bp target site duplication with the sequence "GCTGAGAT" (at 8,871–8,878 bp) and "TTGAAGAA" (at 13,292–13,299 bp) at random sites; this may have been due to a cointegrate (Figure 6).

3.5. Main genomic features of *Providencia rettgeri* pan-genome

OrthoFinder assigned 123,540 genes (98.3% of the total) to 6,649 orthogroups. Fifty percent of all genes were in orthogroups with 30 or more genes (G50 was 30) and were contained in the largest 2042 orthogroups (O50 was 2042). There were 2,591 orthogroups with all species present and 2,442 of these consisted entirely of single-copy genes.

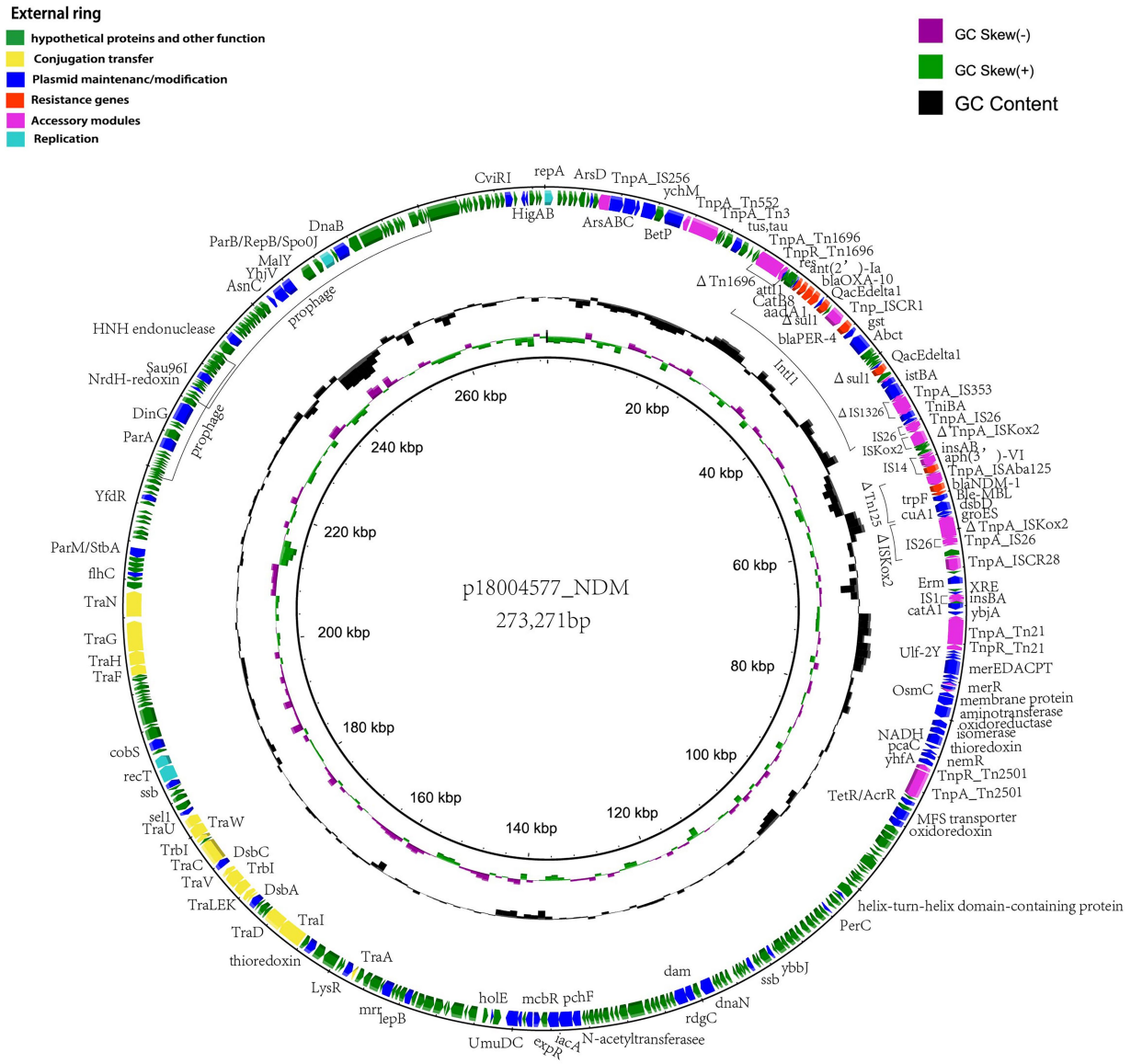

FIGURE 4
Schematic representation of the genetic organization of p18004577_NDM. The outermost ring shows the coding sequences of the plasmid. Each coding sequence is color coded according to its predicted function, as shown in the figure legend. The two inner rings represent the GC content and the GC skew graph, respectively.

The maximum-likelihood phylogenetic tree constructed based on the best amino acid substitution model of 30 genomes single-copy lineal homolog proteins was used to further explore genomic similarities among strains showed in Figure 7A. The analysis of 30 *P. rettgeri* genomes by FastANI showed that higher the association between the microbial species, the higher the ANI values between each other (Figure 7B). The ML phylogenetic tree revealed that strains could be broadly clustered into two major clades base on the genetic distance calculated by the ANI value. The sample sources were scattered among the two clades of phylogenetic trees.

Roary analysis of *P. rettgeri* pan-genome identified 415 core, 756 soft core, 5,744 shell and 12,967 cloud genes. As the number of sequenced genomes increases, the species' pan-genome size converges to a certain value, highlighting the "close" nature of *P. rettgeri* pan-genome. Accordingly, we obtained four different classes of genes belonging to "core" ($29 \leq$ strains ≤ 30), "soft core" ($28 \leq$ strains < 29), "shell" ($4 \leq$ strains < 28), and "cloud" (strains < 4) groups, respectively (Figure 8A). Pan-genomics of the whole dataset was performed, followed by plotting the pan genome matrix. The matrix disclosed the deviance in the presence-absence profile of the 30 *P. rettgeri* strains (Figures 8B–D). The functional enrichment analysis of these 415 core genomes with the results are shown in Supplementary Figure 2.

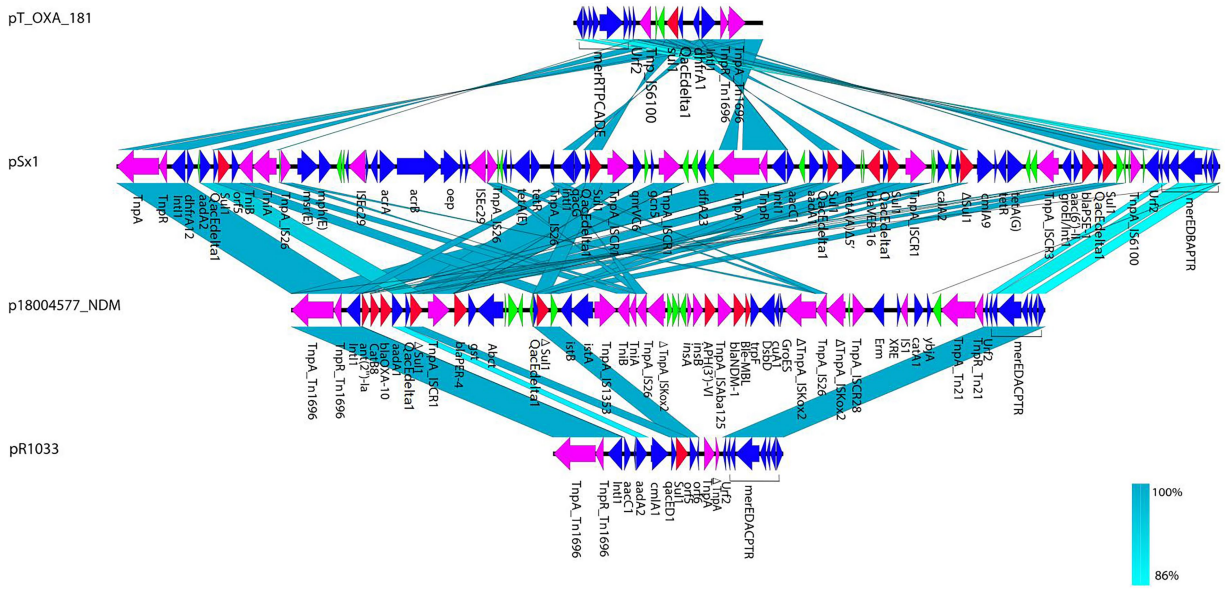

FIGURE 5
Structural comparisons of Tn1696 from p18004577_NDM, pSx1 (accession no. CP013115), pT_OXA_181 (accession no. JQ996150), and pR1033 (accession no. U12338). Arrows indicate the deduced open reading frames (ORFs) and their orientations. Blue arrows were used for ORFs encoding plasmid basic functions and green arrows expressing hypothetical proteins. Resistance genes are indicated by red arrows, while transposon-related genes (tnpA and tnpR) and insertion sequences are indicated by purple arrows.

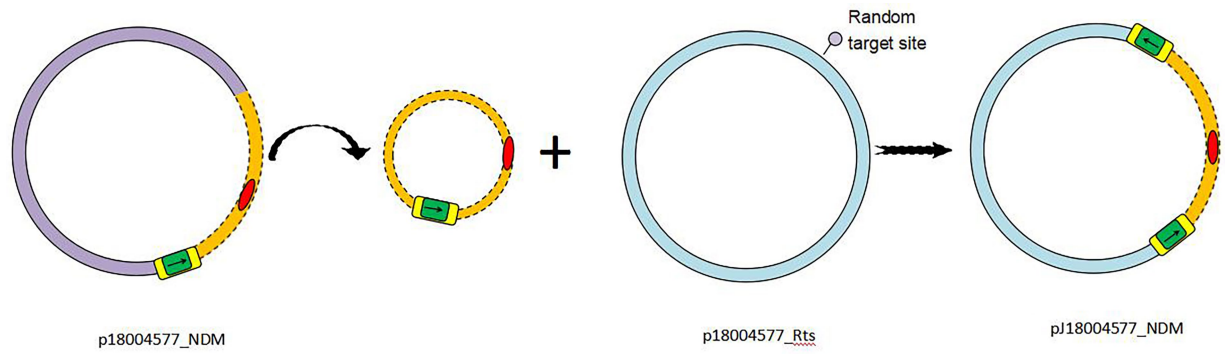

FIGURE 6
Schematic of transconjugant formation. IS26 is shaded green, with the left and right ΔISKox2 shaded yellow. Antibiotic resistance genes are colored red. The p18004577_NDM backbone is colored light purple, whereas the p18004577_Rts backbone is light blue. Small black arrows indicate the orientation of the IS26 and ΔISKox2 genes.

4. Discussion

The first case of carbapenem-resistant *Providencia* was reported in Japan in 2003; since then, carbapenem-resistant *Providencia* has been detected in many countries (Shibata et al., 2003; Abdallah and Balshi, 2018). The first clinical isolate of NDM-1-producing *P. rettgeri* was reported in Israel in 2013 (Gefen-Halevi et al., 2013). The high resistance of *P. rettgeri* to carbapenems is associated with the production of bla_{NDM-1}. Recently, Shen and Huang et al. reported an *NDM-1*, *VIM-1*, and *OXA-10* co-producing *P. rettgeri* strain P138, which has a similar drug resistance spectrum as the strain reported in our study, with resistance to imipenem and ertapenem carbapenems (Shen et al., 2021). The horizontal dissemination of resistance between bacteria can occur *via* conjugative plasmids and integrative conjugative elements (He et al., 2015). In the latest study, Watanabe and Nakano et al. investigated the genetic structure of unique plasmids harboring.

bla_{IMP-70} and $bla_{CTX-M-253}$ in multidrug-resistant *Providencia Rettgeri* and suggested that the cointegration of plasmids in *P. rettgeri* may not be unusual and may play a role in the transmission of clinically relevant b-lactamases (Watanabe et al.,

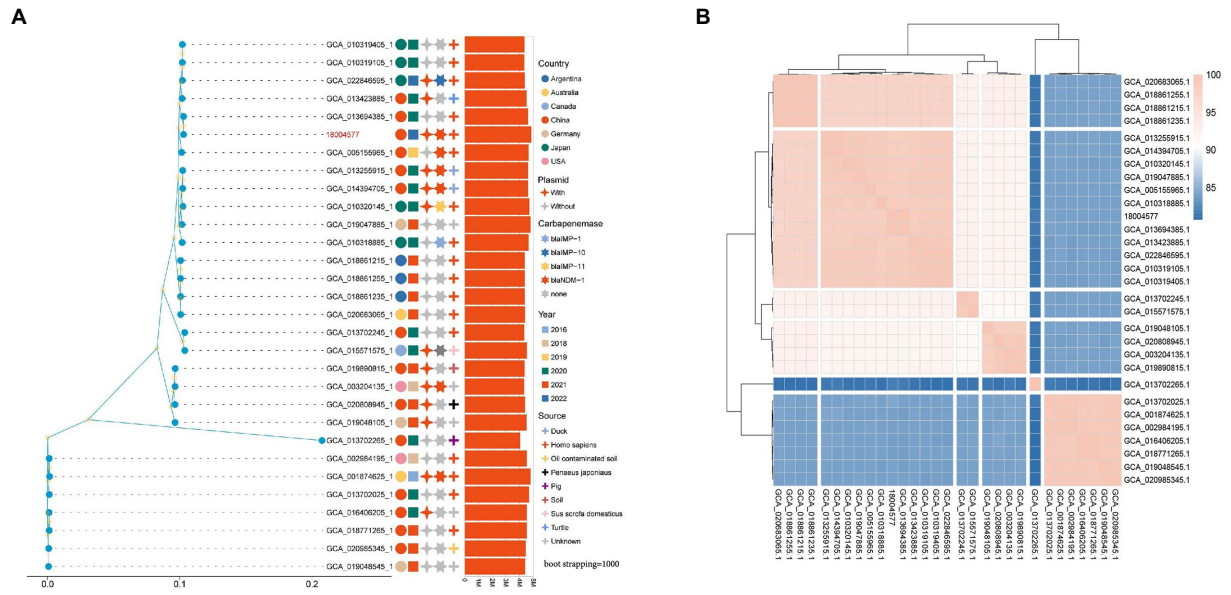

FIGURE 7
(A) Phylogenetic tree of the *Providencia rettgeri* strains. Isolation source, year, and geographical provenience of each strain are reported. The number of plasmids and types of carbapenemase genes were marked with different colors and shapes on the right side of the tree; **(B)** Correlation matrix of average nucleotide identity for the 30 *P. rettgeri* genomes obtained by FastANI.

2022). In our study, one class 1 integron was identified in p18004577_NDM, which harbored multiple resistance genes, such as bla_{OXA-10}, *ant* (2′)-*Ia*, *catB8*, bla_{PER-4}, and two *Δsul1*.

As for insertion sequences, *IS26* elements are known to play a key role in the dissemination of antibiotic resistance genes and are often combined with *Tn125* family transposons (Poirel et al., 2011). *IS6/IS26* family ISs can form cointegrates *via* both copy-in and targeted conservative mechanisms in the recruitment and spread of antibiotic resistance genes for gram-negative bacteria. In p18004577_NDM, the bla_{NDM-1}-containing region was truncated by *Tn125* bracketing with one copy of *ISAba125*, which is likely the original mobilization mechanism of the *en bloc* acquisition of both the bla_{NDM-1} and ble_{MBL} genes (Poirel et al., 2012). Overall, the genetic environment surrounding bla_{NDM-1} involves [*ΔISKox2-IS26-ΔISKox2*]-[*aph*(3′)-*VI*]-bla_{NDM-1}, which occurs in replicative transposition, as confirmed by conjugation experiments. Interestingly, this is the first study that describes its Russian doll insertion structure [*ΔISKox2-IS26-ΔISKox2*], which plays a role similar to that of *IS26* using the "copy-in" mechanism for the mobilization of [*aph*(3′)-*VI*]-bla_{NDM-1}. We tried to detect circular intermediates of [*ΔISKox2-IS26-ΔISKox2*], but did not succeed.

The prototype IncT large plasmid Rtsl was originally isolated from a clinical strain of *Pr. vulgaris* (Terawaki et al., 1967) and expressed pleiotropic thermosensitive phenotypes in autonomous replication (DiJoseph, 1974), conjugative transferability (Terawaki et al., 1967), host cell growth (Terawaki et al., 1967; DiJoseph et al., 1973), and T-even phage restriction (Janosi et al., 1994). In our study, the p18004577_Rst strain shared 99% nucleotide identity with 98% cover with plasmid Rts1, but its temperature-sensitive replication needs further assessment.

In this study, we relied on publicly available complete genome sequences to construct a phylogenetic tree of globally reported *P. rettgeri* strains. The relationship and epidemiological distribution of all of the deposited *P. rettgeri* genomes in GenBank are depicted in Figure 7A Interestingly, globally bla_{NDM-1}-producing *P. rettgeri* isolates were mainly reported in China, whereas bla_{IMP}-producing isolates were found in Japan (Figure 7A and Supplementary Table 1). Meanwhile, carbapenemase producers were dispersedly distributed among all *P. rettgeri* strains. This suggests that the subsequent evolution of carbapenemase-producing *P. rettgeri* strains can be mainly attributed to the acquisition of genetic material through horizontal gene transfer of mobile genetic elements during the spread of these strains globally. However, this study provides a comprehensive pan-genome analysis of *P. rettgeri*. The dissection of *P. rettgeri* pan-genome into the four different gene categories ("core," "soft core," "shell," and "cloud") will facilitate genetic engineering strategies for genomic reduction/optimization. Furthermore, understanding the origin of isolation of each strain and their niche-specific adaptation can surveillance and prevent the spread of resistant strains.

5. Conclusion

The pathogenicity of the multidrug-resistant *P. rettgeri* 18004577 strain reveals the significant mobilome of this pathogen, and the presence of resistance genes, such as bla_{NDM-1}, bla_{PER-4}, and bla_{OXA-10} contribute significantly to carbapenem resistance in *P. rettgeri*. Taken together, our findings enhanced the knowledge of the diversity of pathogenicity, antibiotic resistance, and mobilome of the genus *Providencia*. *Providencia*

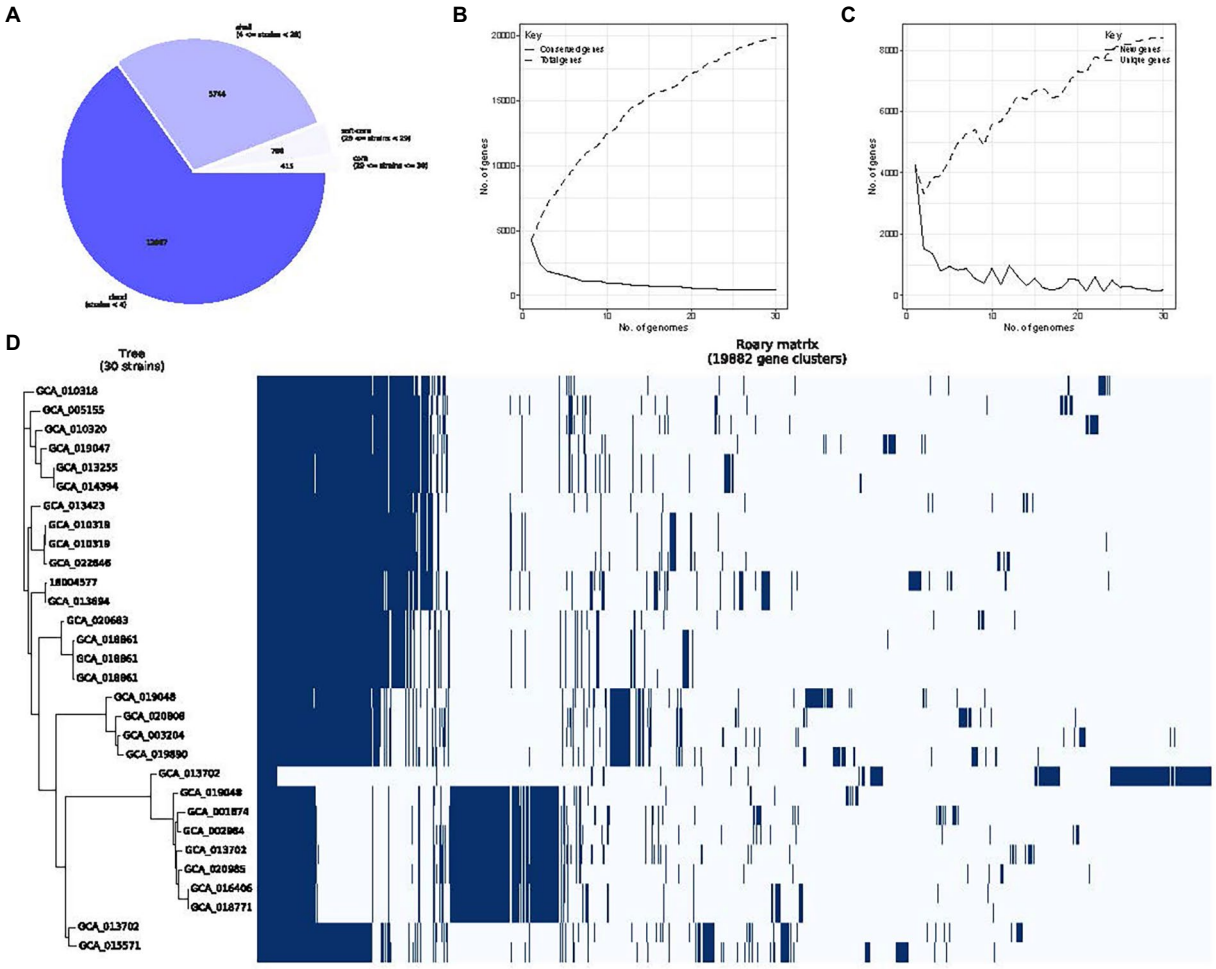

FIGURE 8
Providencia rettgeri pan-genome. **(A)** The number of genes belonging to the core, soft core, shell or cloud of the *P. rettgeri* species is represented as a pie chart; **(B)** Conserved genes frequency vs. genome number; **(C)** Representation of *P. rettgeri* gene content according to the pan-genome variation since the genomes are added in random order to the analysis. The short line represents unique genes; the long line represents new genes; and **(D)** The gene presence-absence profile of *P. rettgeri* strains.

rettgeri could be reservoir carbapenemase genes and can transmit these genes to other organisms *via* horizontal gene transfer. In the future, researchers should aim to increase and enhance the monitoring of carbapenemase and perform combined antimicrobial susceptibility tests to seek an effective therapeutic regimen for infections caused by Carbapenem-resistant Enterobacteriaceae strains. The comparative genomic analysis conducted in this study provides new insights into the genomic content and variability of *P. rettgeri* confirming that the genomic screening of new strains is essential since the bacterial genomes are dynamic entities.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number CP114206.

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

YH contributed to experiment conception and design. YY and MZ conducted bioinformatics analysis and wrote the paper. YB and ZS performed data analysis. RC and YW carried out bacteria identification. XL and QW prepared the tables and figures. YH is responsible for submitting a competing interests' statement on behalf of all authors of the paper. 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

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Detection of *Klebsiella pneumonia* DNA and ESBL positive strains by PCR-based CRISPR-LbCas12a system

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Introduction: *Klebsiella pneumonia* (*K. pneumonia*) is a Gram-negative bacterium that opportunistically causes nosocomial infections in the lung, bloodstream, and urinary tract. Extended-spectrum β-Lactamases (ESBLs)-expressed *K. pneumonia* strains are widely reported to cause antibiotic resistance and therapy failure. Therefore, early identification of *K. pneumonia*, especially ESBL-positive strains, is essential in preventing severe infections. However, clinical detection of *K. pneumonia* requires a time-consuming process in agar disk diffusion. Nucleic acid detection, like qPCR, is precise but requires expensive equipment. Recent research reveals that collateral cleavage activity of CRISPR-LbCas12a has been applied in nucleic acid detection, and the unique testing model can accommodate various testing models.

Methods: This study established a system that combined PCR with CRISPR-LbCas12a targeting the *K. pneumoniae* system. Additionally, this study summarized the antibiotic-resistant information of the past five years' *K. pneumoniae* clinic cases in Luohu Hospital and found that the ESBL-positive strains were growing. This study then designs a crRNA that targets *SHV* to detect ESBL-resistant *K. pneumoniae*. This work is to detect *K. pneumoniae* and ESBL-positive strains' nucleic acid using CRISPR-Cas12 technology. We compared PCR-LbCas12 workflow with PCR and qPCR techniques.

Results and Discussion: This system showed excellent detection specificity and sensitivity in both bench work and clinical samples. Due to its advantages, its application can meet different detection requirements in health centers where qPCR is not accessible. The antibiotic-resistant information is valuable for further research.

KEYWORDS

CRISPR-Cas, nucleic acid detection, *Klebsiella pneumonia* (*K. pneumonia*), Extended-spectrum β-lactamases (ESBL), SHV

Introduction

Klebsiella pneumonia (*K. pneumonia*) is a family of Gram-negative bacteria that causes nosocomial infections in the bloodstream, wound, and urinary tract (Magill et al., 2014). Hypervirulent *K. pneumoniae* strains, such as K1, K2, and K5, have emerged worldwide and caused

severe infections, including liver abscess and pneumonia, with a mortality rate as high as 20–30% (Podschun and Ullmann, 1998).

β-Lactamases (ESBLs) can degrade β-Lactam antibiotics into non-effective compounds, thus resulting in drug-resistant strains (Bush and Bradford, 2016). However, due to the excessive use of β-Lactam antibiotics, the prevalence of ESBL-producing *K. pneumonia* has primarily increased. To date, ESBL-producing *K. pneumonia* contributes to nearly 45% of *K. pneumoniae* nosocomial infections (Miftode et al., 2021) and 43% in the intensive care unit (Paterson et al., 2004; Calbo et al., 2011). More strikingly, ESBL-positive strains result in significantly higher mortality (Miftode et al., 2021); a recent study reported that ESBL-producing *K. pneumonia* is associated with over 55% mortality (Starzyk-Luszcz et al., 2017). Despite various genes encoding ESBLs, most ESBLs were derived from one or two amino acid substitutions of SHV-1 and TEM-1 (Ramdani-Bouguessa et al., 2011; Ben Achour et al., 2014). *SHV*-type ESBLs almost evolved from *SHV-1*; for example, *SHV-2* harbors G238S (Zhong et al., 2021). Over 100 *SHV* variants have been found,[1] and most of which are associated with ESBL-positive strains. Until 2016, the *SHV*-type ESBLs only accounted for 10% ESBLs. However, the majority are found in *K. pneumoniae* (Castanheira et al., 2016). Thus, the detection of *SHV* is valuable in identifying ESBLs in *K. pneumoniae*.

Klebsiella pneumonia colonies derived from clinical samples under 24–48 h of 37°C incubation culture after disk diffusion present features that could be diagnosed by well-trained personnel (Wagner et al., 2016). Usually, 1 week is required to determine the specific antibiotic resistance of bacterial strain (Giske et al., 2022). Despite the low cost and the simplicity of operation, disk diffusion is time-consuming and sometimes produces false-negative results especially for atypical colonies (Johnson et al., 2006; Hutchison et al., 2018). In contrast, nucleic acid detection methods that detect *K. pneumoniae*-specific DNA fragments are more advantageous. For example, quantitative real-time PCR or qPCR is the most widely used one (Hyun et al., 2019). The *K. pneumonia* and specific drug-resistant strains can be determined by qPCR (Yan et al., 2021). For ESBL testing, *SHV-1*, *TEM*, and *CTX-M* gene DNA fragments are used (Souverein et al., 2017). However, qPCR requires expensive equipment, which restricts its application in the healthcare center.

The CRISPR-Cas systems have been discovered to cleave target DNA or RNA under the guidance of crRNA in a base-pairing manner (Yan et al., 2019). Recent research shows that Cas13 and Cas12 exhibit collateral cleavage activity that could degrade probes if crRNA perfectly base-pairs targeted RNAs or DNAs (Chen et al., 2018; Gootenberg et al., 2018). Combined with DNA amplification and signal detection methods, Cas12 and Cas13 have been applied to detect nucleic acid (Li et al., 2019). The detection workflow only requires 37°C incubation and does not rely on complicated equipment. To combat COVID-19, lots of nucleic acid detection workflow based on Cas13 and Cas12 have been developed (Kostyusheva et al., 2021; Nouri et al., 2021). Both Cas12 and Cas13 require no expensive equipment and are compatible with various amplification or detection methods.

Despite these advantages, the CRISPR-Cas12 detection method targeting *K. pneumonia* has not been reported. We established a sensitive nucleic acid detection method based on CRISPR-Cas12 and PCR to detect *K. pneumonia*. It produces results in less than 2 h and requires less expensive equipment (Figure 1A). Furthermore, we tried to identify ESBLs by targeting *SHV* DNA fragments. Compared to the traditional detection methods, this trial may help community healthcare centers to accomplish nucleic acid detection.

Results

Establishment of PCR-LbCas12a detection targeting *Klebsiella pneumonia*

To find a suitable target for nucleic acid detection, we used blast to screen the most relevant gene as a target for nucleic acid detection. We downloaded 14,288 genomic sequences from NCBI and removed the sequences under 5.2 M, for this is a complete genomic DNA size for *K. pneumonia*. As a result, 2,024 sequences survived for further analysis. We found that *IF-2*, *16S RNA*, and *YP_005224572.1* compose 99.70% (2018/2024), 98.86% (2001/2024), and 99.70% (2018/2024) of all the sequences (Figure 1B). Therefore, they are suitable targets for nucleic acid detection. Considering the TTTV PAM sequences required for LbCas12a targeting, we designed five 36-nt long crRNAs which share 19 nt standards nucleotides and have 17 nt unique sequences to base-pair different *K. pneumoniae DNA* targets. K.P.-crRNA-1,2 target 16S RNA, K.P.-crRNA-3,4 target *YP_005224572.1*, and K.P.-crRNA-5 targets *IF-2*. We also screened the *K. pneumonia* genomes and found that these five crRNA targets separately 98.86% (2001/2024), 98.67% (1997/2024), 95.90% (1941/2024), 98.81% (2000/2024), and 99.90% (2022/2024) of the genomes (Figure 1C). To quickly test the detecting efficiency of the crRNAs, chemically synthetic single-stranded DNA targets were added to the LbCas12a system. Compared to the negative control, all crRNAs generated a significantly high level of FAM signals (Figure 1D). Further, the free FAM group generated by digesting probes can generate 488 nm fluorescent under 360 nM UV light exposure (Wang et al., 2022). We photographed the tube after the Cas12a reaction (Figure 1E). The K.P.-crRNA-2 and K.P.-crRNA-4 generate potent and stable FAM signals.

Next, we test the primers to amplify DNA fragments for crRNA-2 and crRNA-4. The amplification efficiency of *YP_005224572.1* primers is far more efficient than that of *16S* (Figure 1F). To test the specificity one step further, we blast the K.P.-crRNA-2,4, *16S*, *YP_005224572.1* in other *Klebsiella* strains (Supplementary Table 1). The results showed that the K.P.-crRNA-2 targets all of them, while the K.P.-crRNA-4 perfectly targets only *Klebsiella oxytoca*. *Klebsiella michiganensis*, *Klebsiella variicola*, and *Klebsiella Africana* have mutations on the targets, which might not be detected. As a result, K.P.-crRNA-4 and the primers targeting the *YP_005224572.1* gene are used to establish this system to detect the *K. pneumoniae* strain (Figure 1A).

PCR-LbCas12a is sensitive and specific in *Klebsiella pneumonia* nucleic acid detection

To explore the minimum amount of DNA sample required for nucleic acid detection, serial diluted standard DNA samples were to test PCR, qPCR, and PCR-LbCas12a techniques. Basic PCR exhibited

1 https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/SHV

Abbreviations: PCR, Polymerase chain reaction; *K. pneumonia*, *Klebsiella pneumonia*; UTI, Urinary tract infection; BSI, Bloodstream infections; ESBL, Extended-spectrum β-lactamases.

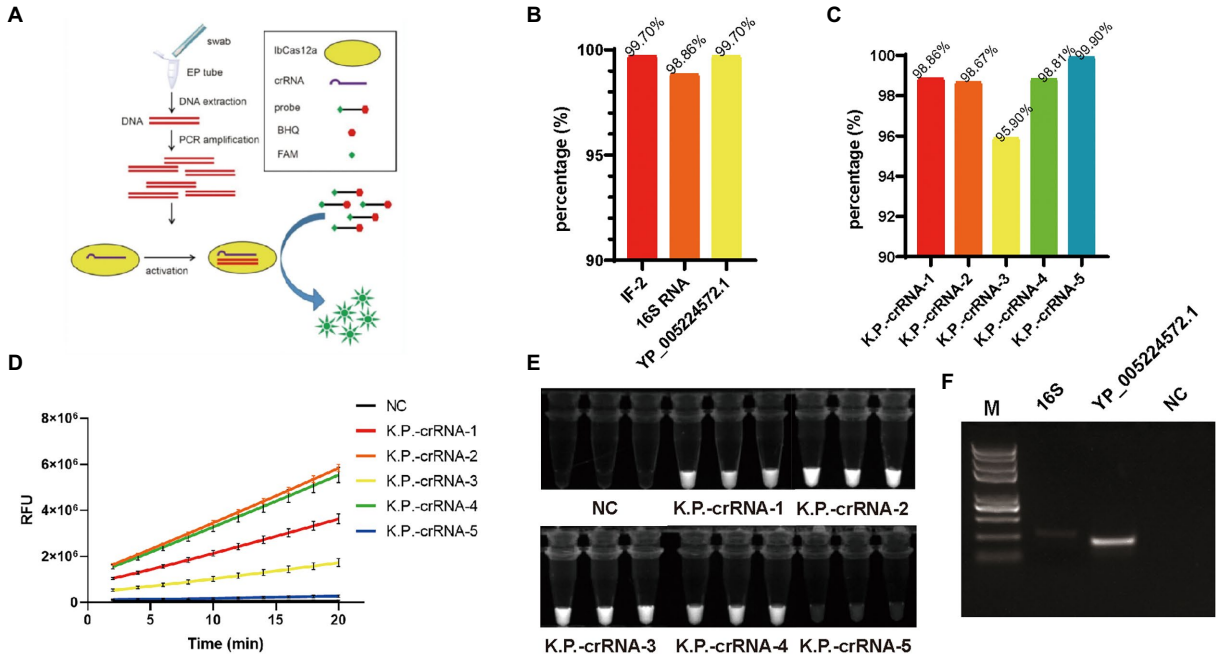

FIGURE 1
(A) Graphical illustration of PCR-LbCas12a detecting *Klebsiella pneumonia* nucleic acid. **(B)** The proportion of *IF-216SRNA* and *YP_005224572.1* in the 2024*K. pneumonia* genomes. **(C)** The percentage of each K.P.-crRNA hitting the 2024*K. pneumonia* genomes. **(D)** FAM signal of five K.P.-crRNA activity in LbCas12a reaction. **(E)** PCR-LbCas12a reaction products of **(D)** were photographed under UV activation. **(F)** The PCR amplification efficiency of primers targeting*16S* and *YP_005224572.1*.

positive signals when target DNA was as few as 10 copies (Figures 2A–C). In contrast, the LbCas12a system and qPCR can display a signal in 40 min when the copy number is as few as one single copy (Figure 2A). Next, to confirm if the detection system's specificity targets only *K. pneumoniae*, we applied PCR-LbCas12a detection in 10 commonly seen pathogens in laboratory department, including *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Shigella dysenteriae*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Corynebacterium striatum*, and *Candida albicans* (fungi).

As a result, after 40 min PCR-Cas12 reactions, only *K. pneumonia* generated a significantly higher FAM signal (Figure 2D). The PCR-LbCas12a detection system is sensitive and specific.

PCR-LbCas12a detects clinic samples

To apply this PCR-LbCas12a detection system to clinic use, we collected 89 sputum samples tested using disk diffusion. Total DNA was extracted from the samples. Then, PCR was processed and followed by LbCas12a incubation at 37°C for 20 min. Results were collected by photographing samples under UV light exposure (Supplementary Figure S1). Most of the positive and the negative samples were identical. Six samples were only positive in disk diffusion assay, and seven samples were only positive in PCR-LbCas12a workflow (Figure 2E). To verify the controversial samples, we PCR amplified them and processed Sanger sequencing. The Sanger sequence results showed that they are nearly 100% identical to the *K. pneumonia YP_005224572.1* gene fragment. Meanwhile, seven samples that are positive failed to be detected in this system. This failure might result from the corruption of the sputum samples, which were not well preserved after disk diffusion for diagnosis.

Detection of *SHV* In ESBL-producing *Klebsiella pneumonia*

To start the task, we looked up the drug-resistant information of *K. pneumonia* in the medical laboratory department's record since 2018. The ESBL-positive strains' percentage is increasing from 22.9% (2018) to 40.3% (2020) and then remains at a relatively high level (Figure 3A). This information suggests that the identification of ESBL-positive strains is valuable. *SHV*-associated ESBLs are the most commonly reported groups, so we designed four crRNAs that target *SHV* to detect ESBLs. We also screened the 184 reported SHV genes by this four crRNAs, and found that SHV-crRNA1-4 target 93.47% (172/184), 93.47% (172/184), 93.47% (172/184), and 98.91% (182/184) of 184 SHV genes (Figure 3B).

Using the PCR-LbCas12a workflow, we tested the four crRNAs efficiencies. Although all the crRNAs are effective (Figures 3C, D), the SHV-crRNA-4 is the most efficient. The PCR-LbCas12a can test as few as one copy of the SHV-1 DNA fragment (Figure 3E).

Next, we detected the clinic samples. We collected 18 *K. pneumoniae* clinic samples that had been tested by disk diffusion susceptibility test. We tried it in our PCR-LbCas12a workflow and compared it with the clinic diagnosis (Figure 3F). All the ESBL (+) samples were positive in our workflow. Additionally, 10 more ESBL (−) were tested positive in PCR-LbCas12a detection. Sanger sequencing results suggest that three of them contain the *SHV-1* DNA fragment; the rest seven samples failed to be detected by PCR.

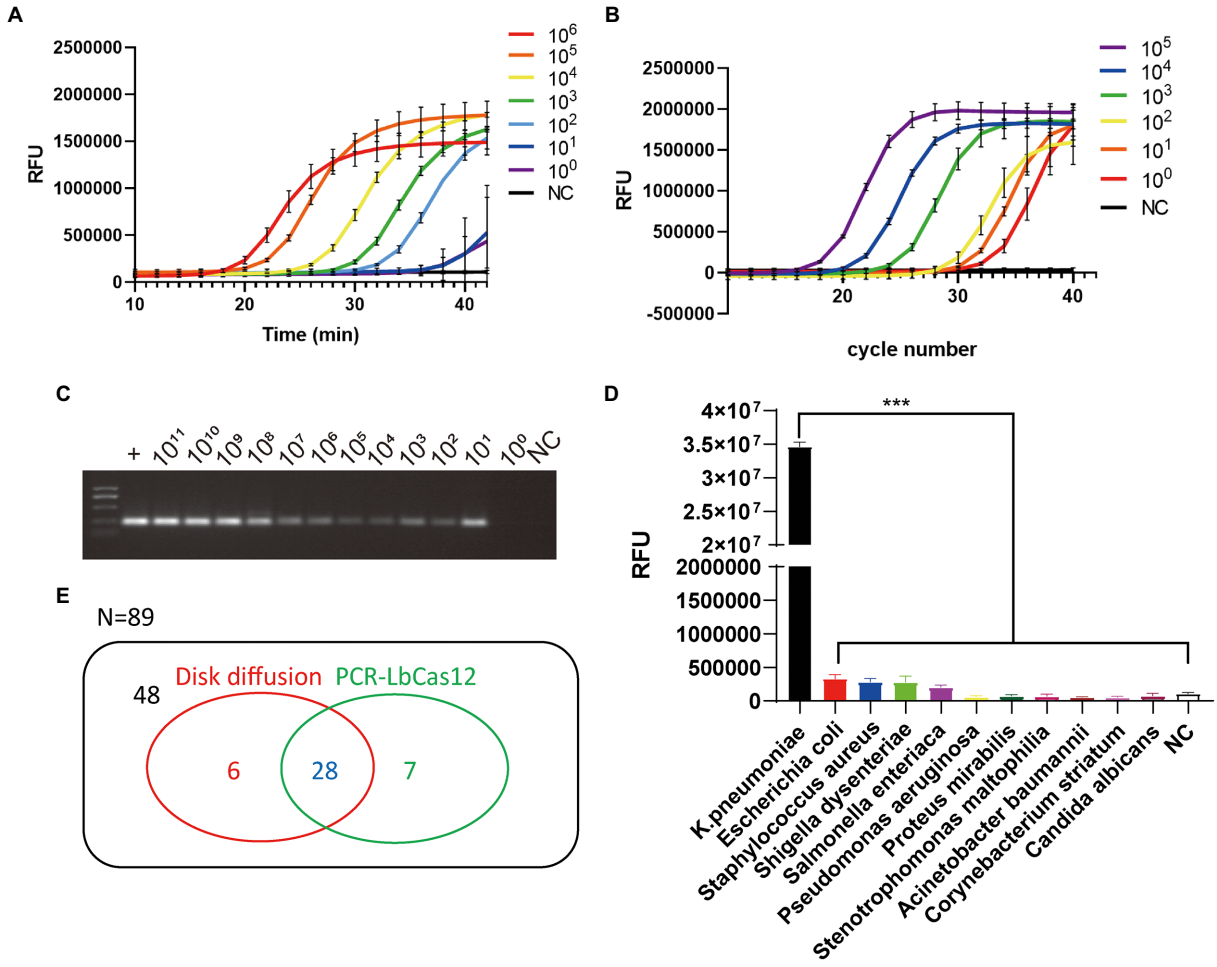

FIGURE 2
(A) The RFU signal generated by the PCR-LbCas12a system detecting the serially diluted standard *YP_005224572.1* DNA. **(B)** The qPCR results of serial diluted standard DNA samples. **(C)** 1% agarose gel electrophoresis of the PCR product from serial diluted standard DNA samples. **(D)** Different pathogens was detected by PCR-Cas12a system. Only *Klebsiella pneumoniae* was successfully detected. Data are mean±s. d. of *n*=3 biological independent experiments. **(E)** Venn diagram of results of testing 89 sputum samples in disk diffusion assay and PCR-LbCas12a workflow.

Discussion

The excessive use of antibiotics increases drug-resistant bacteria. A recent discovery reveals that 73.1% of *K. pneumonia* are resistant to at least one antibiotic (Petrosillo et al., 2019; Sharahi et al., 2021). Multi-drug resistant and extensively drug-resistant *K. pneumonia* strains are increasing (Peng et al., 2020; Yan et al., 2021; Yang et al., 2021). Effectively controlling *K. pneumonia* requires the direct knowledge of drug-resistant information (Ludden et al., 2020).

The most used detection method is the disk diffusion antibiotic susceptibility test (Prastiyanto et al., 2020). However, the sensitivity and accuracy are only about 56% and 65% (Koyuncu and Haggblom, 2009). Furthermore, two rounds of tests are required to identify specific antibiotic information, which costs more time. In comparison, nucleic acid detection is more advantageous in terms of stability and accuracy. qPCR specifically and accurately identifies *K. pneumonia* from bacteria like *E. coli* and *S. aureus* (Kim et al., 2021) and its antibiotic-resistant gene (Castanheira et al., 2021). However, qPCR needs expensive equipment and skilled workers (Corman et al., 2020).

Recently, CRISPR-Cas12-mediated trans-collateral activity was widely applied to nucleic acid detection (Li et al., 2019; Ma et al., 2021; Selvam et al., 2021). The CRISPR-Cas12 detection system is accurate and specific and can also combine various readout and amplification technologies (Ali et al., 2020; Chen et al., 2020; Ramachandran et al., 2020).

In this study, we first established the PCR-LbCas12a system in this study to detect *K. pneumoniae* nucleic acid and *SHV* genes. We used PCR to harvest enough target DNA as a substrate for the LbCas12a reaction. The PCR-LbCas12a detection system can detect as low as only one copy of *K. pneumonia*. The results were highly consistent with the disk diffusion test. The clinic information on drug-resistant *K. pneumonia* showed that the ESBL (+) strains have been increasing over the past 5 years. We also tried the workflow to detect ESBL-resistant strains by detecting *SHV* fragments. The detection of *SHV* is successful. However, most of the positive strains are not ESBL (+) in disk diffusion tests. We speculate that this contradiction may result from the following reasons:

1. The disk diffusion might be too strict about detecting the *K. pneumonia* strains that are less resistant.
2. The *K. pneumonia* samples may not be well preserved to grow on the agar disk.
3. Many of the *SHV* variants do not encode ESBL enzymes.

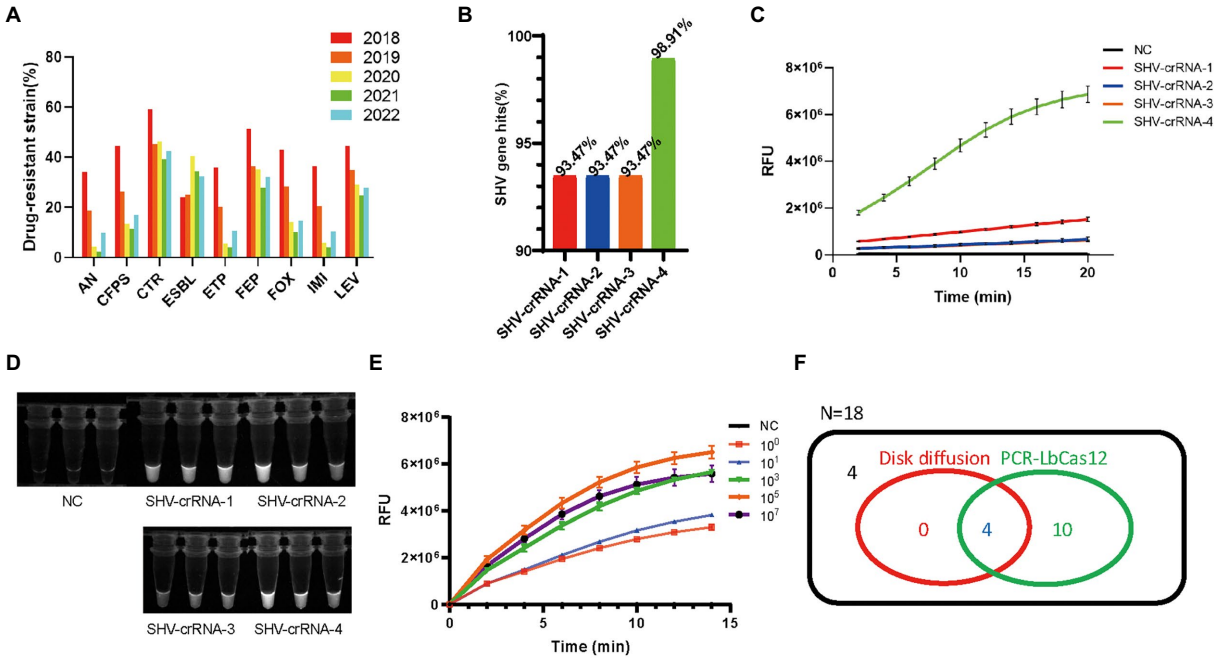

FIGURE 3
(A) The summary of drug-resistant *Klebsiella pneumonia* information in the Medical Laboratory Department of Shenzhen Luohu People's Hospital from January 2018 to June 2022. **(B)** The percentage of *SHV* genes hit by four SHV-crRNAs. FAM signal, **(C)** and photograph under UV, **(D)** of four SHV-crRNAs activity in PCR-LbCas12a reaction. **(E)** The RFU signal generated by the PCR-LbCas12a system detecting the serially diluted standard *SHV* DNA. **(F)** Venn Diagram of results of testing ESBL of 18K. pneumonia samples in PCR-LbCas12a and disk diffusion susceptibility test.

Nevertheless, this workflow exhibits the potential to detect specific DNA fragments. Accurately detecting specific antibiotic-resistant strains needs more adjustment and knowledge of the mechanism. Compared to disk diffusion, PCR-LbCas12a detection, which takes no more than 2 h, is highly advantageous in time-consuming.

Materials and methods

Bioinformatics analysis and scripts

The source is downloaded from NCBI-genome. The R and Python scripts are prepared by Guoyu Peng. The detailed information is on https://github.com/GuoYu-Peng/GANAB_BLCA.

Nucleic acid preparation

crRNAs were designed to target *16sRNA*, *YP_005224572.1*, and *IF-2* gene according to the protocol (Chen et al., 2018). RNA nucleotides were chemically synthesized without 5′-phosphorylation (Transheep, China). crRNA consists of 19 nt common sequences and 17 nt for recognizing target (Li et al., 2018). DNA and RNA sequences used in this manuscript are in the Supplementary material.

DNA extraction and quantification

Clinical samples were swabs of sputa. According to the manufacturer's protocol, swabs were dipped in cell lysate and processed genomic DNA extraction using the DNA extraction Kit (Tianlong science & technology, China). Extracted DNA samples were quantified by NanoDrop (Thermo Fisher Scientific, US) and preserved at −80°C before use.

PCR and qPCR

PCR system was carried out in a 20 μl reaction system in the 0.2 ml EP tube. Each reaction contains 10 μl of PrimeSTAR (TAKARA, Japan) PCR premix, 1 μl of forward primer (10 nM) and 1 μl of reverse primer (10 nM), 10 ng of sample DNA, and ddH_2O to supplement the volume to 20 μl. The PCR reactions were processed for 35 cycles on an Eppendorf thermocycler with denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s. DNA electrophoresis was processed in 1% agarose gel in TAE buffer.

qPCR reactions were processed using Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (Yeasen, China) on QuantStudio Dx (ABI, US). Program started with a 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s qPCR. Each reaction was repeated in three biologically independent experiments.

PCR-LbCas12a detection

The LbCas12a detection was carried out in a 20 μl system. The system contains 2 μl Buffer 3 (NEB, US), 50 nM LbCas12a protein, 60 nM crRNA, and 30 nM labeled probe, and 100 ng purified PCR product. Samples were mixed and then incubated at 37°C, and signals

were obtained from QuantStudio Dx (ABI, US) every minute for 20–60 min. Each reaction was repeated in three biologically independent experiments.

For clinic detection, samples were incubated at 37°C for 20 min, and then photographed under the UV light exposure. Two independent experiments were processed for each sample.

Sample information

Sample information is available in Supplementary Table 2.

Study approval

The Luohu Ethics Committee approved the project [LLBGS (2021) 020].

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 Luohu Ethics Committee approved the project [LLBGS (2021) 020]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ShangW conceived this idea. ShangW, ShanW, and YT processed the experiments. GP analyzed the data. TH, XQ, and JW collected the clinic samples. XW extracted the DNA. YL, SZ and SoW offered the funding and the platform. 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.2023.1128261/full#supplementary-material

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The mobile gene cassette carrying tetracycline resistance genes in *Aeromonas veronii* strain Ah5S-24 isolated from catfish pond sediments shows similarity with a cassette found in other environmental and foodborne bacteria

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Aeromonas veronii is a Gram-negative bacterium ubiquitously found in aquatic environments. It is a foodborne pathogen that causes diarrhea in humans and hemorrhagic septicemia in fish. In the present study, we used whole-genome sequencing (WGS) to evaluate the presence of antimicrobial resistance (AMR) and virulence genes found in *A. veronii* Ah5S-24 isolated from catfish pond sediments in South-East, United States. We found *cphA4*, *dfrA3*, *mcr-7.1*, *valF*, bla_{FOX-7}, and bla_{OXA-12} resistance genes encoded in the chromosome of *A. veronii* Ah5S-24. We also found the tetracycline *tet(E)* and *tetR* genes placed next to the IS*5*/IS*1182* transposase, integrase, and hypothetical proteins that formed as a genetic structure or transposon designated as IS*5*/IS*1182*/*hp*/*tet(E)*/*tetR*/*hp*. BLAST analysis showed that a similar mobile gene cassette (MGC) existed in chromosomes of other bacteria species such as *Vibrio parahaemolyticus* isolated from retail fish at markets, *Aeromonas caviae* from human stool and *Aeromonas media* from a sewage bioreactor. In addition, the IS*5*/IS*1182*/*hp*/*tet(E)*/*tetR*/*hp* cassette was also found in the plasmid of *Vibrio alginolyticus* isolated from shrimp. As for virulence genes, we found the tap type IV pili (*tapA* and *tapY*), polar flagellae (*flgA* and *flgN*), lateral flagellae (*ifgA* and *lfgL*), and fimbriae (*pefC* and *pefD*) genes responsible for motility and adherence. We also found the hemolysin genes (*hylII*, *hylA,* and *TSH*), *aerA* toxin, biofilm formation, and quorum sensing (*LuxS*, *mshA,* and *mshQ*) genes. However, there were no MGCs encoding virulence genes found in *A. veronii* AhS5-24. Thus, our findings show that MGCs could play a vital role in the spread of AMR genes between chromosomes and plasmids among bacteria in aquatic environments. Overall, our findings are suggesting that MGCs encoding AMR genes could play a vital role in the spread of resistance acquired from high usage of antimicrobials in aquaculture to animals and humans.

KEYWORDS

Aeromonas veronii, antimicrobial resistance, mobile gene cassette, virulence, tetracycline, environment, foodborne

1. Introduction

Aeromonas veronii is a Gram-negative bacterium ubiquitously found in different aquatic environments. It was first reported by Hickman-Brenner et al. (1987) as a new species in 1983. It is pathogenic to several fish species that include the top farmed species such as common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis niloticus*), and pangasius (*Pangasius hypophthalmus*) (González-Serrano et al., 2002; Smyrli et al., 2017, 2019; Wang et al., 2022). It causes hemorrhagic septicemia and skin ulcers in fish (Hoai et al., 2019; Tekedar et al., 2020) and diarrhea in humans (Roberts et al., 2006). Strain variations have been linked to virulence leading to studies aimed at identifying the virulence factors associated with mortalities (González-Serrano et al., 2002; Smyrli et al., 2017, 2019; Wang et al., 2022). The high mortalities experienced in aquaculture have led to use of antibiotics, thereby contributing to increase of antimicrobial resistance (AMR) (Roberts et al., 2006). As mentioned in our previous studies (Dubey et al., 2022a,b), the major limitation with most studies aimed at identifying AMR genes in bacteria is that they are mostly done by PCR that only detects AMR genes based on the primers used in the assay. This poses the risk of omitting important AMR genes whose primers are not included in PCR assays. Besides, PCR-based assays do not determine whether the AMR genes are intrinsically encoded in the chromosomes or extrinsically in plasmids. So, the use of whole-genome sequencing (WGS) able to detect all genes and their location in bacteria genomes is a better approach for elucidating the role of different bacteria species in the spread of AMR and virulence genes than PCR-based assays.

The spreading of AMR genes by horizontal transfer is contributing to involvement of bacteria species outside the 12 bacteria families enlisted to pose the greatest AMR threat to human health by the World Health Organization (WHO) (Willyard, 2017). As pointed out by White et al. (2001), the spread of AMR genes is enhanced when they form part of mobile gene cassettes (MGCs) or transposons. The MGCs were first identified as integrated AMR genes found in integrons in the early 1980s (Ward and Grinsted, 1982; Meyer et al., 1983; White et al., 2001). Although studies done this far have focused on cassettes carrying AMR genes, it is likely that the packaging in cassettes includes other genes such as virulence factors. As stated by White et al. (2001), MGCs facilitate horizontal gene transfer using various mechanisms that include mobilization of individual cassettes by integrons (Collis and Hall, 1992), movement of integrons having cassettes by transposases (Brown et al., 1996; Craig, 1996; Minakhina et al., 1999), dissemination of larger transposons carrying integrases (Liebert et al., 1999), and translocation of conjugative plasmids having integrases among bacteria (White et al., 2001). It is likely that most of the AMR genes associated with infections in aquaculture, livestock and humans are part of MGCs (Recchia and Hall, 1995). Yet, gene cassettes conferring resistance to antibiotics used in aquaculture have not been widely investigated as done in mammalian studies. Hence, it is unknown whether the AMR genes selected against drugs like tetracycline, sulphonamide, and trimethoprim widely used in aquaculture are packaged in MGCs. Thus, although previous studies have focused on identifying individual genes associated with resistance, the cassettes responsible for the spread of AMR genes has not been widely investigated for bacteria found in aquaculture.

In the present study we used WGS to profile all AMR and virulence genes found in *A. veronii* Ah5S-24 isolated from pond sediment obtained from the South East, USA by DePaola et al. (1988). Although in the previous study, they detected presence of Oxytetracycline-resistance (OTc^r) and tetracycline-resistance (Tc^r) by selecting for isolates that replicated on MacConkey agar containing oxytetracycline or tetracycline antibiotics, they did not determine whether the resistance gene was located in the chromosome or plasmids. Even though they showed the transfer of OTc^r and Tc^r resistance from the *Aeromonas* isolate to *Escherichia coli*, they did not determine whether the transfer was plasmid mediated or MGC. Thus, we wanted to determine whether the OTc^r and Tc^r resistance in the isolate was encoded in the chromosome or plasmid. We also wanted to determine whether the resistance detected was associated with a tetracycline genetic structure similar to that found in other bacteria species. We anticipate that data presented herein will underscore the importance of screening for MGCs carrying AMR genes from aquatic organisms with potential transmission to animals and humans.

2. Methodology

2.1. Bacteria culture, characterization, and antibiotic diffusion test

A suspected *Aeromonas hydrophila* isolated from pond sediments in the South-Eastern USA by DePaola et al. (1988) in 1988 was retrieved from the −80°C freezer at the Norwegian University of Life Sciences (NMBU), Ås, Norway. The isolate was kindly provided by Dr. Angelo DePaola, Gulf Coast Seafood Laboratory, United States. After thawing, the bacteria isolate was streaked on blood agar and incubated at 10°C for 5–7 days. Single colonies were streaked on tryptone soy agar (TSA) for purification followed by characterization using the Matrix-assisted laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry while DNA was extracted based on manufacturer's protocol (Qiagen, Germany). Identification of the bacteria species was done by PCR using universal 16S rRNA primers 27F and 1492R. Phenotypic characterization of antibiotic resistance was done using the Kirby-Bauer disk diffusion test (Joseph et al., 2011). The commercial antibiotic discs (Neo-Sensitabs™, Rosco) used consisted of Penicillin (PEN-10 μg), Amoxicillin (AMOXY-30 μg), Ampicillin (AMP-10 μg), Ciprofloxacin (CIPR-5 μg), Cefoxitin (CFO-30 μg), Cephalothin (CEP-30 μg), Tetracycline (TET-30 μg), Gentamycin (GEN-10 μg), Rifampicin (RIF-5 μg), Sulfonamide (SULFA-240 μg), Trimethoprim (TRIM-5 μg), Erythromycin (Ery-15 μg), Nitrofurantoin (NI-300 μg), and (Colistin-CO-150 μg)

Table 1 Overview of antibiotic resistence genes detected in the draft genome of *Aeromonas veronii* AhS5-24 together with phenotypic antibiotic susceptibility testing results using disk diffusion assay.

Resistance mechanism	Resistance gene	Antibiotic class	Antibiotic	Results
Antibiotic inactivation	bla_{FOX-7}	Cephamycin	Cefoxitin (CFO30)	R
	bla_{OXA-12}	Cephalosporin	Cephalothin (CEP 30)	R
	cphA4	*β*-lactams	Amoxicillin (AMOXY)	R
Antibiotic efflux	*tet(E)*	Tetracycline	Tetracycline (TET30)	R
	MexB	Sulfonamide, *β*-lactams	Sulfonamide (SULFA)	I
	CRP	Macrolide	Erythromycin (ERY15)	S
Antibiotic target alteration	*mcr*-7.1	Peptide	Colistin (CO150)	S
	vatF	–	–	–
Antibiotic target replacement	*dfrA3*	Diaminopyrimidine	Trimethoprim (TRIM5)	I
Other resistance mechanism		Fluoroquinolone	Ciprofloxin (CIPR5)	S
		Aminoglycoside	Gentamicin (GEN10)	S
		Nitrofuran	Nitrofurantoin (NI300)	S
		Rifamycin	Rifampicin RIF.5	S

(Table 1). A volume of 100 µl containing freshly cultured bacteria diluted at McFarland concentration of 10^8 CFU/ml was spread on Müller Hinton agar followed by putting the antibiotic discs on the bacteria lawn. Next, the plates were incubated at 30°C overnight followed by measuring the susceptibility or resistance based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (Kahlmeter et al., 2006; Cockerill et al., 2012).

2.2. DNA extraction

Genomic DNA (gDNA) was extracted as previously described (Becker et al., 2016) using the MagAttract® HMW DNA kit based on the manufacturer protocols (Qiagen GmbH, Hilden, Germany). Briefly, a 1 ml volume of approximately 2×10^9 CFU/ml of freshly overnight cultured bacteria was spanned in 2 ml Eppendorf tubes followed by suspending the pellets in 180 µl buffer ATL (tissue lysis buffer, Qiagen GmbH, Hilden, Germany). Next, Proteinase K was added to each vial at a concentration of 20 mg/ml followed by incubation at 56°C in an Eppendorf thermomixer for 30 min. Afterward, 4 µl RNase was added and the vials were pulse vortexed followed by adding 15 µl of MagAttract Suspension G and 280 µl Buffer MB to each vial (Tarumoto et al., 2017). The suspension from each tube was transferred onto the MagAttract holder followed by mixing for 1 min on an Eppendorf thermomixer. The magnetic beads having the gDNA were separated on the MagAttract magnetic rack for approximately 1 min. Supernatants were removed without disturbing the beads followed by washing the magnetic beads twice using MW1 and PE buffer (Becker et al., 2016; Tarumoto et al., 2017). The remaining suspension was removed by washing the beads twice using 1 ml RNAase free water (Qiagen GmbH, Hilden, Germany) (Becker et al., 2016). The gDNA was harvested by eluting in 100 µl buffer EB while purity was evaluated using the NanoDrop (Thermo Fisher, United States) and gel electrophoresis using 1% agarose. Quantification of gDNA was carried out using the Qubit double-stranded DNA high-CHS kit following the manufacturer's guidelines (Life Technologies Inc., Carlsbad, CA, United States) (Guan et al., 2020).

2.3. Library preparation, sequencing, and bioinformatics analysis

Library preparation was carried out using Nextera DNA Flex (Tagmentation Illumina Inc. San Diego, CA, United States) while Illumina MiSeq were used with a paired-end read length of 2 × 300 bp. The bacterial raw DNA reads were analyzed using the online Galaxy platform[1] version 21.05. Quality of both forward and reverse raw reads were analyzed using the FastQC Version 0.11.9 software (Bioinformatics B, 2011), while the Trimmometric version 0.38.1 was used to remove the adapters and low-quality reads from paired-end sequences (Bolger et al., 2014). The resulting paired-end sequence reads were *de novo* assembled using SPAdes v. 3.12.0 (Coil et al., 2015) with 33 to 91 k-mers (Bankevich et al., 2012) while genome annotation was done using the prokaryotic genome annotation pipeline (PGAP) (Tatusova et al., 2016) from the National Center for Biotechnology and Information (NCBI) and Prokka (Seemann, 2014). Online Galaxy platform (see Footnote 1) version 21.05 was used for bioinformatic analysis.

2.4. Pangenome analysis

Pangenome analysis of *A. veronii* AhS5-24 together with 30 complete genomes of other *A. veronii* isolates retrieved from the NCBI was carried out using Roary v. 3.13.0 using general feature files 3 (.gff) file generated from Prokka v. 1.14.5. The phylogenetic tree was made using the Phandango software using Gene_presence_absence and Newick files obtained from Roary v. 3.13.0. The average nucleotide identity (ANI) of all 31 *A. veronii* genomes was computed using FastANI v1.3 using *A. veronii* FC951 (CP032839) as a reference strain. Antimicrobial resistance (AMR) genes were identified using staramr version 0.7.2 (Tran et al., 2021) and ABRicate v1.0.1 (Seemann, 2016) in the Comprehensive antimicrobial resistance database (CARD) (Alcock et al., 2020) and staramr v. 0.7.2 with the identification threshold set at 80%. Plasmidfinder v 2.0 (Ullah et al., 2020) was used

1 https://usegalaxy.no/

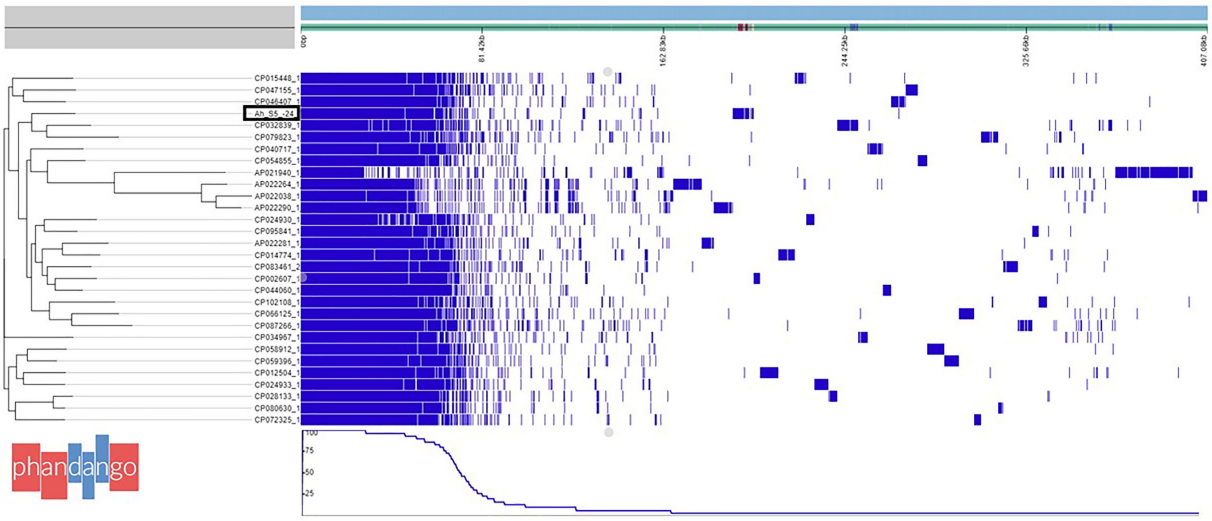

FIGURE 1
Phylogenetic tree based on pangenome analysis of *Aeromonas veronii* AhS5-24 together with 30 complete genomes of other *A. veronii* strains obtained from the National Center for Biotechnology and Information (NCBI). Note that *A. veronii* AhS5-24 is clustered together with *A. veronii* strains FC951 (CP032839) isolated from hospital sewage. A total of 20352 genes were detected in all 31 *A. veronii* genomes by pangenome analysis using the Roary software of which 1,429 genes were core genes, 875 soft core genes while the number of shell and cloud genes was 2,241 and 15,807 genes, respectively.

to identify plasmids in the bacterial genomes while virulence genes were identified using virulence factors database (VFDB). Genome circular maps were created using Proksee.[2]

2.5. Phylogenetic analysis of antimicrobial resistance genes

Phylogenetic comparison of the *tet(E)* and *tetR* genes from strain AhS5-24 with other *A. veronii* isolates was done using the Molecular Evolutionary Genetic Analysis version 7 (MEGA-7) software (Kumar et al., 2016). The *tet(E)* and *tetR* sequences from strain AhS5-24 were retrieved after screening using ABRicate version 1.0.1 followed by comparison with *tet(E)* and *tetR* sequences from other *A. veronii* isolates retrieved from NCBI. Phylogenetic trees were produced using the Neighbor-joining and BioNJ algorithm to a pairwise matrix estimated using JTT model and expressed as number of base substitution per site (Jones et al., 1992).

3. Results

3.1. Genome organization and pangenome analysis

The draft genome of *A. veronii* AhS5-24 showed a high similarity with other *A. veronii* genomes, as shown in Figure 1. The draft genome of strain AhS5-24 had a size of 4,748,224 bp with G + C content of 58.48%. It contained 157 contigs with an N50 value of 115,408. A total of 4,493 genes were predicted with 4,334 genes coding for proteins. The genome contained a total of 108 genes of RNA consisting of 99 tRNA and 5 rRNAs. The total number of genes detected from the 31 *A. veronii* genomes based on pangenome analysis was 20,352 of which 1,429 genes were core-, 875 softcore-, 2,241 shell-, and 15,807 cloud genes. The phylogenetic tree divided the genomes into three groups of which strain AhS5-24 was closely related to the human CP032839 (FC951) and hospital sewage CP079823 (HD6454) isolates (Figure 1). The average nucleotide identity (ANI) analysis using FastANI showed high similarity (>93%) of all 31 *A. veronii* isolates despite coming from different host species and geographic locations. The ANI of strain AhS5-24 was 96.31% similar with the *A. veronii* CF951 (CP032839) human clinical isolate and 96.20% similar with *A. veronii* HD6454 (CP079823) from hospital sewage.

The virulence genes found in *A. veronii* AhS5-24 comprised the motility and adherence genes that included the (i) lateral flagella proteins consisting of *lfgA* and *lfgL*, (ii) polar flagella that were represented by *flgA* and *flgN*, (iii) members of the tap type IV pili that included *tapA*, *tapW* and *tapY*, and (iv) fimbrial adherence determinants that included *pefC* and *pefD* genes (Figure 2). The mannose-sensitive hemagglutinin (MHSA) is encoded by the genes *mshA* and *mshQ* (Figure 2). Genes associated with capsule formation and immune evasion included *ddhA*, *ddhC*, and *wcaG1*. The hemolysin genes detected were *hlyA*, *hylIII*, and thermostable hemolysin (*TSH*) while toxin genes consisted of aerolysin *aerA*. Genes associated with iron acquisition consisted of the Iron *ABC* transporter while biofilm formation and quorum sensing genes were represented by *luxS* and *MshA-Q* pilus. We detected genes belonging to the type II secretion systems (T2SS) represented by *exeA* to *exeN* (Supplementary Table S1) and *vgrG*, which is part of T6SS (Figure 2). Overall, the virulence genes detected belonged to motility, adherence, secretion systems, iron acquisition, biofilm formation, quorum sensing, and immune evasion groups.

2 https://proksee.ca/

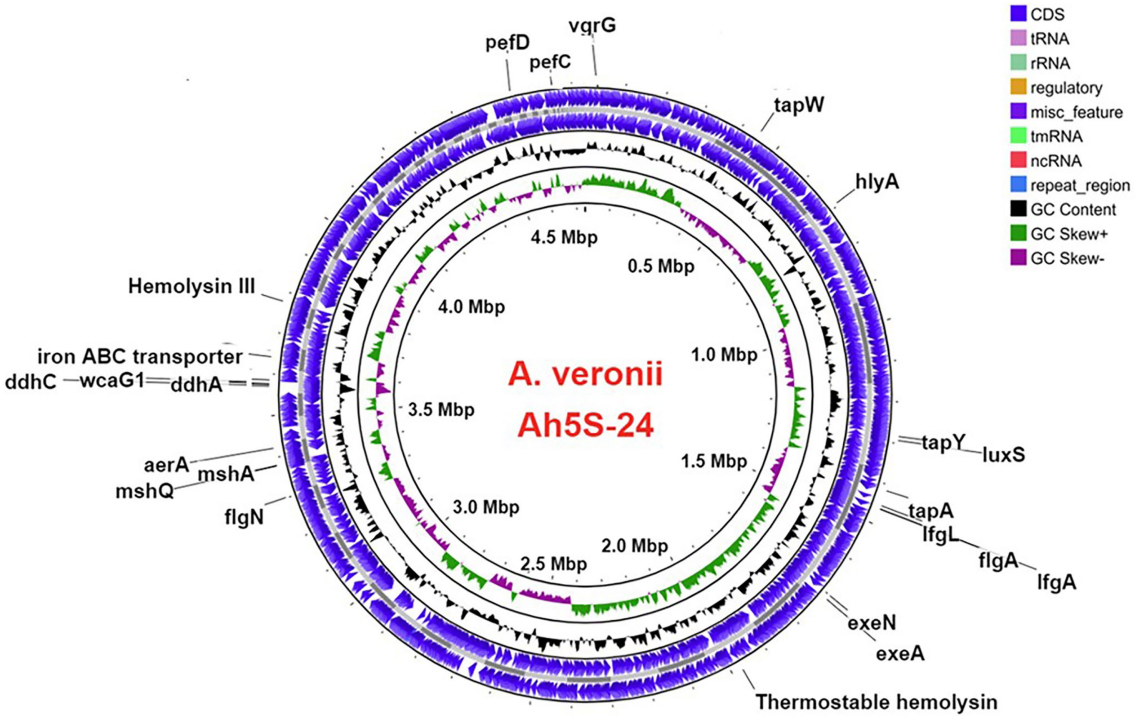

FIGURE 2
Circular map showing the loci for different virulence genes in the genome of *Aeromonas veronii* strain AhS5-24. Motility and adherence genes detected included the lateral flagella *ifgA* and *lfgL* genes; polar flagella *flaA* and *flgN* genes; tap type IV pili *tapA* and *tapY*; fimbriae *pefC* and *pefD* genes. The mannose-sensitive hemagglutinin (MHSA) genes were represented by *mshA* and *mshQ* while the hemolysin genes included *hylA, hylIII,* and *TSH*. Iron acquisition was represented by the iron ABC transporter protein and genes associated with capsule formation included the *ddhA, ddhC,* and *wcaG1* genes. Quorum sensing and biofilm formation genes were represented by *luxS*. The T2SS was represented by *exeA* and *exeN* genes while T6SS was represented by the *vgrG* gene.

3.2. Phenotype characterization of antimicrobial resistance genes

Results of the disk diffusion test showed that strain Ah5S-24 was resistant to CFO30, CEP30, AMOXY30, and TET30, whereas it showed intermediate resistance against SULFA240 and TRIM5 (Table 1). However, it was susceptible to ERY15, CO150, CIPR5, GEN10, NI300, and RIF5. We found an overall correlation kappa score of 82% (Cohen's $k = 0.8235$) with a specificity of 91.66% and sensitivity of 93% between the phenotypic profile based on the disk diffusion test and genotypic profile based on the genes identified using the CARD (Alcock et al., 2020).

3.3. Genotype characterization of antimicrobial resistance genes

Identification of AMR genes using the CARD (Alcock et al., 2020) showed that strain Ah5S-24 encoded multiple AMR genes that included the β-lactamase like $bla_{\text{FOX-7}}$, $bla_{\text{OXA-12}}$, and *cphA4*. Other genes detected included the colistin *crp* and *mcr-7.1* genes as well as the streptogramin A acetyl transferase *vatF* gene (Figure 3). There were no integrase and transposases located near the $bla_{\text{FOX-7}}$, $bla_{\text{OXA-12}}$, *cphA4, crp*, *mcr-7.1,* and *vatF* genes. The trimethoprim *dfrA3* gene was placed together with the sulfurtransferase, DUF2541 family protein, mog, DUF3135 domain-containing protein, threonine exporter protein, and phosphoadenyl-sulfate reductase (Figure 3). The efflux pumps detected included the resistance-nodulation-cell division (RND) *mexB* and *smeD* that were placed next to each other together with the IS*5* transposase (Figure 3).

Our findings show that the repressor of the tetracycline resistance element gene *tetR* was placed next to *tet(E)* together with the IS*5*/IS*1182* transposase, helicase, integrase, tyrosine type recombinase/integrase, and the site-specific integrase all in one cassette (Figure 3). The cassette found in *A. veronii* AhS5-24 showed a high similarity with cassettes found in *Vibrio parahaemolyticus* (MN199028.1) isolated from a fish market, *Vibrio alginolyticus* plasmid (MN865127.1) from shrimp, *Aeromonas caviae* (CP110176) from human stool, and *Aeromonas media* (CP03844.1) from a sewage bioreactor (Figure 4). They all had a similar genetic structure or transposon consisting of the IS*5*/IS*1182* transposase followed by a gene encoding a hypothetical protein (*hp*), *Tet(E)*, *tetR,* and another hypothetical protein (*hp*), thereby forming a MGC designated as 1S*5*/IS*1182/hp/tet(E)/tetR/hp* (Figure 4). Suffice to point out that the cassette from *Vibrio alginolyticus* (MN865127.1) was from a plasmid, while the cassettes from *A. veronii* AhS5-24, *Vibrio parahaemolyticus* (MN199028.1), *A. media* (CP038444.1), and *Aeromonas caviae* (CP110176) were from chromosomes. This findings demonstrate that the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette can be found both in chromosomes and plasmids of different bacteria species. It is noteworthy that the cassette for *Vibrio alginolyticus* plasmid (MN865127.1) had the *IShfr9* transposase, and not the IS*5*/IS*1182* transposase, despite having a

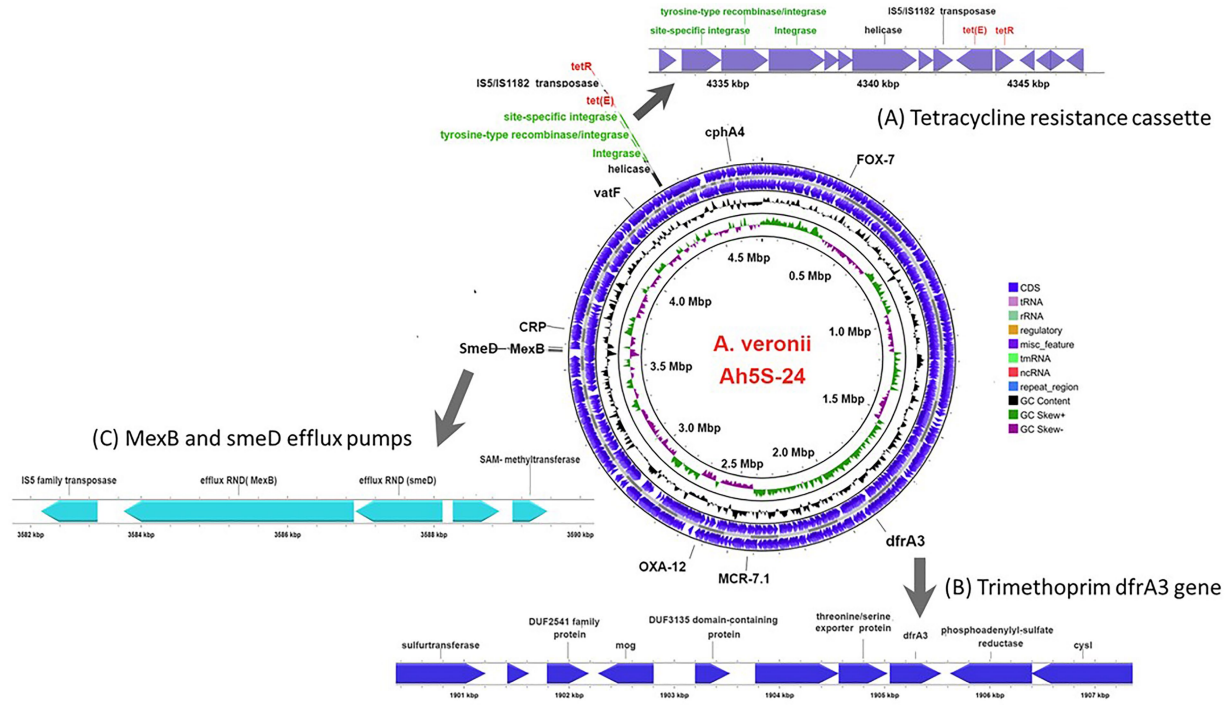

FIGURE 3
Circular genomic map of *Aeromonas veronii* AhS5-24 showing the loci for antimicrobial resistance (AMR) genes. The AMR genes detected included the *β*-lactam bla_{OXA-12} and bla_{FOX-7} genes together with *cphA4*, *dfrA3*, *mcr-7.1,* and *vatF* genes while the efflux pump proteins detected were *CRP*, *smedD,* and *mexB*. The extended linear map **(A)** shows the cassette encoding the site-specific integrase, tyrosine-type recombinase/integrase, integrase, helicase, IS*5*/IS*1182* transposase, *tet(E)* efflux pump protein gene and *tetR* gene designated as IS*5*/IS*1182/hp/tet(E)/tetR/hp*. The extended line map **(B)** shows the linear relationship between the trimethoprim *dfrA3* gene and other genes that includes sulfurtransferase, DUF2541 family protein, mog, DUF3135 domain-containing protein, threonine/serine exporter protein, *dfrA3* and phosphoadenyl-sulfate reductase in the genome of *A. veronii* AhS5-24. The extended map **(C)** shows the linear relationship between the *smeD* and *mexB* efflux pumps in the genome of *A. veronii* AhS5-24.

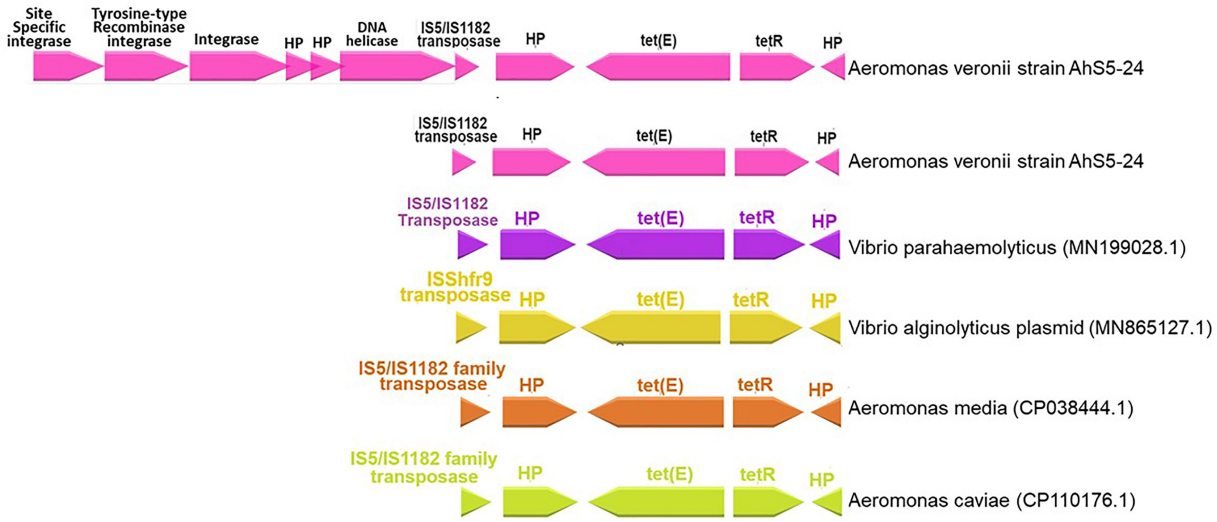

FIGURE 4
Comparison of the IS*5*/IS*1182/hp/tet(E)/tetR/hp* gene cassettes for *Aeromonas veronii* strain AhS5-24 from pond sediments, *Vibrio parahaemolyticus* (MN199028) isolated from retail fish from a market, *Vibrio alginolyticus* plasmid (MN865127.1) from shrimp, *Aeromonas media* (CP038444.1) from sewage bioreactor and *Aeromonas caviae* (CP110176.1) from human stool. Note that all isolates had the hypothetical proteins (*hp*), *tetR*, *tet(E),* and IS*5*/IS*1182* transposase forming a gene cassette designated as IS*5*/IS*1182/hp/tet(E)/tetR/hp*. The uppermost linear map shows *Aeromonas veronii* strain AhS5-24 having the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette linked to the DNA helicase, two hypothetical proteins, integrase, tyrosine-type recombinase/integrase and site-specific integrase.

similar *hp/tet(E)/tetR/hp* component with other bacteria species used in the comparison (Figure 4).

Phylogenetic analysis showed that the *tet(E)* gene from *A. veronii* AhS5-24 had a 100% similarity with *tet(E)* genes from different bacteria species that included *Escherichia coli* (AIL23572.1, CAC20135.1, and WP_20194468.1), *Aeromonas caviae* (BBR12376.1, WP_244056220.1, and WP_201964468.1), *Yersinia ruckeri* (APO36645.1, APO36648.1, and APO36646.1), *Klebsiella pneumoniae* (EIW8806435.1), *Aeromonas* spp. (QEV84027.1 and WP_017780889.1), and *Enterobacter cloacae* (ASF90526.1) (Figure 5). Phylogenetic analysis also showed that the *tetR* gene from *A. veronii* AhS5-24 had a 100% similarity with *tetR* genes from different bacteria species that included *E. coli* (AAA98409.1), Gammaproteobacteria (W_P011899269.1 and WP_017411289.1), *Aeromonas salmonicida* (QJR83010.1), *Aliivibrio salmonicida* (CAC81917.1), and *A. caviae* (WP_223946105.1 and WP_223945860.1) (Figure 6). Altogether, our findings show that *tet(E)* and *tetR* genes were highly similar with those found in different bacteria species.

4. Discussion

In this study, we have shown that the bacteria isolated from pond sediments in the South East USA previously classified as *A. hydrophila* using the API 20E system in DePaola et al. (1988) was characterized as *A. veronii* Ah5S-24 using WGS and pangenome analysis. We have also shown that the T^r and TO^r detected by DePaola et al. (1988) could be linked to the *tetR* and *tet(E)* genes found in the same isolate designated as strain Ah5S-24 in this study. In addition, strain Ah5S-24 encoded several virulence and AMR genes of which tetracycline resistance genes were placed in the same genetic structure with an integrase, transposase and recombinase and can be defined as a transposon. These findings demonstrate that *Aeromonas* spp. isolated from aquatic environments have the potential to transmit AMR genes to other bacteria using transposons carrying different AMR genes.

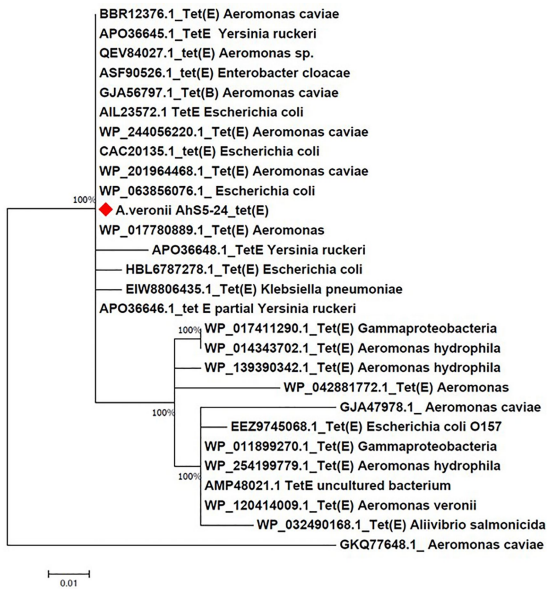

FIGURE 5
Phylogenetic tree comparing the *tet(E)* gene from different bacteria species. Note that the *tet(E) Aeromonas veronii* strain AhS5-24 had 100% similarity with *tet(E)* genes from other bacteria species that include *Aeromonas caviae* (BBR12376.1, GJA56797.1, and WP/244056220.1 and WP_20964468.1), *Yersinia ruckeri* (Apo036645.1, APO36646.1, and APO36648.1), *Enterobacter cloacae* (ASF90526.1), *Escherichia coli* (AIL23572.1, CAC20135.1, WP_063856076.1 and HBL6787278.1), and *Klebsiella pneumoniae* (EIW8806435.1).

Pangenome analysis showed a high similarity of strain Ah5S-24 with other *A. veronii* strains linked to different diseases in aquatic organism and humans. For example, strains CP032839.1 and CP046407.1 shown to be closely related with *A. veronii* Ah5S-24 were from human clinical cases (Ragupathi et al., 2020) and diseased rohu (*Labeo rohita*) (Tyagi et al., 2022), respectively. Besides, *A. veronii* Ah5S-24 had several virulence genes linked to adherence, biofilm formation, quorum sensing, immune evasion, toxins and intracellular secretion systems (TSS) found in other pathogenic *A. veronii* strains (Arechaga and Cascales, 2022). Detection of the *Msh* pili, tap type IV pili, lateral-and polar flagellar genes associated with intestinal adherence, colonization, and biofilm formation (Ro, 2006; Hadi et al., 2012) is suggestive that these genes play a vital role in the pathogenicity of strain Ah5S-24. The presence of the *LuxS* and *mshQ* genes is suggestive that strain Ah5S-24 has the capacity for biofilm formation and quorum sensing as seen in other bacteria species (Enos-Berlage et al., 2005; Trappetti et al., 2011) while presence of the iron ABC transporter is suggestive that strain Ah5S-24 uses this protein in acquiring iron from infected hosts (Delepelaire, 2019). Detection of *ddhA*, *ddhC,* and *wcaG1* associated with capsule formation (Mobine, 2008) is suggestive strain Ah5S-24 has the ability to form a capsule as a defense mechanism against host immune responses while presence of *hylA*, *hylII,I,* and *TSH* together with aerolysin *aerA* is suggestive that these genes might be linked to pore formation and intracellular release of enterotoxins by strain Ah5S-24 as seen in other bacteria species (Honda et al., 1992; Baida and Kuzmin, 1996; Abrami et al., 2000; Maté et al., 2014). Besides, several scientists (Wong et al., 1998; Heuzenroeder et al., 1999; Wu et al., 2007; Castilho et al., 2009) have shown that a combination of *hylA(+)* and *aerA(+)* is a major virulence determinant in *Aeromonas* spp. Castilho et al. (2009) found a high prevalence of hemolytic and cytotoxic *Aeromonas* spp. that had both *hylA(+)* and *aerA*$^+$ from human clinical, food, and environmental samples in Brazil while Wu et al. (2007) showed that the absence of *hlyA(−)* and *aerA(−)* in *Aeromonas* spp. from fish and human samples in Taiwan was associated with low virulence. Heuzenroeder et al. (1999) and Wong et al. (1998) showed that deletion or attenuation of the *hlyA(+)* and *aerA(+)* double mutant significantly reduced the pathogenicity of *A. hydrophila* in mice. They also showed that cytotoxicity to buffalo green monkey kidney cells and hemolysis on horse blood agar was only eliminated in the double and not in the single mutants of *A. veronii*, *A. hydrophila*, and *A. caviae*. Our findings show that *A. veronii* Ah5S-24 had both *hlyA(+)* and *aerA(+),* indicating that it shares the two key virulence determinants with other pathogenic *Aeromonas* spp.

Several studies have shown that *Aeromonas* spp. intrinsically carry various bla_{OXA} genes in their genomes that include the $bla_{OXA\text{-}12}$ gene (Dubey et al., 2022a,b) previously detected in *A. media*, *A. jandaei*, *A. sobria*, *A. dhakensis,* and *A. hydrophila* (Rasmussen et al., 1994; Alksne and Rasmussen, 1997; Hilt et al., 2020; Huang et al., 2020; Dubey et al., 2022a) being in line with its presence in strain Ah5S-24 while $bla_{FOX\text{-}7}$ previously reported in *A. media* and

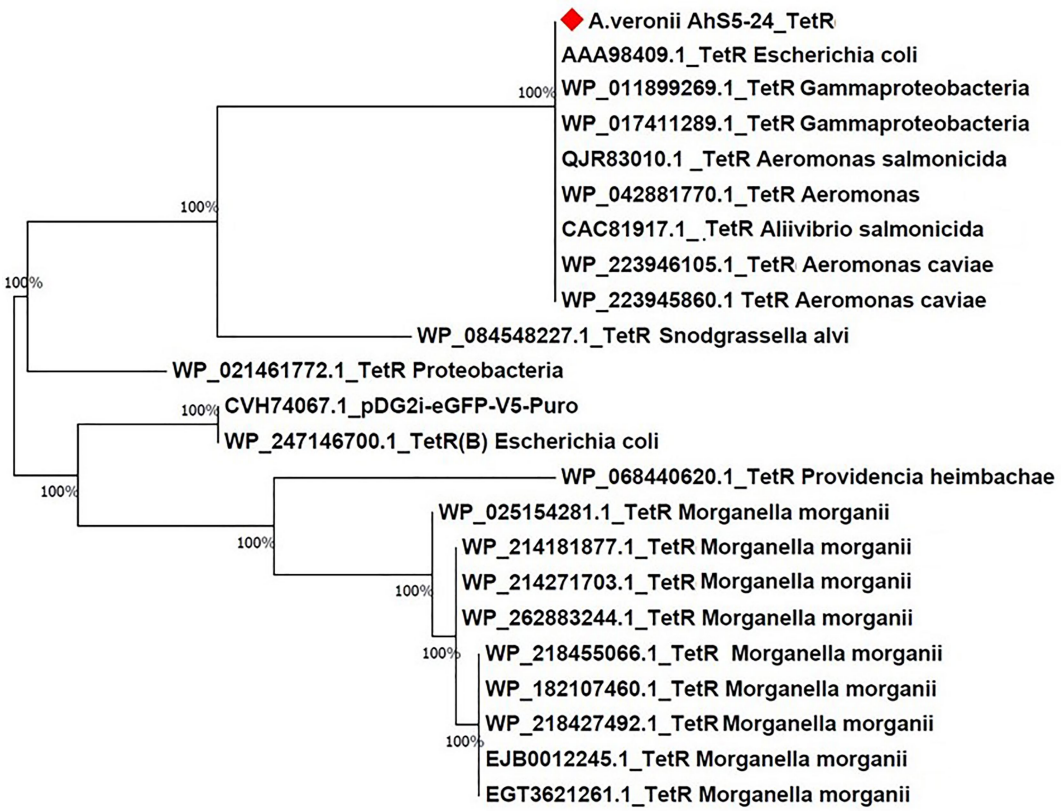

FIGURE 6
Phylogenetic tree comparing the *tetR* gene from different bacteria species. Note the high (100%) similarity between the *tetR* gene of *Aeromonas veronii* strain AhS5-24 with *tetR* genes of *Escherichia coli* (AAA98409.1), Gammaproteobacteria (WP_011899269.1 and WP_017411289), *Aeromonas salmonicida* (QJR83010.1), *Aeromonas* spp. (WP_04288770.1), and *Aeromonas caviae* (WP_223946105.1 and WP_223945860.1).

A. allosaccharophila was also found in strain Ah5S-24 (Ebmeyer et al., 2019). Other AMR genes detected included the *cphA4* gene known to be intrinsically encoded in various *Aeromonas* spp. (Dubey et al., 2022a,b) as well as the colistin-resistance *mcr-7.1* gene also reported from different *Aeromonas* spp. (Dubey et al., 2022a,b). Despite so, the $bla_{\text{OXA-12}}$, $bla_{\text{FOX-7}}$, and *mcr-7.1* genes detected in strain Ah5S-24 were not associated with integrases, recombinases or transposases suggesting that these genes could not be easily transferred or acquired from other bacteria species. Similarly, although trimethoprim and sulfonamide are among the most widely used antibiotics linked to AMR in aquaculture (Gao et al., 2012; Muziasari et al., 2014; Phu et al., 2015), the trimethoprim resistance gene *dfrA3* detected in the present study was not linked to integrases and transposases. Thus, the sulfonamide and trimethoprim resistance observed in the disc diffusion test could have been mediated by the *MexB* and *smeD* pumps that have been associated with resistance of several drugs that include sulfonamide, fluoroquinolone, cephalosporins, carbapenem, and trimethoprim. The trimethoprim and sulfonamide resistance observed on the disc diffusion test was intermediate (I) unlike the tetracycline resistance (R), which was highly expressed suggesting that the impact of trimethoprim and sulfonamide in conferring resistance was not as high as tetracycline in strain AhS5-24. Despite so, we found a high correlation of kappa score of 82% (Cohen's $k = 0.8235$) with a specificity of 91.66% and sensitivity of 93% between the phenotype characterization based on the disc diffusion test and genotypic characterization based on the CARD (Alcock et al., 2020), indicating that the two diagnostic tests were highly in agreement.

Tetracycline is one of the most widely used antibiotics in aquaculture, which has been linked to resistance in farmed aquatic organisms (Seyfried et al., 2010; Tamminen et al., 2011). Thus, it is likely that selection of the Tet E operon in strain AhS5-24 occurred in pond sediments used for aquaculture where tetracycline was used for the treatment of fish diseases. Although the absence of plasmids is suggestive that strain Ah5S-24 had lesser chances of transferring AMR genes to other bacteria, detection of the Tet E operon together with the integrase and IS*5*/IS*1182* transposase suggests that *tetR* and *tet(E)* genes could be transferred or acquired from other bacteria using the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette encoded in strain Ah5S-24. Besides, DePaola et al. (1988) used the same isolate to transfer the OT^r and T^r resistance to *E. coli* suggesting that the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette found in strain AhS5-24 could have been responsible for transferring the tetracycline resistance to *E. coli*. Also, detection of the same cassette in *V. parahaemolyticus*, *V. alginolyticus* (MN199028.1), *A. media* (CP038444.1), *A. caviae* (CP110176.1), and *A. caviae* (CP038445.1) emanating from fish market, shrimp, sewage bioreactor and human stool is suggesting that the IS*5*/IS*1182/hp/tet(E)/tetR/hp* transposon could be involved in interspecies transmission of the *tet(E)* and *tetR* genes in different bacteria species. These findings also suggest that the IS*5*/IS*1182/hp/tet(E)/tetR/hp* transposon might be in existence in different bacteria species found in different aquatic environments hosted by species that include

shrimps, fish, animals and humans. Its presence in *V. parahaemolyticus* (MN199028.1) isolated from retail fish at markets and *V. alginolyticus* (MN865127.1) from shrimps is indicative that it could play a vital role in transmission of *tet(E)* and *tetR* genes to humans through food.

The similarity of the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette found in the chromosomes of strain *A. veronii* AhS5-24, *V. parahaemolyticus* (MN199028.1) and *A. media* (CP038445.1), with the transposon found in the plasmid of *V. alginolyticus* (MN865127.1) is suggesting that the IS*5*/IS*1182/hp/tet(E)/tetR/hp* transposon can be transferable between chromosomes and plasmids of different bacteria species. Also, the high similarity of the *tet(E)* and *tetR* genes detected in strain AhS5-24 with those found in *E. coli*, *K. pneumoniae,* and *Aeromonas* spp. shown in the phylogenetic analysis consolidates our view that *tet(E)* and *tetR* genes could be transmissible between different bacteria species using MGCs. Thus, it is likely that the transfer of the OT^r and T^r resistance to *E. coli* observed by DePaola et al. (1988) was not plasmid mediated but it was done by the IS*5*/IS*1182/hp/tet(E)/tetR/hp* transposon found in strain Ah5S-24. Therefore, our findings indicate that the resistance acquired by different *Aeromonas* spp. in aquatic environments could play a vital role in the transfer of AMR genes to foodborne, environmental, nosocomial and other bacteria species using MGCs. However, future studies should seek to demonstrate the transfer of *tet(E)* and *tetR* genes using the IS*5*/IS*1182/hp/tet(E)/tetR/hp* cassette to other bacteria spp. including nosocomial, foodborne and environmental bacteria.

5. Conclusion

In this study, we have shown that *A. veronii* AhS5-24 is a multidrug-resistant bacterium encoding several AMR and virulence genes. It encoded a tetracycline resistance operon Tet E placed in a transposon designated as IS*5*/IS*1182/hp/tet(E)/tetR/hp* found in different bacteria species inhabiting different aquatic environments and infecting different host species suggesting that the Tet E operon could be transferred to other bacteria. Overall, this study shows that MGCs encoding AMR genes found in bacteria inhabiting aquatic environments could play a vital role in the spread of AMR genes to other bacteria infecting animals and humans.

Data availability statement

The *Aeromonas veronii* whole genome shotgun (WGS) project has the project accession JAJVCX000000000. This version of the project (01) has the accession number JAJVCX010000000 and consists of sequences JAJVCX010000001-JAJVCX010000157.

Author contributions

SD, HS, and HM: conceptualization, methodology, mobilizing resources, supervision, data curation, and bioinformatics analysis. SD, EA-W, BP, AD, HS, and HM: manuscript preparation, editing, and submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AD was employed by Angelo DePaola Consulting LLC.

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.2023.1112941/full#supplementary-material

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PHT427 as an effective New Delhi metallo-β-lactamase-1 (NDM-1) inhibitor restored the susceptibility of meropenem against *Enterobacteriaceae* producing NDM-1

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Introduction: With the increasingly serious problem of bacterial drug resistance caused by NDM-1, it is an important strategy to find effective inhibitors to assist β-lactam antibiotic treatment against NDM-1 resistant bacteria. In this study, PHT427 (4-dodecyl-*N*-1,3,4-thiadiazol-2-yl-benzenesulfonamide) was identified as a novel NDM-1 inhibitor and restored the susceptibility of meropenem against *Enterobacteriaceae* producing NDM-1.

Methods: We used a high throughput screening model to find NDM-1 inhibitor in the library of small molecular compounds. The interaction between the hit compound PHT427 and NDM-1 was analyzed by fluorescence quenching, surface plasmon resonance (SPR) assay, and molecular docking analysis. The efficacy of the compound in combination with meropenem was evaluated by determining the FICIs of *Escherichia coli* BL21(DE3)/pET30a(+)-bla_{NDM-1} and *Klebsiella pneumoniae* clinical strain C1928 (producing NDM-1). In addition, the mechanism of the inhibitory effect of PHT427 on NDM-1 was studied by site mutation, SPR, and zinc supplementation assays.

Results: PHT427 was identified as an inhibitor of NDM-1. It could significantly inhibit the activity of NDM-1 with an IC_{50} of 1.42 μmol/L, and restored the susceptibility of meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1} and *K. pneumoniae* clinical strain C1928 (producing NDM-1) *in vitro*. The mechanism study indicated that PHT427 could act on the zinc ions at the active site of NDM-1 and the catalytic key amino acid residues simultaneously. The mutation of Asn220 and Gln123 abolished the affinity of NDM-1 by PHT427 *via* SPR assay.

Discussion: This is the first report that PHT427 is a promising lead compound against carbapenem-resistant bacteria and it merits chemical optimization for drug development.

KEYWORDS

PHT427, NDM-1 inhibitor, meropenem, bacterial resistance, carbapenemase

1. Introduction

The multi drug resistance (MDR) of bacteria, especially the prevalence and development of multi drug resistant Gram-negative *Enterobacteriaceae*, poses a huge threat to global health and development (Yewale, 2014). According to the Antimicrobial Resistance Review commissioned by the British government, antimicrobial resistance will cause 10 million deaths every year by 2,050 (de Kraker et al., 2016). WHO has announced twelve most important bacteria, nine of which are Gram-negative bacteria, and the three most important are multidrug resistant Gram-negative bacteria, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Tacconelli et al., 2018).

β-lactam antibiotics are the most common and effective drugs in the treatment of Gram-negative bacterial infections. The main drug resistance mechanisms include the expression of β-lactamases which has been studied most, the mutation of penicillin binding protein and the active efflux of drugs (Lima et al., 2020). β-lactamase inhibitors are still the most successful antibiotic adjuvants although they have been used for more than 70 years (González-Bello, 2017). According to the differences between protein sequences, Ambler divided β-lactamases into four types: Ambler A, Ambler B, Ambler C, and Ambler D. The active center of class A, C, and D enzymes have serine residues, also known as serine β-lactamases (SBLs). While the active center of class B enzymes are metal ions (mainly zinc ions), called metallo-β-lactamases (MBLs) (Ambler, 1980). Based on sequence similarity, MBL is divided into three subcategories: B1, B2, and B3 (Garau et al., 2004). B1 subclasses are the most important class of MBLs in clinic, including New Delhi metallo-β-lactamase 1 (NDM-1), which poses a serious threat to human health due to its complex substrate, broad-spectrum function, transferability, etc., (Walsh et al., 2011).

NDM-1 has spread in more than 70 countries worldwide since it was first found in *Klebsiella pneumoniae* isolated from hospitalized patients in 2008 (Yong et al., 2009; Dortet et al., 2014). NDM-1 can hydrolyze almost all β-lactam antibiotics, including carbapenem antibiotics (except aztreonam), therefore it has attracted much attention (Tooke et al., 2019). NDM-1 mainly exists in *K. pneumoniae* and *Escherichia coli* (Poirel et al., 2010). *Enterobacteriaceae* producing NDM-1 was reported in hospital and community acquired infections, including urinary tract infection, sepsis, pulmonary infection, peritonitis, etc., (Poirel et al., 2014). The carbapenem resistant classical *K. pneumoniae* strain carrying bla_{NDM} was first detected in southwest Iran (Saki et al., 2022). In addition, an NDM-1-producing ST25 *K. pneumoniae* strain was isolated from a 9-day old female newborn diagnosed with intracranial infection in China (Zhao et al., 2022). At present, more than 500 NDM-1 inhibitors have been reported, but there is no clinically available NDM-1 inhibitor except the combination of bicyclic borate inhibitor VNRX-5133 and cefepime which has entered the phase III clinical trial (Abdelraouf et al., 2020). Therefore, it is urgent to find an effective drug that inhibits the resistance of NDM-1.

PHT427 was originally designed as an AKT (protein kinase B) inhibitor (Moses et al., 2009), and could also inhibit PtdIns-dependent protein kinase 1 (PDPK1) (Meuillet et al., 2010). However, the effect on Gram-negative bacteria has not been reported. In this study, PHT427 (4-dodecyl-*N*-1,3,4-thiadiazol-2-yl-benzenesulfonamide) was identified as a novel NDM-1 inhibitor through our high-throughput screening model (Jiangxue, 2019). Notably, the combination of PHT427 with meropenem could attenuate meropenem resistance in NDM-1-producing *E. coli*. Fluorescence quenching and SPR assays suggested that PHT427 was able to bind to NDM-1. Asn220 and Gln123 in the active site played a key role in maintaining the stability of PHT427 and NDM-1 binding. Zinc supplementation assay demonstrated that PHT427 exerted its inhibitory activity by chelating zinc ions at the active site of NDM-1 enzyme. In conclusion, our results indicate that PHT427 is a promising anti-Gram-negative bacteria agent which targets NDM-1.

2. Materials and methods

2.1. Bacterial strains and chemicals

The engineered strain *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1} with the bla_{NDM-1} gene (GenBank: AB614355.1) was provided by Professor Xuefu You of the Institute of Medicinal Biotechnology, Chinese Academy of medical sciences (IMB, CAMS). *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2) were supplied by Professor Hui Wang of the Institute of Clinical Laboratory, Peking University People's Hospital. PHT427 was purchased from Shanghai Pottery Biotechnology Co., Ltd. All antibiotics were obtained from the National Institutes for Food and Drug Control (Beijing, China). The Vero cells and HepG2 cells were presented by Professor Yuhuan Li and Qiyang He (IMB, CAMS), respectively. EDTA was purchased by Sangon Biotech (Shanghai) Co., Ltd. $ZnCl_2$ was obtained from J&K Scientific Company. The library of small molecular compounds which contains 13,250 compounds with known biological activities was purchased from Topscience (Shanghai), and the article number was L4000.

2.2. Construction of plasmid

The mutations in bla_{NDM-1} were generated by site-directed mutagenesis using Fast Mutagenesis System (TransGen Biotech, Beijing, China) according to kit instructions as previous described (Li et al., 2017). Key amino acids in NDM-1 were changed to alanines accordingly: Q123A (Gln123 to Ala), D124A (Asp124 to Ala), and N220A (Asn220 to Ala). Positive mutated plasmids were confirmed by DNA Sequencing.

2.3. Expression and purification of NDM-1 wild type and mutated enzyme

The pET30a(+)-bla_{NDM-1} and mutated plasmids were transformed into *E. coli* BL21(DE3) competent cells, respectively. The single clone was cultured in LB medium with 50 μg/mL

kanamycin at 37°C. NDM-1 was expressed by the addition of 0.5 mM IPTG and cultured at 22°C for 21 h when the absorbance at 600 nm reach the range of 0.6–0.8. The harvested bacterial cells were disrupted by pressure and centrifuged at 12,000 rpm for 30 min. The proteins which present in the supernatant were purified through Ni^{2+} ion-affinity chromatography using a linear gradient 45–400 mM imidazole in washing buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.9). The eluted fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining. The protein concentration was measured using Easy II Protein Quantitative Kit (BCA) (TransGen Biotech, Beijing, China), and the purified proteins were stored at −80°C with 50% glycerol.

2.4. NDM-1 inhibitor screening

Nineteen compounds were screened at a final concentration of 20 μmol/L through our previous high-throughput screening model of NDM-1 inhibitors (Jiangxue, 2019). Briefly, the assays were carried out in the 96-well plates containing the following ingredients: 10 mmol/L HEPES (pH 7.5), 2.8 U NDM-1 and 62.5 μmol/L meropenem. The EDTA (20 μmol/L) was used as a positive control, and the negative control group only contained DMSO. The inhibition rate was calculated as follows:

$$\%\text{inhibition} = \left(1-\frac{Ap-As}{Ap-An}\right) \times 100\%$$

in which, *Ap* and *An* represented the average absorbance of positive and negative controls, respectively, and *As* was the absorbance of sample. Compounds were perceived as hits when the 80% inhibition limit was achieved.

2.5. Enzyme inhibition assay

NDM-1 was incubated with a gradient concentration of PHT427 in 10 mmol/L HEPES (pH 7.5) at 37°C for 15 min. EDTA was used as the parallel positive control and DMSO was used as the parallel negative control. Then the substrate meropenem (final concentration of 60 μmol/L) was added to initiate the reaction and the absorbance at OD_{300} nm which could reflect the activity of NDM-1 was recorded using Enspire 2300 multilabel reader (PerkinElmer) at 37°C. The IC_{50} value was analyzed using GraphPad Prism 8.0.

For the test of Q123A, D124A, and N220A, the enzyme inhibition assay briefly goes as follow: NDM-1, Q123A, D124A, and N220A (final concentration of 0.25 μg/mL) were incubated at 37°C for 15 min in the absence of PHT427, respectively. Other procedures were kept strictly the same.

2.6. Fluorescence quenching assay

Fluorescence detection assay was carried out in the 96-well black plate. NDM-1 (final concentration of about 416 μg/mL) was incubated at 37°C for 15 min in the absence or presence of a gradient concentration of PHT427 (31.25–500 μM). The excitation wavelength was 270 nm, and emission spectra were acquired by scanning from 310 to 490 nm using Enspire 2300 multilabel reader (PerkinElmer).

2.7. Surface plasmon resonance (SPR) assay

Surface plasmon resonance was performed using Reichert 2SPR with CM5 chip (Reichert, New York, USA). NDM-1, N220A, and Q123A were diluted to 100 μg/ml at 10 mmol/L sodium acetate buffer at pH 4.5 and then immobilized to a CM5 chip by amine coupling, respectively. PHT427 was dissolved in running buffer (PBST containing 1% DMSO) to different concentration gradients (6.25–50 μM) and then injected into the surface of the protein-coupled chip channels at the flow rate of 25 μL/min. The binding affinity was calculated using TraceDrawer software (Reichert, New York, USA).

2.8. Molecular docking

The crystal structure of NDM-1 solved at a 2.40 Å resolution was derived from the Protein Data Bank (PBD: 4RBS), which is a complex of NDM-1 and meropenem. PHT427 molecular structure was optimized and then docked with the active pocket of NDM-1 using Discovery Studio 2018 software. The docking model was selected with the highest score to analyze the interaction between NDM-1 and PHT427.

2.9. Zinc supplementation assay

The active center of NDM-1 contains two zinc ions, which are necessary for NDM-1 to exert antibiotic hydrolytic activity. In order to investigate whether the inhibition of NDM-1 activity by PHT427 was related to zinc ions, zinc supplementation assay which could test the inhibitory activity of inhibitors against NDM-1 in the presence of Zn^{2+} ions was performed. NDM-1 (final concentration of 0.25 μg/mL) was incubated with PHT427 (20 μM) at 37°C for 15 min in the absence or presence of Zn^{2+} ions (20 μM). EDTA was used as positive control and DMSO was the negative control. Then the substrate meropenem (final concentration of 60 μmol/L) was added to initiate the reaction, and the change in absorbance at 300 nm was monitored on a Enspire 2300 multilabel reader (PerkinElmer) at 37°C for calculation of the inhibition rates.

2.10. Determination of minimum inhibitory concentration (MIC) and checkerboard microdilution assays

The MICs of PHT427 in combination with β-lactam antibiotics to *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 and C2315 were performed using the broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI)[1] guidelines. The bacterial cells were

cultured at 37°C for about 21 h and the results were observed. For checkerboard microdilution assay, meropenem (0.03125-256 μg/mL) was tested in combination with PHT427 (0-400 μmol/L) against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2), in triplicate. Other procedures were kept strictly the same. The fractional inhibitory concentration index (FICI) was determined according to the following equation: FICI = FICA + FICB = CA/MICA + CB/MICB, where MICA and MICB are the MIC values of compounds A and B, respectively, when functioning alone, and CA and CB are the concentrations of compounds A and B at the effective combinations. Synergism was defined when FICI $\leq$ 0.5, indifference was defined when FICI $>$ 0.5 and $<$ 4, and antagonism was defined when FICI $\geq$ 4 (Mathers, 2015). A volume of 624.98 μmol/L was set as the MIC of PHT427 against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1} for the determination of FIC values, and 400 μmol/L was set as the MIC of PHT427 against *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2) for the determination of FIC values.

2.11. Cell cytotoxicity assay

Cell Counting Kit-8 (CCK8) was used to test cell cytotoxicity of the compound. Vero cells (the kidney cells of the African green monkey) and HepG2 cells (human hepatocellular carcinoma) were seeded into 96-well culture plates at the density of 2×10^3 cells per well and 5×10^3 cells per well, respectively. The cells were then treated with varying concentrations of PHT427 (0–200 μM). After 48 h, 20 μL of CCK8 reagent was added to each well and then cultured for 1–2 h. Absorbance was measured at 450 nm using Enspire 2300 multilabel reader (PerkinElmer) using wells without cells as blanks, and the IC_{50} values were calculated using GraphPad prism 8.0. All experiments were performed in triplicate.

2.12. Acute toxicity assay *in vivo*

Kunming male mice (25 g) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). Experimental mice were randomized to cages of 6 per group for this experiment. The mice were divided into three groups, including Control group (0.5% CMC-Na), PHT427 (L) (500 mg/kg in 0.5% CMC-Na), and PHT427 (H) (1,000 mg/kg in 0.5% CMC-Na). Mice administered via gastric gavage and closely monitored over 72 h. The number of surviving mice was recorded. All animal experimental procedures were performed under the regulations of the Institutional Animal Care and Use Committee of the Institute of Medicinal Biotechnology.

2.13. Statistical analysis

All the data were performed on three independent experiments, using GraphPad prism 8.0. The statistical analysis of the results was performed using two tailed *t*-test with ** indicating $P < 0.01$, *** indicating $P < 0.001$, and **** indicating $P < 0.0001$.

3. Results

3.1. PHT427 inhibits the activity of NDM-1

NDM-1 inhibitors are screened from the library of small molecular compounds using our previous high-throughput screening model. Nineteen compounds with an inhibition rate of $\geq$80% were identified. Among them, PHT427 (**Figure 1A**) showed the best inhibitory activity, which could inhibit NDM-1 in a dose-dependent manner with an IC_{50} of 1.42 μmol/L (**Figure 1B**). The molecular formula and molecular weight of PHT427 is $C_{20}H_{31}N_3O_2S_2$ and 409.61 g/mol, respectively.

3.2. The interaction between PHT427 and NDM-1 via fluorescence quenching and SPR assays

The fluorescence quenching and SPR assays were performed to analyze the interaction between PHT427 with NDM-1. We first examined how PHT427 affected the intrinsic tryptophan fluorescence of the NDM-1 enzyme via the fluorescence quenching assay. As shown in **Figure 2A**, NDM-1 enzyme alone (0 μM) exhibited remarkable florescence at about 344 nm when excited at 270 nm, which was a property of its aromatic amino acid residues. When PHT427 (31.25–500 μM) was added, the fluorescence intensity of the NDM-1 was quenched gradually in a concentration dependent manner, and the fluorescence intensity almost vanished in the presence of the high concentration of PHT427 (**Figure 2B**). The results demonstrated that PHT427 was able to bind to NDM-1 by a dose-dependent manner. The aromatic amino acid residues in NDM-1 may undergo a conformational change as a result of this PHT427 binding, which will obstruct the formation of the active center and/or the binding of the substrate. The intrinsic fluorescence of NDM-1 was quenched by the intrinsic fluorescence changes induced by PHT427.

An SPR assay was used for further examining the interaction between PHT427 and NDM-1. CM5 SPR chip was coated with NDM-1 by establishing a covalent bond between amine group of N-terminal amino acid and the carboxyl group of CM5 chip, and then PHT427 with different concentration gradients (6.25–50 μM) were injected into the NDM-1 immobilized chambers. The results showed that PHT427 could bind to NDM-1 with a K_D value of 6.28×10^{-5} M (**Figure 2C**), which suggested a moderate affinity interaction between PHT427 and NDM-1.

3.3. Analysis of the molecular docking results

We used Discovery Studio 2018 software to perform molecular docking between NDM-1 (PBD: 4RBS) and PHT427. As shown in **Figure 3**, the sulfonamide group of PHT427 is an important functional group for the inhibitory activity of NDM-1. PHT427 acts on Zn1 and Zn2 of NDM-1 through the oxygen atom of the sulfonamide group. The other oxygen atom of the sulfonamide group and the nitrogen atom of the five membered heterocycle

10.3389/fmicb.2023.1168052

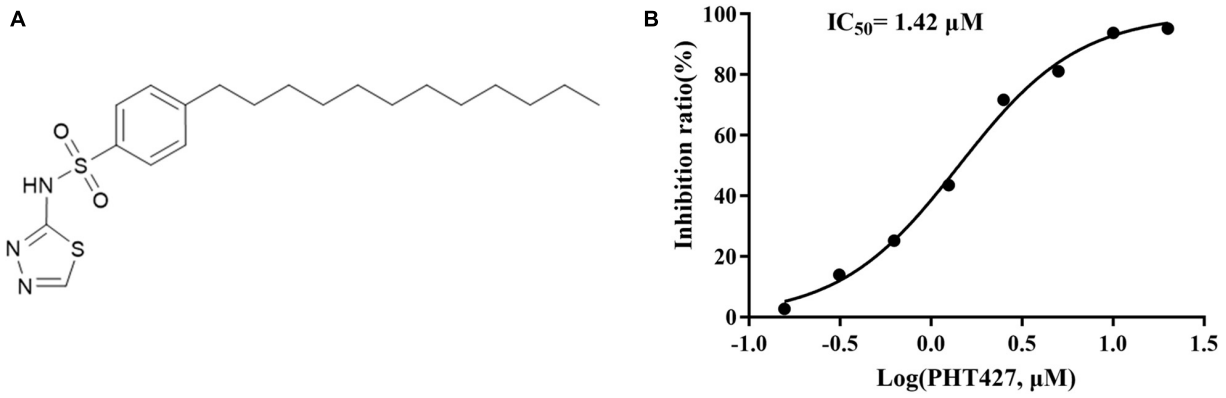

FIGURE 1
Identification of PHT427 as an effective NDM-1 inhibitor. **(A)** The structure of PHT427. **(B)** Enzyme inhibition assay was used to detect the activity of NDM-1.

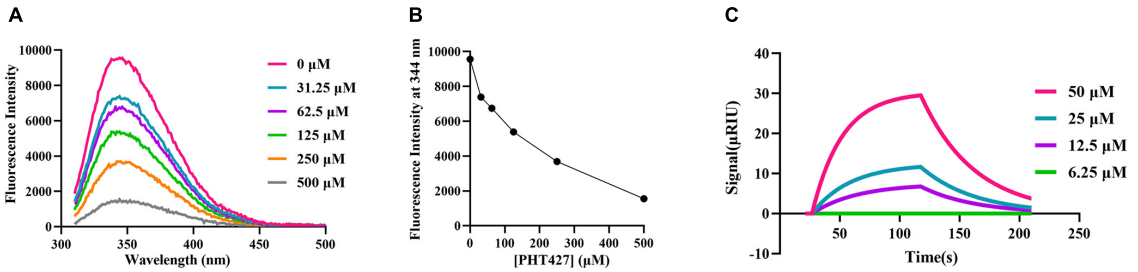

FIGURE 2
Confirming the interaction between PHT427 and NDM-1. **(A)** Variation of the intrinsic fluorescence spectra of NDM-1. The excitation wavelength was 270 nm, and emission spectra were acquired by scanning from 310 to 490 nm. **(B)** PHT427 quenches the intrinsic fluorescence of NDM-1 in a concentration dependent manner. **(C)** Binding sensorgrams of PHT427. NDM-1 was immobilized to a CM5 chip by amine coupling and the figure was generated by SPR analysis software called TraceDrawer.

form a hydrogen bond with Asn220 to improve the stability of NDM-1 and the compound. Meanwhile, the nitrogen atom of the sulfonamide group forms a hydrogen bond with Asp124, and the sulfur atom of the five membered heterocycle also forms a hydrogen bond with Gln123.

3.4. Asn220 mutant and Gln123 mutant are crucial for maintaining the stability of PHT427 and NDM-1 binding

According to the molecular docking results, PHT427 forms hydrogen bond interaction with key amino acids Asn220, Asp124, and Gln123. Here, we mutated Gln123, Asp124, and Asn220 to alanines, respectively (as Q123A, D124A, and N220A, respectively). The enzymatic activity of site-directed mutation of Asn220 and Gln123 to alanines (as N220A, Q123A) under identical conditions is 92.29, 86.11%, respectively, indicating that the mutation does not affect the enzymatic activity. While site-directed mutation of Asp124 to an alanine (as D124A) abolished the enzymatic activity by ∼95% under identical conditions, confirming the importance of this residue for the enzymatic activity (**Figure 4A**). Therefore, N220A and Q123A were used for further research.

To further clarify the importance of Asn220 and Gln123 in maintaining the stability of NDM-1 and PHT427, we subsequently examined the interaction through SPR assay. Compared with NDM-1 and PHT427, the affinity of PHT427 to mutated proteins (N220A, Q123A) were reduced significantly under identical conditions (**Figures 4B, C**). Hence, these results demonstrate that Asn220 and Gln123 in the active site play a pivotal role on maintaining the stability of PHT427 and NDM-1 binding.

3.5. PHT427 exerts its inhibitory activity by chelating zinc ions at the active site of NDM-1 enzyme

Metal ions are the key cofactor of NDM-1 catalytic function. Zinc ion deficiency causes the inactivation of NDM-1. It has been reported that EDTA exert inhibitory activity only by chelating zinc ions of NDM-1 (Wang et al., 2021). Therefore, a zinc supplementation assay was used to investigate whether PHT427 could exert an inhibitory effect on the NDM-1 enzyme by chelation of zinc ions. As shown in **Figure 5**, PHT427 (20 μmol/L) and the positive control EDTA (20 μmol/L) exhibited good inhibitory activity against NDM-1 enzyme. However, the addition of zinc ions (20 μmol/L) caused that the inhibitory activity of EDTA to NDM-1

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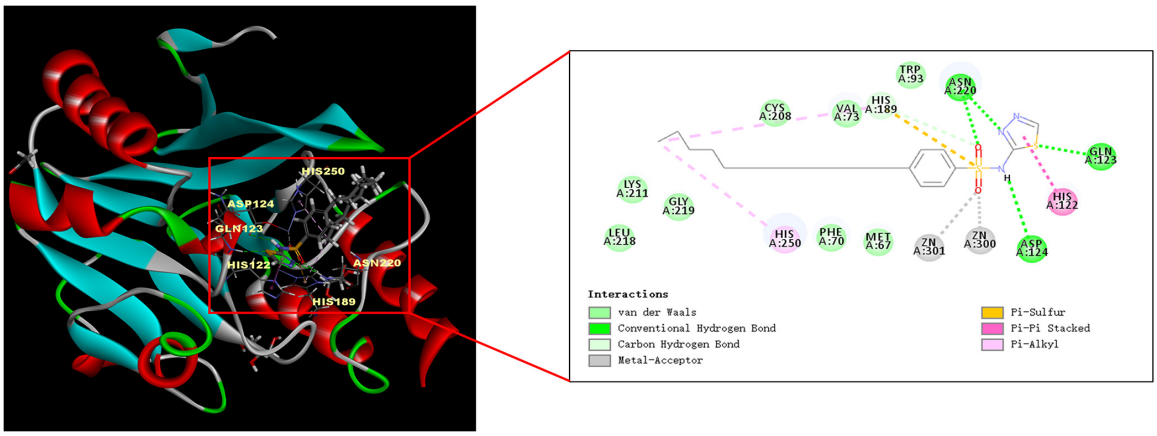

FIGURE 3
Molecular docking of NDM-1 and PHT427. The active pocket of NDM-1 bound to PHT427.

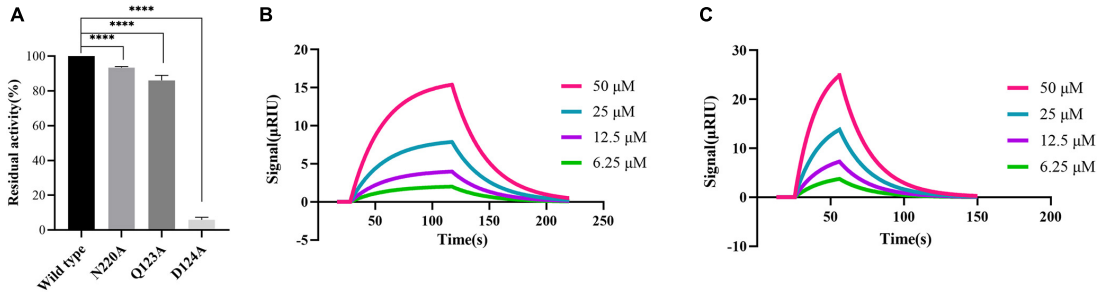

FIGURE 4
Proving the importance of Asn220 and Gln123 for maintaining the stability of PHT427 and NDM-1 binding. **(A)** Residual activity of wild-type NDM-1, N220A, Q123A and D124A at concentration of 0.25 μg/mL. **** was indicated as $P < 0.0001$. **(B)** Demonstration of the interaction between N220A and PHT427 by SPR, with a K_D value of 8.96×10^{-4} M. **(C)** Demonstration of the interaction between Q123A and PHT427 by SPR, with a K_D value of 1.24×10^{-4} M.

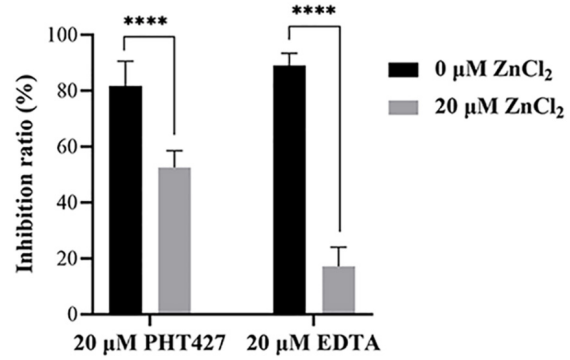

FIGURE 5
Zinc supplementation assay. Inhibitory activity of NDM-1 enzyme with PHT427, EDTA, PHT427 + $ZnCl_2$, and EDTA + $ZnCl_2$, respectively. **** was indicated as $P < 0.0001$.

significant decreased about ~70%, and that of PHT427 to NDM-1 abolished by ~30% under identical conditions, suggesting that PHT427 could exert its inhibitory activity by chelating zinc ions at the active site of the NDM-1 enzyme. Our results showed that, unlike EDTA, PHT427 may not only exert inhibitory activity by chelating zinc ions of NDM-1, but also through other mechanisms.

3.6. PHT427 restores the susceptibility of meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2) *in vitro*

We tested the MICs of β-lactam antibiotics combined with PHT427 at a gradient concentration against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}. As shown in Table 1, the results showed the MICs of penicillin, ampicillin, ceftazidime, meropenem and biapenem were decreased by 16-, 16-, 8-, 128-, and 16-fold, respectively, in combination with PHT427 at concentration of 39.06 μmol/L.

To verify the above conclusion, we further evaluated the synergistic effect of the combination of PHT427 with meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing

TABLE 1 MIC (μg/mL) of antibiotics against *E.coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}.

Strain	Antibiotics	MIC (μg/mL)	
		Alone	Combination (Reduction fold)
E.coli BL21(DE3)/pET30a(+)−bla_{NDM-1}	Penicillin	256	16 (16)
	Ampicillin	>256	16 (16)
	Ceftazidime	256	32 (8)
	Meropenem	32	0.25 (128)
	Biapenem	4	0.25 (16)

PHT427 in combination with antibiotics was tested at a final concentration of 39.06 μmol/L.

NDM-1 and KPC-2) by checkerboard microdilution assays. PHT427 combined with meropenem can reduce the MIC of meropenem (from 16 μg/mL to 0.25 μg/mL) against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}. The FICI was 0.04, which indicated the synergistic interaction between them (Synergy is defined for FIC index $\leq$ 0.5) (**Figure 6A**). In addition, the synergistic effects were also evaluated on *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2), and the FIC index values were 0.38 and 0.50, respectively (**Figures 6B, C**). In summary, PHT427 could restore the susceptibility of meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2) *in vitro*.

4. Discussion

The emergence and spread of carbapenem resistant *Enterobacteriaceae* (CRE) has posed a great threat to human and animal health (Zhang et al., 2017). NDM-1 has spread rapidly worldwide since its discovery. Food producing animals in China are important hosts of NDM positive *E. coli* (Kuang et al., 2022). The clinical and microbiological data which were collected from 96 ICUs in 78 hospitals in Henan Province, China, showed that the positive rate of carbapenem resistant *Enterobacteriaceae* (CRE) in intensive care units (ICUs) was very high (Guo et al., 2022). In addition, pathogens producing carbapenemase also appeared in coastal waters, and an NDM-1-positive *E. coli* strain belonging to the international clone sequence type (ST) 162 was identified in a pygmy sperm whale (*Kogia breviceps*) (Sellera et al., 2022). Therefore, it is urgent to develop a new antimicrobial agent against carbapenem-resistant bacteria. In the past 20 years, only six new antibiotics have been approved, and all of them are ineffective against Gram-negative bacteria (Butler et al., 2017). Combination therapies, including combination I (antibiotic + antibiotic), combination II (antibiotic + non-antibiotic) and combination III (non-antibiotic + non-antibiotic) offer a promising pipeline for the discovery and development of new anti-infective regimens in the post-antibiotic era (Liu et al., 2021). Non-antibiotic compounds can enhance antibiotic activity by blocking resistance, enhancing intracellular antibiotic accumulation, complementing bactericidal mechanisms, inhibiting signaling and regulatory pathways, or enhancing the host response to bacterial infection (Liu et al., 2019). Combination II (antibiotic + non-antibiotic) has been viewed as a more practical and efficient option than developing novel antibiotics for monotherapy (Olsen, 2015; Czaplewski et al., 2016). Moreover, combination II approach prolongs the life of well-established and clinically validated antibiotics, and significantly shortens development time and cost, while ensuring the safety of drugs (Liu et al., 2021). The outstanding success is inhibitors of β-lactamases, such as amoxicillin-clavulanic acid pair, ceftazidime-avibactam, ceftolozane-tazobactam, meropenem-vaborbactam, imipenem-relebactam etc., (Yahav et al., 2020). Cefepime in combination with VNRX-5133 has shown highly effective antibacterial activity against carbapenem-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*, and is currently in a phase III clinical trial (Liu et al., 2019; Vázquez-Ucha et al., 2020).

PHT427 inhibited pleckstrin homology domain targeting AKT and the PtdIns-3-kinase/PtdIns-dependent protein kinase 1 (PDPK1)/Akt (protein kinase B). It was also effective for pancreatic cancer, breast cancer, non-small cell lung cancer (Moses et al., 2009; Meuillet et al., 2010; Miller et al., 2021). Besides, PHT427 had greater additive activity than paclitaxel in breast cancer and erlotinib in non-small cell lung cancer (Meuillet et al., 2010), and improved the treatment of Mia PaCa-2 pancreatic cancer through poly(lactic-co-glycolic acid) (PLGA) nanoparticles (Kobes et al., 2016). Meanwhile, PHT427 has also been reported in the research of microbial system. It was identified as a new antimicrobial agent against *Staphylococcus aureus* causing bovine mastitis, which effectively inhibited the FeoB activity and the growth of Gram-positive food borne bacteria (Shin et al., 2021). However, its inhibitory activity against drug-resistant Gram-negative bacteria has not been reported. In this study, PHT427, a NDM-1 inhibitor, was screened from the library of small molecule compounds based on our previous high-throughput screening model, with an IC_{50} of 1.42 μmol/L. Additionally, we confirmed the interaction between PHT427 and NDM-1 via fluorescence quenching and SPR assays.

The reported NDM-1 inhibitors are mainly divided into three categories according to their interactions with the NDM-1 protein. The first species of inhibitors act on the zinc ion of NDM-1 active site directly, such as Ethylenediamine derivatives. The second type of inhibitors block the binding of NDM-1 to the substrate by acting on the amino acid residues of NDM-1, like Magnolol and its derivatives. The third kind of inhibitors act on the zinc ions at the active site of NDM-1 and the catalytic key amino acid residues at the same time, and they are considered to be the most potential NDM-1 inhibitors, such as captopril and its derivatives (Linciano et al., 2019; Wang et al., 2021). Our molecular docking results showed that PHT427 belonged to the third category of NDM-1 inhibitors. PHT427 acts on Zn1 and Zn2 of NDM-1 through the oxygen atom of the sulfonamide group and forms hydrogen bond interaction with key amino acids Asn220, Asp124, and Gln123. Asp124 participates in the coordination of Zn2, and Zn1 is connected to Zn2 through the side chain of Asp124 (Skagseth et al., 2017; Linciano et al., 2019). Gln123 and Asp124 form hydrogen bond interaction with oxygen atoms adjacent to hydrophobic β-lactam R groups and hydrophilic pores for substrate bonding (Groundwater et al., 2016). Asn220 participates in substrate recognition and hydrolysis, and can stabilize tetrahedral intermediates product by combining with Zn1 to form oxygen anion pore. In addition, Asn220 can

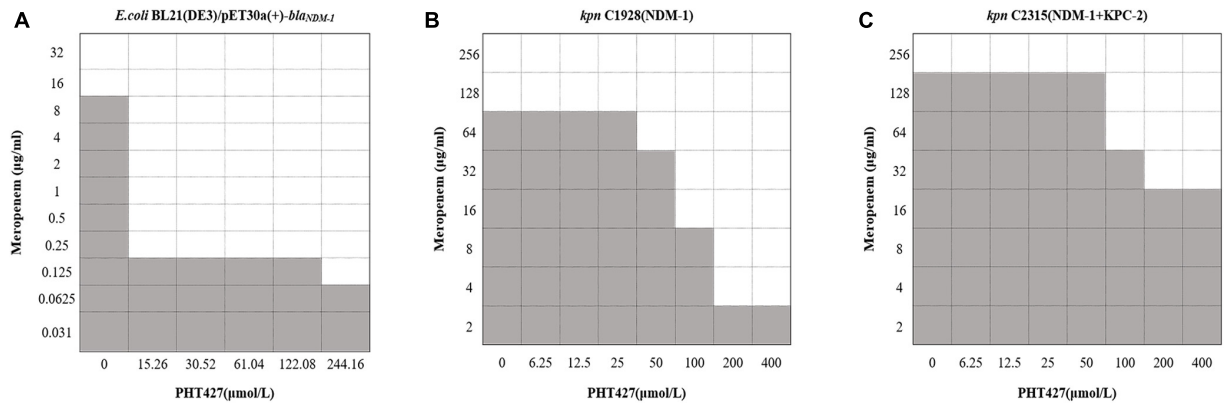

FIGURE 6
PHT427 restored the susceptibility of meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *Klebsiella pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2). Checkerboard microdilution assays for PHT427 in conjunction with meropenem against. **(A)** *E.coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}. **(B)** *Klebsiella pneumoniae* clinical strain C1928 (producing NDM-1). **(C)** *Klebsiella pneumoniae* clinical strain C2315 (producing NDM-1 and KPC-2).

form hydrogen bond with carbonyl oxygen of the substrate (Groundwater et al., 2016). We mutated Gln123, Asp124, and Asn220 to alanines, respectively (as Q123A, D124A, and N220A, respectively), and the results showed that N220A and Q123A significantly reduced the interaction compared with NDM-1-wild type. This supported our molecular docking results. Moreover, the mutation of Asp124 reduced the enzymatic activity significantly, which indicated that the importance of this residue for the enzymatic activity. Zinc supplementation assay demonstrated that PHT427 exerted its inhibitory activity by chelating zinc ions at the active site of NDM-1 enzyme. Therefore, the mechanism study showed that PHT427 acts on the zinc ions at the active site of NDM-1 and the catalytic key amino acid residues simultaneously. Additionally, checkerboard microdilution and MIC assays showed that PHT427 was effective for *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, and more importantly, it had strong inhibitory activity on *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2). Meanwhile, the combination of PHT427 and meropenem had synergistic effect. However, the synergism effect of PHT427 with meropenem against clinical strains was inferior to the *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1} isolate. This could be explained by the more complicated drug resistance mechanism of clinical strains, including expression of β-lactamases, the mutation of penicillin binding protein and the active efflux of drugs efflux pumps, etc., (Lima et al., 2020). This phenomenon was consistent with the reported that the synergism effect of 3-Bromopyruvate with β-lactam antibiotics against clinical strains was inferior to the *E. coli* BL21 isolate (Kang et al., 2020). Based on the above results, we believe that PHT427 could be combined with antibiotics to treat carbapenem resistant strains. Furthermore, PHT427 displayed low cytotoxicity with an IC_{50} value of 104.70 ± 3.33 μmol/L for Vero cells, and an IC_{50} value of 76.92 ± 0.57 μmol/L for HepG2 cells (**Supplementary Figure 1**). *In vivo* acute toxicity assay was performed using male Kunming mice. The surviving number of PHT427 (L) group is 5/6 and that of PHT427 (H) group is 5/6, indicating the safety of the compound in animals. PHT427 showed relatively good safety and does not cause significant changes in body weight and blood biochemistry after oral administration for more than 5 days (Meuillet et al., 2010). Therefore, our results showed that PHT427 is a promising lead compound against carbapenem-resistant bacteria and it merits chemical optimization for drug development. However, we have not evaluated whether PHT427 combined with existing antibiotics can effectively treat carbapenem-resistant bacterial infection *in vivo*, which will be further studied in the future.

5. Conclusion

Our research showed that PHT427 was a novel and effective NDM-1 inhibitor. PHT427 could restore the susceptibility of meropenem against *E. coli* BL21(DE3)/pET30a(+)-bla_{NDM-1}, *K. pneumoniae* clinical strains C1928 (producing NDM-1) and C2315 (producing NDM-1 and KPC-2) *in vitro*. The mechanism study showed that PHT427 acts on the zinc ions at the active site of NDM-1 and the catalytic key amino acid residues simultaneously, and Asn220 and Gln123 at the active site were vital for maintaining the stability of PHT427 and NDM-1 binding.

Data availability statement

The datasets presented in this article are not readily available because some of these data are needed for follow-up research. Requests to access the datasets should be directed to YL, liuys@imb.pumc.edu.cn.

Ethics statement

All animal experimental procedures were performed under the regulations of the Institutional Animal Care and Use Committee of the Institute of Medicinal Biotechnology, and Approval Number is IMB-20230310$D_3$02.

Author contributions

XL and QW conducted to the research and wrote the manuscript. YG, CL, SL, TL, and CX helped with the experimental process. JZ performed the checkerboard microdilution assay. XL and JH analyzed the data. XL, XW, and YL 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.

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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.2023.1168052/full#supplementary-material

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Global prevalence and antibiotic resistance in clinical isolates of *Stenotrophomonas maltophilia*: a systematic review and meta-analysis

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Introduction: *Stenotrophomonas maltophilia* is a little-known environmental opportunistic bacterium that can cause broad-spectrum infections. Despite the importance of this bacterium as an emerging drug-resistant opportunistic pathogen, a comprehensive analysis of its prevalence and resistance to antibiotics has not yet been conducted.

Methods: A systematic search was performed using four electronic databases (MEDLINE via PubMed, Embase, Scopus, and Web of Science) up to October 2019. Out of 6,770 records, 179 were documented in the current meta-analysis according to our inclusion and exclusion criteria, and 95 studies were enrolled in the meta-analysis.

Results: Present analysis revealed that the global pooled prevalence of *S. maltophilia* was 5.3 % [95% CI, 4.1–6.7%], with a higher prevalence in the Western Pacific Region [10.5%; 95% CI, 5.7–18.6%] and a lower prevalence in the American regions [4.3%; 95% CI, 3.2–5.7%]. Based on our meta-analysis, the highest antibiotic resistance rate was against cefuroxime [99.1%; 95% CI, 97.3–99.7%], while the lowest resistance was correlated with minocycline [4·8%; 95% CI, 2.6–8.8%].

Discussion: The results of this study indicated that the prevalence of *S. maltophilia* infections has been increasing over time. A comparison of the antibiotic resistance of *S. maltophilia* before and after 2010 suggested there was an increasing trend in the resistance to some antibiotics, such as tigecycline and ticarcillin-clavulanic acid. However, trimethoprim-sulfamethoxazole is still considered an effective antibiotic for treating *S. maltophilia* infections.

KEYWORDS

***Stenotrophomonas maltophilia*, prevalence, antibiotic resistance, global, meta-analysis**

Introduction

Stenotrophomonas maltophilia is an environmental Gram-negative bacillus that has been the subject of extensive research over the last two decades due to its status as the only known species of *Stenotrophomonas* to cause opportunistic infections in humans (1). Before the 1970s, this bacterium was underestimated and was considered a rare opportunistic pathogen with low invasiveness. However, advances in medical interventions and pharmacological treatments have led to an increase in the population of immunocompromised patients, such as those undergoing chemotherapy, organ transplantations, or complex surgeries, who are prone to infection with this bacterium. In addition, the development of diagnostic methods in clinical microbiology resulted in more precise identification of this pathogen. Therefore, the number of reported *S. maltophilia* infections has increased, and it is recognized as an emerging nosocomial pathogen (2). *S. maltophilia* causes infections of the soft tissue, urinary tract, eye, and wound. In addition, it causes pneumonia, bacteremia, sepsis, endocarditis, osteochondritis, mastoiditis, and meningitis (3). Predisposing factors associated with *S. maltophilia* infections include underlying malignancy, indwelling devices, chronic respiratory disease, particularly cystic fibrosis, immune compromisation, prolonged antibiotic use, and long-term hospitalization or admission to an intensive care unit (ICU) (3, 4). The treatment of infections caused by this bacterium presents several challenges. Distinguishing colonization from invasive infections is problematic, and physicians often fail to recognize their associated risk factors and clinical characteristics, which leads to delayed antibiotic prescription and high mortality (5).

Because of the high-level intrinsic resistance of *S. maltophilia* to several classes of antibiotics, there are restricted therapeutic choices for its infections. This bacterium can resist the β-lactam antibiotics (most notably carbapenems) by producing ß-lactamase enzymes, including L1 and L2. It also disrupts the action of aminoglycosides by hydrolyzing enzymes such as acetyl-transferases or modifying the structure of lipopolysaccharide. In addition, low membrane permeability and the overproduction of efflux pumps are other mechanisms that render *S. maltophilia* resistant to a broad range of antibiotics (2, 6). Additionally, they can acquire resistance genes and genetic mutations (7, 8), further limiting the choice of effective antimicrobials. This increasing prevalence of drug-resistant *S. maltophilia* has presented one of the biggest challenges in treating patients in recent years (3, 9).

The Infectious Diseases Society of America (IDSA) has approved a guideline document with recommendations for treating *S. maltophilia* infections (10). Trimethoprim-sulfamethoxazole (TMP/SMX) is the antibiotic of choice for treating these infections, but its use is limited by allergy, intolerance, and increased resistance (11). Other drugs with good susceptibility impact include ticarcillin-clavulanate, ceftazidime, and fluoroquinolones, although resistance to these drugs has been reported. Tetracyclines such as minocycline, tigecycline, and doxycycline are also efficacious in treating *S. maltophilia* infections, and their efficacy has been reported in different geographic areas (3, 12).

The main objective of this study was to assess the global prevalence of *S. maltophilia* and its resistance to commonly used antibiotics. We conducted this systematic review of global human infections due to *S. maltophilia* over the last 31 years.

Methods

Search strategy and selection criteria

Four electronic databases, including MEDLINE (via PubMed), Embase, Web of Science, and Scopus, were systematically searched using different combinations of the following keywords: "*Stenotrophomonas maltophilia*" OR "*Xanthomonas maltophilia*" AND "antibiotic resistance" AND "minimum inhibitory concentration" AND "disk agar diffusion" AND "multilocus sequence typing" AND "E-test" AND "antimicrobial resistance gene". The databases were searched up to 20 October 2019 without any start time limitation.

The study was carried out based on the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (13). Two distinct reviewers applied the inclusion and exclusion criteria for article selection and screened the titles and abstracts of all studies; then, two autonomous researchers qualified the screened papers. Any disagreements between the reviewers were resolved by consensus.

Inclusion criteria

Articles were included if they reported the prevalence of *S. maltophilia* isolation among diverse patients in combination with the antibiotic resistance rates of the isolates to various antibiotics, or reported only the antibiotic resistance rates of the isolates. Only articles about the clinical isolates of *S. maltophilia* were enrolled, and studies on the environmental isolates were not considered.

Exclusion criteria

Conference papers were not evaluated as they did not provide sufficient information for quality assessment. Dissertations and theses were excluded. Articles with unrelated topics, duplicates or overlapping studies, reviews, meta-analyses or systematic reviews, case reports, brief reports, notes, editorials, correspondence, short communications, and letters to the editors were not included. Studies with languages other than English or with unavailable full text were dismissed. Studies that evaluated species other than *S. maltophilia* or tested a total isolate <10 were not assessed. Articles that reported antibiotic resistance as MIC 90 or those that evaluated the combinatorial effects of antibiotics were not enrolled. Studies that considered *S. maltophilia* a Gram-negative bacterium and reported a total antibiotic resistance rate in Gram-negative bacteria were excluded. Articles were removed if they tested only the resistant isolates or reported only the prevalence of *S. maltophilia* infection.

Study selection and data extraction

Two independent researchers read the included articles in full text and extracted the following details: first author's name, year of study, year of publication, location of the study (country and region), sample size (N/total), type of samples, antibiotic susceptibility testing methods used (agar dilution, broth microdilution, broth macrodilution, E-test, disk agar diffusion [DAD], MIC test strip, Vitek, Phoenix, and Microscan), the antibiotic resistance rate of isolates against various antibiotics, frequency of resistance genes, and frequency of different sequence types. Any discrepancy between the two reviewers was settled by consensus.

Quality assessment

Two reviewers separately evaluated the quality of the included studies using the Joanna Briggs Institute (JBI) critical appraisal checklist for studies reporting prevalence data (14). This scale rates each criterion out of 1, with a total score ranging from 0 to 10. Studies with a score of ≥5 were classified as high quality.

Meta-analysis

The meta-analysis was carried out using Comprehensive Meta-Analysis (CMA) software version 2.0 (Biostat, Englewood, NJ). A random-effect model was used for meta-analysis and to pool the estimations. The prevalence of the investigated phenomenon was presented as a forest plot diagram, which shows the estimated prevalence and its relevant 95% confidence interval (CI). Heterogeneity between studies was reported by I^2 statistics. An I^2 between 0 and 25% suggests low heterogeneity, 25–50% indicates moderate heterogeneity, 50–75% represents substantial heterogeneity, and 75–100% shows considerable heterogeneity. Subgroup meta-analysis was employed to compare the prevalence of *S. maltophilia* based on WHO-defined regions and 5-year time intervals. In addition, the antibiotic resistance rates of isolates were compared based on world regions and whether they were reported before or after 2010. To assess the potential risk of publication bias, Begg's rank correlation and Egger's weighted

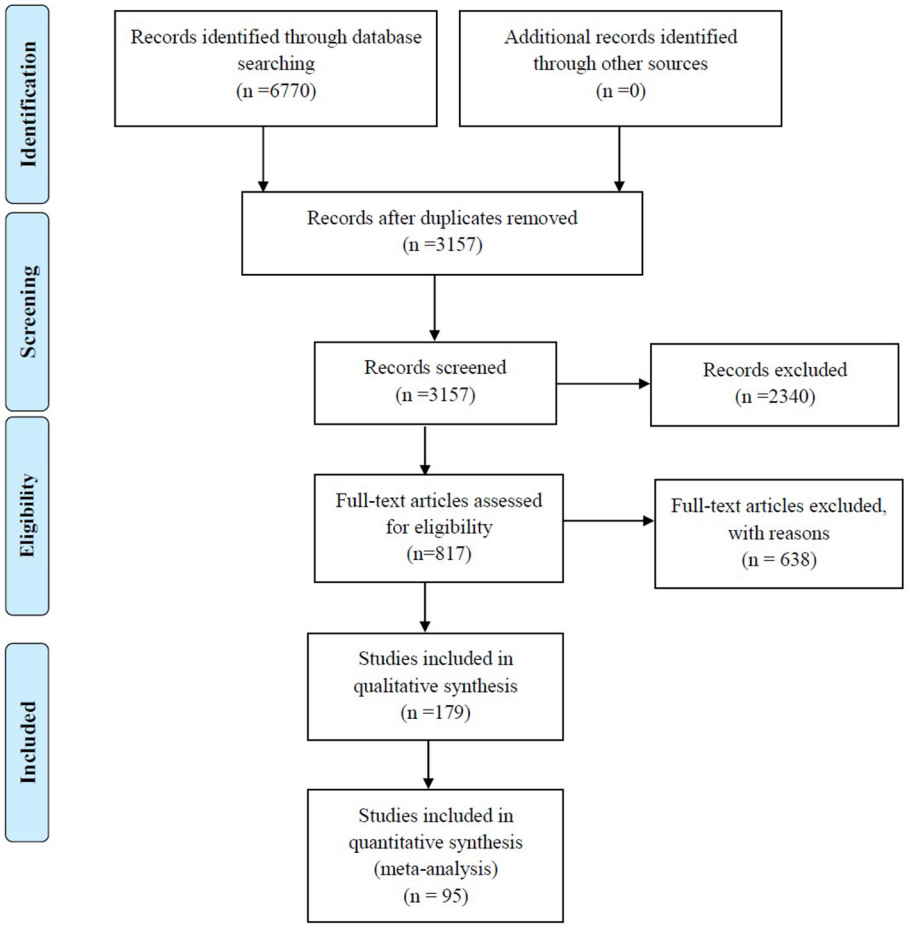

FIGURE 1
Summary of the literature search and study selection.

TABLE 1 Characteristics of the studies that reported *Stenotrophomonas maltophilia* isolation in different parts of the world.

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
1	Al-Lawati et al. (16)	ND	2000	Oman	Eastern Mediterranean Region (EMR)	Not-determined (ND)	9/100	Respiratory (7), wound (1), others (1)	Hospitalized patients (ICU)	6
2	Asaad et al. (17)	2012-2013	2013	Saudi Arabia	EMR	Cross-sectional	26/125	Clinical samples	Hospitalized patients	7
3	Bostanghadiri et al. (18)	2016-2017	2019	Iran	EMR	Cross-sectional	164/164	Blood (137), cough swabs (16), nose/throat secretions (9), sputum (1), CSF (1)	Hospitalized patients	4
4	Cunha et al. (19)	1995	1997	Saudi Arabia	EMR	Prospective	27/1132	Clinical samples	Nosocomial infection	6
5	Ebrahim-Saraie et al. (20)	2015-2016	2019	Iran	EMR	Retrospective	44/44	Clinical samples	NICU, ICU, SUR, transplant, general medicine	5
6	El Tahawy and Khalaf (21)	1999-2000	2001	Saudi Arabia	EMR	ND	35/499	Clinical samples	ICU, surgery, pediatric, gynecology	7
7	Jamali et al. (22)	2008-2009	2011	Iran	EMR	ND	100/2300	Blood(100)	Hospitalized patients	7
8	Khalili et al. (23)	2007-2010	2012	Iran	EMR	ND	281/1745	Clinical samples	Hospitalized patients	6
9	Morsi et al. (24)	2013-2015	2016	Egypt	EMR	Cross-sectional	32/32	Urine (1), sputum (7), endotracheal aspirates (15), blood (3), pus (6)	Hospitalized patients	6
10	Qadri et al. (25)	ND	1991	Saudi Arabia	EMR	ND	31/3144	Clinical samples	ND	7
11	Qadri et al. (26)	ND	1992	Saudi Arabia	EMR	ND	28/1205	Clinical samples	ND	7
12	Qadri et al. (27)	ND	1993	Saudi Arabia	EMR	ND	67/1294	Clinical samples	Hospitalized patients	7
13	Qadri et al. (28)	1992	1993	Saudi Arabia	EMR	Cross-sectional	22/563	Clinical samples	Hospitalized patients	7
14	Qadri et al. (29)	ND	1992	Saudi Arabia	EMR	ND	36/922	Clinical samples	Hospitalized patients	7
15	Cha et al. (30)	2006-2014	2016	South Korea	Western Pacific Region (WPR)	Cross-sectional	127/127	Blood (127)	Bacteremia	6
16	Chang et al. (31)	2002	2004	Taiwan	WPR	Cross-sectional	93/93	Sputum (54), wounds (14), central venous catheter (8), urine (5), bile (4), blood (4), throat swabs (2), cerebrospinal fluid (1), eye (1)	ND	5
17	Chen et al. (32)	2002-2006	2010	Taiwan	WPR	Retrospective	67/1307	Blood (67)	Hospitalized patients (hematological malignancy)	7
18	Cho et al. (33)	2009-2014	2015	South Korea	WPR	Retrospective	31/31	Blood (31)	Hospitalized patients (hematological malignancy)	5

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
19	Cho et al. (34)	2009	2012	South Korea	WPR	ND	33/33	Clinical samples	Hospitalized patients	5
20	Chung et al. (35)	2010	2013	South Korea	WPR	ND	206/206	Clinical samples	ND	5
21	Chung et al. (9)	2009-2010	2015	South Korea	WPR	ND	252/252	Clinical samples	ND	5
22	Fu et al. (36)	ND	2003	China	WPR	ND	323/3905	Clinical samples	ND	7
23	Fujita et al. (37)	1988-1992	1996	Japan	WPR	ND	10/10	Upper respiratory tract (10)	Patients with pneumonia	5
24	Friedman et al. (38)	1988-1997	2002	Australia	WPR	Retrospective	45/45	Blood (45)	Patients with bacteremia	4
25	Hsueh et al. (39)	1999-2003	2005	Taiwan	WPR	ND	451/1006	Clinical samples	ND	6
26	Hu et al. (40)	2006-2008	2011	China	WPR	ND	102/102	Clinical samples	ICU, surgery, oncology, neurology, respiratory care, geriatrics	6
27	Hu et al. (41)	2005-2014	2016	China	WPR	ND	300/300	Clinical samples	ND	6
28	Hu et al. (42)	2010-2011	2014	China	WPR	ND	83/83	Clinical samples	Hospitalized patients	6
29	Hu et al. (43)	2005-2014	2018	China	WPR	ND	300/300	Clinical samples	Hospitalized patients	6
30	Ismail et al. (44)	2011-2012	2017	Malaysia	WPR	ND	84/84	Clinical samples	ND	6
31	Jean et al. (45)	2013-2014	2017	Taiwan	WPR	ND	39/799	Clinical samples	Hospitalized patients	6
32	Kanamori et al. (46)	2009-2010	2015	Japan	WPR	ND	181/181	Clinical samples	Hospitalized patients, community patients	6
33	Liaw et al. (47)	2002-2003	2010	Taiwan	WPR	ND	30/70	Sputum (30)	Sputum, wound, central venous catheter, urine, blood, cerebrospinal fluid, eye	7
34	Liu et al. (48)	2008-2013	2016	Taiwan	WPR	Retrospective	50/378	Blood (50)	Bloodstream infection (BSI)	7
35	Lan et al. (49)	2011-2013	2017	Vietnam	WPR	ND	11/1017	Blood (11)	BSI	7
36	Neela et al. (50)	2008	2012	Malaysia	WPR	ND	64/64	Tracheal aspirate (25), peritoneal fluid (1), bronchoalveolar lavage (1)	ICU, neurology, psychiatric, dermatology wards	6
37	Ning et al. (51)	2007-2011	2013	China	WPR	ND	17/127	Sputum (17)	Patients with VAP in a pediatric ICU	6
38	Rhee et al. (52)	2007-2011	2013	South Korea	WPR	ND	121/121	Clinical samples	ND	6
39	Shi et al. (53)	2003-2006	2009	China	WPR	Cross-sectional	48/323	Blood (48)	Hospitalized (liver transplant)	7

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
40	Sun et al. (54)	2006-2012	2016	China	WPR	Cross-sectional	51/51	Pus (7), intravascular catheter (7), postoperative and burn wound (7), bronchial secretions/lavage (6), urinary catheter (6), urine (5), sputum (4), bile (4), blood (3), ascitic fluid (2)	Hospitalized patients with invasive infections	6
41	Tan et al. (55)	2004	2006	Singapore	WPR	Cross-sectional	17/ 102	Clinical samples	ND	7
42	Tanimoto et al. (56)	2005	2013	Japan	WPR	ND	66/66	Clinical samples	ND	6
43	Wang et al. (57)	1998	2000	China	WPR	Cross-sectional	50/440	Clinical samples	ND	7
44	Wang et al. (58)	1999-2003	2004	Taiwan	WPR	Cross-sectional	50/50	Blood (50)	Hospitalized patients (bacteremia)	6
45	Wei et al. (59)	2013	2016	China	WPR	Cross-sectional	80/80	Respiratory tract specimens (63), catheter-related specimens (10), urine (4), blood (3)	ND	6
46	Wu et al. (60)	1998-2008	2012	Taiwan	WPR	Cross-sectional	377/377	Respiratory tract (256), blood (48), others (73)	Hospitalized (ICU)/outpatient patients (60)	6
47	Watanabe et al. (61)	1994-2011	2014	Australia	WPR	Comparative analysis	40/40	Clinical samples	ND	6
48	Xu et al. (62)	2005-2008	2010	China	WPR	ND	12/258	Clinical samples	Neonate patients (NICU)	7
49	Yuk-Fong Liu et al. (63)	1993-1994	1995	Taiwan	WPR	ND	28/366	Clinical samples	Hospitalized patients (ICU)	7
50	Zhao et al. (64)	2015	2017	China	WPR	Cross-sectional	400/400	Sputum (315), throat swab (30), urine (25), secretions (15), bile (10), blood (5)	Hospitalized patients	5
51	Zhao et al. (65)	2012-2014	2016	China	WPR	Cross-sectional	450/450	Clinical samples	Hospitalized patients	6
52	Zhao et al. (66)	2012-2015	2018	China	WPR	Cross-sectional	450/450	Respiratory tract specimens (450)	Hospitalized patients	6
53	Zhang et al. (67)	ND	2012	China	WPR	Cross-sectional	442/442	Clinical samples	ND	6
54	Chawla et al. (68)	2009-2011	2013	India	South-East Asia Region (SEAR)	Retrospective	15/33	Respiratory samples (15)	Respiratory tract infection	7
55	Chawla et al. (69)	2012-2013	2014	India	SEAR	Retrospective	33/33	Sputum (17), endotracheal aspirates (16)	Patients with lower respiratory tract infection (LRTI)	6

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
56	Garg et al. (70)	2014-2016	2019	India	SEAR	ND	5/3414	Clinical samples	ND	5
57	Gunasekar et al. (71)	2017	2018	India	SEAR	ND	12/240	ND	ND	7
58	Kaur et al. (72)	2012-2013	2015	India	SEAR	ND	106/106	Clinical samples	Hospitalized patients	6
59	Nayyar et al. (73)	2015-2016	2017	India	SEAR	Retrospective	23/2734	Blood (15), urine (4), tracheal aspirate (4)	Pediatric patients	6
60	Paopradit et al. (74)	2014-2015	2017	Thailand	SEAR	ND	64/64	Sputum (36), blood (9), tissue (6), pus (1), urine (1), body fluid (9), bronchial wash (2)	Patients on the ICU, respiratory care unit (RCU), medicine (MED), surgical, pediatric, emergency room, eye wards	6
61	Tantisiriwat et al. (75)	2014-2015	2017	Thailand	SEAR	Cross-sectional	33/ 1288	Sputum, urine, pus, blood	ND	6
62	Averbuch et al. (76)	2001-2014	2017	Israel	European Region (EUR)	Retrospective	10/116	Blood (10)	Hospitalized children (malignancies and solid tumors)	7
63	Averbuch et al. (77)	2014-2015	2017	Israel	EUR	Non-interventional prospective	31/704	Blood (31)	Patients with hematopoietic stem cell transplant (HSCT)	7
64	Bousquet et al. (78)	2003-2010	2014	France	EUR	Retrospective	45/723	Blood (45)	Hematological malignancies	5
65	Canton et al. (79)	1991- 1998	2002	Spain	EUR	ND	98/127	Respiratory secretion, Sputum	Hospitalized patients (CF and non-CF)	5
66	Chen et al. (80)	1991	1993	UK, Ireland	EUR	ND	21/6724	Clinical materials except feces	Hospitalized patients	6
67	De Dios Caballero et al. (81)	2013	2015	Spain	EUR	Prospective, multicenter, observational	49/339	Sputum (49)	CF patients	7
68	Cikman et al. (82)	2006-2012	2016	Turkey	EUR	Retrospective	118/118	Tracheal aspirate (67), blood (17), sputum (10), wound (10), ear (3), CSF (2), paracentesis (2), pleural fluid (2), urine (2), puncture fluid (2), catheter (1)	ND	5
69	Di Bonaventuraa et al. (83)	2001	2002	Italy	EUR	ND	19/223	Respiratory tract specimen, blood, urine, skin and wound swabs	Neutropenic patients with hematological malignancies	6
70	Di Bonaventuraa et al. (84)	ND	2004	Italy	EUR	ND	50/50	Clinical samples	Neutropenic patients with hematological malignancies	6
71	Djordjevic et al. (85)	2009-2015	2017	Serbia	EUR	Cohort	38/850	Sputum, BAL, tracheal samples	Medical-Surgical ICU/HAP and VAP	7

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
72	Esposito et al. (86)	2003-2014	2017	Italy	EUR	ND	91/91	Sputum samples (91)	CF patients	5
73	Frank et al. (87)	1996-1997	2000	Germany	EUR	ND	52/52	Tracheal secretions, wound, blood, urine, biopsy, puncture fluid	ND	6
74	Fadda et al. (88)	1997-1999	2004	Italy	EUR	ND	307/307	Respiratory tract samples (307)	Hospitalized patients	6
75	Gajdacs et al. (2)	2008-2017	2019	Hungary	EUR	Retrospective	579/579	Tracheal aspirates, sputum, BAL, pleural and pericardial puncture	Septicemia, hematological malignancies and solid tumors, pneumonia, pleuritis, CF, meningitis, etc.	4
76	Galani et al. (89)	2004-2006	2008	Greece	EUR	ND	36/778	Clinical samples	ND	6
77	Garcia-Rodriguez et al. (90)	1992	1995	Spain	EUR	ND	21/2426	Clinical samples	ND	6
78	Garcia-Rodriguez et al. (91)	1991	1989	Spain	EUR	ND	42/42	Clinical samples	ND	5
79	Garcia-Rodriguez et al. (92)	ND	1991	Spain	EUR	ND	18/18	Clinical samples	ND	5
80	Gesu et al. (93)	2000	2003	Italy	EUR	ND	124/4003	Clinical samples	ND	6
81	Glupczynski et al. (94)	1996-1997	2001	Belgium	EUR	ND	73/73	Clinical samples	ICU patients	6
82	Glupczynski et al. (94)	1998-1999	2001	Belgium	EUR	ND	48/48	Clinical samples	ICU patients	6
83	Gómez-Garces et al. (95)	1996-2006	2009	Spain	EUR	ND	80/228	Clinical samples	ND	7
84	Goncalves-Vidigal et al. (96)	2009-2011	2011	Germany	EUR	ND	65/65	Sputum (65)	CF patients	6
85	Gordon et al. (97)	ND	2010	UK	EUR	ND	13/13	Sputum, blood	ND	4
86	Gospodarek et al. (98)	1994-1995	1997	Poland	EUR	ND	27/27	Wound smears, pus, intubation tube	Intensive therapy, urologic, neurology, surgery	5
87	Gramegna et al. (99)	2001-2010	2018	UK	EUR	ND	34/193	Sputum (34)	CF patients	7
88	Grillon et al. (100)	ND	2016	France	EUR	ND	40/120	Clinical samples	ND	7
89	Grohs et al. (101)	ND	2017	France	EUR	ND	12/58	Respiratory samples (12)	CF patients	7

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
90	Guembe et al. (102)	2003-2007	2008	Spain	EUR	ND	7/572	Wound, abscesses	Patients with intra-abdominal infection	7
91	Gulmez et al. (103)	2005	2010	Turkey	EUR	ND	25/25	Clinical samples	Hospitalized patients	5
92	Gulmez et al. (104)	1998-2003	2005	Turkey	EUR	ND	205/205	Clinical samples	Hospitalized patients	6
93	Guriz et al. (105)	1995-2005	2008	Turkey	EUR	ND	33/33	Blood (33)	Hospital-acquired bacteremia	6
94	Hohl et al. (106)	ND	1991	Switzerland	EUR	ND	33/33	Clinical samples	ND	6
95	Hombach et al. (107)	2010-2011	2012	Germany	EUR	ND	160/3713	Clinical samples	ND	7
96	Hoban et al. (108)	1997-1999	2001	16 European countries	EUR	ND	578/21464	Clinical samples	ND	7
97	Juhász et al. (109)	2009-2011	2014	Hungary	EUR	ND	100/160	Clinical samples	Hospitalized patients	7
98	Klietmann et al. (110)	1986-1989	1991	Germany	EUR	ND	234/130033	Clinical samples	ND	7
99	Koseoglu et al. (111)	1998-2001	2014	Turkey	EUR	ND	40/40	Clinical samples	Pediatric patients	6
100	Kucukates et al. (112)	2000-2002	2005	Turkey	EUR	ND	16/367	Clinical samples	Hospitalized patients (coronary and surgical ICUs)	7
101	Lakatos et al. (113)	1993-2013	2014	Switzerland	EUR	ND	27/27	Blood (27)	Bacteremia	4
102	Lanzafame et al. (114)	ND	2005	Italy	EUR	ND	64/495	ND	Patients hospitalized in intensive care, onco-hematological, surgical, burn and transplant units	7
103	Livermore et al. (115)	1991 and 2001	2003	UK, Ireland	EUR	ND	23/5031	Clinical samples	Hospitalized patients	6
104	Livermore et al. (116)	2008-2012	2014	UK	EUR	ND	40/170	ND	CF patients	7
105	Madi et al. (117)	2013-2015	2016	Serbia	EUR	Retrospective	88/88	Clinical samples	CF, non-CF outpatients and inpatients	6
106	McKnight et al. (118)	ND	2005	Ireland	EUR	ND	10/60	Sputum (10)	CF patients	7
107	Micozzi et al. (119)	1987-1996	2000	Italy	EUR	Retrospective	26/26	Blood (26)	Patients with hematologic malignancies (bacteremia)	5
108	Milne et al. (120)	2001-2010	2012	UK	EUR	ND	80/80	Respiratory samples (80)	CF patients	6

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
109	Pasargiklian et al. (121)	1993	1996	Italy	EUR	ND	25/303	Broncho aspirate (25)	ICU patients	7
110	Samonis et al. (122)	2005-2010	2012	Greece	EUR	Retrospective	68/81	Bronchial secretions/lavage (23), sputum (15), pus (8), blood (7), intravascular catheter tip (4), urine (4), ascitic fluid (3), bile (3), contact lenses (3), cornea (1), peritoneal dialysis fluid (1), throat swab (1), bone (1)	Hospitalized/outpatient patients (5.9%)	7
111	Samonis et al. (123)	2008	2010	Greece	EUR	Retrospective	21/594	Blood, lower respiratory tract, pus, normally sterile fluids, central venous catheter tips, stool, ophthalmic specimens, upper respiratory tract, genital tract	Hospitalized/outpatient patients (10.3%)	7
112	Schmitz et al. (124)	1997-1998	1999	Austria, Belgium, France, Germany, Greece, Italy, Netherlands, Poland, Portugal, Spain, Switzerland	EUR	Cross-sectional	106/9682	Blood, respiratory tract, wound, urine	ND	7
113	Traub et al. (125)	ND	1987	Germany	EUR	ND	14/14	Clinical samples	ND	4
114	Traub et al. (126)	1986-1997	1998	Germany	EUR	ND	96/96	Clinical samples	ICU patients	6
115	Tripodi et al. (127)	ND	2001	Italy	EUR	ND	50/50	Clinical samples	ND	6
116	Tunger et al. (128)	2003-2005	2007	Turkey	EUR	Retrospective	35/35	Blood (35)	Hospitalized patients (bacteremia)	6
117	Usarek et al. (129)	2011-2014	2016	Poland	EUR	Retrospective	26/26	Blood (26)	Hospitalized patients (blood infection)	4
118	Valenza et al. (130)	2006	2008	Germany	EUR	Cross-sectional	70/464	Sputum (70)	CF patients	7
119	Adams-Sapper et al. (131)	2007-2009	2012	USA	Region of the Americas (AMR)	Cross-sectional	9/376	Blood (9)	Hospitalized patients, outpatients, jail clinics (bloodstream infection)	6
120	Alcaraz et al. (132)	2004-2012	2018	Argentina	AMR	Cross-sectional	63/63	Respiratory specimens, blood, renal biopsy, peritoneal fluids, urine	Non-CF patients exposed to invasive devices	5

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TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
121	Blondeau et al. (133)	1994-1995	1999	Canada	AMR	ND	31/1518	Clinical samples	ND	7
122	Church et al. (134)	1999-2009	2012	Canada, USA	AMR	ND	90/90	Blood (62), lower respiratory tract specimen (19), peritoneal fluid (5), cerebrospinal fluid (4)	Hospitalized patients (invasive infections)	6
123	Denisuik et al. (135)	2007-2016	2018	Canada	AMR	National surveillance	238/8130	Respiratory specimen, blood, wound, urine	Patients with respiratory infections, urine, wound and BSIs.	7
124	Flamm et al. (136)	2015	2019	USA	AMR	ND	102/2254	Clinical samples	ND	7
125	Flores-Treviño et al. (137)	2006-2013	2014	Mexico	AMR	ND	119/119	Respiratory tract, blood, wound	ICU Patients	6
126	Forrester et al. (138)	ND	2018	USA	AMR	ND	13/93	Respiratory specimens (13)	CF patients	7
127	Fuchs et al. (139)	1994	1996	USA	AMR	ND	74/74	Clinical samples	ND	6
128	Gerlach et al. (140)	ND	1992	USA	AMR	ND	76/3416	Clinical samples	ND	7
129	Herrera-Heredia et al. (141)	2007-2015	2017	Mexico	AMR	ND	196/196	Clinical samples	ND	6
130	Hoban et al. (142)	1997-1999	2003	Canada, USA	AMR	ND	110/4536	Clinical samples	ND	7
131	Isenberg et al. (143)	1996-1997	1999	USA	AMR	ND	20/60	Clinical samples	ND	7
132	Jones et al. (144)	1995-1996	1997	USA	AMR	ND	18/270	Blood (18)	Nosocomial BSI	7
133	Jones et al. (145)	1997	1999	Canada, USA, Latin America	AMR	ND	177/23000	Clinical samples	ND	7
134	Karlowsky et al. (146)	2010-2012	2013	Canada	AMR	ND	174/9758	Clinical samples	ND	7
135	Karlowsky et al. (147)	2009-2009	2011	Canada	AMR	ND	79/4546	Clinical samples	ND	7
136	Karlowsky et al. (148)	2000-2000	2002	USA	AMR	ND	94/3099	Clinical samples	ND	7
137	Krueger et al. (149)	ND	2001	USA	AMR	ND	23/23	Urine, sputum, wound	ND	5
138	Mutnick et al. (150)	2000-2001	2013	USA	AMR	ND	54/1992	ND	Hospitalized patients in the oncology center (bloodstream, respiratory, urinary, skin and soft tissues infections)	7

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10.3389/fmed.2023.1163439

TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
139	Nicodemo et al. (151)	2000-2002	2004	Brazil	AMR	ND	70/70	Respiratory (47), urine (6), biopsy tissues (4), blood (3) and others (10)	Hospitalized patients	6
140	Passerini De Rossi et al. (152)	2004-2008	2009	Argentina	AMR	ND	32/32	Clinical samples	Patients with device-associated nosocomial infection	6
141	Poulos et al. (153)	ND	1995	Canada, USA	AMR	ND	31/31	Clinical samples	ND	5
142	Rizek et al. (154)	ND	2015	Brazil	AMR	ND	48/153	Blood (48)	ND	7
143	Rolston et al. (155)	ND	2003	USA	AMR	Cross-sectional	40/924	Clinical samples	Hospitalized patients (cancer patients)	7
144	Rolston et al. (156)	ND	1997	USA	AMR	Cross-sectional	30/716	Clinical samples	Hospitalized patients (cancer patients)	7
145	Rutter et al. (157)	2010-2014	2016	USA	AMR	Cross-sectional	45/542	Respiratory samples (45)	Hospitalized patients (CF patients)	7
146	Sader et al. (158)	2015-2017	2018	USA	AMR	Cross-sectional	311/6091	Trans tracheal aspiration, bronchoalveolar lavage, protected brush samples, qualified sputum samples	Hospitalized patients (pneumonia patients)	7
147	Sader et al. (159)	ND	1993	USA	AMR	ND	10/853	Clinical samples	Hospitalized patients (septicemia)	6
148	Sahm et al. (160)	1999	2001	USA	AMR	Cross-sectional	123/3368	Clinical samples	ND	7
149	San Gabriel et al. (161)	1996- 2001	2004	USA	AMR	Cross-sectional	955/955	Respiratory samples (955)	CF patients	6
150	Sattler et al. (162)	1992-1998	2000	USA	AMR	Retrospective	51/51	Blood (32), conjunctiva (3), urine (3), skin and soft tissue (3), surgical site or wound (3), paranasal sinus (3), other sites (4)	ND	6
151	Travassos et al. (163)	ND	2004	Brazil	AMR	ND	39/39	ND	Hospitalized/outpatient patients (9)	6
152	Spierer et al. (164)	2000–2013	2018	USA	AMR	Retrospective	15/58	Corneal (15)	Keratitis patients	7
153	Zhanel et al. (165)	2007-2009	2011	Canada	AMR	Cross-sectional	245/18538	Blood, urinary tract, respiratory tract, wound	Inpatients and outpatients	7
154	Zhanel et al. (166)	2014–2015	2018	Canada	AMR	Cross-sectional	118/4637	Blood, urinary tract, respiratory tract, wound	ND	7

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TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
155	Zhanel et al. (167)	2005-2006	2008	Canada	AMR	Cross-sectional	108/3931	Blood, urine, wound/tissue, respiratory tract	Hospitalized patients (ICU)	7
156	Chow et al. (168)	2002	2006	China, Taiwan, Korea, Australia, Thailand, Malaysia, USA, Spain, Germany, Belgium, Italy, Mexico, Puerto Rico, Guatemala, Argentina, Ecuador, Venezuela	Multiple regions	Prospective	36/3134	ND	Patients with intra-abdominal infections	7
157	Corlouer et al. (169)	2013-2014	2017	France, Spain, Tunisia	Multiple regions	Collection study	83/83	Sputum (16), tracheal aspiration (10), protected distal specimen (7), bronchoalveolar lavage (2), blood (18), urine (9), suppuration (8), central arterial/venous catheter (4), others (9)	CF patients, solid cancer, hematological malignancy and organ transplant	5
158	Diez-Aguilar et al. (170)	2003-2016	2019	Netherlands, Ireland, Spain, USA, Australia	Multiple regions	Cross-sectional	106/286	Respiratory samples (106)	CF patients	7
159	Farrell et al. (171)	2005-2010	2014	Europe, Israel, Turkey	Multiple regions	ND	420/60084	Clinical samples	Hospitalized patients	7
160	Farrell et al. (172)	2003-2008	2010	Asia-pacific, Europe, Latin America, North America	Multiple regions	ND	1586/1586	Clinical samples	Bloodstream and respiratory tract infections	6
161	Fedler et al. (173)	2004	2006	North America, Latin America, Europe	Multiple regions	ND	53/3537	Clinical samples	Pediatric patients	7
162	Flamm et al. (174)	2013	2016	USA, Europe-Mediterranean, Latin America, Asia-pacific	Multiple regions	ND	464/464	Clinical samples	ND	6

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TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
163	Frei et al. (175)	ND	1994	USA, Canada, Brazil, Japan, Spain, Switzerland	Multiple regions	ND	61/61	Clinical samples	ND	6
164	Fritsche et al. (176)	2000-2004	2005	Asia, Australia, Europe, North America, South America	Multiple regions	ND	57/10763	ND	Patients with community-acquired respiratory tract infections	7
165	Gales et al. (2001b)	1997-1999	2001	Asia-pacific, Europe, Latin America, Canada, USA	Multiple regions	The SENTRY Antimicrobial Surveillance Program	842/70067	Blood, Respiratory, wound, urine	BSIs (objective A), pneumonia in hospitalized patients (objective C), skin/soft-tissue infections (objective D), and urinary tract infections (objective E)	7
166	Gales et al. (177)	2001-2004	2006	Asia-pacific, Europe, Latin America, Canada, USA	Multiple regions	ND	1256/13808	Clinical samples	ND	7
167	Gales et al. (178)	2002-2005	2008	Asia-pacific, Europe, Latin America, Canada, USA	Multiple regions	ND	763/763	Blood, respiratory tract samples	ND	6
168	Hoban et al. (179)	ND	1993	6 countries	Multiple regions	ND	61/6064	Clinical samples	ND	7
169	Jones et al. (180)	1997-2001	2003	Asia-pacific, Europe, Latin America, US, Canada	Multiple regions	ND	1488/18569	Clinical samples	ND	7
170	Liu et al. (181)	2003-2010	2012	Taiwan, Thailand, Vietnam, Philippines, Hong Kong, China, Malaysia, Singapore, South Korea, Australia, New zealand	Multiple regions	Prospective	204/20710	Tissue, wound, fluid obtained from paracentesis or percutaneous aspiration of abscesses	Patients with intra-abdominal infections (IAI)	7

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TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
171	Renteria et al. (182)	2007-2012	2014	Egypt, Morocco, Mauritius, Namibia, South Africa, Tunisia, Israel, Jordan, Lebanon, Oman, Saudi Arabia	Multiple regions	ND	16/2245	Body fluids, stomach, large and small colon, rectum, liver, gall bladder, pancreas, other intra-abdominal organs	Hospitalized patients	7
172	Sader et al. (183)	2011-2014	2016	Argentina, Brazil, Chile, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Panama, Peru, Venezuela	Multiple regions	Cross-sectional	141/13494	Clinical samples	ND	7
173	Sader et al. (184)	2009–2012	2014	USA, Belgium, France, Germany, Greece, Ireland, Italy, Poland, Portugal, Spain, Sweden, UK, Turkey, Israel	Multiple regions	Cross-sectional	330/8201	Trans tracheal aspiration, bronchoalveolar lavage, protected brush samples, qualified sputum samples	Hospitalized patients (Pneumonia patients)	7
174	Sader et al. (185)	2011	2013	USA, Canada, Belgium, Czech Republic, France, Germany, Greece, Ireland, Israel, Italy, Poland, Portugal, Romania, Russia, Slovakia,	Multiple regions	Cross-sectional	362/362	Clinical samples	Hospitalized patients (BSI, respiratory tract infections, wound and skin infections)	7

(Continued)

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TABLE 1 (Continued)

	References	Time of study	Time of publication	Country	WHO regions	Type of study	Sample size (N/total)	Type of samples	Patients	Quality score
				Slovenia, Spain, Sweden, Turkey, United Kingdom, Ukraine, Argentina, Brazil, Chile, Mexico, Australia, China, Hong Kong, India, Japan, Korea, Malaysia, New Zealand, Singapore, Taiwan, Thailand						
175	Sader et al. (186)	2000–2004	2005	ND	Multiple regions	Cross-sectional	131/9093	Clinical samples	Hospitalized patients (ICU)	7
176	Thomson et al. (187)	ND	1999	USA, Czech Republic, Hungary, Spain, Sweden, the United Kingdom, Australia	Multiple regions	ND	16/296	ND	ND	6
177	Toleman et al. (188)	1998–2003	2007	ND	Multiple regions	Cross-sectional	1744/1744	ND	ND	6
178	Tsiodras et al. (189)	1993-1997	2000	USA, Switzerland	Multiple regions	Retrospective case series	69/1279	Clinical samples	Hospitalized patients	7
179	Yamane et al. (190)	1992	1994	USA, Canada, Brazil, Japan, Switzerland, Spain	Multiple regions	Cross-sectional	61/889	Clinical samples	ND	7

10.3389/fmed.2023.1163439

TABLE 2 Meta-analysis of the global prevalence rate of *Stenotrophomonas maltophilia* isolation from clinical samples.

	No. of studies	Prevalence of *S. maltophilia* isolation [95% CI]	N/total	Heterogeneity test, I^2	Heterogeneity test, *P*-value	Begg's test	Egger's test
Overall	95	5.3 [4.1–6.7]	11557/561463	99.428	0.000	0.017	0.367

regression methods in combination with a funnel plot were used ($P < 0.05$ was regarded as indicative of a statistically notable publication bias) (15).

Results

A total of 6,770 records were identified through searches of the four aforementioned electronic databases (Figure 1). After removing the 3,613 duplicates, 3,157 unique records were screened based on titles and abstracts, and 2,340 articles were excluded, such as studies with non-relevant topics ($n = 1,245$), repetitive articles ($n = 470$), reviews ($n = 234$), systematic reviews ($n = 3$), case reports ($n = 64$), letters to the editors ($n = 60$), conference abstracts ($n = 111$), editorials ($n = 9$), short surveys ($n = 10$), correspondence ($n = 2$), notes ($n = 12$), reports ($n = 3$), a book ($n = 1$), articles with a total sample of <10 strains ($n = 17$), non-English studies ($n = 47$), and articles that studied environmental samples ($n = 21$). In addition, 30 articles were removed because their full texts were not available. The eligibility of 817 full-text articles was assessed and, ultimately, 179 studies met the inclusion criteria and were enrolled in the qualitative analysis. Of these, 95 studies reporting the prevalence of *S. maltophilia* infection were selected for quantitative analysis (meta-analysis). The characteristics of the 179 included studies are summarized in Table 1.

Overall, 179 studies conducted during the 31-year period between 1986 and 2017 were included. The articles had a wide geographical distribution, and the studies featured in them were carried out in different parts of the world. According to the World Health Organization's (WHO) regions, most studies were from the European Region ($n = 57$, 32%), followed by the West-Pacific Region ($n = 39$, 22%), the Region of the Americas ($n = 37$, 21%), the Eastern Mediterranean Region ($n = 14$, 8%), and the South-East Asian Region ($n = 8$, 4%). There was no independent study from the African Region. Twenty-four studies (13%) were conducted across different continents and were, therefore, classified as multiple region studies and did not conform to the WHO categories (Table 1).

The studies had very different sample sizes, ranging from 10 to 130,033. A total of 580,963 samples were examined, of which 25,596 were positive for *S. maltophilia*. Of the 179 studies, only 58 reported the types and details of examined specimens (5,106 samples). The most frequent sources of *S. maltophilia* isolation were respiratory samples ($n = 3,434$, 67%) and blood ($n = 1,223$, 24%) (Table 1). The qualities of all the reviewed studies were evaluated using the JBI critical appraisal checklist. Of the 95 studies included in the meta-analysis, 78 (82%) scored seven, 16 (17%) scored six, and one (1%) scored five. Therefore, all the studies enrolled in the meta-analysis had a high-quality score (a score of five or more) (Table 1).

Prevalence of *Stenotrophomonas maltophilia* by WHO regional offices

Based on the meta-analysis, the pooled prevalence rate of global *S. maltophilia* infection was estimated to be 5.3 % [95% CI, 4.1–6.7%] (Table 2 and Figure 2). Egger's test did not demonstrate publication bias ($P > 0.05$). However, Begg's test showed evidence of publication bias in the 95 analyzed studies ($P = 0.017$). Additionally, the corresponding funnel plot indicated publication bias (Supplementary File 1). Results demonstrated high heterogeneity ($I^2 = 99.428\%$; $P = 0.000$) among the selected studies (Table 2).

Subgroup meta-analysis based on the publication period of the studies (from 1991 to 2019) revealed that the prevalence rate of *S. maltophilia* isolation had an increasing trend over time, from 1.7% [95% CI, 0.7–4%] between 1991 and 1995 to 6.5% [95% CI, 4.1–10.1%] between 2016 and 2019. The highest prevalence rate [7.7%; 95% CI, 4.3–13.4 %] was observed between 2011 and 2015 (See Figure 3 and Table 3) (Supplementary File 1).

Subgroup meta-analysis based on the world regions defined by WHO revealed that the highest prevalence of *S. maltophilia* infections occurred in the Western Pacific Region [10.5%; 95% CI, 5.7–18.6%] and the European Region [7.9%; 95% CI, 4.3–14%]. The lowest prevalence occurred in the Region of the Americas [4.3%; 95% CI, 3.2–5.7%] (see Table 3 and Figure 4).

Evaluation of the regional prevalence of *S. maltophilia* isolation based on the publication time of studies (from 1991 to 2019) showed an overall increasing trend. In the Western Pacific Region, the prevalence rate of *S. maltophilia* decreased from 2006 to 2010; however, the prevalence rates in the European Region and the Regions of America increased after this time interval (Figure 5 and Supplementary File 1).

The antibiotic resistance rate of *Stenotrophomonas maltophilia*

The susceptibility of *S. maltophilia* isolates to various antibiotics was determined using various methods, including broth micro-dilution, broth macro-dilution, agar dilution, disk agar diffusion (DAD), E-test, and automated methods (e.g., VITEK, Phoenix, and micro-scan systems). Broth micro-dilution was the most frequently used assay. The standards used for interpreting the results of susceptibility assays varied, with different breakpoints used, such as those of the Clinical and Laboratory Standards Institute (CLSI), National Committee for Clinical Laboratory Standards

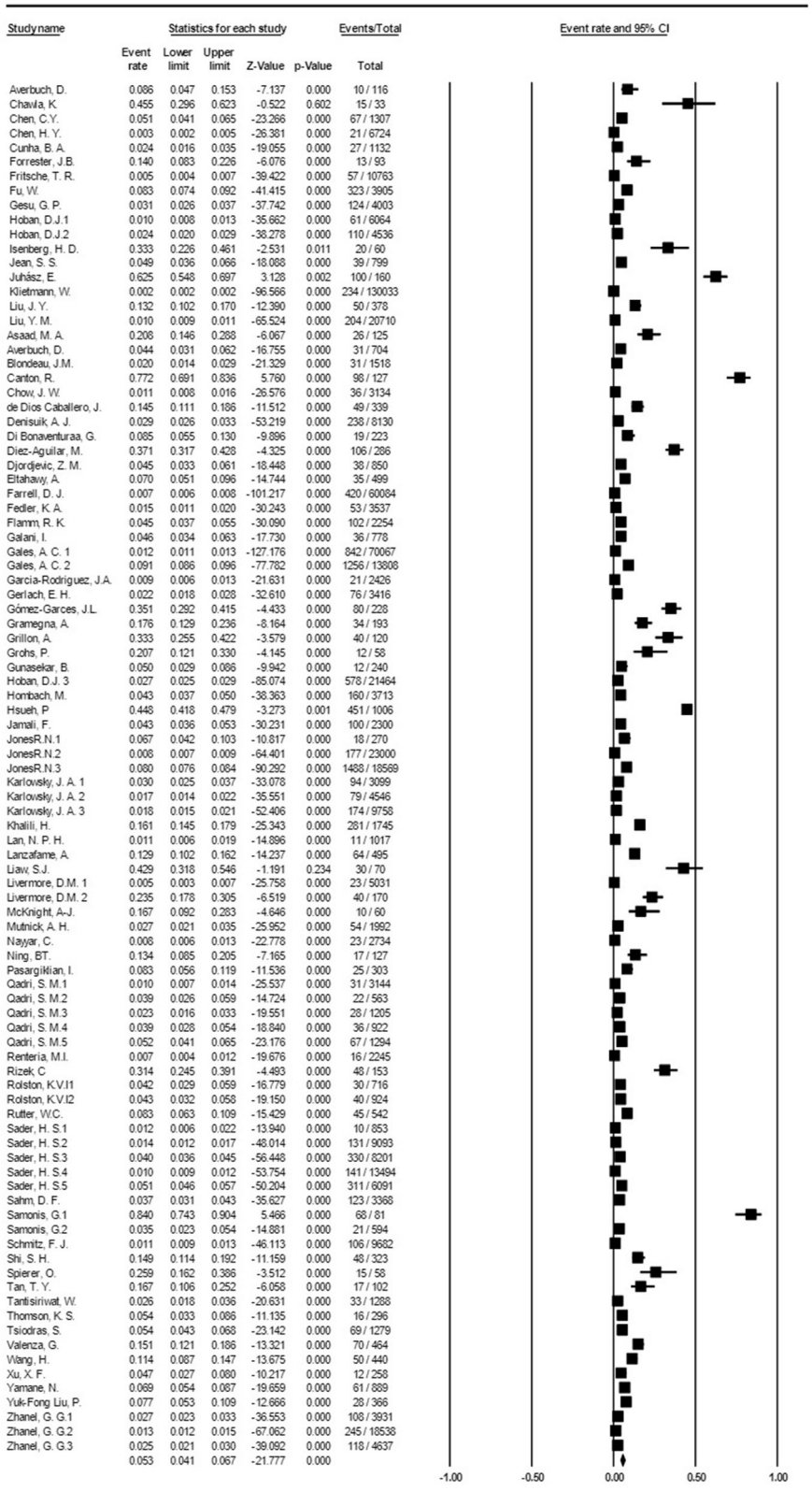

FIGURE 2
Forest plot diagram of the global prevalence rate of *S. maltophilia* isolation from clinical samples. The middle point of each line indicates the prevalence rate, and the length of the line indicates the 95% confidence interval of each study.

(NCCLS), European Committee on Antimicrobial Susceptibility Testing (EUCAST), U.S. Food and Drug Administration (FDA), British Society for Antimicrobial Chemotherapy (BSAC), TRUST, and Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (Supplementary File 2).

As shown in Table 4, the highest resistance rates of *S. maltophilia* isolates were to cefuroxime [99.1%; 95% CI, 97.3–99.7%], cefoxitin [96.5%; 95% CI, 80.9–99.4%], ampicillin [96.1%; 95% CI, 92.8–97.9%], imipenem [94.9%; 95% CI, 92.3–96.7%], and meropenem [93.3%; 95% CI, 87.2–96.6%], while the lowest resistance rates were to doxycycline [5.7%; 95% CI, 3.3–9.7%] and minocycline [4.8%; 95% CI, 2.6–8.8%].

A comparison of antibiotic resistance rates of *S. maltophilia* before and after 2010 (Figure 6) revealed an increasing trend for some antibiotics, such as chloramphenicol (12.3%), TMP/SMX (11.6%), ceftazidime (8.6%), and levofloxacin (1.8%). Conversely, the resistance rate against minocycline (2.2%) decreased.

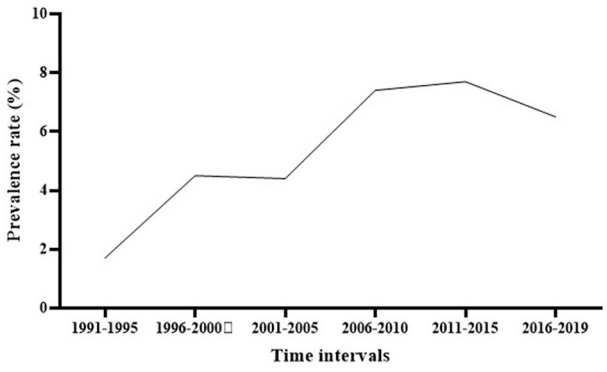

FIGURE 3
The global prevalence of *S. maltophilia* isolation based on the publication time of studies.

The results of the subgroup meta-analysis based on the world regions and antibiotic resistance rates, presented in Figures 7–9, as well as in Supplementary File 1, showed that the highest resistance rate across all regions was to ceftazidime, while the lowest rate was to minocycline.

Discussion

Although *S. maltophilia* shows limited invasiveness in immunocompetent individuals, it can lead to severe infections in immunocompromised patients. Moreover, its high intrinsic resistance to a large number of antimicrobial agents results in treatment failure and mortality in patients infected by this microorganism (191–194). Thus, the undertaking of a first systematic review and meta-analysis addressing the prevalence rate of isolation and antibiotic resistance rates of *S. maltophilia* in different regions of the world may be of great value in managing infections caused by this bacterium.

Based on the present meta-analysis, most studies were reported from the European Region (n = 57, 32%), while in a similar investigation (12), the majority of cases were reported and managed in the United States of America (n = 72, 27.7%). The differences between the inclusion and exclusion criteria applied in these two studies may explain the differing results. In the current study, the global prevalence rate of *S. maltophilia* isolation from clinical samples was 5.3%, and according to the WHO classification, the highest prevalence rate of *S. maltophilia* isolation was observed in the Western Pacific Region (10.5%), followed by the European Region (7.9%), which may be due to their long-shared land border. Among the reasons for the discrepancies in the prevalence of *Stenotrophomonas maltophilia* infection in different world regions, we can mention the following: disparate health policies in each country affect the importance of pathogens, so, in some countries, *Stenotrophomonas maltophilia* is still considered an

TABLE 3 Subgroup meta-analysis of the global prevalence rate of *Stenotrophomonas maltophilia* isolation from clinical samples.

Subgroups		No. of studies	Prevalence of *S. maltophilia* isolation [95% CI]	N/total	Heterogeneity test, I^2	Heterogeneity test, *P*-value	Begg's test	Egger's test
Time of publication	1991-1995	13	1.7 [0.7–4.0]	696/157899	99.155	0.000	0.502	0.036
	1996-2000	11	4.5 [2.2–8.8]	569/38696	98.493	0.000	0.119	0.003
	2001-2005	17	4.4 [2.7–7.1]	4159/156226	99.525	0.000	0.232	0.983
	2006-2010	13	7.4 [4.5–12.1]	1834/28534	98.529	0.000	0.951	0.620
	2011-2015	20	7.7 [4.3–13.4]	2465/135819	99.517	0.000	0.047	0.011
	2016-2019	20	6.5 [4.1–10.1]	1383/43283	98.555	0.000	0.381	0.157
World regions	Asia (Total)	27	7.1 [4.6–10.7]	1879/27322	98.71	0.000	0.738	0.025
	Asia (EMR)*	10	4.7 [2.6–8.6]	653/12929	98.146	0.000	0.858	0.035
	Asia (SEAR)	4	5.2 [1.1–20.9]	83/4295	97.709	0.000	0.308	0.237
	Asia (WPR)	13	10.5 [5.7–18.6]	1143/10098	98.823	0.000	0.760	0.301
	EUR	29	7.9 [4.3–14]	2173/190229	99.453	0.000	0.586	0.008
	AMR	26	4.3 [3.2–5.7]	2593/105324	98	0.000	0.0325	0.0148

*EMR, Eastern Mediterranean Region; SEAR, South-East Asia Region; WPR, Western Pacific Region; EUR, European Region; AMR, Regions of the Americas.

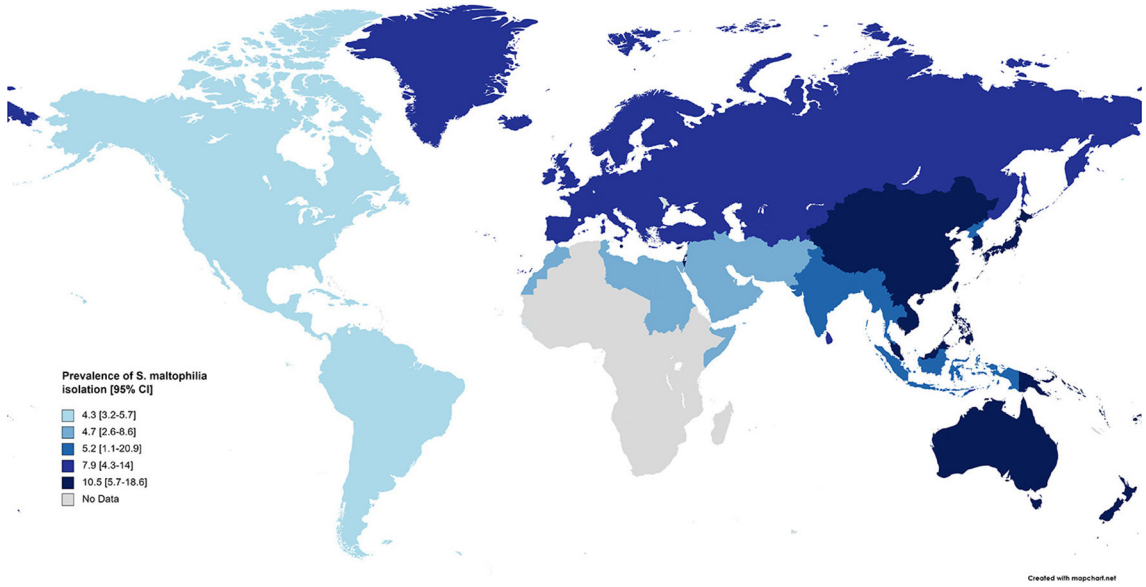

FIGURE 4
Prevalence of *S. maltophilia* isolated from clinical samples, by WHO regions.

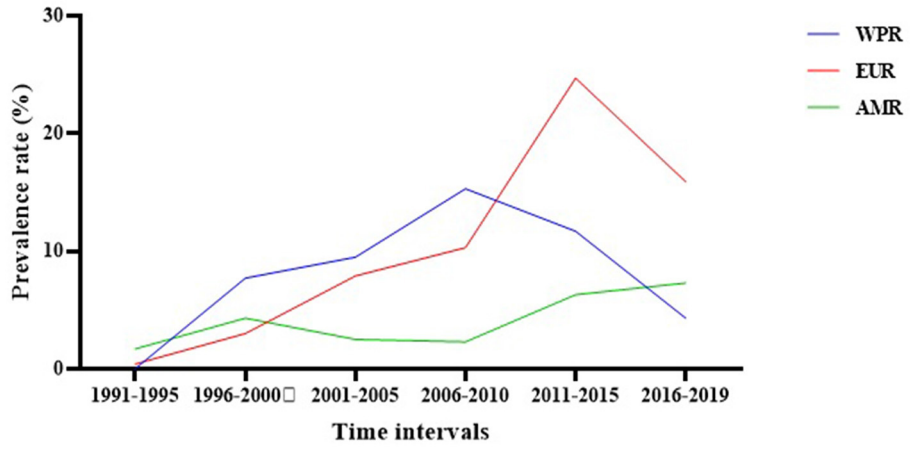

FIGURE 5
The regional prevalence of *S. maltophilia* isolation based on the publication time of studies.

unimportant opportunistic pathogen, so few studies have been reported. For example, most of the cases were documented in European (195), Asian (86), and American (196) countries, while there was no relevant study performed in the African continent. This difference can cause publication bias and affect the overall results. Additionally, the differences in health levels of various countries and the numbers and types of examined patients all influence the reported prevalence of *Stenotrophomonas maltophilia*.

In this meta-analysis, among different clinical samples, respiratory samples were the most frequent source (67%), followed by blood samples (24%). This finding is consistent with other studies, in which *S. maltophilia* was most commonly associated with respiratory tract infections, followed by bloodstream infections (74, 197). However, in another systematic review, blood was the most prevalent site of *S. maltophilia* isolation (12). In a large study performed in the USA and fifteen centers in European countries in 2012, 6.3% of the isolates obtained from respiratory tract infections were identified as *S. maltophilia*. These data suggest that the rate of respiratory tract infections caused by *S. maltophilia* is increasing (3, 198). The bacterium's capability for adherence to plastic surfaces and biofilm formation on hospital devices, such as those inserted into the respiratory tract, may explain its high rate in the aforementioned samples (199, 200). For example, among patients with ventilator-associated pneumonia (VAP), the most common nosocomial infection in mechanically ventilated patients, *S. maltophilia* is the probable causative pathogen (196, 201). Moreover, its adaptation to the airways of individuals with cystic fibrosis (CF) has led it to being recognized as an emerging multi-drug resistant opportunistic pathogen (86).

The prevalence rate of infections caused by this bacterium increased from 1.7% to 6.5% during the 31 investigated years,

10.3389/fmed.2023.1163439

TABLE 4 Total antibiotic resistance rates of *Stenotrophomonas maltophilia* strains in the world.

Antibiotic	No. of studies	Antibiotic resistance rate [95% CI]	N/total	Heterogeneity test, I^2	Heterogeneity test, *P*-value	Begg's test	Egger's test
Penicillins							
Ampicillin	6	96.1 [92.8–97.9]	358/367	41.721	0.127	1.000	0.509
Ticarcillin	14	67.6 [53.5–79.1]	1126/1616	93.177	0.000	1.000	0.982
Piperacillin	29	72.5 [64.1–79.5]	2167/3108	93.636	0.000	0.652	0.251
Cephalosporins							
Ceftazidime	120	53.7 [49.8–57.5]	8445/17526	94.850	0.000	0.561	0.005
Cefoprazone	6	53 [29.6–75.2]	248/747	96.172	0.000	0.707	0.141
Cefepime	39	59.5 [50.7–67.8]	2310/4120	95.313	0.000	0.260	0.414
Cefoxitin	8	96.5 [80.9–99.4]	263/276	84.133	0.000	0.107	0.010
Cefotaxime	19	89.5 [77.8–95.4]	1093/1546	95.747	0.000	0.401	0.018
Ceftriaxone	24	91.2 [83.3–95.5]	1253/1588	91.399	0.000	0.172	0.051
Cefuroxime	6	99.1 [97.3–99.7]	528/529	0.000	0.796	0.132	0.663
β-lactam/β-lactamase inhibitor							
Amoxicillin/clavulanate	10	91 [73.5–97.4]	562/621	90.444	0.000	0.858	0.141
Ampicillin/sulbactam	4	91.7 [15.2–99.9]	128/372	93.917	0.000	1.000	0.004
Ticarcillin/clavulanate	54	33.2 [27.7–39.2]	3406/12314	96.699	0.000	0.665	0.137
Cefoprazone/sulbactam	7	30.7 [16.7–49.5]	165/936	92.308	0.000	0.229	0.040
Piperacillin/tazobactam	49	62.9 [55.6–69.6]	3135/5195	94.150	0.000	0.869	0.568
Carbapenems							
Meropenem	39	93.3 [87.2–96.6]	2574/3149	95.578	0.000	0.004	0.00024
Imipenem	64	94.9 [92.3–96.7]	4399/5203	92.250	0.000	0.013	0.000
Monobactams							
Aztreonam	24	84.1 [68.8–92.7]	1457/2662	97.164	0.000	0.711	0.038
Aminoglycosides							
Amikacin	59	69.8 [63.2–75.7]	3874/5783	94.439	0.000	0.432	0.483
Gentamicin	53	73.4 [66.4–79.3]	3077/4256	92.875	0.000	0.240	0.993
Tobramycin	26	81 [74.5–86.2]	1921/2483	88.506	0.000	0.122	0.179
Netilmicin	8	73.2 [46.2–89.7]	353/490	94.806	0.000	0.265	0.443
Fluoroquinolones							
Ciprofloxacin	100	47.6 [42.6–52.5]	4888/9660	93.837	0.000	0.114	0.628
Levofloxacin	72	19.7 [16.4–23.4]	2250/14141	94.656	0.000	0.046	0.607
Moxifloxacin	12	17.5 [9.8–29.2]	218/1858	93.896	0.000	0.890	0.224
Ofloxacin	16	29.9 [22.1–39]	546/1697	89.733	0.000	0.558	0.241
Gatifloxacin	7	10.9 [5.9–19.4]	220/2809	94.490	0.000	1.000	0.487
Norfloxacin	9	66.9 [45.3–83.1]	324/458	90.688	0.000	0.465	0.349
Trovafloxacin	6	16.3 [5.9–37.7]	153/1190	95.506	0.000	0.707	0.748
Tetracyclines							
Tetracycline	13	58.6 [45.2–70.8]	1398/2432	95.208	0.000	0.450	0.987
Doxycycline	10	5.7 [3.3–9.7]	189/2312	88.180	0.000	0.283	0.112
Minocycline	18	4.8 [2.6–8.8]	172/3018	91.488	0.000	0.288	0.00040
Tigecycline	18	11.8 [7–19.1]	474/3849	95.745	0.000	0.404	0.317

(Continued)

TABLE 4 (Continued)

Antibiotic	No. of studies	Antibiotic resistance rate [95% CI]	N/total	Heterogeneity test, I^2	Heterogeneity test, *P*-value	Begg's test	Egger's test
Chloramphenicol	29	46.9 [37.2–56.9]	2507/5223	97.284	0.000	0.735	0.719
Polymyxins							
Colistin	19	48.4 [31.6–65.5]	911/1768	95.839	0.000	1.000	0.213
High-dose colistin	5	27.3 [10.8–53.7]	488/1826	96.376	0.000	0.806	0.386
Polymyxin B	8	18 [11.8–26.5]	819/3896	94.518	0.000	1.000	0.411
Sulfonamides							
Trimethoprim/ sulfamethoxazole	93	14.7 [11.7–18.3]	2968/20084	96.824	0.000	0.611	0.010
Phosphonic antibiotics							
Fosfomycin	6	32.3 [12.4–61.7]	223/818	97.308	0.000	1.000	0.759

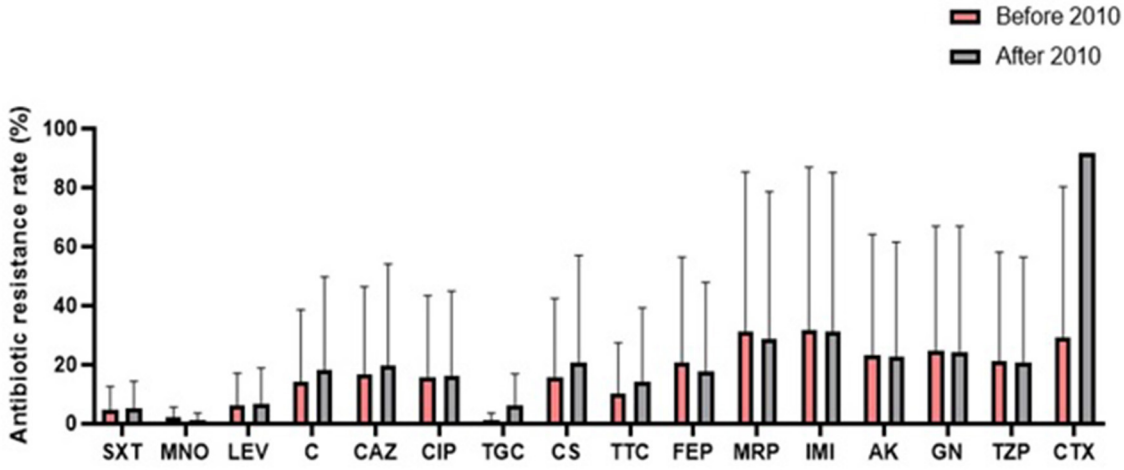

FIGURE 6
Comparison of the global antibiotic resistance rates of *S. maltophilia* before and after 2010 (SXT, trimethoprim-sulfamethoxazole; MNO, minocycline; LEV, levofloxacin; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; TGC, tigecycline; CS, colistin; TTC, ticarcillin-clavulanic acid; FEP, cefepime; MRP, meropenem; IMI, imipenem; AK, amikacin; GN, gentamicin; TZP, piperacillin-tazobactam; CTX, cefotaxime).

suggesting that it is emerging as an opportunistic pathogen, particularly among immunocompromised hosts. This rapid rise may be due to its resistance to a wide range of antimicrobial agents, as well as the increased focus on this bacterium as a cause of infection. The treatment of *S. maltophilia* infections is challenging due to the difficulty of differentiating colonization from infection and the intrinsic resistance of this bacterium to multiple classes of antibiotics. The WHO has classified *S. maltophilia* as one of the leading multidrug-resistant organisms in hospital settings (202). Additionally, recent antibiotic treatment and other known factors associated with acquiring *S. maltophilia* infections demonstrate specific features of this bacterium (195).

Based on our data, the highest and the lowest global resistant rates were to cefuroxime and minocycline, respectively (Figure 3). The lowest resistance to TMP-SMX was observed in the EMR (4.5%) and AMR (13.1%), while in other geographical regions, resistance was higher than 20%. Consequently, TMP-SMX may be the first choice for treatment based on antibiotic susceptibility and therapeutic success (3, 60, 203). Fortunately, in the present study, a comparison of global antibiotic resistance rates of *S. maltophilia* before and after 2010 (Figure 4) confirmed the effectiveness of this medication for treating infections of this opportunistic organism. However, there is not always a logical correlation between laboratory sensitivity and clinical results. Other antibiotics for treating *Stenotrophomonas* infections include fluoroquinolones, tetracyclines, and selected β-lactams, such as ceftazidime and ticarcillin/clavulanate. However, the development of resistance to some of these antibiotics renders them unreliable.

Fluoroquinolones are prescribed for treating infections caused by TMP-SMX-resistant *S. maltophilia* and for patients for whom this drug has adverse effects. Studies comparing treatments with fluoroquinolones and TMP-SMX have proposed that levofloxacin has similar effectiveness with fewer adverse effects than TMP-SMX (204, 205). Our study indicates that resistance rates to levofloxacin vary geographically, ranging from 6.4% in EMR to 15%—22% in EUR, AMR, and WPR, and up to 26% in SEAR. However, the rapid emergence of resistance against quinolones *in vitro* and *in vivo* is of concern when levofloxacin is used to treat *S. maltophilia* infections.

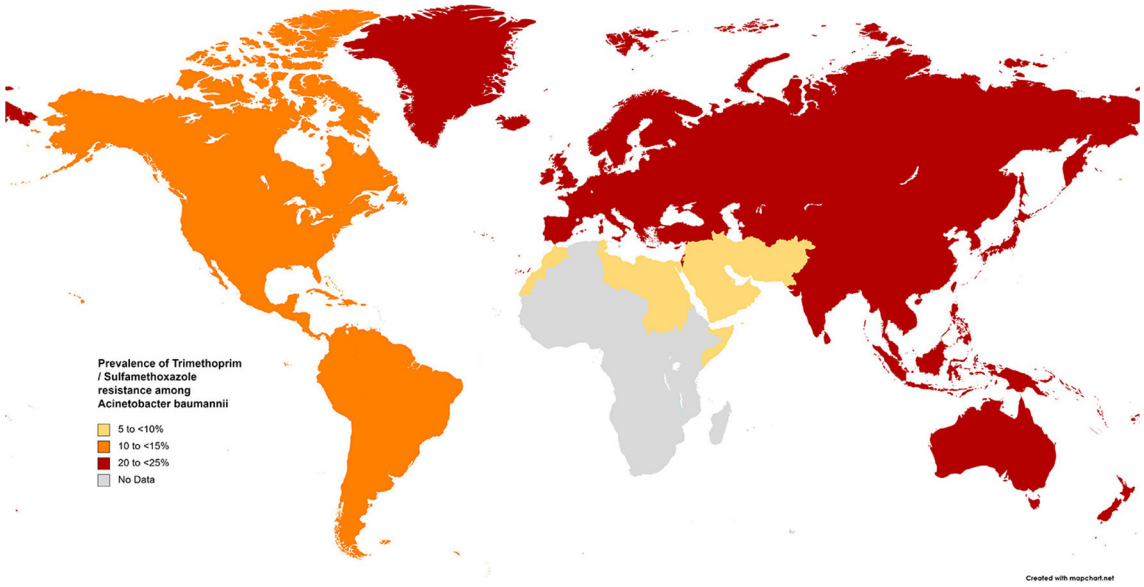

FIGURE 7
Prevalence of trimethoprim/sulfamethoxazole resistance in *S. maltophilia* isolated from clinical samples, by WHO regions.

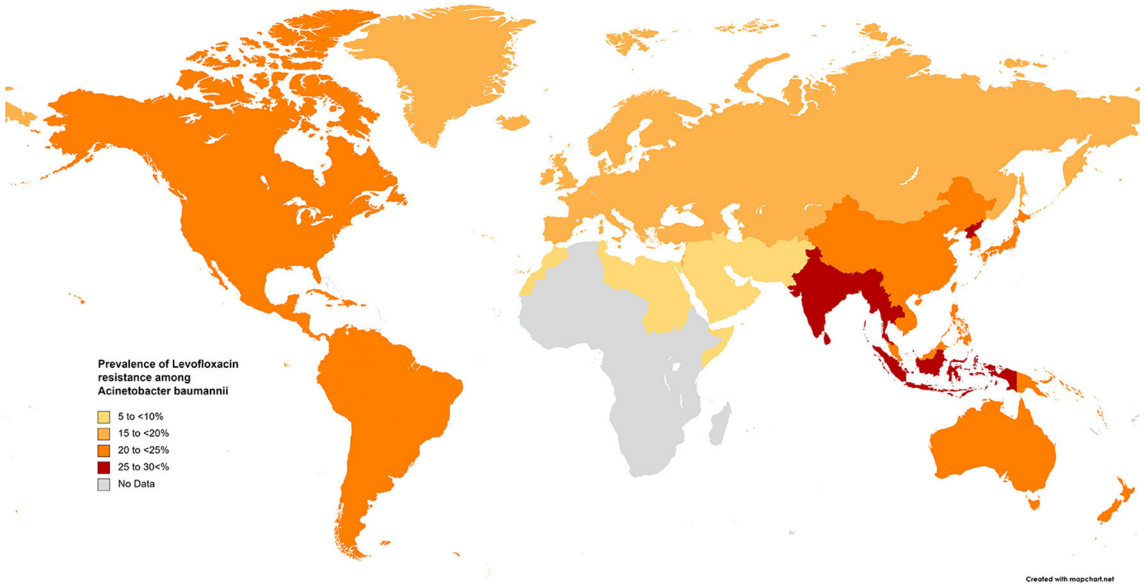

FIGURE 8
Prevalence of levofloxacin resistance in *S. maltophilia* isolated from clinical samples, by WHO regions.

In surveillance studies of the efficacy of tigecycline and related tetracycline antibiotics, minocycline was found to be effective against *S. maltophilia* (206). In this study, resistance to minocycline was <10% in all geographical areas and global resistance to tigecycline was 11.8%. A comparison of the antibiotic resistance rates of *S. maltophilia* before and after 2010 revealed an increase in resistance to tigecycline from 4.1% to 18.6%. Several studies have revealed that minocycline is not inferior to TMP-SMX and may even be more suitable than TMP-SMX in terms of susceptibility. These results suggest that minocycline and TMP-SMX may be the first-line therapy in *S. maltophilia* infections, even in TMP-SMX-resistant strains (59).

Ceftazidime and ticarcillin/clavulanate have previously been reported as the most effective β-lactam drugs against *S. maltophilia*. However, reduced sensitivity to ceftazidime has been documented in recent studies. Owing to β-lactamase production, a high resistance rate to β-lactams such as cefuroxime, cefoxitin, imipenem, and meropenem (> 90%, Table 4) has been observed, thus reducing their role in the treatment of *S. maltophilia* infections (207). According to this analysis, ceftazidime has a high

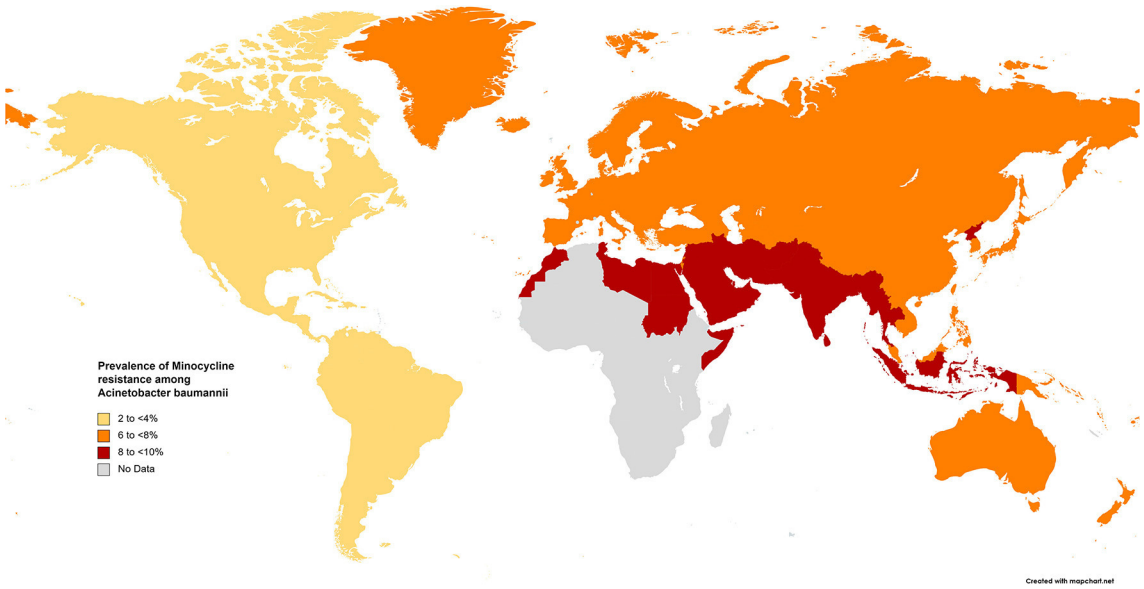

FIGURE 9
Prevalence of minocycline resistance in *S. maltophilia* isolated from clinical samples, by WHO regions.

resistance rate in all regions classified by the WHO (AMR, 56.4%; EMR, 42.9%; SEAR, 65.1%; WPR, 52.6%). Our study suggests that the rate of resistance to ticarcillin/clavulanate globally is 33.2%. Therefore, these current resistance rates to ceftazidime and ticarcillin/clavulanate render them unreliable. However, the use of ceftazidime in combination with other antibiotics (typically vancomycin, amikacin, TMP-SMX, or fluoroquinolones) is an effective treatment for infections caused by *S. maltophilia* (13). A systemic literature review by Gibb and Wong (208) offers recommendations for a treatment strategy for *Stenotrophomonas* infection based on current evidence. The first-line drugs suggested are TMP-SMX, fluoroquinolones, and tetracyclines.

Our study presents several limitations. First, a large number of the included studies (84 articles) evaluated a specific number of *S. maltophilia* isolates but did not report the prevalence rate of isolation; thus, these studies were not included in the meta-analysis, which could affect the pooled prevalence rate of *S. maltophilia* isolation and the antibiotic resistance rates. Second, the number of published studies reporting the resistance mechanism of strains isolated from clinical samples (see Supplementary File 2) is relatively small, and the specific genes conferring antibiotic resistance in these isolates remain unclear. Third, a few studies used typing methods to evaluate *S. maltophilia* isolates (see Supplementary File 2), so we could not report the most prevalent types of this bacterium at the global and regional levels.

Conclusion

In conclusion, despite the undeniable clinical impact of *S. maltophilia*, compared with other Gram-negative species, this bacterium is remarkably understudied. Thus, collecting and analyzing data related to different aspects of *S. maltophilia* may assist in improving the clinical management of challenges caused by this bacterium. This meta-analysis presents the global antibiotic resistance of *S. maltophilia* over the last 31 years and demonstrates different rates of resistance in world geographical regions, as well as the growing trend of resistance to most antibiotics. The variations in antibiotic resistance of *S. maltophilia* isolates in different regions may be the result of the use of different protocols for patient treatment. Additionally, the improper and experimental use of antibiotics plays an important role in increasing resistance, leading to an increased risk of treatment failure. To address this issue, it is necessary to carry out antibiotic sensitivity tests before prescribing antibiotics and implementing an antimicrobial stewardship program for every hospital, as well as provide continuous training for clinicians about their performance in the hospital environment. Finally, collecting and preparing local sensitivity patterns will be effective in allowing the selection of the optimal empiric treatment for *S. maltophilia* infections.

Author contributions

MB contributed to the study design, data extraction, data analysis, design and production of figures, and wrote and revised the final manuscript. AS-M contributed to the study design, data extraction, data analysis, and writing of the manuscript. GB contributed to the data analysis and statistical analysis, designed and produced figures, and writing of the manuscript. EE contributed to the study design, data extraction, and writing of the manuscript. LJ contributed to the study design and the writing and revision of the final manuscript. RB contributed to the study design, data analysis and interpretation, and the writing of the manuscript. ME designed the study, oversaw the analysis, and wrote and revised the final manuscript. FJ designed the study, was the arbiter for the study searches and data

extraction, and wrote and revised the final 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/fmed.2023.1163439/full#supplementary-material

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