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Enterobacteriaceae antimicrobial agents and resistance: Relationship with the therapeutic approach, volume II

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Enterobacteriaceae antimicrobial agents and resistance: Relationship with the therapeutic approach, volume II

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Editorial: Enterobacteriaceae antimicrobial agents and resistance: relationship with the therapeutic approach, volume II

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multi-drug resistant *Enterobacteriaceae*, innovative therapies, association of antibiotics, plasmids, humoral immunity

Editorial on the Research Topic
Enterobacteriaceae antimicrobial agents and resistance: relationship with the therapeutic approach, volume II

Antimicrobial resistance (AMR) is one of the major global health issues worldwide (Hu et al., 2016). The situation is becoming increasingly drastic, especially in the last years, due to the increase of new antibacterial agents that, in the long run, lose their effectiveness, becoming useless or even harmful for infections treatments, thereby leading to a greater resistance selection (Laxminarayan et al., 2016). Consequently, antibiotics become ineffective, increasing the risk of disease spread, severe illness, and death (Palacios-Baena et al., 2021). New strategies should be taken into account in the therapeutic approach through enhancing host immunity mechanisms or relying on new compounds, such as essential oils (Oliva et al., 2018), N-acetylcysteine (Oliva et al., 2021), bacteriophages (Qin et al., 2021), vaccines (Diago-Navarro et al., 2017), or combination therapy (Mascellino et al., 2021) (Figure 1).

The presence of enzymes, such as extended-spectrum β-lactamase (ESBL) and carbapenemases (KPCs, Metallo β-lactamases, and OXA), constitutes the principal resistance mechanism to antibiotics (Scudeller et al., 2021).

A new metallo-β-lactamase called SZM-1 was identified and characterized in Shenzhen Bay, South China, by Fang et al.

This Shenzhen metallo-β-lactamase, belonging to an *Arenimonas* metagenome-assembled genome recovered from the river sediment in the Shenzhen Bay area, shows carbapenemase activity and confers an increasing resistance toward carbapenems in the carrier bacteria.

In order to test the most common resistance genes (*blaKPC, blaNDM-1, blaIMP-1 group, and blaVIM*), Kim et al. developed a novel method, loop-mediated isothermal amplification (LAMP)-based assay using eight reference Gram-negative bacterial strains. The results of the LAMP assay were compared to those of conventional PCR and appeared quite similar, with a good specificity and sensitivity, thus making this method appropriate for detecting the above four β-lactamase genes. The low complexity and low cost of this

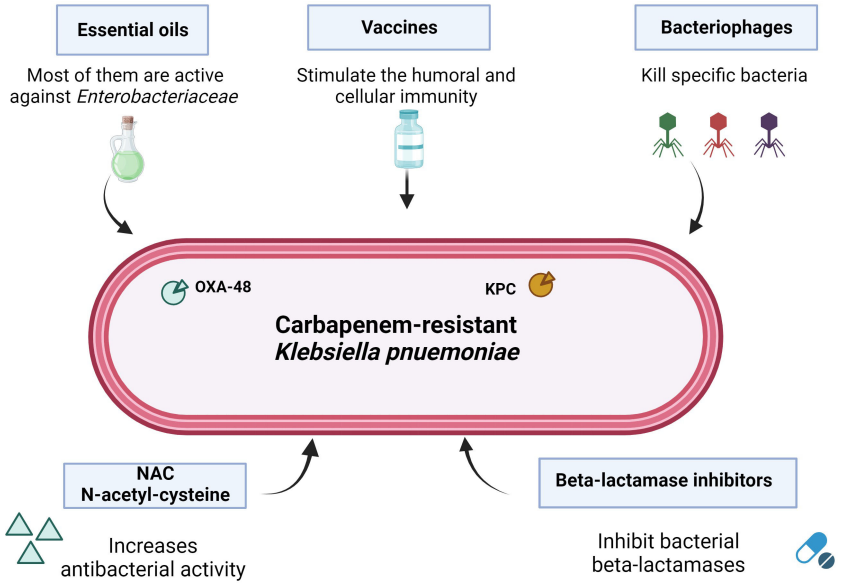

FIGURE 1
Activity of different compounds against resistant *Klebsiella pneumoniae* (figure made by Biorender.com; accessed on December 15).

assay may be suitable for most types of laboratories and may be beneficial for low-income countries.

Nosocomial infections by multidrug-resistant (MDR) organisms are among the main causes of morbidity and mortality in patients hospitalized in intensive care units (ICUs). For this purpose, the study of Li et al., which deals with the less frequent bacterium of *Enterobacter bugandensis*, is useful, because this microorganism is mainly involved in nosocomial infections, and MDR strains have been isolated from various environments. This emerging pathogen causes serious problems in nosocomial infections treatment. Among the various mechanisms of antimicrobial resistance, active efflux pumps are a well-known system that eliminates clinically relevant antimicrobial agents, rendering specific pathogens resistant to the activity of multiple drugs. The authors studied the activity of TolC, an outer membrane component of different efflux pumps, in respect to the susceptibility to a range of 26 antimicrobial agents in *E. bugandensis* strains, leading to excellent results that can be used as a reference for the future emergence of MDR isolates belonging to this species.

Two interesting articles based on the association of β-lactams together with β-lactamase inhibitor activity are included in this Research Topic. The first one by Kang et al. evaluated the efficacy of ceftazidime–avibactam and aztreonam–avibactam against bloodstream infections or lower respiratory tract infections caused by extensive drug-resistant or pan-drug-resistant *Pseudomonas aeruginosa*. Both associations turned out to be effective against this microorganism. The second article by Zhang et al. examined the activity of piperacillin–tazobactam (PTZ) compared to meropenem (MER), which is commonly used to treat complicated urinary tract infections. Both MER and PTZ were active (≈ 80% eradication rate); however, the adverse events were found to be more common in MER than in PTZ.

Klebsiella pneumoniae, Acinetobacter baumannii, and *Pseudomonas aeruginosa* appear to be the most commonly implied microorganisms in antimicrobial resistance. Carbapenem-resistant *Klebsiella pneumoniae* is detected frequently in bloodstream infections, causing high mortality rates especially in ICUs, and in hospital-acquired infections (Li et al.).

In the same way, the presence of carbapenem-resistant *Acinetobacter baumannii* (CRAB) is very common, as reported by Huang et al. in China. CRAB is highly refractory to conventional clinical antibiotics, with the exception of cefoperazone/sulbactam. Particularly, the presence of OXA-23 and OXA-51 resistance genes is common in these strains, and together with TEM β -lactamase, they are most the dominantly involved factors making the multidrug-resistant CRAB the main pathogen of nosocomial infections all over the world.

The articles of Shang et al. and Hadjadj et al. describe the colonization and outbreak of resistant germs.

In the first one, it was noticed that extended-spectrum β-lactamase-producing *Enterobacteriaceae* colonization does not interfere, following infections by ESBL, with either ESBL Gram-negative bacteria or liver transplant outcomes. Consequently, this colonization cannot be considered a risk factor for possible post-transplant Gram-negative infections, even if some previous literature data provide conflicting results mainly for Gram-positive bacteria (Russell et al., 2008).

In the second article, the outbreak of carbapenem-resistant *Enterobacteriaceae i*n a thoracic oncology unit was taken into consideration. The study included *Citrobacter freundii* and *E. hormaechei* (both belonging to the *Enterobacter cloacae complex)* producing OXA-48 carbapenemase in hospitalized patients. The *blaOXA-48* gene was disseminated by both clonal and plasmid spread. The origin of this outbreak appears to have

been both external and internal to the ward, meaning that this cross-infection had to be managed with the implementation of appropriate prevention and control measures.

Lastly, an effective new strategy for fighting antimicrobial resistance (AMR) could rely on increasing host immunity by making people less receptive to infections and more ready to combat them. This interesting topic has been elucidated by Liang et al. This article has demonstrated that host defense plays a critical role in killing *K. pneumoniae* and, hence, sheds light on the mechanisms underlying bacterial immunity, such as mechanical barriers, innate immune cells, and cellular and humoral immunity, in order to provide new strategies for the clinical treatment of *K. pneumoniae* infection. Understanding host immune mechanisms would facilitate the comprehension of the pathogenesis and could provide new ideas for future treatment of this infection in the era of antibiotics.

In conclusion, AMR is currently a crucial issue in the management of infectious diseases worldwide (Jamal et al., 2020). New antibiotics, antimicrobial associations, and combinations between beta-lactams and beta-lactamases inhibitors are taken into account, even if bacterial resistance and the therapy failures are always lurking (Bhatnagar et al., 2021). Appropriate prevention and control measures should be greatly improved (van Seventer and Hochberg, 2017). This Research Topic addresses an increasing problem that requires drastic solutions for limiting the further spread of resistant bacteria. Alternative therapeutic programs could be proposed, such as using N-acetyl-cysteine, bacteriophages, and, more importantly vaccines, stimulating the immune response that can recognize different bacterial antigenic epitopes in order to promote pathogen elimination.

Author contributions

MM: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. AO: Data curation, Investigation, Methodology, Writing – review & editing. SB: Formal analysis, Visualization, Writing – review & editing.

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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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References

Bhatnagar, A., Ransom, E. M., MaChado, M. J., Boyd, S., Reese, N., Anderson, K., et al. (2021). Assessing the in *vitro* impact of ceftazidime on aztreonam/avibactam susceptibility testing for highly resistant MBL-producing enterobacterales. *J. Antimicrob. Chemother.* 76 (4), 979–983. doi: 10.1093/jac/dkaa531

Diago-Navarro, E., Calatayud-Baselga, I., Sun, D., Khairallah, C., Mann, I., Ulacia-Hernando, A., et al. (2017). Antibody-based immunotherapy to treat and prevent infection with hypervirulent klebsiella pneumoniae. *Clin. Vaccine Immunol.* 24, e00456–e00416. doi: 10.1128/cvi.00456-16

Hu, F. P., Guo, Y., Zhu, D. M., Wang, F., Jiang, X. F., Xu, Y. C., et al. (2016). Resistance trends among clinical isolates in China reported from CHINET surveillance of bacterial resistance 2005-2014. *Clin. Microbiol. Infect.* 22 Suppl 1, S9–14. doi: 10.1016/j.cmi.2016.01.001

Jamal, A. J., Mataseje, L. F., Brown, K. A., Katz, K., Johnstone, J., Muller, M. P., et al. (2020). Carbapenemase-producing enterobacterales in hospital drains in southern Ontario, Canada. *J. Hosp. Infect.* 106, 820–827. doi: 10.1016/j.jhin.2020.09.007

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Rottingen, J. A., Klugman, K., et al. (2016). Access to effective antimicrobials: a worldwide challenge. *Lancet* 387 (10014), 168–175. doi: 10.1016/S0140-6736(15)00474-2

Mascellino, M. ,. T., Biswas, S., and Oliva, A. (2021). Editorial: enterobacteriaceae antimicrobial agent and resistance: relationship with the therapeutic approach. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.728371

Oliva, A., Bianchi, A., Russo, A., Ceccarelli, G., Cancelli, F., Aloj, F., et al. (2021). Effect of N-Acetylcysteine Administration on 30-Day Mortality in Critically Ill Patients with Septic Shock Caused by Carbapenem-Resistant Klebsiella pneumoniae and Acinetobacter baumannii: A Retrospective Case-Control Study. *Antibiotics* 10 (3), 271. doi: 10.3390/antibiotics10030271

Oliva, A., Costantini, S., De Angelis, M., Garzoli, S., Bozovic, M., Mascellino, M. T., et al. (2018). High Potency of Melaleuca alternifolia Essential Oil against Multi-Drug Resistant Gram-Negative Bacteria and Methicillin-Resistant Staphylococcus aureus. *Molecules* 23 (10), 2584–2597. doi: 10.3390/molecules23102584

Palacios-Baena, Z. R., Giannella, M., Manissero, D., Rodriguez-Bano, J., Viale, P., Lopes, S., et al. (2021). Risk factors for carbapenem-resistant gram-negative bacterial infections: A systematic review. *Clin. Microbiol. Infect.* 27 (2), 228–235. doi: 10.1016/j.cmi.2020.10.016

Qin, J., Wu, N., Bao, J., Shi, X., Ou, H., Ye, S., et al. (2021). Heterogeneous *klebsiella pneumoniae* co-infections complicate personalized bacteriophage therapy. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.608402

Russell, D. L., Flood, A., Zaroda, T. E., Acosta, C., Riley, M. M., Busuttil, R. W., et al. (2008). Outcomes of colonization wth MRSA and VRE among liver transplant candidates and recipients. *Am. J. Transplant.* 8, 1737–1743. doi: 10.1111/j.1600-6143.2008.02304.x

Scudeller, L., Righi, E., Chiamenti, M., Bragantini, D., Menchinelli, G., Cattaneo, P., et al. (2021). Systematic review and meta-analysis of in vitro efficacy of antibiotics against carbapenem-resistant Gram-negative bacilli. *Int. J. Antimicrob. Agents* 57 (5), 106344. doi: 10.1016/j.ijantimicag.2021.106344

van Seventer, J. M., and Hochberg, N. S. (2017). Principles of infectious diseases: transmission, diagnosis, prevention, and control. *Int. Encyclopedia Public Health* 2017, 22–39. doi: 10.1016/B978-0-12-803678-5.00516-6

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Identification and characterization of a novel metallo β-lactamase, SZM-1, in Shenzhen Bay, South China

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Metallo β-Lactamases (MBLs) degrade most clinical β-lactam antibiotics, especially Carbapenem, posing a huge threat to global health. Studies on environmental MBLs are important for risk assessment of the MBLs transmission among connected habitats, and between environment and human. Here, we described a novel metallo β-Lactamases, named SZM-1 (Shenzhen metallo-β-lactamase), from an *Arenimonas* metagenome-assembled genome recovered from the river sediment in the Shenzhen Bay area, south China. Phylogenetic analysis, primary sequence comparison, structural modeling suggested that the SZM-1 belongs to B1 MBL family, likely harboring a typical di-zinc catalytic center. Furthermore, the gene encoding the MBLs was cloned into *Escherichia coli* TOP10 for Carba NP test and antimicrobial susceptibility test. The results indicated that the SZM-1 had carbapenemase activity, and conferred the carrier to increased resistance toward carbapenems. Taken together, our results raise alarms about the emergence and spread of the SZM-1, and suggest further surveillance, especially in hospital settings and clinical isolates, to determine whether bla_{SZM-1} is a mobilizable antibiotic resistance.

KEYWORDS

metallo β-lactamase, metagenomic sequencing, carbapenem-resistance, SZM-1, carbapenemase

Introduction

Carbapenem is one of the last-resort β-lactam antibiotics for the treatment of serious bacterial infections that caused by gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*. Nevertheless, multiple molecular mechanisms that confer bacterial resistance phenotype become the major

barrier to the clinical application of the drug (Kelly et al., 2017; Aurilio et al., 2022). As one of such mechanisms, metallo-β-lactamases (MBLs), can directly degrade β-lactam ring of the carbapenem and most other β-lactam antibiotics, through their zinc dependent catalytic active center (Abbas, 2021). Despite the development of efficient treatment options for infections caused by carbapenem-resistant Gram-negatives, such as ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, and cefiderocol, only the later has demonstrated *in vitro* activity against MBL-producers (Mojica et al., 2022). Therefore, MBLs are still an alarming clinical problem, and the identification of new MBL genes important for understanding the epidemiology of this global threat.

To date, a majority of representative MBL types, for example NDM-1, IMP-1, VIM-1, and most their variants, have been firstly detected in clinical bacterial strains (Walsh et al., 1994; Lauretti et al., 1999; Poirel et al., 2000; Yong et al., 2009; Munita and Arias, 2016). While the wide distribution of the MBLs in cultured-based clinical isolates, increasing evidences have highlighted their environmental source, such as water environment (Deshmukh et al., 2011; Dziri et al., 2018; Fonseca et al., 2018). One reason for the close attention is that the environmental microorganisms represent a significant reservoir of novel antibiotic resistance genes (ARGs), including such MBL genes (Hernando-Amado et al., 2019). Besides, the transferable features of MBLs, which are mediated *via* mobile genetic elements, pose an urgent need for assessing the risk of the environment–human transmission (Bebrone, 2007).

Currently, metagenomic sequencing and analysis methods make it possible to globally monitor the MBL genes in a wide range of environments (Gudeta et al., 2016; Park et al., 2018; Hendriksen et al., 2019). For instance, several studies have used the metagenome-assembled genomes (MAGs) to identify bacterial hosts for the ARGs in both clinical and natural environments, providing new insights into the linkage of microbial community and the ARGs (Ma et al., 2016; Jia et al., 2019; Zhang et al., 2022). Moreover, methods for recovering MAGs from samples are considered important methodological extensions to traditional microbiological techniques and provide unprecedented access to uncultured organisms diversity, broadening the tree of life (Castelle and Banfield, 2018; Liu et al., 2021). Therefore, the metagenomic binning can contribute to a better understanding of the MBLs and provides important insights into the potential MBLs phylogenetic origin.

This study describes the identification of a novel subclass B1 MBL, SZM-1 (Shenzhen metallo-β-lactamase), in estuary sediment metagenomes from Shenzhen Bay area, one of the most developed areas with expanding population in south China (Yan et al., 2019).

Materials and methods

Metagenomic library construction

Three estuary sediment samples were collected at Dasha river of Shenzhen Bay in south China, and total DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany). Metagenomic sequence data were acquired using Illumina HiSeq sequencing with 150-bp paired-end reads at Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China). The resulting raw sequencing reads were further dereplicated (100% identity over 100% length) and trimmed using sickle[1]. Remaining high-quality metagenomic reads were *de novo* metagenome assembled using MEGAHIT (Li et al., 2016) with parameters "–min-contig-len 1000 –k-min 21 –k-max 141 –k-step 12 –merge-level 20,0.95." To perform gene prediction and annotation, the prokaryote annotation tool Prokka (Seemann, 2014) combined with the BLAST program[2] was used.

Identification of the SZM-1 in metagenome-assembled genomes

Metallo β-Lactamases were predicted in the metagenomic assemblies using ABRicate (v1.0.1)[3] with the following thresholds: ≥70% DNA identity, ≥80% DNA coverage (Zankari et al., 2012). Our search for the putative MBLs obtained an IMP-1 like protein (share 81.03% sequence identify), designated SZM-1, encoding by an open reading frame of 699 bp (designated as bla_{SZM-1}). Annotation of mobile elements was carried out using ISfinder[4]. To find out the bacterial host of the bla_{SZM-1}, genome binning of the metagenome-assembled genomes (MAGs) was carried out using MetaBAT (Kang et al., 2019) with 12 sets of flags inducing different sensitivity and specificity combinations. CheckM (Parks et al., 2015) was used to calculate the completeness and contamination of MAGs. The ARG-carrying bin was selected for taxonomy classification with GTDB-Tk package (Chaumeil et al., 2019). The MAGs and bla_{SZM-1}-carrying contigs were taxonomically classified using the default parameters of Kraken2 (Wood and Salzberg, 2014) and Kaiju (Tovo et al., 2020). Only bla_{SZM-1}-carrying contigs that are longer than 10 kb were considered; taxonomic affiliation of the contig is agreed with the overall taxonomy of the MAG (Zhang et al., 2022).

1 https://github.com/najoshi/sickle
2 http://blast.ncbi.nlm.nih.gov/Blast.cgi
3 https://github.com/tseemann/abricate
4 https://www-is.biotoul.fr/

Bioinformatics analysis of the SZM-1

All the protein sequences were obtained from NCBI database,[5] except the *bla*$_{SZM-1}$ gene. Phylogenetic analysis was conducted using CLC Genomics Workbench. The protein sequences were aligned using MEGA7 (Kumar et al., 2016).

In silico structure modeling of the SZM-1

The structure model for SZM-1 was built using the AlphaFold 2 of Colab server[6]. The multiple sequence alignment model, model type, pair mode, and the number of recycle were set as "UniRef + Environmental," "auto," "unpaired + paired," and "3," respectively. For each protein sequence, the one with the highest IDDT score of the resulting five models was used here.

Cloning of the bla$_{SZM-1}$ gene

The sequence of *bla*$_{SZM-1}$ was codon optimized, synthesized (BGI Genomics Co., Ltd.) and cloned into a pHSG398 vector (without signal peptide) using restriction-free seamless cloning method. Briefly, the *bla*$_{SZM-1}$ and the pHSG398 (TaKaRa Bio Co. Ltd., Japan) was amplified by PCR, respectively, using primers F-szm-1 (5′-ATGACCATGATTACGAATATGACCGCCGCAGGTGCAGA-3′)/R-szm-1 (5′-CCCGGGTACCGAGCTCGATTAATCATTC GGCAGCGGCGG-3′) and F-HSG (5′-TCGAGCTCGGTA CCCGGGGATC-3′)/R-HSG (5′-ATTCGTAATCATGGTCATA GCTG-3′) to construct the pHSG398-SZM-1. The resulting PCR fragments were purified and assembled with In-Fusion HD Cloning Kit (TaKaRa Bio Co. Ltd., Japan). To construct pHSG398-NDM-1, the full length of the NDM-1 (NCBI accession number: KX999121) was cloned into pHSG398 vector using restriction-free seamless cloning method with primers F-ndm-1 (5′- AGCTAT GACCATGATTACGAATATGCCGGGTTTCGGGGCAG-3′)/ R-ndm-1 (5′- CCGGGTACCGAGCTCGATCAGCGCAGCTT GTCGGCC-3′) and F-HSG (5′-TCGAGCTCGGTACCCGG GGATC-3′)/R-HSG (5′-ATTCGTAATCATGGTCATAGCTG-3′). Finally, the resulting recombinant plasmids were transformed into *Escherichia coli* TOP10 strains, respectively, that were used for further Carba NP test and resistance phenotype assay.

5 https://www.ncbi.nlm.nih.gov

6 https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb

Carba NP test

The Carba NP test was performed using the bacterial colonies according to the modified protocol as previously described (Pasteran et al., 2015).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted using liquid broth dilution tests as recommended by CLSI guidelines (Clinical and Laboratory Standards Institute [CLSI], 2017). with Mueller-Hinton Broth (Oxoid Co. Ltd., UK). The *E. coli* TOP10 strains carrying the pHSG398 vector with a synthesized NDM-1 gene insert or no insert were used as positive and a negative control, respectively.

Nucleotide sequence accession number

The metagenome-assembled genomes generated and analyzed during the current study are available in the NCBI (accession number: PRJNA848622).

Results

Identification of SZM-1 as a novel subclass B1 metallo-β-lactamase

The genes encoding SZM-1 were identified in two assembled contigs with lengths of 13,122 and 4,117 bp, respectively (**Figure 1**). Except for *bla*$_{SZM-1}$, other ORFs showed weak or no significant similarity to known sequences in NCBI-nr database. Binning analysis yielded a positive MAG for the *bla*$_{SZM-1}$ gene, which consists of 89 contigs, including the 13,122 bp contig (genome completeness: 93.07%; contamination: 1.75%). In this MAG, no mobile genomic elements have been detected. Besides, the *bla*$_{SZM-1}$-carrying MAG was taxonomically classified as genus *Arenimonas* within phylum Proteobacteria. The *bla*$_{SZM-1}$ gene locates in a conserved genetic environment, no mobile element was identified in its vicinity (**Figure 1**), and the GC content of MAG (61.9%) and the *bla*$_{SZM-1}$-carrying contig (65.7%) are at the same level. Meanwhile, the taxonomic affiliation of *bla*$_{SZM-1}$-carrying contig agreed with the overall taxonomy of the MAG. Therefore, it can be concluded with high certainty that Arenimonas is the recent origin of *bla*$_{SZM-1}$. Given that the SZM-1 shows high amino acid sequence identified to subclass B1 MBL1, and in order to phylogenetical analysis of the SZM-1, a maximum likelihood phylogenetic tree was constructed for the representative subclass

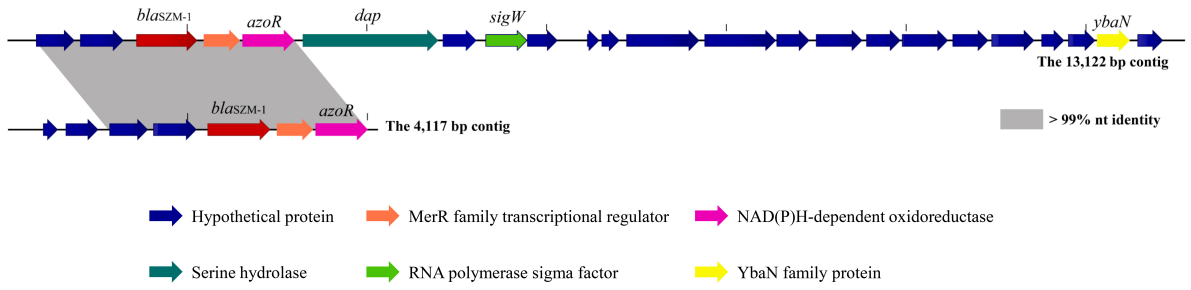

FIGURE 1
Genetic context surrounding the bla_{SZM-1} gene. The genetic contexts of bla_{SZM-1} on the 13,122-bp contig and the 4,117-bp contig are shown for comparison. The orfs that encode hypothetical proteins with unknown functions are shown in blue. Regions of >99% homology are marked with gray shading. The arrows indicate the positions and directions of transcription for each gene.

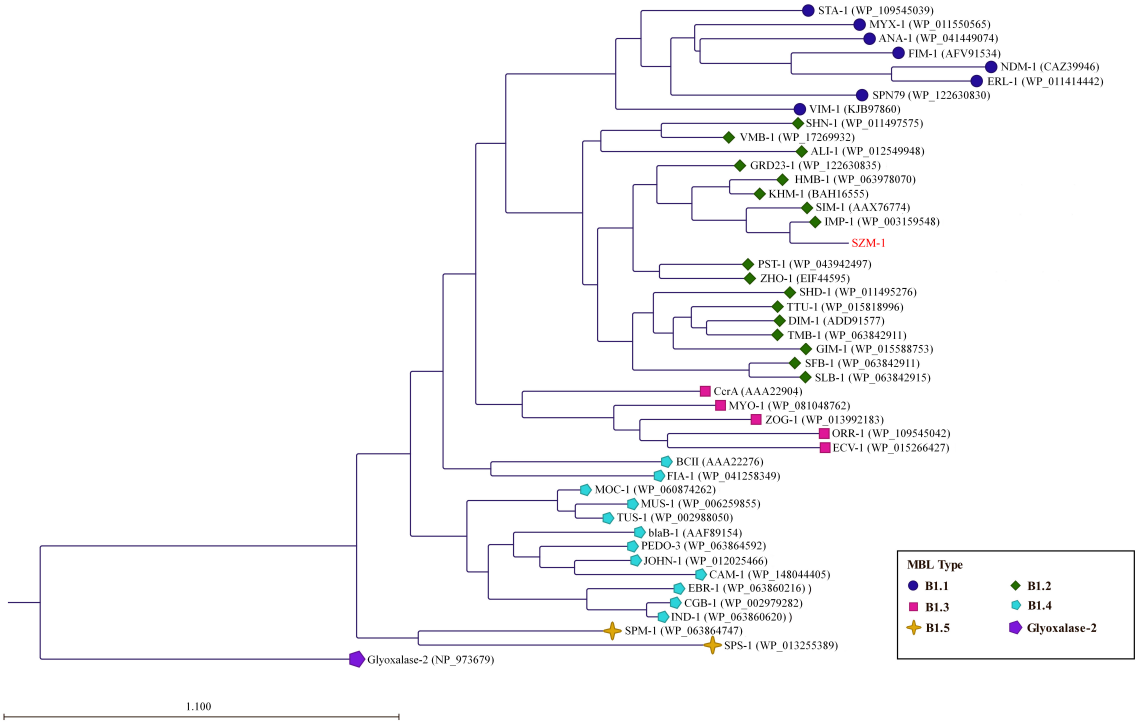

FIGURE 2
Phylogenetic analysis of the SZM-1. The phylogenetic tree was constructed with amino acid sequences of the representative MBL B1 subclasses (B1.1–B1.5) and Glyoxalase-2 (used as an outgroup). The tree was created by the Neighbor-Joining method (protein distance measure model: Jukes-Cantor; protein substitution model: WAG; bootstrap replicates: 1000). The horizontal bar indicates the number of amino acid substitution for site.

B1 MBLs, SZM-1, and Glyoxalase-2 (used as an outgroup). As shown in the B1 MBLs tree, the SZM-1 was located close to the IMP-1 clade within the B1.2 branch; this tree topology clearly demonstrates that the SZM-1 belongs to subclass B1.2 MBLs (**Figure 2**). Nonetheless, analysis of amino acid sequence of the SZM-1 indicates the absence of the typical N-terminal signal peptide, implying the inefficient export of the novel MBL to periplasmatic space (Derman et al., 1993).

Structural conservation of di-zinc catalytic center of SZM-1

As previously described, the B1 MBLs have been characterized by mono-zinc or di-zinc dependent catalytic center for their enzymic activity (Bebrone, 2007; Llarrull et al., 2008). To characterize the zinc catalytic center of SZM-1, detailed sequence comparison of the SZM-1, as well as other B1 MBLs was performed. The primary sequence analysis revealed

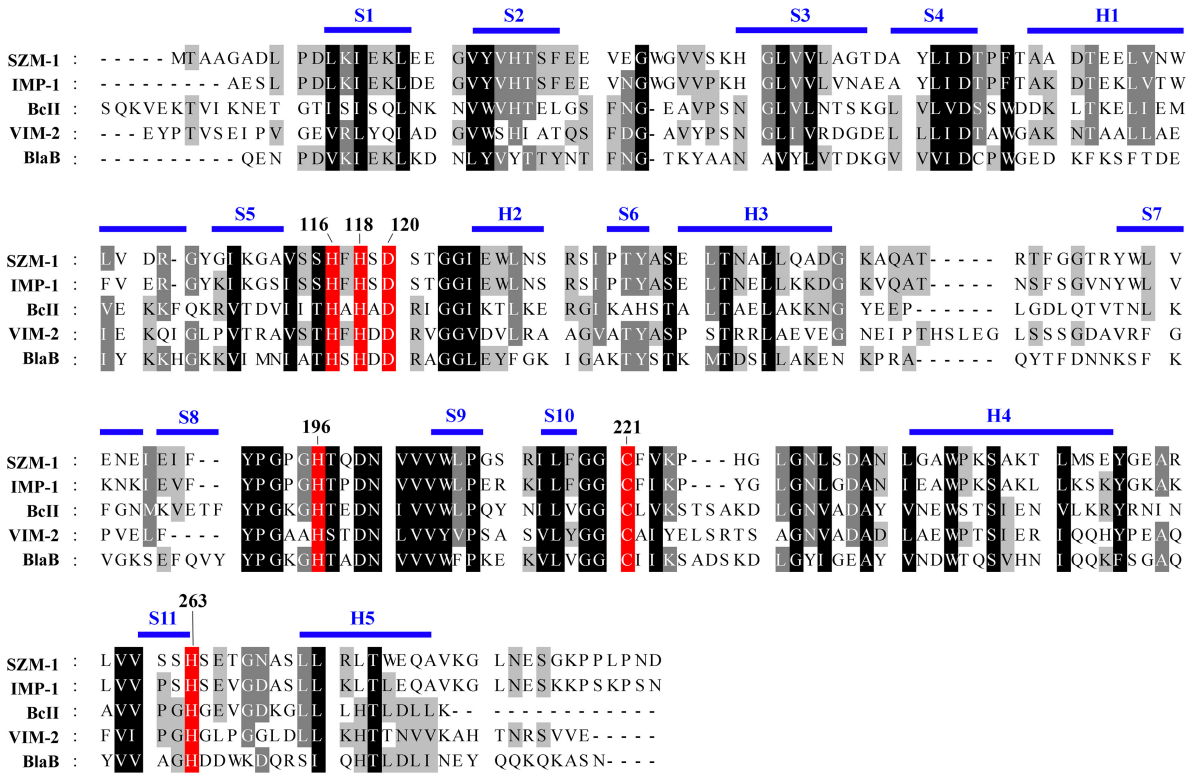

FIGURE 3
Amino acid sequence alignment of SZM-1 and the representative class B1 MBLs. The conserved class B residues are highlighted with red color. The representative class B1 MBLs include IMP-1 (NCBI accession number: WP 003159548.1), BcII (WP 000742468.1), VIM-2 (WP 003108247.1), and BlaB (WP 029729112.1). Conserved secondary-structure (S, β-strand; H, Helix) are indicated above the sequences. The key residues involved in the coordination of the zinc iron are numbered according to the standard MBL numbering system.

that the SZM-1 harbors conserved zinc-interacting residues that are well-characterized in B1 MBLs (**Figure 3**).

Moreover, the structure of the full-length SZM-1 was predicted by the AlphaFold 2 of Colab server, and was further compared with the IMP-1 structure (PDB accession number: 1 ddk). The structural model display that the SZM-1 adopts a canonical αββα structure that resemble the IMP-1 structure with root-mean-square deviation (RMSD) value 0.446. Remarkably, according to the standard MBL numbering system, the conformation of the SZM-1 zinc-interacting active center, consisting of residues His116, His118, Asp120, His196, Cys221, and His263, is closely similar to that of the IMP-1 structure, strongly suggesting that its enzymatic activity is di-zinc dependent (**Figure 4**; Garau et al., 2004).

Carbapenem-resistance phenotype of SZM-1

The *E. coli* TOP10 carrying bla_{SZM-1} showed carbapenemase activity by Carba NP test. Furthermore, when compared with the negative control, the bla_{SZM-1} carrier manifested a significant increase in MIC of Imipenem (>8-fold), while a slight increase of Meropenem (>2-fold; **Table 1**). In contrast, there was no difference in the MICs of aztreonam between the bla_{SZM-1} carrier and the negative control. Besides, expression of bla_{SZM-1} conferred *E. coli* TOP10 with reduced susceptibility to other tested β-lactams, including ampicillin (MIC = 64 mg/liter), cefotaxime (MIC = 4 mg/liter), ceftriaxone (MIC = 32 mg/liter), ceftazidime (MIC = 64 mg/liter), amoxycillin/clavulanic acid (MIC = 32 mg/liter), cefoxitin (MIC = 32 mg/liter), and piperacillin/tazobactam (MIC = 4 mg/liter). Together, these data reveal the carbapenem-resistance risks of the SZM-1.

Discussion

In this work, we reported a novel metallo β-lactamase, SZM-1, in Dasha river of Shenzhen Bay by shotgun metagenomic sequencing. The combined phylogenetic analysis, primary sequence alignment, and structural modeling indicate that the SZM-1 is a typical B1 MBL that likely employs a di-zinc dependent catalytic center. Moreover, these computational

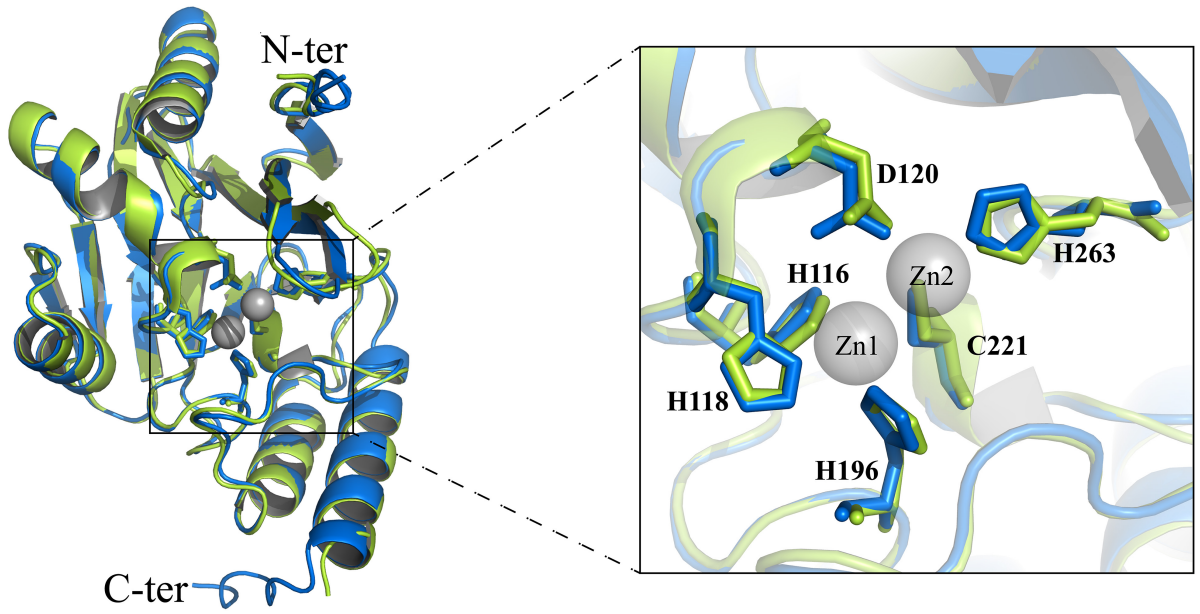

FIGURE 4
Structural analysis of the zinc-dependent active site of SZM-1. Structural comparison of active sites between IMP-1 (green) and SZM-1 (blue). The key residues (according to the standard MBL numbering system) involved in the coordination of the zinc iron are marked. The two zinc ions of the active site are marked by gray color.

TABLE 1 Antimicrobial drug susceptibility profile.

Antibiotic	MIC (mg/liter)		
	E. coli TOP10/ pHSG398-NDM-1	*E. coli* TOP10/ pHSG398-SZM-1	*E. coli* TOP10/ pHSG398-
Ampicillin	>128	64	0.5
Cefotaxime	>128	4	0.25
Ceftriaxone	>128	32	0.25
Ceftazidime	>128	64	0.5
Amoxycillin/ clavulanic acid	>128	32	0.25
Cefoxitin	>128	32	0.5
Piperacillin/ tazobactam	>128	4	0.5
Imipenem	>64	2	< 0.25
Meropenem	>64	0.5	< 0.25
Aztreonam	<0.25	<0.25	< 0.25

results are best compatible with the carbapenemase activity of the SZM-1, and the carbapenem-resistance phenotype of the bla_{SZM-1} carrier.

To the best of our knowledge, while the *Arenimonas* has been previously reported as a host of ARGs, this is the first-time characterization of a new MBL in this genus (Chen et al., 2019). Unlike the plasmid-borne IMP-1 and other mobile B1 MBLs, the absence of mobilizable elements in the genomic context of the bla_{SZM-1} seemingly does not contribute to the transmission of bla_{SZM-1} between organisms (Pongchaikul and Mongkolsuk, 2022). Nonetheless, given the foreign mobilizable elements may insert at genomic location around the bla_{SZM-1}, transmission risk of such gene in hospital settings and other human living environments cannot be eliminated, especially when one notices the expanding population in Shenzhen Bay. Besides, the expansion of such MBLs has the potential to alter the structure of the bacterial population, leading to the emergence of antibiotic resistance environments (Hernando-Amado et al., 2019).

The MIC results indicated that reduce susceptibility of SZM-1 carrier to imipenem and meropenem, are comparable to that of IMP-1, which may be underpinned by the similar structures of di-zinc dependent catalytic center (Janda and Abbott, 2021). However, the SZM-1 displays reduced tolerance to β-lactam antibiotic, such as cefotaxime, suggesting the important roles of non-catalytic residues in the MBL activity. From a One Health perspective, the identification of the SZM-1 puts more emphasis on important roles of metagenomic analyses in the identification and detection of antibiotic resistance determinants from environmental microbiomes (Hernando-Amado et al., 2019). Our result also raised the awareness of the urgent for assessing the potential risk of novel MBLs in non-clinical ecosystems.

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

RX and RZH conceived, designed the study, and reviewed the manuscript. LF, ZbL, and ZyL performed the experiments, analyzed the data, and drafted the manuscript. All authors revised the manuscript and approved the final 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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References

Abbas, F. M. (2021). Metallo-β-lactamases: A review. *Ann. Rom. Soc. Cell Biol.* 25, 1308–1316.

Aurilio, C., Sansone, P., Barbarisi, M., Pota, V., Giaccari, L. G., Coppolino, F., et al. (2022). Mechanisms of action of carbapenem resistance. *Antibiotics* 11:421. doi: 10.3390/antibiotics11030421

Bebrone, C. (2007). Metallo-β-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. *Biochem. Pharmacol.* 74, 1686–1701. doi: 10.1016/j.bcp.2007.05.021

Castelle, C. J., and Banfield, J. F. (2018). Major new microbial groups expand diversity and alter our understanding of the tree of life. *Cell* 172, 1181–1197. doi: 10.1016/j.cell.2018.02.016

Chaumeil, P. A., Mussig, A. J., Hugenholtz, P., and Parks, D. H. (2019). GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics* 36, 1925–1927. doi: 10.1093/bioinformatics/btz848

Chen, H., Chen, R., Jing, L., Bai, X., and Teng, Y. (2019). A metagenomic analysis framework for characterization of antibiotic resistomes in river environment: Application to an urban river in Beijing. *Environ. Pollut.* 245, 398–407. doi: 10.1016/j.envpol.2018.11.024

Clinical and Laboratory Standards Institute [CLSI] (2017). *Performance standards for antimicrobial susceptibility testing: M100*, 27th Edn. Wayne, PA: CLSI.

Derman, A. I., Puziss, J. W., Bassford, P. J. Jr., and Beckwith, J. (1993). A signal sequence is not required for protein export in prlA mutants of *Escherichia coli*. *EMBO J.* 12, 879–888. doi: 10.1002/j.1460-2075.1993.tb05728.x

Deshmukh, D. G., Damle, A. S., Bajaj, J. K., Bhakre, J. B., and Patwardhan, N. S. (2011). Metallo-beta-lactamase-producing clinical isolates from patients of a tertiary care hospital. *J. Lab. Physicians* 3, 93–97. doi: 10.4103/0974-2727.86841

Dziri, O., Dziri, R., Maraoub, A., and Chouchani, C. (2018). First report of SHV-148-type ESBL and CMY-42-type AmpC beta-lactamase in *Klebsiella pneumoniae* clinical isolates in Tunisia. *Microb. Drug Resist.* 24, 1483–1488. doi: 10.1089/mdr.2018.0073

Fonseca, E. L., Andrade, B. G., and Vicente, A. C. (2018). The resistome of low-impacted marine environments is composed by distant metallo-β-lactamases homologs. *Front. Microbiol.* 9:677. doi: 10.3389/fmicb.2018.00677

Garau, G., Garcia-Saez, I., Bebrone, C., Anne, C., Mercuri, P., Galleni, M., et al. (2004). Update of the standard numbering scheme for class B beta-lactamases. *Antimicrob. Agents Chemother.* 48, 2347–2349. doi: 10.1128/AAC.48.7.2347-2349.2004

Gudeta, D. D., Bortolaia, V., Pollini, S., Docquier, J.-D., Rossolini, G. M., Amos, G. C., et al. (2016). Expanding the repertoire of carbapenem-hydrolyzing metallo-ß-lactamases by functional metagenomic analysis of soil microbiota. *Front. Microbiol.* 7:1985. doi: 10.3389/fmicb.2016.01985

Hendriksen, R. S., Munk, P., Njage, P., Van Bunnik, B., Mcnally, L., Lukjancenko, O., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10:1124. doi: 10.1038/s41467-019-08853-3

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martinez, J. L. (2019). Defining and combating antibiotic resistance from One Health and global health perspectives. *Nat. Microbiol.* 4, 1432–1442. doi: 10.1038/s41564-019-0503-9

Janda, J. M., and Abbott, S. L. (2021). The changing face of the family *Enterobacteriaceae* (Order: "Enterobacterales"): New members, taxonomic issues, geographic expansion, and new diseases and disease syndromes. *Clin. Microbiol. Rev.* 34:e00174-20. doi: 10.1128/CMR.00174-20

Jia, S. Y., Wu, J. L., Ye, L., Zhao, F. Z., Li, T., and Zhang, X. X. (2019). Metagenomic assembly provides a deep insight into the antibiotic resistome alteration induced by drinking water chlorination and its correlations with bacterial host changes. *J. Hazard. Mater.* 379:120841. doi: 10.1016/j.jhazmat.2019.120841

Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., et al. (2019). MetaBAT 2: An adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7:e7359. doi: 10.7717/peerj.7359

Kelly, A. M., Mathema, B., and Larson, E. L. (2017). Carbapenem-resistant *Enterobacteriaceae* in the community: A scoping review. *Int. J. Antimicrob. Agents* 50, 127–134. doi: 10.1016/j.ijantimicag.2017.03.012

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lauretti, L., Riccio, M. L., Mazzariol, A., Cornaglia, G., Amicosante, G., Fontana, R., et al. (1999). Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. *Antimicrob. Agents Chemother.* 43, 1584–1590. doi: 10.1128/AAC.43.7.1584

Li, D., Luo, R., Liu, C. M., Leung, C. M., Ting, H. F., Sadakane, K., et al. (2016). MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods* 102, 3–11. doi: 10.1016/j.ymeth.2016.02.020

Liu, Y., Makarova, K. S., Huang, W. C., Wolf, Y. I., Nikolskaya, A. N., Zhang, X. X., et al. (2021). Expanded diversity of Asgard archaea and their relationships with eukaryotes. *Nature* 593, 553–557. doi: 10.1038/s41586-021-03494-3

Llarrull, L. I., Tioni, M. F., and Vila, A. J. (2008). Metal content and localization during turnover in *B. cereus* metallo-beta-lactamase. *J. Am. Chem. Soc.* 130, 15842–15851. doi: 10.1021/ja801168r

Ma, L. P., Xia, Y., Li, B., Yang, Y., Li, L. G., Tiedje, J. M., et al. (2016). Metagenomic assembly reveals hosts of antibiotic resistance genes and the shared resistome in pig, chicken, and human feces. *Environ. Sci. Technol.* 50, 420–427. doi: 10.1021/acs.est.5b03522

Mojica, M. F., Rossi, M. A., Vila, A. J., and Bonomo, R. A. (2022). The urgent need for metallo-beta-lactamase inhibitors: An unattended global threat. *Lancet Infect. Dis.* 22, e28–e34. doi: 10.1016/S1473-3099(20)30868-9

Munita, J. M., and Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiol. Spectr.* 4, 1–24. doi: 10.1128/microbiolspec.VMBF-0016-2015

Park, K. S., Kim, T. Y., Kim, J. H., Lee, J. H., Jeon, J. H., Karim, A. M., et al. (2018). PNGM-1, a novel subclass B3 metallo-β-lactamase from a deep-sea sediment metagenome. *J. Glob. Antimicrob. Resist.* 14, 302–305. doi: 10.1016/j.jgar.2018.05.021

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015). CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* 25, 1043–1055. doi: 10.1101/gr.186072.114

Pasteran, F., Tijet, N., Melano, R. G., and Corso, A. (2015). Simplified protocol for carba NP test for enhanced detection of carbapenemase producers directly from bacterial cultures. *J. Clin. Microbiol.* 53, 3908–3911. doi: 10.1128/JCM.02032-15

Poirel, L., Naas, T., Nicolas, D., Collet, L., Bellais, S., Cavallo, J.-D., et al. (2000). Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid-and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob. Agents Chemother.* 44, 891–897. doi: 10.1128/AAC.44.4.891-897.2000

Pongchaikul, P., and Mongkolsuk, P. (2022). Comprehensive analysis of imipenemase (IMP)-type metallo-beta-lactamase: A global distribution threatening asia. *Antibiotics* 11:236. doi: 10.3390/antibiotics11020236

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Tovo, A., Menzel, P., Krogh, A., Cosentino Lagomarsino, M., and Suweis, S. (2020). Taxonomic classification method for metagenomics based on core protein families with Core-Kaiju. *Nucleic Acids Res.* 48:e93. doi: 10.1093/nar/gkaa568

Walsh, T. R., Hall, L., Assinder, S. J., Nichols, W. W., Cartwright, S. J., Macgowan, A. P., et al. (1994). Sequence analysis of the L1 metallo-beta-lactamase from *Xanthomonas maltophilia*. *Biochim. Biophys. Acta* 1218, 199–201. doi: 10.1016/0167-4781(94)90011-6

Wood, D. E., and Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:R46. doi: 10.1186/gb-2014-15-3-r46

Yan, H., He, X., Lei, Y., Wang, Y., Su, H., and Jiang, S. (2019). Land use-induced change in trophic state of Shenzhen Bay (South China) over the past half-century. *Mar. Pollut. Bull.* 145, 208–213. doi: 10.1016/j.marpolbul.2019.05.046

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., et al. (2009). Characterization of a new metallo-beta-lactamase gene, $bla_{(NDM-1)}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob. Agents Chemother.* 53, 5046–5054. doi: 10.1128/AAC.00774-09

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, Z. Y., Zhang, Q., Wang, T. Z., Xu, N. H., Lu, T., Hong, W. J., et al. (2022). Assessment of global health risk of antibiotic resistance genes. *Nat. Commun.* 13:1553. doi: 10.1038/s41467-022-29283-8

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Host defense against the infection of *Klebsiella pneumoniae*: New strategy to kill the bacterium in the era of antibiotics?

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Klebsiella pneumoniae (*K. pneumoniae*) is a typical gram-negative iatrogenic bacterium that often causes bacteremia, pneumonia and urinary tract infection particularly among those with low immunity. Although antibiotics is the cornerstone of anti-infections, the clinical efficacy of β-lactamase and carbapenems drugs has been weakened due to the emergence of drug-resistant *K. pneumoniae*. Recent studies have demonstrated that host defense plays a critical role in killing *K. pneumoniae*. Here, we summarize our current understanding of host immunity mechanisms against *K. pneumoniae*, including mechanical barrier, innate immune cells, cellular immunity and humoral immunity, providing a theoretical basis and the new strategy for the clinical treatment of *K. pneumoniae* through improving host immunity.

KEYWORDS

***Klebsiella pneumoniae*, infection, immunity, clinical treatment, antibiotics**

1 Introduction

Klebsiella pneumoniae, a common gram-negative facultative anaerobic bacterium, widely exists not only in the natural soil and water but also in human and animal respiratory tract and intestinal tract (Paczosa and Mecsas, 2016). According to the 20-year-Antimicrobial Surveillance Program (from 1997 to 2016), *K. pneumoniae* (7.7%)

ranks the third place in the most common pathogens, which is the leading cause of bloodstream infection (Diekema et al., 2019). It is easy to colonize on the surface of the human gastrointestinal and respiratory mucosa and cause pneumonia, urinary tract infections (UTIs), bacteremia and liver abscess in clinic (Paczosa and Mecsas, 2016; Choby et al., 2020). Elders, newborns and tumor patients with low immunity are generally susceptible to *Klebsiella* (Chew et al., 2017).

Klebsiella has several subspecies, including *K. pneumoniae subsp. pneumoniae*, *K. ozaenae subsp. ozaenae* and *K. rhinoscleromatis*. *K. pneumoniae* causes more than 95% of the common clinical cases of *Klebsiella* infection. *K. pneumoniae* strains are usually classified as classical, *hypervirulent K. pneumoniae* (*hvKp*) and multidrug resistant (MDR). Classical *K. pneumoniae* is composed of opportunistic strains often associated with hospital infection. Highly pathogenic strains, including *hvKp* are considered community-acquired bacteria that infect people of all ages, including healthy people (Wang et al., 2020). *hvKp* is a variant of *K. pneumoniae*, which is more virulent than classical *K. pneumoniae*. *hvKp* is generally resistant to the third- and fourth-generation cephalosporins and has a stronger metastatic ability. The most common metastatic sites are the eyes, lung and central nervous system, which have become the focus of clinical microbial research (Paczosa and Mecsas, 2016; Russo and Marr, 2019). Furthermore, *K. pneumoniae* is also known for its antibiotic-resistant genes, which can spread to other gram-negative bacteria. Indeed, many antibiotic-resistance genes commonly detected in multidrug-resistant organisms were first described in *K. pneumoniae* (Holt et al., 2015). Among the isolates of *K. pneumoniae* reported in the European Centre for Disease Prevention and Control, more than one-third of *K. pneumoniae* are resistant to one or more antimicrobial agents, including fluoroquinolones, the third-generation cephalosporins and aminoglycosides (Bengoechea and Sa Pessoa, 2019). The invasiveness of *K. pneumoniae* depends on its capsule, lipopolysaccharide (LPS), fimbriae and siderophores (Rodríguez-Medina et al., 2019). With the prevalence of *hvKp* and MDR, whether human will find an effective way against *K. pneumoniae* remains a mystery. Here, we focus on the relevant immune mechanism against *K. pneumoniae* to provide new clues for the clinical treatment of *K. pneumoniae* infection.

2 Innate Immune responses to *K. pneumoniae*

K. pneumoniae infection can be thought of as the outcome of interactions between *K. pneumoniae* and the host, including innate immunity and adaptive immunity. Innate immunity is the first line against *K. pneumoniae* infection, involving various barriers, innate immune cells and molecules.

2.1 The function of barriers

2.1.1 Respiratory barriers

K. pneumoniae is one of the most common floras causing hospital-acquired infections and lower respiratory tract infections in the intensive care units (Saharman et al., 2020). When *K. pneumoniae* invades host, the mechanical barrier provides the immediate protection. As the interface between the host and *K. pneumoniae*, the respiratory tract and its epithelial cells play an active role as a mechanical barrier. Adult microbiota activates the defense of upper respiratory tract through interleukin (IL)-17A, while *K. pneumoniae* could overcome this obstacle to establish colonization through encapsulation (Sequeira et al., 2020). The adhesion factors with various physiological functions are present in cell wall and other structures of *K. pneumoniae* (Na et al., 2014). The colonization of *K. pneumoniae* could damage small airway epithelial cells and increase the level of tumor necrosis factor (TNF)-α in lung, and the upregulation of TNF-α could significantly exacerbate epithelial cell injury (Xu and Xu, 2005).

Additionally, the mucus ciliary layer of respiratory tract can adhere to or remove the bacteria or other particles entering the respiratory tract, while changes in the thickness, properties and cilia clearance of mucus influence the dismissal of *K. pneumoniae* by respiratory tract. Lung infection with *K. pneumoniae* could lead to massive infiltration of inflammatory cells, resulting in a progressive decrease in local defenses (Zheng et al., 2014). The outer membrane protein A of *Klebsiella pneumoniae* (KPOmpA) affects the expression of adhesion molecules and the secretion of cytokine in bronchial epithelial cells (BECs). It has been proved that KPOmpA can bind tightly to human BEC cell line BEAS-2B and primary cultures of BECs, activating the nuclear factor kappa B (NF-κB) signal pathway, thus stimulating the host defense. In addition, BECs exert internalized clearance of *K. pneumoniae* that invades the respiratory tract (Pichavant et al., 2003).

Therefore, mucus and ciliated epithelial cells in the respiratory system can effectively hinder the invasion of *Klebsiella* and eliminate it in multiple ways. However, the infection of *Klebsiella* could trigger an inflammatory response in the respiratory tract, leading to the accumulation of inflammatory cells, which can disrupt the mechanical barrier of the ciliary layer of respiratory tract and disturb the host defense (Figure 1).

2.1.2 Urinary barriers

Catheter-associated urinary tract infections (CAUTIs) is one of the most common nosocomial infections and complications of indwelling catheters (Maunders et al., 2022). *K. pneumoniae* is prone to UTIs through catheters, accounting for 2-6% of hospital UTIs (Li et al., 2014; Maunders et al., 2022). However, the mechanical force created by the flow of urine can remove

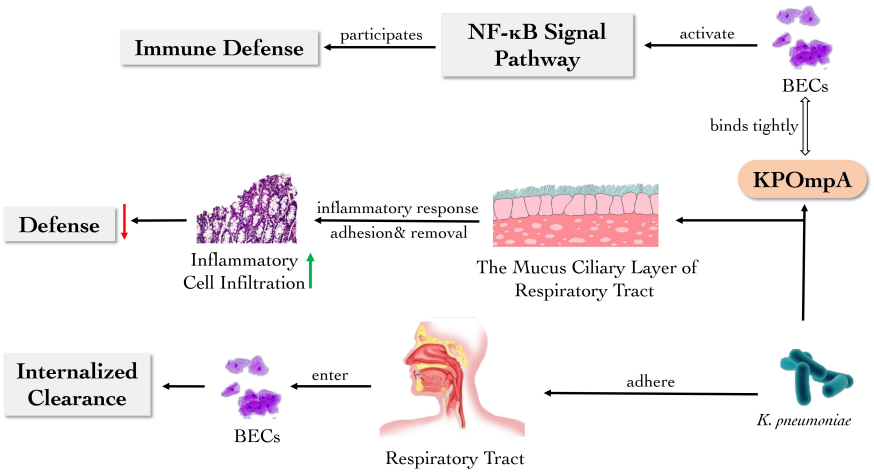

FIGURE 1
The main role of respiratory barriers against *K. pneumoniae*. *K. pneumoniae* can be eliminated by BECs and the mucus cilia layer of the respiratory tract. KPOmpA from *K. pneumoniae* binds tightly to BECs, thereby activating NF-κB signal pathway to participate in the host defense. However, *K. pneumoniae* and its products cause airway inflammation and weaken airway defense.

pathogens normally, which acts as an essential barrier for the colonization of bacteria. Furthermore, the pH value of urine is a critical factor in the colonization and proliferation of pathogenic bacteria in the urinary tract, and the alteration of pH value may play an important role in the treatment and prevention of *Klebsiella* on UTIs (Yang et al., 2014; Wasfi et al., 2020). Meanwhile, the bladder smooth muscle activity could significantly increase the positive rate of *Klebsiella* in urine, which allows for flushing *Klebsiella in vivo* (Burnett et al., 2021). It is known that *K. pneumoniae* may adhere to the host cell surface with the help of various adhesion factors such as the *K. pneumoniae* MrkD adhesin, colonizing the host and causing infections (Jagnow and Clegg, 2003; Li et al., 2009). Fortunately mechanical forces such as urine activity, bladder contraction, and the alteration of pH value in urine are capable of weakening the colonization of *K. pneumoniae* (Figure 2).

2.1.3 Digestive barriers

Studies have shown that the main anti-*Klebsiella* effect of digestive system comes from gut microbiota. The gut microbiota consists of diverse bacterial communities that perform various functions and influence the host's overall health, including nutrient metabolism, immune system regulation and natural defense against infection (Al Bander et al., 2020). During the *K. pneumoniae* infection, there is a complex interaction between the host and gut microbiota.

Researches have shown that in the early stage of *K. pneumoniae* infection, the richness and composition of gut

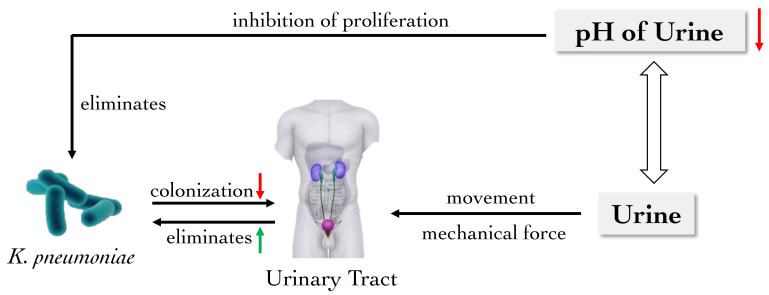

FIGURE 2
The main role of urinary barrier against *K. pneumoniae*. The mechanical force generated by the flow of urine removes *K. pneumoniae* from the urinary tract, which is also an important physical barrier to reduce bacterial colonization. Moreover, the reduction of urinary pH inhibits the proliferation of *K. pneumoniae* in the urinary tract.

microbiota changes, especially the numbers of *Lactobacillus reuteri* and *Bifidobacterium pseudolongum* decrease significantly (Wu et al., 2020; Wolff et al., 2021). Among the gut microbiota, *Bacteroidetes* can strengthen the intestinal immune barrier through IL-36 and macrophages to prevent the colonization and transmission of *K. pneumoniae* (Sequeira et al., 2020).

Short-chain fatty acids (SCFA), fermentation products of intestinal flora, including acetic acid, butyric acid and propionic acid, play a pivotal role in resisting the colonization and inflammation of *K. pneumoniae*. Vornhagen et al. observed that SCFA could directly inhibit bacterial growth through intracellular acidification in a dose-dependent manner. SCFA also reduces epithelial oxygenation and stimulate the expression of antimicrobial peptides in the gut microbiota, thus weakening pathogen colonization. Further, SCFA affects intestinal homeostasis to induce gut microbiota to produce metabolites, thereby decreasing the fitness of *K. pneumoniae* lacking functional plasmid encoding tellurite TeO3-2-resistance (Ter) operons in the intestinal tract (Vornhagen et al., 2021). Another research also showed that the G protein-coupled receptor 43 (GPR43) combined with acetate could upregulate the activity of neutrophils and alveolar macrophages, which reduce the number of bacteria in the airway in the early stage of infection, and promote inflammation regression to reduce lung injury in the late stage of infection. These results indicate that GPR43 plays a significant role in the "gut–lung axis" as a sensor of the host gut microbiota activity. Increasing SCFA will probably be a new way to promote inflammation resolution in clinical practice (Galvão et al., 2018). Aside from that, butyrate and tryptophan decomposition metabolites are able to enhance gut integrity and stimulate innate lymphoid cells group 3 (ILC3) to produce IL-22. Gut microbiota also could reduce intestinal permeability and increase the epithelial defense mechanism to form a mucosal barrier. Therefore, they maintain the stability of the intestinal environment (Shi et al., 2017). In the case of liver abscess induced by *K. pneumoniae*, relevant studies have discovered that antibiotic treatment before *K. pneumoniae* infection weakens the protective effect of intestinal flora in mice. Surprisingly, after fecal transplantation, the concentrations of chemokine (C-X-C motif) ligand 1 protein (CXCL1), TNF-α, monocyte chemoattractant protein-1 (MCP-1), IL-1β, IL-6 and IL-17 in mice serum were recovered and liver injury was alleviated (Zheng et al., 2021).

In a word, gut microbiota and its metabolites is essential in *K. pneumoniae* colonization and inflammatory response (Figure 3). Administration of exogenous SCFA could be sufficient to reduce fitness of *K. pneumoniae*. However, whether other substances also have impacts and how these microorganisms and metabolites interact with the host remains need to be further explored.

2.2 The function of innate immune cells

2.2.1 Dendritic cells

Dendritic cells (DCs) of the lung are situated in close proximity to alveolar epithelium and resident alveolar macrophages, playing a specific role as antigen-presenting cells (APCs) (Von Wulffen et al., 2007). There are several subtypes of DCs. Plasmacytoid DCs (pDCs) can produce interferon (IFN)-α and sense the damaged skin to heal wounds. $CD103^{+}$ DCs, $CD11b^{hi}$ DCs and monocyte-derived DCs (MoDCs) can act as migratory DCs to promote the activation of naïve $CD4^{+}$ and $CD8^{+}$ T cells in lymph nodes (Hackstein et al., 2012; Plantinga

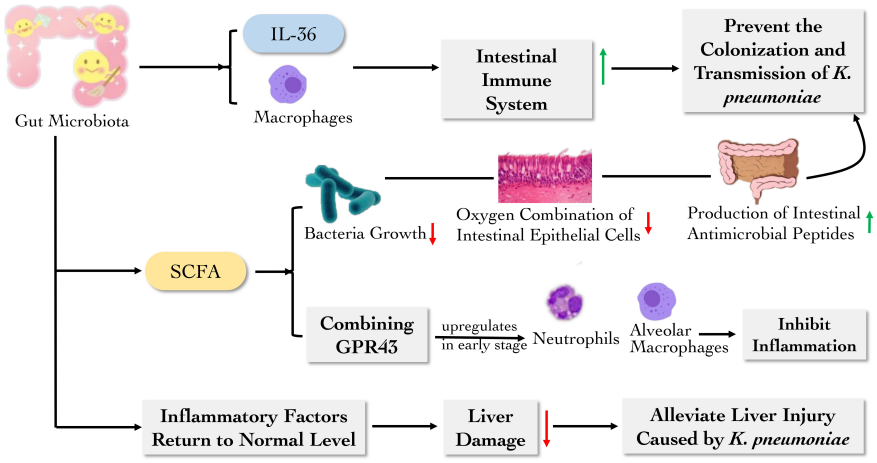

FIGURE 3
The main role of digestive barrier against *K. pneumoniae*. The gut microbiota and their metabolites can reduce the colonization and transmission of *K. pneumoniae* and inhibit inflammation by regulating the activity of immune cells and inflammatory factors.

et al., 2013). Hackstein et al. discovered a rapid increase of activated $CD103^+$ DC, $CD11b^+$ DC and MoDC within 48 h post infection of *K. pneumoniae*. The *K. pneumoniae*-infected animals showed that in respiratory DC subpopulations there were elevated IFN-α in pDC, elevated IFN-γ, IL-4 and IL-13 in $CD103^+$ DC and IL-19 and IL-12p35 in $CD11b^+$ DC subsets in comparison to $CD11c^+$ MHC-class II^{low} cells indicating distinct functional roles. $CD103^+$ DC and $CD11b^+$ DC subsets represented the most potent naïve $CD4^+$ T helper cell activators in the infection model of *K. pneumoniae* (Hackstein et al., 2013) (Figure 4). Therefore, the novel insight into the activation of respiratory DC subsets during *K. pneumonia* infection is provided.

2.2.2 Macrophages

Pulmonary macrophages are derived from monocytes, which mainly stimulate other immune cells acting as APCs and secreting immune molecules. The latest experiments showed that capsular polysaccharide (CPS) derived from carbapenem-resistant *K. pneumoniae KN2* serotype can stimulate J774A.1 mouse macrophage to release TNF-α and IL-6 *in vitro*. The CPS also exerts an immune response through TLR4 in human embryonic kidney-293 (HEK-293) cells (Lee et al., 2022). Melissa and Kovach observed that the clearance rate of *K. pneumoniae* in IL-36γ-deficient mice was decreased and the mortality of the mouse was increased, which confirmed that IL-36γ is related to the anti-*Klebsiella* effect. Further, it is proved that pulmonary macrophages secreted IL-36γ in a non-Golgi-dependent manner, playing a critical role in innate mucosal immunity of lung (Kovach et al., 2016; Kovach et al., 2017). The chemokine-mediated transportation of mononuclear phagocytes also is essential in the defense against bacterial pneumonia. In the *K. pneumoniae*-infected mouse model, the deletion of chemotactic cytokines receptor 2 (CCR2) could reduce all the monocyte phagocyte subsets and change the phenotype of pulmonary macrophages, reducing the amount of M1 macrophages and TNF in lung (Chen et al., 2013). Myeloid but not neutrophil-specific hypoxia-inducible factor (HIF)-1α-deficient mice increased bacterial loads in the lungs and distant organs after infection of *K. pneumoniae* as compared to control mice, pointing to a role of HIF-1α in macrophages. What's more, alveolar and lung interstitial macrophages from myeloid-specific HIF-1α-deficient mice produced a lower level of immunity, suggesting the importance of HIF-1α expressed in lung macrophages in protective innate immunity during pneumonia caused by *K. pneumoniae* (Otto et al., 2021).

Collectively, the researches above indicate that pulmonary macrophage is essential in innate immunity by secreting cytokine such as IL-36, TNF-α and IL-6 after *K. pneumoniae* infection, while CCR2 and HIF-1α play an auxiliary role in the anti-*K. pneumoniae* activity of macrophages (Figure 4). These discoveries provide new ideas for the clinical treatment of pneumonia caused by *Klebsiella* infection.

2.2.3 Neutrophils

Neutrophils are the first line against a variety of infectious pathogens. Neutrophils could kill pathogens by phagocytosis and neutrophil extracellular traps (NETs). NETs is one of the primary defensive mechanisms of neutrophils against *carbapenemase resistant hypervirulent K. pneumoniae* (*CR-*

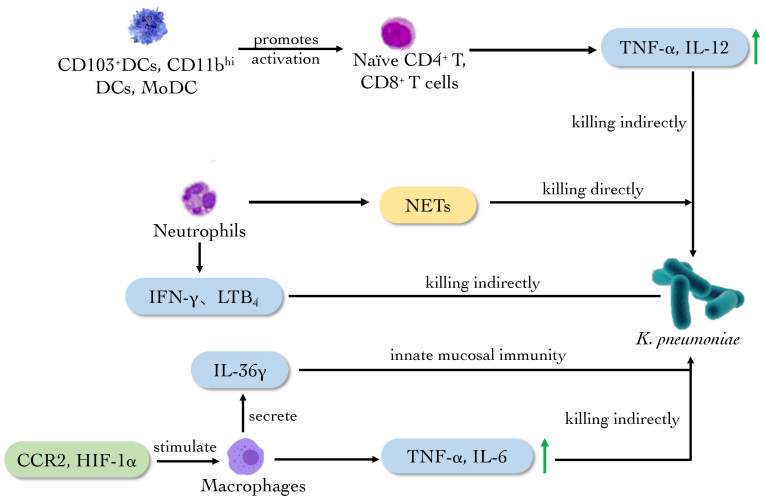

FIGURE 4
The main role of innate immune cells against *K. pneumoniae*. Neutrophils produce NETs to eliminate *K. pneumoniae* directly. Meanwhile, $CD103^+$DCs, $CD11b^{hi}$ and MODCs promote the activation of naïve T cells. CCR2 and HIF-1a activate macrophages to secrete cytokines such as TNF-α, IL-6 and IL-36γ to eradicate the bacterium.

hvKp). Through the scanning electron microscope test, Jin et al. found that the NETs in type 2 diabetes patients had lost its smooth and regular shape, which may lead to the defection of congenital immune response for the patients against *CR-hvKp*. The study confirmed the direct killing effect of NETs to *CR-hvKp* (Jin et al., 2020). More recently researchers compared the concentration of cytokines in regular diet group of mice and high-fat diet group infected with *K. pneumoniae*, discovering that the concentrations of IL-1β, IL-6, IL-17, IFN- γ, CXCL2 and TNF-α were much lower in the high-fat diet group, meanwhile, the number of neutrophils was reduced, and the functions including the phagocytosis, killing ability and production of the reactive oxygen intermediates (ROI) were impaired significantly, which proved the critical role of neutrophils in anti-*K. pneumoniae* effect (Mancuso et al., 2022).

Further studies found that the expression of CXCL5 in IL-17-deficient epithelium decreased, while intranasal injection of recombinant CXCL5 in mice could restore neutrophils' recruitment and bacterial clearance (Chen et al., 2016). It was also discovered that the number of neutrophils decreased and the production of leukotriene B_4 (LTB_4), reactive oxygen species (ROS) and reactive nitrogen species (RNS) decreased in CXCL1-/- mice infected with *K. pneumoniae* (Batra et al., 2012). Meanwhile, subsequent experiments on depleted neutrophils showed that neutrophils were the main source of LTB_4 in the lungs after infection (Batra et al., 2012). The above researches reveal the important role of CXCL1 in the expression of ROS and RNS produced by neutrophils, the regulation of host immunity to *K. pneumoniae* infection, and the curative effect of LTB_4 on the recruitment of neutrophils. In addition, more studies have shown that IL-33 can enhance host defense during bacterial pneumonia through the combined function of neutrophils and inflammatory monocytes (Ramirez-Moral et al., 2021).

Evidences *in vitro* and *in vivo* above indicate that neutrophils play an anti-*K. pneumonia's* role mainly through NETs and secreting corresponding molecules such as LTB_4, ROS and RNS (Figure 4). What's more, the administration of CXCL5 and LTB_4 can restore the activity of neutrophils, providing a new direction for the clinical treatment of *K. pneumoniae*.

2.2.4 Innate Lymphoid Cells

All T lymphocytes including T_H17 and γδT subsets participating in innate immunity derive from common lymphoid progenitor in bone marrow, differentiate and mature in thymus. γδ17 cell is a subset of γδT cells which produce large quantities of IL-17A in the presence of IL-23 and IL-1β. γδ17 cell expresses the type I IL-4R. And IL-4 signaling increases STAT6 phosphorylation in γδT cells. Whereas IL-4 inhibits the production of IL-17A by γδ17 cell. *K. pneumoniae* infection of STAT6 knockout mice shows a higher amount of γδ17 cell compared to that of wild-type mice, demonstrating that STAT6 signaling negatively regulates γδ17 cell that play a front-line role in mucosal immunity against *K. pneumoniae* (Bloodworth et al., 2016). Recently, Mackel et al. evaluated the role of T cells in protection against classical *K. pneumoniae* reinfection and demonstrated that mice lacking T cells were unable to establish a protective response. However, mice individually deficient in either of the major T cell subsets, γδ or αβ (classical T cells), effectively mounted a protective response, indicating either subset alone was sufficient to mediate protection against the reinfection of *K. pneumoniae* (Mackel et al., 2022) (Figure 5). The researches above demonstrate the imperative contribution of innate T cells to protective immunity against classical *K. pneumoniae* and will guide further inquiries into host effector responses required to control *K. pneumoniae* infection.

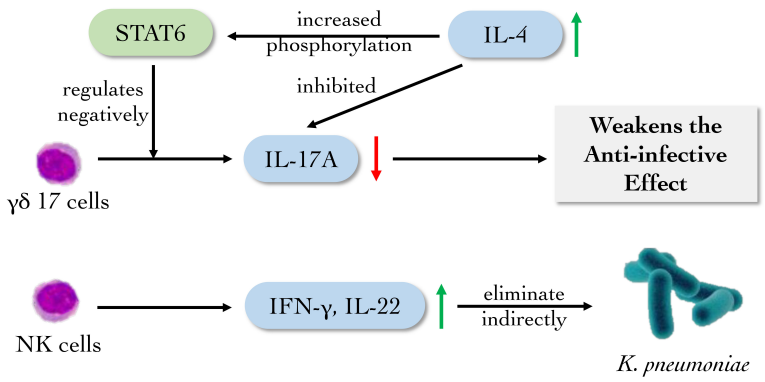

FIGURE 5
The main role of innate lymphoid cells against *K. pneumoniae*. NK cells and its subsets mainly eliminate *K. pneumoniae* indirectly by producing cytokines. γδ17 cells facilitate the eradication of *K. pneumoniae* by secreting IL17A, which can be suppressed by high level of IL-4.

Nature killer (NK) cells, which belong to innate lymphoid cells (ILCs), are found to recognize and eliminate "altered self" as cytotoxic lymphocytes, which also take part as the source of early inflammatory cytokines in the innate immune system (Pallmer and Oxenius, 2016; Myers and Miller, 2021). After activation, they secrete perforin and TNF to kill "allosome" substances nonspecifically. It was found that after infection with *K. pneumoniae*, the survival rate of IL-22$^{-/-}$ mouse was lowered while the survival rate of Rag2$^{-/-}$ mouse had no significant changes compared with wild-type mouse. Simultaneously, Rag2$^{-/-}$Il2rg$^{-/-}$ mice failed to produce IL-22. NK cells and T cells may produce IL-22 and have conventional host defense against *K. pneumoniae*, which were confirmed with Rag2$^{-/-}$ Il2rg$^{-/-}$ C57BL/6 mice (Xu et al., 2014). Type I IFN receptor (*Ifnar*) 1-deficient mice infected with *K. pneumoniae* failed to activate NK cells to produce IFN-γ, which caused the weakening of NK cell killing effect. Meanwhile, exogenous IFN-γ can recover the level of IFN-γ in *Ifnar1*$^{fl/fl}$ (Ifnar1^{tm1Uka})-CD11c^{Cre}, *Ifnar1*$^{fl/fl}$-LysM^{Cre} and *Ifnar1*$^{fl/fl}$-MRP8^{Cre} mice on C57BL/6 background (Ivin et al., 2017). These data identify NK cell-intrinsic type I IFN signaling as essential driver of *K. pneumoniae* clearance, and reveal a specific target for future therapeutic exploitations (Figure 5).

2.3 The function of innate immune molecules

2.3.1 TLRs

Innate immunity depends on signals produced by pattern recognition receptors (PRRs). Toll-like receptors (TLRs) is the earliest PRRs, which can recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) in microorganisms (O'neill et al., 2013). When the ligand binds to TLRs, myeloid differentiation factor88 (MyD88) and Toll/IL-1R (TIR) domain-containing adaptor protein (TIRAP) are recruited into the TLR complex to activate MAPK and NF-κB signal pathways to produce cytokines and chemokines. This cascade reaction is called MyD88-dependent pathway. In the lung against *K. pneumoniae* TIRAP is a critical mediator of antibacterial defense (Jeyaseelan et al., 2006). Besides, the activation of TLRs also recruits other adapter proteins, such as TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). This pathway activates NF-κB and type I IFN, which is called TRIF-dependent pathway. Both TRIF-dependent and MyD88-dependent signaling contributes to host defense against pulmonary *Klebsiella* infection (Cai et al., 2009). The TLR-mediated innate immune responses control bacterial growth at the infection site, thus minimizing bacterial transmission. Currently, 12 TLRs from mice and 10 TLRs from human have been identified (Baral et al., 2014).

Among TLRs, TLR2 and TLR4 play important roles in *K. pneumoniae* infection. TLR2 transmits signal mainly by forming heterodimers with TLR1 or TLR6 to resist external pathogens. Meanwhile, TLR4 could induce host defense against gram-negative bacterial pulmonary infection by sensing bacterial LPS (Baral et al., 2014). Compared with wild type (WT) mice, Jeon et al. found that the survival time of TLR4 knock-out (KO) and TLR2/4 double KO (DKO) mice infected with 5×10^3 CFU *K. pneumoniae* was significantly shortened. The mRNA levels of TNF-α, MCP-1 and inducible nitric oxide synthase (iNOS) in TLR2/4 DKO mice were substantially lower than those in the WT group, indicating that TLR2 and TLR4 play a synergistic role in innate immune response during *K. pneumoniae* infection (Jeon et al., 2017).

Meanwhile, by analyzing the gene expression profiles in the lung of C57BL/6 mice (resistant to bacterial transmission), 129/SVJ mice (susceptible), C3H/HeJ mice (susceptible and TLR4 signal deficient) and their respective control strains C3H/HeN mice (moderately resistant), it was found that the most significant number of TLR4-dependent induced genes were expressed in C57BL/6 and C3H/HeN mice after infection with *K. pneumoniae*. These genes include cytokines and chemokine genes needed for neutrophil activation or recruitment, growth factor receptors, MyD88 and adhesion molecules. The results indicated that in the early stage of infection, the TLR4 signal controlled the expression of most genes in lung to cope with gram-negative bacterial infection (Schurr et al., 2005).

At the same time a variety of TLRs expressed on DCs, such as TLR9, can trigger the cascade signal response of proinflammatory cytokines, leading to the production of TNF-α, IL-12 and other proinflammatory cytokines in large quantities to resist the invasion of *K. pneumoniae* (Bhan et al., 2007; Von Wulffen et al., 2007). Interestingly, though the initiation of most TLRs depends on MyD88, Adam et al. discovered that the inflammatory response induced by *K. pneumoniae* does not depend on MyD88 in lung epithelial cells and platelets (De Stoppelaar et al., 2015; Anas et al., 2017).

Collectively, TLR2 and TLR4 signaling could improve the levels of TNF-α, MCP-1, iNOS and other proinflammatory cytokines to indirectly eliminate the bacteria during the early stage of *K. pneumoniae* infection (Figure 6).

2.3.2 NLRs

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are classical pattern recognition receptors highly expressed in first-reactive cells such as neutrophils, macrophages and DC cells. They can regulate various signal pathways, including MyD88 and TLRs containing adaptor molecular 1-dependent pathways. Besides these regulatory effects, some NLRs also are assembled into polymer protein complexes called inflammasome (Ravi Kumar et al., 2018; Ghimire et al., 2020).

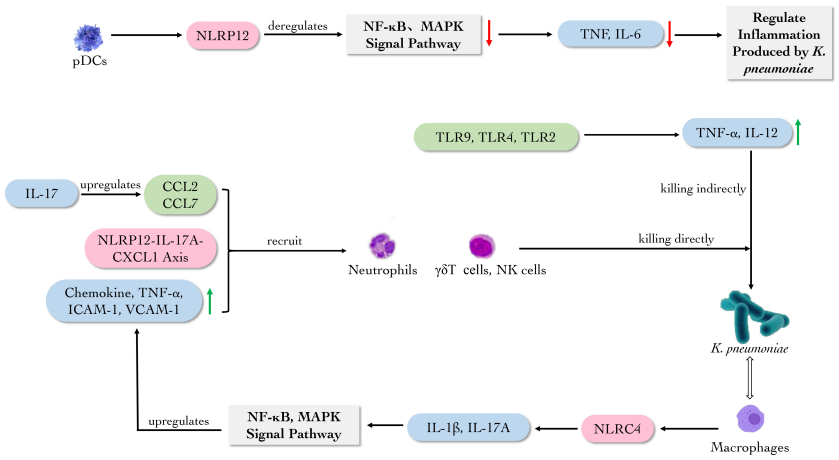

FIGURE 6
The main role of immune molecules against *K. pneumoniae*. pDCs secrete NLRP12 participating NF-κB and MAPK signal pathways to regulate the inflammation caused by *K. pneumoniae*. While TLR2, TLR4 and TLR9 promote the production of TNF-α and IL-12. Macrophage-derived NLRC4 induces the production of IL-1 and IL-17A in lung, activating NF-κB and MAPK signal pathways to upregulate the production of chemokine, TNF-α, ICAM-1 and VCAM-1, which can recruit neutrophils. Meanwhile, NLRP12-IL-17A-CXCL1 axis, CCL2 and CCL7 recruit neutrophils, γδT cells and NK cells to eliminate *K. pneumoniae*.

Nucleotide-binding oligomerization domain, leucine- rich repeat and pyrin domain-containing (NLRP) is one category of NLRs. Studies have shown that NLRP6 and NLRP12 can act as a negative regulator of the NF-κB and mitogen-activated protein kinase (MAPK) signal pathways to attenuate intestinal inflammation during the infection of *K. pneumoniae* (Ghimire et al., 2020). NLRP6 and the adaptor protein, apoptosis-associated speck-like protein (ASC) -mediated inflammasome activation are thought to shape the composition of the commensal gut microbiota, controlling the gut microbiota and the immune response to systemic and intestinal infections (Elinav et al., 2011). However, the finding was challenged by later research (Mamantopoulos et al., 2017). It is meaningful that during the infection of *K. pneumoniae*, *NLRP6* gene-deficient mice show the low levels of neutrophil recruitment, CXC chemokine and granulocyte factor (Cai et al., 2021).

Furthermore, bone marrow-derived DCs (BMDCs) lacking NLRP12 could induce the production of TNF-α and IL-6 (Allen et al., 2013; Tuladhar and Kanneganti, 2020). It was found that intratracheal injection of IL-17A^{+} CD4 T cells or CXCL1^{+} macrophages could prolong the survival of *Nlrp12*$^{-/-}$ mice and recruit neutrophils to eliminate *K. pneumoniae*. It was revealed that the NLRP12-IL-17A-CXCL1 axis *in vivo* could play a vital role in removing extracellular bacteria by recruiting neutrophils (Cai et al., 2016). And another study found that IL-17 can upregulate the expression of CCR2 ligands CCL2 and CCL7, promoting the recruitment of neutrophils and enhancing the anti-bacterial activity in C57BL/6 mice (Xiong et al., 2015).

NLRC4, another inflammasome, is also essential for the clearance of *K. pneumoniae* and neutrophil-mediated lung inflammation (Xiong et al., 2015; Xiong et al., 2016). Macrophage-derived NLRC4 can induce the production of IL-1 and IL-17A from NK cells and γδT cells in lung, activating NF-κB and MAPK signal pathways to regulate the production of neutrophil chemokine, TNF-α, the expression of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in lung homogenates, which could recruit neutrophils and hinder the colonization of *K. pneumoniae* (Cai et al., 2012).

These evidences revealed that NLRP6 and NLRP12 could act as negative regulators of NF-κB and MAPK signal pathways to deregulate the inflammation caused by *K. pneumoniae*. Meanwhile, NLRs prevent the colonization of *K. pneumoniae* by recruiting neutrophils (Figure 6).

3 Adaptive Immune Response to *K. pneumoniae*

It is known that innate immunity and adaptive immunity are equally crucial in resisting pathogen invasion, the adaptive immune response is slower and more specific. Another prominent feature for adaptive immunity is the production of immunologic memory which is activated rapidly during reinfection, resulting in protective response (Bonilla and Oettgen, 2010; Opoku-Temeng et al., 2022). Adaptive

immunity includes T cell-mediated cellular response and B cell-mediated humoral response (Bonilla and Oettgen, 2010).

3.1 T Cell-mediated Immune Response

As *K. pneumoniae* is a typical extracellular bacterium, the effect of cellular immunity on *K. pneumoniae* is relatively limited (Opoku-Temeng et al., 2022). Lee et al. found that T-helper (T_H) lymphocytes played a prominent role in the defense of *K. pneumoniae* through secreting cytokines such as IL-17 and IFN-γ (Lee et al., 2015). It was found that resident memory T cells (T_{RM}) also played an anti-*Klebsiella* role through lung mucosal immunity. Vesely et al. discovered that the lung long-lived CD4 T_{RM} cells derived from T_H17 cells could rapidly release IFN-γ or release IL-4 later to better control infections of CR-hvKp or contribute to the pathology associated with the hypersensitivity (Amezcua Vesely et al., 2019). Meanwhile, the newly studied vaccine can drive lung T_{RM} cells to provide immunity against *Klebsiella via* fibroblast IL-17R signaling (Iwanaga et al., 2021).

3.2 Humoral Immune Response

It is different from cellular immunity, humoral immunity exerts an enormous function in host defense against *K. pneumoniae* infection. Banerjee et al. had isolated cross-reactive anti-CPS antibodies poly-immunoglobulin G (poly-IgG) from the plasma of patients infected with *carbapenem-resistant K. pneumoniae* (*CR-Kp*) strain sequence type 258 (ST258), which indicated poly-IgG could promote the phagocytic function to different serotype *CR-Kp* strains. Still, the protective efficacy was reversed when CPS-specific antibodies (Abs) were depleted (Banerjee et al., 2021). Diago et al. isolated K1-CPS-specific IgG Abs and found that in mouse liver, monoclonal antibodies (mAbs) 4C5 and 19A10 reduced the transmission of *CR-hvKp* with *in vivo* microscope (Diago-Navarro et al., 2017). Subsequently, two anti-CPS IgG mAbs 17H12 and 8F12 were obtained by Diago et al. from the mouse infection model. The two mAbs can promote extracellular processes to kill *CR-Kp*, including the enhancement of biofilm inhibition, the deposition of complement and NETs, reducing bacterial transmission to organs (Diago-Navarro et al., 2018). Motley and his fellows also isolated two anti-CPS mAbs, murine IgG3 (mIgG3) and murine IgG1 (mIgG1), revealing that mIgG3 had better complement-mediated serum bactericidal activity than mIgG1, and promoting neutrophil-mediated killing at a concentration below mIgG1 through enzyme-linked immunosorbent assay and flow cytometry. In contrast, mIgG1 had better activity in enhancing the phagocytosis of macrophages (Motley et al., 2020).

Kobayashi et al. tested CPS-specific rabbit Abs and found that CPS2-specific Abs can promote phagocytosis and the pernicious effect of human neutrophils to ST258 (Kobayashi et al., 2018). Observing the interaction of African green monkey complement and antibodies with hyper mucoviscosity (HMV) or non-HMV *K. pneumoniae*, the results demonstrate that interaction of cellular and humoral immune elements plays a role in the *in vitro* killing of *K. pneumoniae*, particularly HMV isolates. However, low levels of IgG2 titers may lead to a diminished sterilization effect (Soto et al., 2016). The increased prevalence of *K. pneumoniae* LPS O2 serotype strains in all significant drug resistance groups correlates with a paucity of anti-O2 antibodies in human B cell repertoires. It has been identified that human mAbs to O antigen, including a rare anti-O2 specific antibody, is highly protective in mouse infection models, even against heavily encapsulated strains (Pennini et al., 2017).

Furthermore, an isolated antibody B39 targeting conserved epitope binds to *K. pneumoniae* LPS O1 and O1/O2 antigens could promote the conditioning phagocytosis of human macrophages and the clearance of macrophage-associated bacteria when evaluating them by high-volume image (Berry et al., 2022). At the same time, Lee. et al. found that *K. pneumoniae* EV vaccination conferred protection against *K. pneumoniae* infection by inducing EV-reactive Abs and IFN-γ^+ T-cell responses. It indicates that *K. pneumoniae* EV vaccination depends on both humoral and cellular immunity (Lee et al., 2015). Similarly, stable artificial bacterial bionic vesicles (BBVs) were successfully induced and efficiently taken up by DCs to stimulate DCs' maturation. Therefore, as a *K. pneumoniae* vaccine, BBVs could induce bacterial-specific humoral and cellular immune responses to reduce lung inflammation and its bacterial load (Li et al., 2021).

Collectively, these studies strongly suggest the critical role of humoral immunity, which is underestimated in clinical applications in terms of antibiotic therapy. Due to the protective potential of anti-CPS, *K. pneumoniae* CPS is a popular target for immune prevention and/or treatment, and the O antigen of LPS and EVs are also viable targets (Figure 7). In addition, antibody-based clinical treatment strategies may have the capacity to address antibiotic-refractory bacteria in the future.

4 Discussion and conclusion

Over the past decade, *K. pneumoniae* has emerged as a significant clinical and public health threat due to the increasing prevalence of healthcare-associated infections caused by multidrug-resistant strains that produce extended-spectrum β-lactamases and/or carbapenemases. Here, the immune mechanisms associated with the resistance to *K. pneumoniae*,

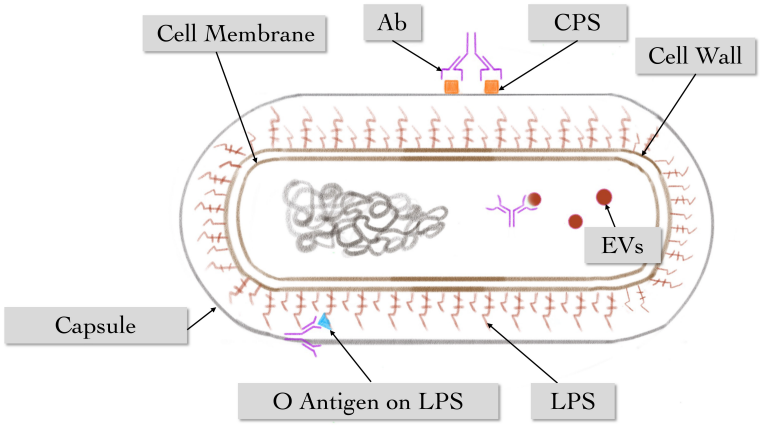

FIGURE 7
The main role of humoral immune response against *K. pneumoniae*. Multiple antibodies can act on different antigenic epitopes of *K. pneumoniae*, including vesicles, O-antigen on LPS, and antigen on CPS.

including innate immunity, cellular immunity and humoral immunity have been reviewed (Table 1). The mechanical barriers play a preliminary role in anti-colonization, while humoral immunity antibodies can recognize different antigenic epitopes of *K. pneumoniae* to promote the elimination of the pathogen. At the same time, multiple immune cells are activated and able to secrete relevant immune factors to aggregate and destroy the infected cells when the organism is infected with *Klebsiella*, demonstrating the diversity and effectiveness of immunity. On the other hand, the immune evasion and pathogenicity of *K. pneumoniae* also reflect the vital role of immunity against *K. pneumoniae*. The current treatments of *hvKp* infection are controlling infection source and aggressive antibiotic therapy. However, due to the diminished effectiveness of conventional clinical treatment against antibiotic-resistant and highly pathogenic strains (Choby et al., 2020b), improving individuals' immunity against *K. pneumoniae* infection may become a new direction for clinical therapy in the future. For the aim above, some measures, for example, regulating the gut microbiota, increasing SCFA, supplying specific antibody and so on, should be taken.

It is put forward that the clearance and weakening of *K. pneumoniae* colonization by host mechanical barrier can be exploited by increasing the expression of *mucin5b* gene in respiratory mucus, maintaining normal renal urinary function and avoiding dysbiosis of the intestinal flora (Roy et al., 2014). It is confirmed that an essential aspect of *Klebsiella* infection biology is the thwarting of TLR-dependent activation of host defense responses controlled by NF-κB and MAPKs (Jeyaseelan et al., 2006; Cai et al., 2009; Regueiro et al., 2011; Frank et al., 2013; Ravi Kumar et al., 2018). We hypothesize that *Klebsiella* may target the cells responsible for producing immune cytokines and blockade the signaling pathways required for the production of the cytokines. Immune cells including DCs, macrophages and neutrophils are all capable of secreting cytokines and regulating inflammatory signal pathways. Therefore, enhancing inflammatory signaling pathways such as NF-κB and MAPKs by selectively inhibiting or enhancing the secretory function of immune cells is a direction of the future research (Deets and Vance, 2021). Further, drug development and clinical trials on antibodies that can effectively bind the antigenic epitopes of *K. pneumoniae* is also a powerful anti-infection tool (Motley et al., 2020).

When it comes to the host defense, anti-*K. pneumoniae* vaccines have to be mentioned. Anti-*K. pneumoniae* vaccine based on surface-exposed bacterial antigens is a promising alternative. CPS of *K. pneumoniae* has long been regarded as a vital virulence factor that promotes resistance to phagocytosis and serum bactericidal activity. Thus, CPS has been targeted previously for the development of therapeutics and vaccines (Opoku-Temeng et al., 2019). However, the high variability in capsular serotypes limits vaccine coverage, and glycoconjugate vaccines are manufactured using intricate chemical methodologies to covalently attach purified polysaccharides to carrier proteins, which is widely considered technically challenging (Feldman et al., 2019). Joy et al. developed a preclinical model of pneumonia in mice and found that non-capsular antigens may also elicit protective immunity (Twentyman et al., 2020). As a vital virulence factor, Outer Membrane Vesicles (OMV) could induce specific adaptive immune responses while displaying intrinsic adjuvant properties. However, the side effects of the OMV vaccine, the complexity of OMV composition, and the multiple antigens in variable concentrations hinder the mass production of OMV vaccines (Martora et al., 2019). In addition, the protective efficacy of ribosome-based vaccine formulations is

TABLE 1 Studies on the mechanism of host defense against *K. pneumoniae (Kp)* infection.

			Mechanism of host defense against *Kp*		Reference
			Mechanism	**Influence**	
Innate immune response	Barriers	respiratory barriers	respiratory epithelial cells	Internalize and eliminate	(Xu and Xu, 2005)
			IL-17A	Activate the defense of upper respiratory tract	(Sequeira et al., 2020)
		urinary barriers	Mechanical force of urine flow	Prevent the colonization of *Kp* on urethra	(Maunders et al., 2022)
			Urine pH↓	Affect the colonization and proliferation of bacteria	(Yang et al., 2014; Wasfi et al., 2020)
			Mechanical force of bladder contraction	Conducive to *Kp* removal	(Burnett et al., 2021)
		digestive barriers	IL-36, macrophages	Prevent the colonization and transmission of bacteria	(Sequeira et al., 2020)
			SCFA	Prevent the growth and colonization of bacteria	(Vornhagen et al., 2021)
			Combined SCFA with GPR43	Reduce the number of *Kp* and control the inflammatory response	(Galvão et al., 2018)
			Mucosal barrier	Inhibit inflammation	(Shi et al., 2017)
			Intestinal flora	Reduce liver injury	(Zheng et al., 2021)
	Innate immune cells	dendritic cells	pDCs, $CD103^+$DC, MoDCs↑	Stimulate $CD4^+$ and $CD8^+$ naïve T cells	(Hackstein et al., 2013)
		macrophages	Release TNF-α and IL-6	Promote inflammation	(Lee et al., 2022)
			IL-36γ	Promote innate mucosal immunity in lung	(Kovach et al., 2016; Kovach et al., 2017)
			CCR2	Increase macrophages and TNF	(Chen et al., 2013)
			HIF-1α	Auxiliary the production of TNF	(Otto et al., 2021)
		neutrophils	NETs	Kill *CR-hvKP* directly	(Jin et al., 2020)
			IL-1β, IL-6, IL-17, IFN-γ, CXCL2 and TNF-α↑	Enhance ability to swallow and kill *Kp*	(Mancuso et al., 2022)
			CXCL5 and LTB_4	Restore the activity of neutrophils	(Batra et al., 2012; Chen et al., 2016)
		Innate lymphoid cells	STAT6 signal	IL-17A↓	(Bloodworth et al., 2016)
			NK cells	Generate IFN-γ and clear *Kp*	(Ivin et al., 2017)
	Immune molecules	TRIF	recruited into the TLR complex to activates NF-κB and type I IFN	Recruit neutrophils, activate MAPKs	(Cai et al., 2009);
		MyD88	recruited into the TLR complex to activate MAPK and NF-κB signal pathways	control bacterial growth at the infection site, thus minimizing bacterial transmission	(Jeyaseelan et al., 2006; Cai et al., 2009)
		TLRs	TLR4 senses bacterial LPS	Against gram-negative bacteria	(Baral et al., 2014)
			TLR2 and TLR4 improve the levels of TNF-α, MCP-1 and iNOS	indirectly eliminate *Kp*	(Schurr et al., 2005; Jeon et al., 2017)
			TLR9 triggers the proinflammatory cascade signal	Stimulate the production of TNF-α and IL-12	(Bhan et al., 2007; Von Wulffen et al., 2007)
		NLRs	NLRP6 and NLRP12 can act as a negative regulator of the NF-κB and MAPK pathways	attenuate the intestinal inflammation	(Ghimire et al., 2020)
			NLRP12-IL-17A-CXCL1 axis	Recruit neutrophils	(Cai et al., 2016)
			IL-17 upregulate the expression of CCL2 and CCL7	Recruit neutrophils	(Xiong et al., 2015; Xiong et al., 2016)
			NLRP12 reduce the production of TNF-α and IL-6	Attenuate the inflammation caused by *Kp*	(Allen et al., 2013; Tuladhar and Kanneganti, 2020)

(Continued)

TABLE 1 Continued

			Mechanism of host defense against *Kp*		Reference
			Mechanism	**Influence**	
			NLRC4 induce the production of IL-1β and IL-17A, activating MAPK and NF-κB signaling pathways	Recruit neutrophils	(Cai et al., 2012)
Adaptive immune response	Cell-mediated immune response		Th lymphocytes secrete IL-17 and IFN-γ	plays a prominent role in the defense of *Kp*	(Lee et al., 2015)
			CD4 T_{RM} cells	Release IFN-γ or IL-4	(Amezcua Vesely et al., 2019)
			lung T_{RM} cells *via* fibroblast IL-17R signaling	provide immunity against *Kp*	(Iwanaga et al., 2021)
	Humoral immune response	MAbs	CPS antigen	Strengthen biofilm inhibition, complement deposition and NETs	(Soto et al., 2016; Diago-Navarro et al., 2017; Diago-Navarro et al., 2018; Kobayashi et al., 2018; Motley et al., 2020; Banerjee et al., 2021)
			O antigen of LPS	Promote the regulation and phagocytosis of macrophages	(Pennini et al., 2017; Berry et al., 2022)
			Vesicle antigen	Elicit EV reactive antibodies and produce IFN-γ T cell response	(Lee et al., 2015)

controversial since many include surface protein contaminations, which may be significant contributors to the protective responses (Pregliasco et al., 2009). Remarkably recombinant outer membrane proteins (OMPs) are promising vaccine candidates against *K. pneumoniae*, alone or combined with other antigens. When administered as a carrier in combination with respiratory syncytial virus subgroup A (RSV-A), OMP could induce IgA, IgG1, and IgG2a production, which provided the protection against *K. pneumoniae* infection (Libon et al., 2002). Moreover, the O antigen on LPS is a highly immunogenic molecule and an essential virulence factor for *K. pneumoniae*. However, the high toxicity of LPS is the main limiting factor related to this type of vaccine. Thus, a delicate balance between immunogenicity and toxicity must be considered (Clarke et al., 2020). Although there are no vaccines available against *K. pneumoniae* infection in clinic, it is a great pleasure that K. pneumoniae vaccines are feasible and a promising strategy to prevent infections and reduce the antimicrobial resistance burden worldwide.

In summary, there is still a considerable gap in our understanding of the pathogenesis of *K. pneumoniae*. However, in-depth knowledge of the host-immune mechanism will facilitate understanding its pathogenesis and provide new ideas for future diagnosis and treatment of *K. pneumoniae* infection in the era of antibiotics.

Author contributions

ZL reviewed all the literature, collected data, and drafted the manuscript. YW, YL, JZ and LY drafted partly and made important suggestions for the amendments. YS conceived the review and drafted partly. XL drafted partly and reviewed the manuscript. YZ and XY contributed substantially by giving insightful comments and suggestions during the creation of the manuscript. YS, XL and XY were responsible for funding. 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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References

Al Bander, Z., Nitert, M. D., Mousa, A., and Naderpoor, N. (2020). The gut microbiota and inflammation: An overview. *Int. J. Environ. Res. Public Health* 17, 7618. doi: 10.3390/ijerph17207618

Allen, I. C., Mcelvania-Tekippe, E., Wilson, J. E., Lich, J. D., Arthur, J. C., Sullivan, J. T., et al (2013). Characterization of NLRP12 during the *in vivo* host immune response to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. *PloS One* 8, e60842. doi: 10.1371/journal.pone.0060842

Amezcua Vesely, M. C., Pallis, P., Bielecki, P., Low, J. S., Zhao, J., Harman, C. C. D., et al (2019). Effector T_H17 cells give rise to long-lived T cells that are essential for an immediate response against bacterial infection. *Cell* 178, 176-1188. doi: 10.1016/j.cell.2019.07.032

Anas, A. A., Claushuis, T., Mohan, R. A., Christoffels, V. M., Aidinis, V., Florquin, S., et al (2017). Epithelial myeloid-differentiation factor 88 is dispensable during *Klebsiella pneumonia*. *Am. J. Respir. Cell Mol. Biol.* 56, 648–656. doi: 10.1165/rcmb.2016-0190OC

Banerjee, K., Motley, M. P., Diago-Navarro, E., and Fries, B. C. (2021). Serum antibody responses against carbapenem-resistant *Klebsiella pneumoniae* in infected patients. *mSphere* 6, e01335-20. doi: 10.1128/mSphere.01335-20

Baral, P., Batra, S., Zemans, R. L., Downey, G. P., and Jeyaseelan, S. (2014). Divergent functions of toll-like receptors during bacterial lung infections. *Am. J. Respir. Crit. Care Med.* 190, 722–732. doi: 10.1164/rccm.201406-1101PP

Batra, S., Cai, S., Balamayooran, G., and Jeyaseelan, S. (2012). Intrapulmonary administration of leukotriene b (4) augments neutrophil accumulation and responses in the lung to *Klebsiella* infection in CXCL1 knockout mice. *J. Immunol.* 188, 3458–3468. doi: 10.4049/jimmunol.1101985

Bengoechea, J. A., and Sa Pessoa, J. (2019). *Klebsiella pneumoniae* infection biology: living to counteract host defences. *FEMS Microbiol. Rev.* 43, 123–144. doi: 10.1093/femsre/fuy043

Berry, S. K., Rust, S., Caceres, C., Irving, L., Bartholdson Scott, J., Tabor, D. E., et al (2022). Phenotypic whole-cell screening identifies a protective carbohydrate epitope on *Klebsiella pneumoniae*. *MAbs* 14, 2006123. doi: 10.1080/19420862.2021.2006123

Bhan, U., Lukacs, N. W., Osterholzer, J. J., Newstead, M. W., Zeng, X., Moore, T. A., et al (2007). TLR9 is required for protective innate immunity in gram-negative bacterial pneumonia: role of dendritic cells. *J. Immunol.* 179, 3937–3946. doi: 10.4049/jimmunol.179.6.3937

Bloodworth, M. H., Newcomb, D. C., Dulek, D. E., Stier, M. T., Cephus, J. Y., Zhang, J., et al (2016). STAT6 signaling attenuates interleukin-17-producing γδ T cells during acute *Klebsiella pneumonia*e infection. *Infect. Immun.* 84, 1548–1555. doi: 10.1128/IAI.00646-15

Bonilla, F. A., and Oettgen, H. C. (2010). Adaptive immunity. *J. Allergy Clin. Immunol.* 125, S33–S40. doi: 10.1016/j.jaci.2009.09.017

Burnett, L. A., Hochstedler, B. R., Weldon, K., Wolfe, A. J., and Brubaker, L. (2021). Recurrent urinary tract infection: Association of clinical profiles with urobiome composition in women. *Neurourol. Urodyn.* 40, 1479–1489. doi: 10.1002/nau.24707

Cai, S., Batra, S., Del Piero, F., and Jeyaseelan, S. (2016). NLRP12 modulates host defense through IL-17A-CXCL1 axis. *Mucosal. Immunol.* 9, 503–514. doi: 10.1038/mi.2015.80

Cai, S., Batra, S., Shen, L., Wakamatsu, N., and Jeyaseelan, S. (2009). Both TRIF- and MyD88-dependent signaling contribute to host defense against pulmonary *Klebsiella* infection. *J. Immunol.* 183, 6629–6638. doi: 10.4049/jimmunol.0901033

Cai, S., Batra, S., Wakamatsu, N., Pacher, P., and Jeyaseelan, S. (2012). NLRC4 inflammasome-mediated production of IL-1β modulates mucosal immunity in the lung against gram-negative bacterial infection. *J. Immunol.* 188, 5623–5635. doi: 10.4049/jimmunol.1200195

Cai, S., Paudel, S., Jin, L., Ghimire, L., Taylor, C. M., Wakamatsu, N., et al. (2021). NLRP6 modulates neutrophil homeostasis in bacterial pneumonia-derived sepsis. *Mucosal. Immunol.* 14, 574–584. doi: 10.1038/s41385-020-00357-4

Chen, K., Eddens, T., Trevejo-Nunez, G., Way, E. E., Elsegeiny, W., Ricks, D. M., et al. (2016). IL-17 receptor signaling in the lung epithelium is required for mucosal chemokine gradients and pulmonary host defense against *K. pneumoniae*. *Cell Host Microbe* 20, 596–605. doi: 10.1016/j.chom.2016.10.003

Chen, L., Zhang, Z., Barletta, K. E., Burdick, M. D., and Mehrad, B. (2013). Heterogeneity of lung mononuclear phagocytes during pneumonia: contribution of chemokine receptors. *Am. J. Physiol. Lung Cell Mol. Physiol.* 305, L702–L711. doi: 10.1152/ajplung.00194.2013

Chew, K. L., Lin, R. T. P., and Teo, J. W. P. (2017). In Singapore: Hypervirulent infections and the carbapenemase threat. *Front. Cell Infect. Microbiol.* 7. doi: 10.3389/fcimb.2017.00515

Choby, J. E., Howard-Anderson, J., and Weiss, D. S. (2020). Hypervirulent klebsiella pneumoniae-clinical and molecular perspectives. *J. Intern. Med.* 287, 283–300. doi: 10.1111/joim.13007

Clarke, B. R., Ovchinnikova, O. G., Sweeney, R. P., Kamski-Hennekam, E. R., Gitalis, R., Mallette, E., et al. (2020). A bifunctional O-antigen polymerase structure reveals a new glycosyltransferase family. *Nat. Chem. Biol.* 16, 450–457. doi: 10.1038/s41589-020-0494-0

Deets, K. A., and Vance, R. E. (2021). Inflammasomes and adaptive immune responses. *Nat. Immunol.* 22, 412–422. doi: 10.1038/s41590-021-00869-6

De Stoppelaar, S. F., Claushuis, T., Jansen, M. P. B., Hou, B., Roelofs, J. J. T. H., Van't Veer, C., et al. (2015). The role of platelet MyD88 in host response during gram-negative sepsis. *J. Thromb. Haemost.* 13, 1709–1720. doi: 10.1111/jth.13048

Diago-Navarro, E., Calatayud-Baselga, I., Sun, D., Khairallah, C., Mann, I., Ulacia-Hernando, A., et al. (2017). Antibody-based immunotherapy to treat and prevent infection with *hypervirulent klebsiella pneumoniae*. *Clin. Vaccine Immunol.* 24, e00456-16. doi: 10.1128/cvi.00456-16

Diago-Navarro, E., Motley, M. P., Ruiz-Peréz, G., Yu, W., Austin, J., Seco, B. M. S., et al. (2018). Novel, broadly reactive anticapsular antibodies against *carbapenem-resistant klebsiella pneumoniae* protect from infection. *mBio* 9, e00091-18. doi: 10.1128/mBio.00091-18

Diekema, D. J., Hsueh, P.-R., Mendes, R. E., Pfaller, M. A., Rolston, K. V., Sader, H. S., et al. (2019). The microbiology of bloodstream infection: 20-year trends from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* 63, e00355-19. doi: 10.1128/AAC.00355-19

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., et al. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145, 745–757. doi: 10.1016/j.cell.2011.04.022

Feldman, M. F., Mayer Bridwell, A. E., Scott, N. E., Vinogradov, E., Mckee, S. R., Chavez, S. M., et al. (2019). A promising bioconjugate vaccine against *hypervirulent klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. U. S. A.* 116, 18655–18663. doi: 10.1073/pnas.1907833116

Frank, C. G., Reguerio, V., Rother, M., Moranta, D., Maeurer, A. P., Garmendia, J., et al. (2013). *Klebsiella pneumoniae* targets an EGF receptor-dependent pathway to subvert inflammation. *Cell Microbiol.* 15, 1212–1233. doi: 10.1111/cmi.12110

Galvão, I., Tavares, L. P., Corrêa, R. O., Fachi, J. L., Rocha, V. M., Rungue, M., et al. (2018). The metabolic sensor GPR43 receptor plays a role in the control of *Klebsiella pneumoniae* infection in the lung. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.00142

Ghimire, L., Paudel, S., Jin, L., and Jeyaseelan, S. (2020). The NLRP6 inflammasome in health and disease. *Mucosal. Immunol.* 13, 388–398. doi: 10.1038/s41385-020-0256-z

Hackstein, H., Kranz, S., Lippitsch, A., Wachtendorf, A., Kershaw, O., Gruber, A. D., et al. (2013). Modulation of respiratory dendritic cells during *Klebsiella pneumonia* infection. *Respir. Res.* 14, 91. doi: 10.1186/1465-9921-14-91

Hackstein, H., Wachtendorf, A., Kranz, S., Lohmeyer, J., Bein, G., and Baal, N. (2012). Heterogeneity of respiratory dendritic cell subsets and lymphocyte populations in inbred mouse strains. *Respir. Res.* 13, 94. doi: 10.1186/1465-9921-13-94

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., et al. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proc. Natl. Acad. Sci. U. S. A.* 112, e3574–e3581. doi: 10.1073/pnas.1501049112

Ivin, M., Dumigan, A., De Vasconcelos, F. N., Ebner, F., Borroni, M., Kavirayani, A., et al. (2017). Natural killer cell-intrinsic type I IFN signaling controls *Klebsiella pneumoniae* growth during lung infection. *PloS Pathog.* 13, e1006696. doi: 10.1371/journal.ppat.1006696

Iwanaga, N., Chen, K., Yang, H., Lu, S., Hoffmann, J. P., Wanek, A., et al. (2021). Vaccine-driven lung T_{RM} cells provide immunity against *Klebsiella via* fibroblast IL-17R signaling. *Sci. Immunol.* 6, eabf1198. doi: 10.1126/sciimmunol.abf1198

Jagnow, J., and Clegg, S. (2003). *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiol. (Reading)* 149, 2397–2405. doi: 10.1099/mic.0.26434-0

Jeon, H.-Y., Park, J.-H., Park, J.-I., Kim, J.-Y., Seo, S.-M., Ham, S.-H., et al. (2017). Cooperative interactions between toll-like receptor 2 and toll-like receptor 4 in murine *Klebsiella pneumoniae* infections. *J. Microbiol. Biotechnol.* 27, 1529–1538. doi: 10.4014/jmb.1704.04039

Jeyaseelan, S., Young, S. K., Yamamoto, M., Arndt, P. G., Akira, S., Kolls, J. K., et al. (2006). Toll/IL-1R domain-containing adaptor protein (TIRAP) is a critical mediator of antibacterial defense in the lung against *Klebsiella pneumoniae* but not *Pseudomonas aeruginosa*. J. *Immunol* 177, 538–547. doi: 10.4049/jimmunol.177.1.538

Jin, L., Liu, Y., Jing, C., Wang, R., Wang, Q., Wang, H., et al. (2020). Neutrophil extracellular traps (NETs)-mediated killing of *carbapenem-resistant hypervirulent* (*CR-hvKP*) are impaired in patients with diabetes mellitus. *Virulence* 11, 1122–1130. doi: 10.1080/21505594.2020.1809325

Kobayashi, S. D., Porter, A. R., Freedman, B., Pandey, R., Chen, L., Kreiswirth, B. N., et al. (2018). Antibody-mediated killing of carbapenem-resistant ST258 *Klebsiella pneumoniae* by human neutrophils. *mBio* 9, e00297-18. doi: 10.1128/mBio.00297-18

Kovach, M. A., Singer, B., Martinez-Colon, G., Newstead, M. W., Zeng, X., Mancuso, P., et al. (2017). IL-36γ is a crucial proximal component of protective type-1-mediated lung mucosal immunity in gram-positive and -negative bacterial pneumonia. *Mucosal. Immunol.* 10, 1320–1334. doi: 10.1038/mi.2016.130

Kovach, M. A., Singer, B. H., Newstead, M. W., Zeng, X., Moore, T. A., White, E. S., et al. (2016). IL-36γ is secreted in microparticles and exosomes by lung macrophages in response to bacteria and bacterial components. *J. Leukoc. Biol.* 100, 413–421. doi: 10.1189/jlb.4A0315-087R

Lee, W. H., Choi, H. I., Hong, S. W., Kim, K. S., Gho, Y. S., Jeon, S. G., et al. (2015). Vaccination with *Klebsiella pneumoniae*-derived extracellular vesicles protects against bacteria-induced lethality *via* both humoral and cellular immunity. *Exp. Mol. Med.* 47, e183. doi: 10.1038/emm.2015.59

Lee, I. M., Huang, T.-Y., Yang, F.-L., Johansson, V., Hsu, C.-R., Hsieh, P.-F., et al. (2022). A hexasaccharide from capsular polysaccharide of *carbapenem-resistant klebsiella pneumoniae KN2* is a ligand of toll-like receptor 4. *Carbohydr. Polym.* 278, 118944. doi: 10.1016/j.carbpol.2021.118944

Libon, C., Haeuw, J. F., Crouzet, F., Mugnier, C., Bonnefoy, J. Y., Beck, A., et al. (2002). *Streptococcus pneumoniae* polysaccharides conjugated to the outer membrane protein a from *Klebsiella pneumoniae* elicit protective antibodies. *Vaccine* 20, 2174–2180. doi: 10.1016/s0264-410x(02)00149-4

Li, Y., Han, W. Y., Li, Z. J., and Lei, L. C. (2009). *Klebsiella pneumoniae* MrkD adhesin-mediated immunity to respiratory infection and mapping the antigenic epitope by phage display library. *Microb. Pathog.* 46, 144–149. doi: 10.1016/j.micpath.2008.11.006

Li, W., Hu, Y., Zhang, Q., Hua, L., Yang, Z., Ren, Z., et al. (2021). Development of drug-resistant vaccine *via* novel vesicle production technology. *ACS Appl. Mater. Interfaces* 13, 32703–32715. doi: 10.1021/acsami.1c06701

Li, B., Zhao, Y., Liu, C., Chen, Z., and Zhou, D. (2014). Molecular pathogenesis of *Klebsiella pneumoniae*. *Future Microbiol.* 9, 1071–1081. doi: 10.2217/fmb.14.48

Mackel, J. J., Morffy Smith, C., Wasbotten, R. K., Twentyman, J., and Rosen, D. A. (2022). Classical and γδ T cells are each independently sufficient to establish protection against a classical strain of. *Klebsiella pneumoniae*. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.974175

Mamantopoulos, M., Ronchi, F., Van Hauwermeiren, F., Vieira-Silva, S., Yilmaz, B., Martens, L., et al. (2017). *Nlrp6*- and ASC-dependent inflammasomes do not shape the commensal gut microbiota composition. *Immunity* 47, 339–348.e334. doi: 10.1016/j.immuni.2017.07.011

Mancuso, P., Curtis, J. L., Weitzel, A. M., Griffin, C. A., Bouchard, B., Freeman, C. M., et al. (2022). Diet-induced obesity in mice impairs host defense against pneumonia *in vivo* and glucose transport and bactericidal functions in neutrophils *in vitro*. *Am. J. Physiol. Lung Cell Mol. Physiol.* 322, L116–L128. doi: 10.1152/ajplung.00008.2021

Martora, F., Pinto, F., Folliero, V., Cammarota, M., Dell'annunziata, F., Squillaci, G., et al. (2019). Isolation, characterization and analysis of pro-inflammatory potential of *Klebsiella pneumoniae* outer membrane vesicles. *Microb. Pathog.* 136, 103719. doi: 10.1016/j.micpath.2019.103719

Maunders, E. A., Ganio, K., Hayes, A. J., Neville, S. L., Davies, M. R., Strugnell, R. A., et al. (2022). The role of ZntA in *Klebsiella pneumoniae* zinc homeostasis. *Microbiol. Spectr.* 10, e0177321. doi: 10.1128/spectrum.01773-21

Motley, M. P., Diago-Navarro, E., Banerjee, K., Inzerillo, S., Fries, B. C., and Fries, B. C. (2020). The role of IgG subclass in antibody-mediated protection against *carbapenem-resistant klebsiella pneumoniae*. *mBio* 11, e02059-20. doi: 10.1128/mBio.02059-20

Myers, J. A., and Miller, J. S. (2021). Exploring the NK cell platform for cancer immunotherapy. *Nat. Rev. Clin. Oncol.* 18, 85-100. doi: 10.1038/s41571-020-0426-7

Na, L., Bo, F., Wang, G. X., Shi, X. F., Zheng, S., Ren, L., et al. (2014). Studies affecting the role of *Klebsiella pneumoniae* in adherence to epithelial cells. *Sichuan J. Physiol. Sci.* 36, 3–6.

O'neill, L., Golenbock, D., and Bowie, A. G. (2013). The history of toll-like receptors-redefining innate immunity. *Nat. Rev. Immunol.* 13, 453–460. doi: 10.1038/nri3446

Opoku-Temeng, C., Kobayashi, S. D., and Deleo, F. R. (2019). *Klebsiella pneumoniae* capsule polysaccharide as a target for therapeutics and vaccines. *Comput. Struct. Biotechnol. J.* 17, 1360–1366. doi: 10.1016/j.csbj.2019.09.011

Opoku-Temeng, C., Malachowa, N., Kobayashi, S. D., and Deleo, F. R. (2022). Innate host defense against *Klebsiella pneumoniae* and the outlook for development of immunotherapies. *J. Innate Immun.* 14, 167–181. doi: 10.1159/000518679

Otto, N. A., Pereverzeva, L., Leopold, V., Ramirez-Moral, I., Roelofs, J. J. T. H., Van Heijst, J. W. J., et al. (2021). Hypoxia-inducible factor-1 in macrophages, but not in neutrophils, is important for host defense during-induced pneumosepsis. *Mediators. Inflamm.* 2021, 9958281. doi: 10.1155/2021/9958281

Paczosa, M. K., and Mecsas, J. (2016). *Klebsiella pneumoniae*: Going on the offense with a strong defense. *Microbiol. Mol. Biol. Rev.* 80, 629–661. doi: 10.1128/MMBR.00078-15

Pallmer, K., and Oxenius, A. (2016). Recognition and regulation of T cells by NK cells. *Front. Immunol.* 7. doi: 10.3389/fimmu.2016.00251

Pennini, M. E., De Marco, A., Pelletier, M., Bonnell, J., Cvitkovic, R., Beltramello, M., et al. (2017). Immune stealth-driven O2 serotype prevalence and potential for therapeutic antibodies against multidrug resistant *Klebsiella pneumoniae*. *Nat. Commun.* 8, 1991. doi: 10.1038/s41467-017-02223-7

Pichavant, M., Delneste, Y., Jeannin, P., Fourneau, C., Brichet, A., Gosset, P., et al. (2003). Outer membrane protein a from *Klebsiella pneumoniae* activates bronchial epithelial cells: implication in neutrophil recruitment. *J. Immuno.* 171, 6697–6705. doi: 10.4049/jimmunol.171.12.6697

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira,, Toussaint, W., et al. (2013). Conventional and monocyte-derived CD11b (+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38, 322–335. doi: 10.1016/j.immuni.2012.10.016

Pregliasco, F., Terracciano, L., Marcassa, S., Zava, D., and Anselmi, G. (2009). Rationale for the clinical use of a ribosome-component immune modulator. *Allergy Asthma Proc.* 30 Suppl 1, S5–12. doi: 10.2500/aap.2009.30.3249

Ramirez-Moral, I., Blok, D. C., Bernink, J. H., Garcia-Laorden, M. I., Florquin, S., Boon, L., et al. (2021). Interleukin-33 improves local immunity during gram-negative pneumonia by a combined effect on neutrophils and inflammatory monocytes. *J. Pathol.* 253, 374–383. doi: 10.1002/path.5601

Ravi Kumar, S., Paudel, S., Ghimire, L., Bergeron, S., Cai, S., Zemans, R. L., et al. (2018). Emerging roles of inflammasomes in acute pneumonia. *Am. J. Respir. Crit. Care Med.* 197, 160–171. doi: 10.1164/rccm.201707-1391PP

Regueiro, V., Moranta, D., Frank, C. G., Larrarte, E., Margareto, J., March, C., et al. (2011). *Klebsiella pneumoniae* subverts the activation of inflammatory responses in a NOD1-dependent manner. *Cell Microbiol.* 13, 135–153. doi: 10.1111/j.1462-5822.2010.01526

Rodríguez-Medina, N., Barrios-Camacho, H., Duran-Bedolla, J., and Garza-Ramos, U. (2019). *Klebsiella variicola*: an emerging pathogen in humans. *Emerg. Microbes Infect.* 8, 973–988. doi: 10.1080/22221751.2019.1634981

Roy, M. G., Livraghi-Butrico, A., Fletcher, A. A., Mcelwee, M. M., Evans, S. E., Boerner, R. M., et al. (2014). *Muc5b* is required for airway defence. *Nature* 505, 412–416. doi: 10.1038/nature12807

Russo, T. A., and Marr, C. M. (2019). Hypervirulent klebsiella pneumoniae. *Clin. Microbiol. Rev.* 32, e00001-19. doi: 10.1128/CMR.00001-19

Saharman, Y. R., Karuniawati, A., Sedono, R., Aditianingsih, D., Goessens, W. H. F., Klaassen, C. H. W., et al. (2020). Clinical impact of endemic NDM-producing *Klebsiella pneumoniae* in intensive care units of the national referral hospital in Jakarta, Indonesia. *Antimicrob. Resist. Infect. Control* 9, 61. doi: 10.1186/s13756-020-00716-7

Schurr, J. R., Young, E., Byrne, P., Steele, C., Shellito, J. E., Shellito, J. E., et al. (2005). Central role of toll-like receptor 4 signaling and host defense in experimental pneumonia caused by gram-negative bacteria. *Infect. Immun.* 73, 532–545. doi: 10.1128/IAI.73.1.532-545.2005

Sequeira, R. P., Mcdonald, J., Marchesi, J. R., and Clarke, T. B. (2020). Commensal bacteroidetes protect against *Klebsiella pneumoniae* colonization and transmission through IL-36 signalling. *Nat. Microbiol.* 5, 304–313. doi: 10.1038/s41564-019-0640-1

Shi, N., Li, N., Duan, X., and Niu, H. (2017). Interaction between the gut microbiome and mucosal immune system. *Mil. Med. Res.* 4, 14. doi: 10.1186/s40779-017-0122-9

Soto, E., Marchi, S., Beierschmitt, A., Kearney, M., Francis, S., VanNess, K., et al. (2016). Interaction of non-human primate complement and antibodies with *hypermucoviscous klebsiella pneumoniae*. *Vet. Res.* 47, 40. doi: 10.1186/s13567-016-0325-1

Tuladhar, S., and Kanneganti, T.-D. (2020). NLRP12 in innate immunity and inflammation. *Mol. Aspects Med.* 76, 100887. doi: 10.1016/j.mam.2020.100887

Twentyman, J., Morffy Smith, C., Nims, J. S., Dahler, A. A., Rosen, D. A., and Rosen, D.A. (2020). A murine model demonstrates capsule-independent adaptive immune protection in survivors of *Klebsiella pneumoniae* respiratory tract infection. *Dis. Model. Mech.* 13, dmm043240. doi: 10.1242/dmm.043240

Von Wulffen, W., Steinmueller, M., Herold, S., Marsh, L. M., Bulau, P., Seeger, W., et al. (2007). Lung dendritic cells elicited by fms-like tyrosine 3-kinase ligand amplify the lung inflammatory response to lipopolysaccharide. *Am. J. Respir. Crit. Care Med.* 176, 892–901. doi: 10.1186/1465-9921-14-91

Vornhagen, J., Bassis, C. M., Ramakrishnan, S., Hein, R., Mason, S., Bergman, Y., et al. (2021). A plasmid locus associated with *Klebsiella* clinical infections encodes a microbiome-dependent gut fitness factor. *PloS Pathog.* 17, e1009537. doi: 10.1371/journal.ppat.1009537

Wang, G., Zhao, G., Chao, X., Xie, L., Wang, H., and Wang, H. (2020). The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. *Int. J. Environ. Res. Public Health* 17, 6278. doi: 10.3390/ijerph17176278

Wasfi, R., Abdellatif, G. R., Elshishtawy, H. M., and Ashour, H. M. (2020). First-time characterization of viable but non-culturable proteus mirabilis: Induction and resuscitation. *J. Cell Mol. Med.* 24, 2791–2801. doi: 10.1111/jcmm.15031

Wolff, N. S., Jacobs, M. C., Wiersinga, W. J., and Hugenholtz, F. (2021). Pulmonary and intestinal microbiota dynamics during gram-negative pneumonia-derived sepsis. *Intensive Care Med. Exp.* 9, 35. doi: 10.1186/s40635-021-00398-4

Wu, T., Xu, F., Su, C., Li, H., Lv, N., Liu, Y., et al. (2020). Alterations in the gut microbiome and cecal metabolome during *Klebsiella pneumoniae*-induced pneumosepsis. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.01331

Xiong, H., Carter, R. A., Leiner, I. M., Tang, Y.-W., Chen, L., Kreiswirth, B. N., et al. (2015). Distinct contributions of neutrophils and CCR2^{+} monocytes to pulmonary clearance of different *Klebsiella pneumoniae* strains. *Infect. Immun.* 83, 3418–3427. doi: 10.1128/IAI.00678-15

Xiong, H., Keith, J. W., Samilo, D. W., Carter, R. A., Leiner, I. M., Pamer, E. G., et al. (2016). Innate Lymphocyte/Ly6C(hi) monocyte crosstalk promotes *Klebsiella pneumoniae* clearance. *Cell* 165, 679–689. doi: 10.1016/j.cell.2016.03.017

Xu, X., Weiss, I. D., Zhang, H. H., Singh, S. P., Wynn, T. A., Wilson, M. S., et al. (2014). Conventional NK cells can produce IL-22 and promote host defense in *Klebsiella pneumoniae* pneumonia. *J. Immunol.* 192, 1778–1786. doi: 10.4049/jimmunol.1300039

Xu, Y., and Xu, H. (2005). Small airway epithelial cell injury induced by *Klebsiella pneumoniae* in rats and its relationship with intrapulmonary alpha tumor necrosis factor. *Chin. J. Biochem. Drugs* 1, 12–14. doi: 10.3969/j.issn.1005-1678.2005.01.0012-03

Yang, L., Wang, K., Li., H., Denstedt, J. D., and Cadieux, P. A. (2014). The influence of urinary pH on antibiotic efficacy against bacterial uropathogens. *Urology* 84, 731.e731–737. doi: 10.1016/j.urology.2014.04.048

Zheng, D., Cao, R., Lin, X., and Wang, J. (2014). Effect of airborne PM2.5 exposure on the inflammatory response to *Klebsiella pneumoniae* in rat lungs. *Basic Med. Clin.* 34, 1110–1112. doi: 10.16352/j.issn.1001-6325.2014.08.017

Zheng, Y., Ding, Y., Xu, M., Chen, H., Zhang, H., Liu, Y., et al. (2021). Gut microbiota contributes to host defense against *Klebsiella pneumoniae*-induced liver abscess. *J. Inflamm. Res.* 14, 5215–5225. doi: 10.2147/jir.S334581

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Outbreak of carbapenem-resistant enterobacteria in a thoracic-oncology unit through clonal and plasmid-mediated transmission of the bla_{OXA-48} gene in Southern France

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Background: Carbapenemase-producing *Enterobacteriaceae* (CPE) represent an increasing threat to public health, especially in hospitals.

Objectives: To investigate an outbreak of CPE in a thoracic-oncology unit by using whole genome sequencing (WGS) and to describe the control measures taken to limit the epidemic, including fecal microbiota transplantation (FMT).

Methods: A retrospective study between December 2016 and October 2017 was performed to investigate an outbreak of CPE in a thoracic-oncology unit at the North Hospital in Marseille, France. The isolates were identified, and antimicrobial susceptibility tests were performed. All CPE were sequenced using MiSeq and/or MinIon technologies. Nucleotide variations between plasmids and similarity within the same species were investigated. The origin of this outbreak, its spread, and the decolonization of patients in the ward were also studied.

Results: Four *Citrobacter freundii*, one *Enterobacter cloacae* and four *E. hormaechei* OXA-48 carbapenemase producers were isolated in eight patients hospitalized the same year in a thoracic-oncology ward. The bla_{OXA-48} gene was present in a Tn*1999.2* transposon located in IncL/M plasmids, with single nucleotide variants (SNV) ranging from 0 to 5. All *C. freundii* strains belonged to the same ST22 and had more than 99.6% similarity between them. Two strains of *E. hormaechei* ST1007 were almost identical at 99.98%, while

the others belonged to a different ST (ST98, ST114, ST133). No single source was identified. FMT resulted in decolonization in 4/6 patients.

Conclusions: WGS demonstrated the dissemination of the *bla*$_{OXA-48}$ gene by both clonal (*C. freundii* ST22 and *E. hormaechei* ST1007) and plasmid spread (pOXA-48 IncL/M). The origin of this outbreak appeared to be both external and internal to the ward. This evidence of cross-infection supports the urgent need for the implementation of infection control measures to prevent CPE dissemination.

KEYWORDS

whole genome sequencing, carbapenemase, *bla*$_{OXA-48}$ gene, IncL/M plasmid, fecal microbiota transplantation

Introduction

Carbapenems are broad-spectrum antibiotics that play a major role in the treatment of severe infections caused by Gram-negative bacteria. The global spread of carbapenem-resistant *Enterobacteriaceae* is becoming a public health issue (Jamal et al., 2020). The rise of carbapenem resistance in *Enterobacteriaceae* is mainly due to the acquisition of carbapenem-hydrolyzing enzymes (carbapenemases) (Tilahun et al., 2021). Genes encoding carbapenemases may be incorporated into the bacterial chromosome, but are mostly located on mobile elements, such as plasmids or transposons that are transferable between bacterial strains and species (San Millan, 2018). Hence, clinical outbreaks are usually complex, involving various factors of gene propagation by clones, plasmids, or transposons (Brehony et al., 2019).

Carbapenemase type OXA-48 first appeared in the mid-2000s in Turkey and has since been found in many European countries and worldwide (Hidalgo et al., 2019). In France, it is the most common enzyme among carbapenemase-producing *Enterobacteriaceae* (CPE) (Emeraud et al., 2020). The *bla*$_{OXA-48}$ gene is thought to originate from the chromosome of environmental *Shewanella* strains (Tacão et al., 2018). Its rapid dissemination between species is due to its nesting in a transposon (Tn*1999*) that is carried primarily by IncL/M type plasmids (Shankar et al., 2020).

Controlling outbreaks in hospital wards is necessary to limit the spread of multidrug-resistant bacteria. The colonization of patients by CPE can interfere with proper care. CPE colonization can also have an impact on the initiation of chemotherapy in cancer patients, as it has been associated with a lower survival rate in patients undergoing induction chemotherapy (Ballo et al., 2019). Thus, a strategy to restore a healthy gut microbiota and to eliminate the CPE reservoir such as fecal microbiota transplantation (FMT) has been implemented. FMT is a validated therapy that is highly effective against recurrent *Clostridium difficile* infections (Yoon et al., 2019). In addition, recent studies have shown that FMT was an efficient strategy for sustained CPE eradication. (Decraene et al., 2018; Saïdani et al., 2019). However, there could be other reservoirs of CPE. For instance, the survival of CPE on surfaces also allows the resumption of epidemics months after the initial case (Mateos et al., 2020).

During nosocomial outbreaks, the transmission of pathogens in a ward can be studied by whole genome sequencing of the bacteria of interest, a highly discriminatory typing technique. The genetic relationship between strains, as well as the presence of antibiotic resistance genes and their genetic support, can be determined very accurately and completely (Jamal et al., 2020). However, short-read sequencing (Illumina technology) is not always suitable for locating antibiotic resistance genes on plasmids, whereas long-read sequencing (Nanopore technology) can solve this problem. Therefore, a hybrid assembly of both highly accurate Illumina data and Nanopore data allow the reconstitution of plasmids and their in-depth analysis (George et al., 2017). In our study, these technologies were used to investigate the source and mode of dissemination of the *bla*$_{OXA-48}$ gene in different *Enterobacteriaceae* during an outbreak in an adult thoracic-oncology unit in Marseille, France. The management of this epidemic by clinicians and the decolonization of patients are also described.

Methods

Study design and bacterial strains

This retrospective study was conducted to investigate an outbreak of CPE in six patients that occurred between October and December 2017 in a thoracic-oncology center at the North

hospital of Marseille, France. To perform this investigation, we also extracted medical records from two other patients that had been positive for CPE in the same ward, one in December 2016 and one between August and October 2017 (Figure 1; Table 1). From these eight patients, nine strains were isolated, including eight from rectal swabs and one from a urine sample. Patient 2 carried two different CPE isolates. An epidemiological investigation was conducted to understand the source of this outbreak. The mode of dissemination of the *bla*$_{OXA-48}$ gene and the relatedness between isolates were studied using whole-genome sequencing.

Decolonization by fecal microbiota transplantation

As previously noted, FMT was performed in six patients (Patients 2 to 7) (Saïdani et al., 2019). Briefly, patients who underwent FMT were previously sampled for various colonization sites (urine, pharynx, nasopharynx, and rectum) and additional potential sites (gastrostomy, skin, wounds, etc.). This mapping of CPE-colonized sites was established over three consecutive days prior to the FMT protocol. Eight days before FMT, a 3-day nasopharyngeal decolonization (in case of nasopharyngeal carriage) was performed using 0.12% chlorhexidine gluconate as local treatment of the mouth (gargling) and nasal cavities (swab applications). Five days before FMT, patients received an initial bowel lavage (until stools became watery and clear). An oral non-absorbable combination of antibiotics comprising colistin 6 MIU every 6h and amikacin 200 mg every 6h was then administered, replaced by other antibiotics in case of resistance, according to isolated CPE antibiotic susceptibility. One day prior to FMT, the patient received a second bowel lavage (until stools became watery and clear) and was given a proton-pump inhibitor (pantoprazole 40 mg twice a day for 48h).

Fecal microbiota transplantation was only considered for CPE-colonized patients for whom rehabilitation, surgery or chemotherapies were indicated and were likely to be delayed, based on the argument that CPE carriage would lead to a consequent loss of opportunity.

Phenotypic and molecular analyses

All strains were isolated on chromID® CARBA SMART agar (bioMérieux, Marcy-l'Etoile, France), except for P7700 on Columbia agar with 5% sheep blood (bioMérieux). Isolates were identified by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF) (Microflex, Brüker Daltonics, Bremen, Germany) (Seng et al., 2009). Antimicrobial susceptibility testing (AST) was performed using the disc diffusion method on a panel of 13 antibiotics (i2a, Montpellier, France): amoxicillin, amoxicillin-clavulanic acid, cefepime, ceftriaxone, piperacillin-tazobactam, ertapenem, imipenem, fosfomycin, doxycycline, trimethoprim-sulfamethoxazole, amikacin, gentamicin and ciprofloxacin, according to EUCAST recommendations (version 3.1). The minimum inhibitory concentration (MIC) of ertapenem and imipenem was determined by the E-test method (bioMérieux, Marcy l'Etoile, France). The β-CARBA test (Bio-Rad, Hercules, CA, USA) was used to detect carbapenemase production.

Real-time PCR (RT-PCR) was performed in all strains to confirm the presence of genes encoding carbapenem

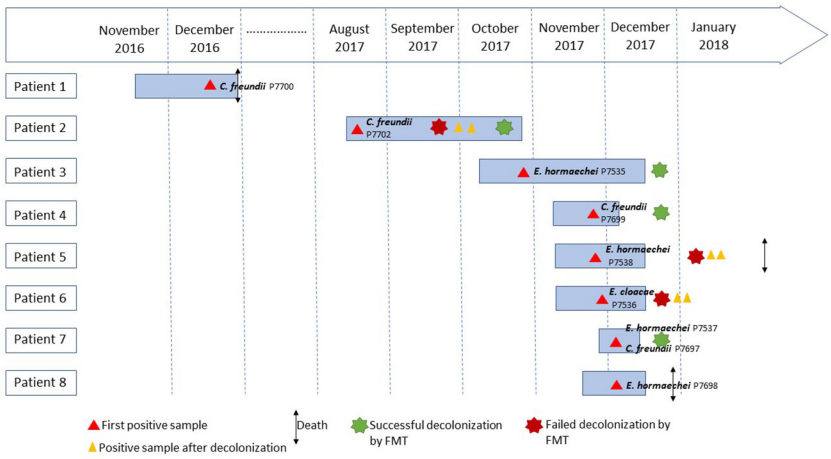

FIGURE 1
Timeline of patients with carbapenemase-producing *Enterobacteriaceae*.

TABLE 1 Clinical information on patients with OXA-48 carbapenemase.

Patient	CPE	Sex	Age	Main diagnosis	Isolation date	Date of admission	Date of dischearge	Hospitalization days	Antibiotic susceptibility testing		Colonization	Decolonization method	CPE decolonization
									Susceptible phenotype	Resistant phenotype			
Patient 1	*C. freundii* P7700	M	59	Lung adenocarcinoma	18/12/2016	21/11/2016	30/12/2016	39	FOF, AK, FEP, ERT (1), IPM (1)	AMX, AMC, CRO, TPZ, CIP, DO, GEN, SXT	U	Dead before FMT	/
Patient 2	*C. freundii* P7702	F	64	Recurrent hemoptysis	17/08/2017	14/08/2017	27/10/2017	74	FOF, AK, FEP, ERT (0.5), IPM (0.5)	AMX, AMC, CRO, TPZ, CIP, DO, GEN, SXT	R/U	FMT by gastric way (2 times)	Failure of the first attempt but success of the second
Patient 3	*E. hormaechei* P7535	F	73	Lung adenocarcinoma	27/10/2017	10/10/2017	19/12/2017	40	FOF, AK, GEN, FEP, DO, SXT	AMX, AMC, CRO, TPZ, CIP, ERT (2), IPM (2)	R	FMT by gastric way	Success
Patient 4	*C. freundii* P7699	F	56	Urothelial carcinoma	22/11/2017	12/11/2017	02/12/2017	20	FOF, AK, FEP, ERT (1), IPM (1)	AMX, AMC, CRO, TPZ, CIP, DO, GEN, SXT	R	FMT by gastric way	Success
Patient 5	*E. hormaechei* P7538	M	71	Laryngeal carcinoma	22/11/2017	11/11/2017	15/12/2017	34	FOF, AK, ERT (1), IPM (1)	AMX, AMC, CRO, TPZ, CIP, FEP, DO, SXT, GEN	R/C	FMT by gastric way	Failure
Patient 6	*E. cloacae* P7536	F	28	Pleurisy on gastropleural fistula	30/11/2017	13/11/2017	17/12/2017	34	FOF, AK	AMX, AMC, CRO, TPZ, CIP, FEP, DO, SXT, GEN, ERT (4), IPM (2)	R	FMT by rectal way	Failure
Patient 7	*C. freundii* P7697	F	57	Lung adenocarcinoma	7/12/2017	27/11/2017	13/12/2017	16	FOF, AK, FEP, ERT (1), IPM (1)	AMX, AMC, CRO, TPZ, CIP, DO, GEN, SXT	R/U	FMT by gastric way	Success
	E. hormaechei P7537								FOF, AK, FEP, DO, SXT, GEN	AMX, AMC, CRO, TPZ, CIP, ERT (2), IPM (2)			
Patient 8	*E. hormaechei* P7698	M	63	Small cell lung carcinoma	7/12/2017	18/11/2017	16/12/2017	28	FOF, AK	AMX, AMC, CRO, TPZ, CIP, FEP, DO, SXT, GEN, ERT (>32), IPM (6)	R/U/C	Dead before FMT	/

CPE, Carbapenemase-producing Enterobacteriaceae; R, Rectal; U, Urinary; C, Cutaneous; FMT, Fecal Microbiota Transplantation; FOF, fosfomycin; AK, amikacin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CRO, ceftriaxone; TPZ, piperacillin-tazobactam; CIP, ciprofloxacin; FEP, cefepime; DO, doxycycline; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; ERT, ertapenem; IPM, imipenem; (MIC en µg/mL).

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hydrolyzing enzymes (bla_{NDM}, bla_{KPC}, bla_{OXA-48}, bla_{VIM}) using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Targeted genes were detected using specific primers and Taqman probes (Yousfi et al., 2019).

Genomic analyses

Total genomic DNA (gDNA) was extracted using a EZ1 DNA kit and the BioRobot EZ1 (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Then, the gDNA was quantified by a Qubit assay (Life Technologies, Carlsbad, CA, USA) and its quality was controlled by Bioanalyser systems (Agilent, Santa Clara, CA, USA). All CPEs were sequenced in 2x250 bp paired-end reads in MiSeq (Illumina Inc., San Diego, CA, USA), of which two *Citrobacter freundii* strains (P7697, P7699) and two *Enterobacter* sp. isolates (P7536, P7538) were also sequenced using MinIon technology (Oxford Nanopore Technologies Inc., United Kingdom). The runs performed by the MinION technology were done using Ligation Sequencing Kit (Oxford Nanopore Technologies Inc., United Kingdom) and the libraries were loaded on a flow cell R9.4.1. Spades software (Bankevich et al., 2012) was used to assemble the Illumina generated sequencing data and also for the assembly of the mixed Nanopore-Illumina data. Genome annotation, antibiotic resistance gene, and plasmid screening were performed with RAST (Aziz et al., 2008), Resfinder (Bortolaia et al., 2020) and PlasmidFinder (Carattoli et al., 2014), respectively. The CPE sequence type (ST) was performed *in silico* using multilocus sequence typing (MLST) analysis on the Center for Genomic Epidemiology website (https://www.genomicepidemiology.org/).

The genetic environment of the bla_{OXA-48} genes was reconstructed by comparing the sequences of the genes surrounding this gene to the NCBI database using the blastX parameter. The complete plasmid sequences of strains P7697, P7699, P7536 and P7538 carrying bla_{OXA-48} genes were compared with the reference plasmid CP027039.1 using CGViewServer software (Stothard et al., 2017). Variant calling on the snippy tool version 4.6.0 was used to detect single nucleotides variants (SNV), both between bla_{OXA-48} plasmids and between strains of the same species. Pangenome analysis was performed with Roary (Page et al., 2015) and visualized using Phandango software (Hadfield et al., 2018). The percent similarity of the *C. freundii* strains and *Enterobacter* sp. strains was calculated by pairwise comparison of their average nucleotide identity based on Blast (ANIb) and using JSpecies (Richter et al., 2016).

Genomes of P7535, P7536, P7537, P7538, P7697, P7698, P7699, P7700 and P7702 strains have been submitted to GenBank under accession numbers JAGDEG000000000, CP071788- CP071792, JAGDEH000000000, CP071830-CP071833, CP071834-CP071838, JAGDEI000000000, CP071907-CP071913, JAGDEJ000000000, and JAGDEK000000000, respectively (Table 2).

Ethics

No sampling was performed for research purposes. Phenotypic, molecular and genomic analyses were performed on bacteria isolated for diagnostic as routine care for epidemiological investigation of the outbreak and infection control intervention. According to European General Data Protection Regulation No. 2016/679, the study was registered under N° 2022-28 in the APHM register.

Results

Outbreak control and decolonization by FMT

Overall, eight CPE carriers in a thoracic-oncology ward were identified (Table 1; Figure 1). The detection of CPE in patient 3 led to the implementation of measures to control the transmission of the outbreak according to current French recommendations (Lepelletier et al., 2015). Initially, the patient was placed on contact isolation precautions, associated with enhanced environmental disinfection, and a training for the healthcare staff on reduction of the risk of cross-transmission was done. In addition, screening of 49 contact patients hospitalized in the thoracic-oncology ward was performed. Five (5/49, 10.2%) new secondary cases (patients 4 to 8) were detected in the thoracic-oncology unit (Table 1; Figure 1). Isolation and prevention policies were also applied to these patients.

Before this outbreak, two patients carried a CPE at different times. The detection of CPE in patient 1 was confirmed after he died of his oncological illness on the ward, one year before the outbreak. Consequently, no isolation measures were taken. Patient 2 was hospitalized two months before the outbreak and was still hospitalized when patient 3 was detected positive. Patient 2 had been hospitalized abroad within 12 months, so he was placed on contact isolation precautions on arrival in the ward and was evaluated for the carriage of CPE according to the recommendations of the French High Committee for Public Health (Lepelletier et al., 2015). Isolation measures were maintained after the finding of *C. freundii* OXA-48 carriage.

In addition to hand hygiene and contact isolation policies, treatment by selective personalized decolonization and FMT was

TABLE 2 Genomic analysis of *C. freundii* and *Enterobacter* sp. strains.

Isolate	ST	Plasmid replicate	ARGs	Genbank accession number
E. hormaechei P7535	1007	Chr- IncFIB	bla_{ACT-15}, bla_{LAP-2}, qnrS1, fosA	JAGDEG000000000
		IncL/M	***bla*$_{OXA-48}$**	
E. cloacae P7536	98	Chr	bla_{ACT-16}, aadA1, sul1, catA1, fosA	CP071792
		IncFII	bla_{OXA-9}, bla_{TEM-1A}, aac(6')Ib-cr, aadA1	CP071790
		IncHI2	bla_{OXA-1}, bla_{TEM-1A}, $bla_{CTX-M-15}$, qnrB1, aac(3)-IIa, aac(6')Ib-cr, aph(3'')-Ib, aph(6)-Id, aadA1, catA1, catB3, sul2, dfrA14, tet(A)	CP071791
		IncA	aac(6')Ib-cr, aadA1, catB2, sul1	CP071789
		IncL/M	***bla*$_{OXA-48}$**	CP071788
E. hormaechei * P7537	1007	Chr– IncFIB	bla_{ACT-15}, bla_{LAP-2}, qnrS1, fosA	JAGDEH000000000
		IncL/M	***bla*$_{OXA-48}$**	
E. hormaechei P7538	133	Chr	bla_{ACT-7}, fosA	CP071833
		IncFIB	None	CP071831
		IncHI2	bla_{OXA-1}, bla_{TEM-1B}, $bla_{CTX-M-15}$, qnrB1, aac(6')Ib-cr, aph(3'')-Ib, aph(6)-Id, aadA1, catA1, catB3, sul2, dfrA14, tet(A)	CP071832
		IncL/M	***bla*$_{OXA-48}$**	CP071830
E. hormaechei P7698	114	Chr- IncFIB	bla_{OXA-1}, bla_{TEM-1B}, bla_{DHA-1}, bla_{ACT-16}, qnrB2, qnrB4, fosA, mph(A), aac(3)-IId, aac(6')Ib-cr, aadA1, catB3, sul1, dfrA1, ARR-3, tet(D)	JAGDEI000000000
		IncL/M	***bla*$_{OXA-48}$**	
*C. freundii** P7697	22	Chr	bla_{CMY-48}, aadA1, dfrA1	CP071834
		pKPC-CAV1193	bla_{SHV-12}	CP071838
		IncA	None	CP071837
		Plasmid	bla_{OXA-1}, mph(A), aac(6')Ib-cr, catB3, sul1, sul2, ARR-3, tet(D)	CP071836
		Unlocated	bla_{TEM-1B}, bla_{OXA-10}, aac(3)-IId, aac(6')Ib-cr	/
		IncL/M	***bla*$_{OXA-48}$**	CP071835
C. freundii P7699	22	Chr	bla_{CMY-48}, aadA1, dfrA1	CP071907
		IncA	aac(6')Ib-cr, aadA1, catB2, sul1	CP071911
		pKPC-CAV1193	bla_{SHV-12}	CP071913
		IncHI1A	None	CP071912
		Plasmid	bla_{OXA-1}, mph(A), aac(6')-Ib-cr, catB3, sul1, ARR-3	CP071909
		Plasmid	bla_{TEM-1B}, catA2, sul2, tet(D)	CP071910
		IncL/M	***bla*$_{OXA-48}$**	CP071908
C. freundii P7700	22	Chr-pKPC-CAV1193- IncA	bla_{CMY-48}, bla_{OXA-1}, bla_{TEM-1B}, bla_{SHV-11}, aac(3)-IId, aac(6')Ib-cr, aadA1, mph(A), catB3, catB2, sul1, sul2, dfrA1, ARR-3, tet(D)	JAGDEJ000000000
		IncL/M	***bla*$_{OXA-48}$**	
C. freundii P7702	22	Chr- pKPC-CAV1193- IncA	bla_{CMY-48}, bla_{OXA-1}, bla_{TEM-1B}, bla_{SHV-11}, aac(3)-IId, aac(6')Ib-cr, aadA1, mph(A), catB3, catB2, sul1, sul2, dfrA1, ARR-3, tet(D)	JAGDEK000000000
		IncL/M	***bla*$_{OXA-48}$**	

*Strains isolated from the same patient.
Bold means Informations concerning the IncL/M plasmid.

performed in 6/8 patients so that they could receive chemotherapy. Four out of six patients had a successful FMT (patient 2, 3, 4, 7) confirmed by three negative consecutive CPE controls after transplantation. Two successive FMTs were required to decolonize patient 2 (Table 1; Figure 1).

A 12-month follow-up did not detect additional CPE carriers in this thoracic-oncology ward.

Isolates and antibiotic susceptibility testing

Four *C. freundii* strains and five *E. cloacae* strains were isolated from eight clinical samples. All CPE were sensitive to fosfomycin and amikacin but resistant to amoxicillin, amoxicillin-clavulanic acid, ceftriaxone, piperacillin-

tazobactam, and ciprofloxacin. All strains of *C. freundii* had the same resistance phenotype (doxycycline, gentamicin, trimethoprim-sulfamethoxazole resistant and cefepime sensitive). Concerning the *Enterobacter* strains, two phenotypes were observed. The isolates P7535 and P7537 were sensitive to cefepime, doxycycline, trimethoprim-sulfamethoxazole and gentamicin, while the remaining (P7536, P7538, P7698) were not (Table 1).

Ertapenem and imipenem MICs of *C. freundii* strains varied from 0.5 to 1 µg/mL and 0.5 to 1 µg/mL respectively, while ertapenem and imipenem MICs of *Enterobacter* isolates ranged respectively from 1 to >32 µg/mL and 1 to 6µg/mL (Table 1). The β-CARBA test was positive for all isolates that carried only the bla_{OXA-48} carbapenemase gene.

Resistome

All strains of *C. freundii* had a median length of 5.2Mb and a median GC of 51.7% while the median length and GC of *Enterobacter* isolates ranged between 4.98 to 5.1Mb and between 55 to 55.1%, respectively. RT-PCR and genome analysis confirmed that OXA-48 carbapenemase was produced by all strains. Resistome analysis showed the presence of genes encoding resistance to different families of antibiotics such as β-lactams, quinolones, aminoglycosides, sulfonamides, cyclins, and chloramphenicol (Table 2).

The gene encoding the OXA-48 enzyme was found in a 63kb IncL/M plasmid, whereas the other antibiotic resistance genes were present on other plasmid types or in the chromosome (Table 2). In all CPE, the bla_{OXA-48} gene was in a Tn*1999.2* transposon (IS10A-lysR-bla_{OXA-48}-IS1-IS10A) (Figure 2). Comparison of the complete OXA-48 plasmids with all CPE showed the quasi-similarity of the outbreak plasmids, with presence of 0 to 5 SNV.

Annotation of genomes on NCBI allowed reclassification of P7535, P7537, P7538, P7698 isolates as *Enterobacter hormaechei* strains (Table 2). According to MLST analysis, all *C. freundii* strains belonged to the same ST22 sequence type, while *Enterobacter* isolates had different ST, except P7535 and P7537, which were ST1007 (Figure 3; Table 2). *E. hormaechei* strains P7535 and P7537 had 99.98% ANIb similarity, while the similarity between the other *Enterobacter* isolates varied from 96.1 to 98.5%. *The C. freundii* strains also shared high similarity rates, from 99.64 to 99.98%. Pangenome and SNV analysis confirmed the same origin for *E. hormaechei* isolates P7535 and P7537, whereas the distance of *C. freundii* isolates indicated that they were related but appear to have diverged over time (Figure 3).

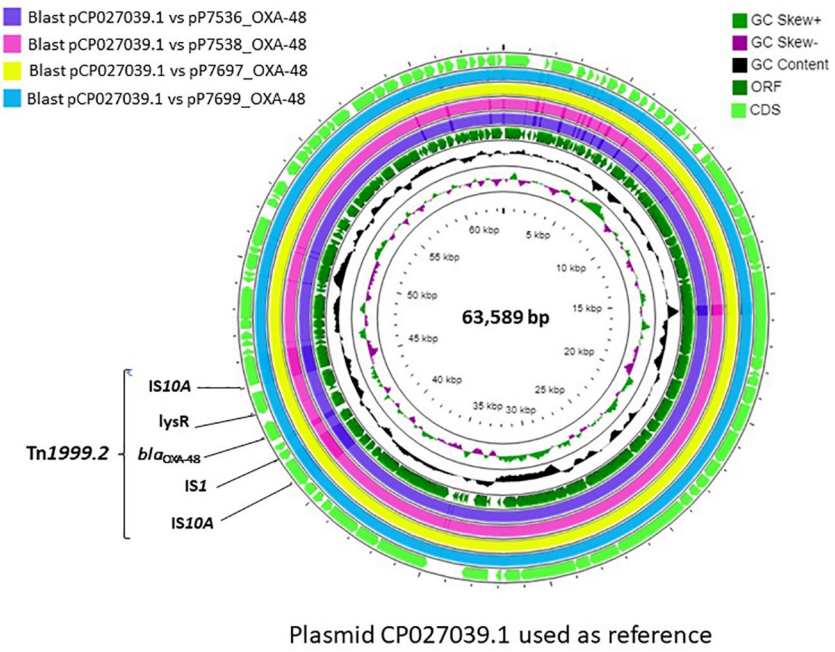

FIGURE 2
Genetic environment of the bla_{OXA-48} gene and circular representation of the complete OXA-48 plasmids.

10.3389/fcimb.2022.1048516

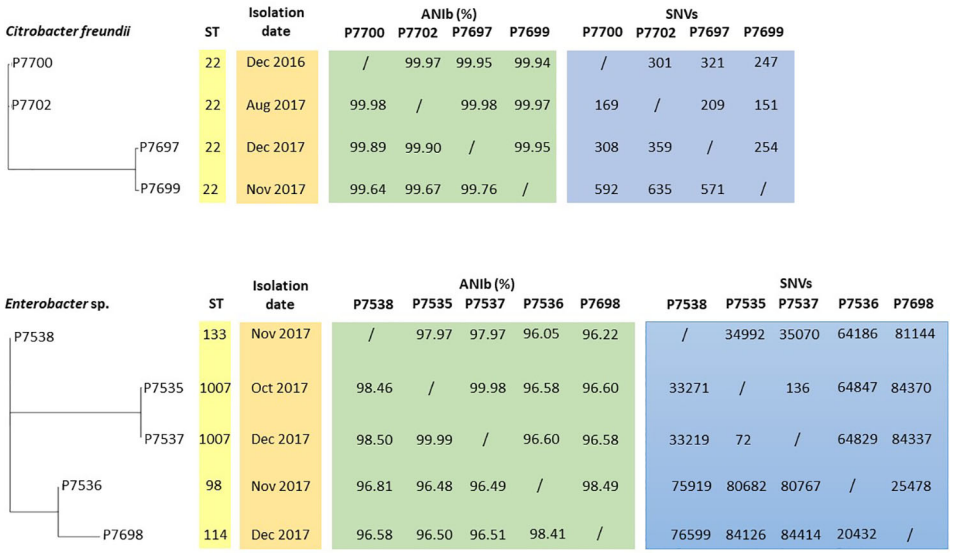

FIGURE 3
Phylogenetic tree based on the pangenome of *C. freundii* and *Enterobacter* sp. strains associated with ST, isolation date, ANIb and SNV data.

Discussion

The emergence and spread of CPE in the hospital setting are a major concern for clinicians. In this study, among the CPE, we isolated *C. freundii* and *E. hormaechei*, which belong to the *E. cloacae* complex and are identified as *E. cloacae* by most biochemical methods (Mateos et al., 2020). The presence of *C. freundii* producing OXA-48 carbapenemase is emerging in clinical settings where recent reports from Spain warned of an increase in its incidence. In Germany, the ST22 clone of OXA-48 carbapenemase producing *C. freundii* is increasingly noted in the hospital setting (Villa et al., 2017; Lalaoui et al., 2019; Yao et al., 2021). Recently in the north of France, an outbreak of OXA-48 carbapenemase -producing *Enterobacteriaceae* species, including *Enterobacter* sp. and *C. freundii* ST22, has been reported in a haematological ward (Jolivet et al., 2021).

IncL/M plasmids are known to be major carriers of the bla_{OXA-48} gene, which is very commonly inserted into a Tn1999-type transposon (Giani et al., 2012). Tn*1999.2*, present in all of our strains, has been found in different species of *Enterobacteriaceae* in Europe (Hidalgo et al., 2019). In our case, the same IncL/M OXA-48 plasmid was propagated among the different strains of *C. freundii* and *Enterobacter* sp. The horizontal transfer of conjugative plasmid is a key factor in the spread of antibiotic resistance genes in different clones and species of *Enterobacteriaceae*. It allows the evolution of resistance in certain bacterial clones due to an efficient bacterial-plasmid association (San Millan, 2018). This phenomenon was observed in a Spanish hospital in which a *Klebsiella pneumoniae* ST11 clone carrying OXA-48 carbapenemase was identified in 44 patients (Branas et al., 2015). In our case, similar clones of *C. freundii* circulated in the ward for one year, and the same strain of *E. hormaechei* spread between patients 3 and 7.

In our study, all patients colonized by CPE were hospitalized in the thoracic-oncology ward. Rectal or urinary colonization with CPE and prolonged hospital stays are risk factors for infections by these pathogens (Mateos et al., 2020). Spontaneous decolonization of intestinal carriage in patients colonized with CPE is a common but slow event (Zimmerman et al., 2013). FMT is a validated procedure that allows reduction of the time of colonization and thus accelerates medical management for patients (Saïdani et al., 2019). In our case, CPE colonization of cancer patients led to the postponement of chemotherapy treatment due to a higher risk of CPE infections in immunocompromised patients. Therefore, the benefits of FMT was to reduce the delay in the management of these patients, thus reducing their loss of opportunity (Saïdani et al., 2019).

The source of initial contamination of patient 1, at the origin of this outbreak, has not been identified to date, but an environmental track is probable and should not be neglected. The hospital environment and especially inanimate surfaces have often been identified as a reservoir for multidrug-resistant bacterial outbreaks (Decraene et al., 2018). Many Gram-negative species can survive on these surfaces for as long as several months (Pantel et al., 2016). One reason for the persistent transmission of OXA-48 *C. freundii* in a hematology department was the presence of contaminated toilets which constituted a potential reservoir (Jolivet et al., 2021). In our

case, the same clone of *C. freundii* was present in four different patients, hospitalized in different rooms, at different times, which is worrying. Therefore, the origins of this clone seem to be both external and internal to the unit. On the one hand, it could be due to the persistence of CPE in the department after the first patient died in 2016. Before the outbreak, patients were not systematically screened on this ward, unless they came from another hospital. On the other hand, the outbreak could have started with patient 2, despite the implementation of isolation precaution procedures. In particular, hand hygiene, the use of gloves, protective clothing and single-use sterile consumables, the excreta management as well as reinforced disinfection of the environment and technical areas are essential in the reduction of the cross-transmission risk (Lepelletier et al., 2015). Systematic screening and isolation of patients upon entry to the unit may be an efficient way to solve this issue and to allow the best possible control of CPE dissemination, although these measures are cumbersome to implement. (Pantel et al., 2014). These procedures are even more important in wards caring for immunocompromised patients (Nicolas-Chanoine et al., 2019). The spread of the same OXA-48 plasmid through different bacterial species and clones has been established. Nevertheless, the measures of control, implemented in accordance with the recommendations of the French High Committee for Public Health after the detection of this outbreak, together with the implementation of an FMT procedure for CPE carriers, promptly controlled this outbreak (Lepelletier et al., 2015).

Conclusion

Whole genome sequencing demonstrated several modes of transmission of OXA-48 carbapenemase through specific clones, plasmids, and transposons. The emergence and spread of CPE over a period of one year in our hospital is a worrisome development. This study highlights the necessity of investigating the source of contamination and controlling it as soon as possible to avoid the persistence of risky clones within a unit. Despite the precautions taken in the thoracic-oncology ward, this outbreak occurred, demonstrating the difficulty in prevention and control. Decolonization by FMT is an effective procedure that allowed a rapid resumption of oncological treatments. Nevertheless, it is necessary to remain vigilant and to continue epidemiological surveillance, especially when new patients are admitted to a unit, particularly in wards with immunosuppressed patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JAGDEG000000000, https://www.ncbi.nlm.nih.gov/genbank/, CP071788-CP071792, https://www.ncbi.nlm.nih.gov/genbank/, JAGDEH000000000, https://www.ncbi.nlm.nih.gov/genbank/, CP071830-CP071833, https://www.ncbi.nlm.nih.gov/genbank/, CP071834-CP071838, https://www.ncbi.nlm.nih.gov/genbank/, JAGDEI000000000, https://www.ncbi.nlm.nih.gov/genbank/, CP071907-CP071913, https://www.ncbi.nlm.nih.gov/genbank/, JAGDEJ000000000, https://www.ncbi.nlm.nih.gov/genbank/, JAGDEK000000000.

Ethics statement

No sampling was performed for research purposes. Phenotypic, molecular and genomic analyses were performed on bacteria isolated for diagnostic as routine care for epidemiological investigation of the outbreak and infection control intervention. According to European General Data Protection Regulation No. 2016/679, the study was registered under N° 2022-28 in the APHM register.

Author contributions

LH wrote the manuscript, performed the experiments, and analyzed the data. NS, CH, PA and PB performed medical examinations, collected and analyzed data. NC performed medical examinations and helped draft the manuscript. J-MR reviewed the manuscript. SB designed the study, drafted, and revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

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

Publisher's note

References

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* 9, 75. doi: 10.1186/1471-2164-9-75

Ballo, O., Tarazzit, I., Stratmann, J., Reinheimer, C., Hogardt, M., Wichelhaus, T. A., et al. (2019). Colonization with multidrug resistant organisms determines the clinical course of patients with acute myeloid leukemia undergoing intensive induction chemotherapy. *PLoS One* 14, e0210991. doi: 10.1371/JOURNAL.PONE.0210991

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Branas, P., Villa, J., Viedma, E., Mingorance, J., Orellana, M. A., and Chaves, F. (2015). Molecular epidemiology of carbapenemase-producing *klebsiella pneumoniae* in a hospital in Madrid: Successful establishment of an OXA-48 ST11 clone. *Int. J. Antimicrob. Agents* 46, 111–116. doi: 10.1016/J.IJANTIMICAG.2015.02.019

Brehony, C., McGrath, E., Brennan, W., Tuohy, A., Whyte, T., Brisse, S., et al. (2019). An MLST approach to support tracking of plasmids carrying OXA-48-like carbapenemase. *J. Antimicrob. Chemother.* 74, 1856–1862. doi: 10.1093/jac/dkz136

Carattoli, A., Zankari, E., Garcia-Fernandez, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Decraene, V., Phan, H. T. T., George, R., Wyllie, D. H., Akinremi, O., Aiken, Z., et al. (2018). A Large, refractory nosocomial outbreak of Klebsiella pneumoniae carbapenemase-producing *escherichia coli* demonstrates carbapenemase gene outbreaks involving sink sites require novel approaches to infection control. *Antimicrob. Agents Chemother.* 62 (12), e01689-18. doi: 10.1128/AAC.01689-18

Emeraud, C., Biez, L., Girlich, D., Jousset, A. B., Naas, T., Bonnin, R. A., et al. (2020). Screening of OXA-244 producers, a difficult-to-detect and emerging OXA-48 variant? *J. Antimicrob. Chemother.* 75, 2120–2123. doi: 10.1093/jac/dkaa155

George, S., Pankhurst, L., Hubbard, A., Votintseva, A., Stoesser, N., Sheppard, A. E., et al. (2017). Resolving plasmid structures in Enterobacteriaceae using the MinION nanopore sequencer: Assessment of MinION and MinION/illumina hybrid data assembly approaches. *Microb. Genomics* 3 (8), e000118. doi: 10.1099/mgen.0.000118

Giani, T., Conte, V., Di Pilato, V., Aschbacher, R., Weber, C., Larcher, C., et al. (2012). Escherichia coli from Italy producing OXA-48 carbapenemase encoded by a novel Tn1999 transposon derivative. *Antimicrob. Agents Chemother.* 56, 2211–2213. doi: 10.1128/AAC.00035-12

Hadfield, J., Croucher, N. J., Goater, R. J., Abudahab, K., Aanensen, D. M., and Harris, S.R. (2018). Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics* 34, 292–293. doi: 10.1093/BIOINFORMATICS/BTX610

Hidalgo, L., de Been, M., Rogers, M. R. C., Schürch, A. C., Scharringa, J., van der Zee, A., et al. (2019). Sequence-based epidemiology of an OXA-48 plasmid during a hospital outbreak. *Antimicrob. Agents Chemother.* 63 (12), e01204-19. doi: 10.1128/AAC.01204-19

Jamal, A. J., Mataseje, L. F., Brown, K. A., Katz, K., Johnstone, J., Muller, M. P., et al. (2020). Carbapenemase-producing enterobacterales in hospital drains in southern Ontario, Canada. *J. Hosp. Infect.* 106, 820–827. doi: 10.1016/j.jhin.2020.09.007

Jolivet, S., Couturier, J., Vuillemin, X., Gouot, C., Nesa, D., Adam, M., et al. (2021). Outbreak of OXA-48-producing enterobacterales in a haematological ward associated with an uncommon environmental reservoir, francs 2016 To 2019. *Eurosurveillance* 26, 1. doi: 10.2807/1560-7917.ES.2021.26.21.2000118

Lalaoui, R., Djukovic, A., Bakour, S., Hadjadj, L., Sanz, J., Salavert, M., et al. (2019). Genomic characterization of citrobacter freundii strains coproducing OXA-48 and VIM-1 carbapenemase enzymes isolated in leukemic patient in Spain. *Antimicrob. Resist. Infect. Control* 8, 167. doi: 10.1186/s13756-019-0630-3

Lepelletier, D., Berthelot, P., Lucet, J. C., Fournier, S., Jarlier, V., Grandbastien, B., et al. (2015). French Recommendations for the prevention of "emerging extensively drug-resistant bacteria" (eXDR) cross-transmission. *J. Hosp. Infect.* 90, 186–195. doi: 10.1016/J.JHIN.2015.04.002

Mateos, M., Hernández-García, M., del Campo, R., Martínez-García, L., Gijón, D., Morosini, M. I., et al. (2020). Emergence and persistence over time of carbapenemase-producing enterobacter isolates in a Spanish university hospital in Madrid, spain, (2005–2018). *Microb. Drug Resist* 27 (7), 895–903. doi: 10.1089/mdr.2020.0265

Nicolas-Chanoine, M. H., Vigan, M., Laouénan, C., Robert, J., Laurans, C., Vachée, A., et al. (2019). Risk factors for carbapenem-resistant enterobacteriaceae infections: a French case-control-control study. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 383–393. doi: 10.1007/S10096-018-3438-9/TABLES/6

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/BIOINFORMATICS/BTV421

Pantel, A., Marchandin, H., Prère, M. F., Boutet-Dubois, A., Brieu-Roche, N., Gaschet, A., et al. (2014). Faecal carriage of carbapenemase-producing gram-negative bacilli in hospital settings in southern France. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 899–904. doi: 10.1007/S10096-014-2298-1

Pantel, A., Richaud-Morel, B., Cazaban, M., Bouziges, N., Sotto, A., and Lavigne, J. P. (2016). Environmental persistence of OXA-48–producing *klebsiella pneumoniae* in a French intensive care unit. *Am. J. Infect. Control* 44, 366–368. doi: 10.1016/J.AJIC.2015.09.021

Richter, M., Rosselló -Móra, R., Oliver Glöckner, F., and Peplies, J. (2016). JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 32, 929–931. doi: 10.1093/bioinformatics/btv681

Saïdani, N., Lagier, J. C., Cassir, N., Million, M., Baron, S., Dubourg, G., et al. (2019). Faecal microbiota transplantation shortens the colonisation period and allows re-entry of patients carrying carbapenamase-producing bacteria into medical care facilities. *Int. J. Antimicrob. Agents* 53, 355–361. doi: 10.1016/J.IJANTIMICAG.2018.11.014

San Millan, A. (2018). Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends Microbiol.* 26, 978–985. doi: 10.1016/j.tim.2018.06.007

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.-E., Rolain, J. M., et al. (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* 49, 543–551. doi: 10.1086/600885

Shankar, C., Muthuirulandi Sethuvel, D. P., Neeravi, A. R., Venkatesan, M., Devanga Ragupathi, N. K., Anandan, S., et al. (2020). Identification of plasmids by PCR based replicon typing in bacteremic Klebsiella pneumoniae. *Microb. Pathog.* 148, 104429. doi: 10.1016/j.micpath.2020.104429

Stothard, P., Grant, J. R., and Van Domselaar, G. (2017). Visualizing and comparing circular genomes using the CGView family of tools. *Brief. Bioinform* 20 (4), 1576–1582. doi: 10.1093/bib/bbx081

Tacão, M., Araújo, S., Vendas, M., Alves, A., and Henriques, I. (2018). Shewanella species as the origin of blaOXA-48 genes: insights into gene diversity, associated phenotypes and possible transfer mechanisms. *Int. J. Antimicrob. Agents* 51, 340–348. doi: 10.1016/J.IJANTIMICAG.2017.05.014

Tilahun, M., Kassa, Y., Gedefie, A., and Ashagire, M. (2021). Emerging carbapenem-resistant enterobacteriaceae infection, its epidemiology and novel treatment options: A review. *Infect. Drug Resist.* 14, 4363–4374. doi: 10.2147/IDR.S337611

Villa, J., Arana, D. M., Viedma, E., Perez-Montarelo, D., and Chaves, F. (2017). Characterization of mobile genetic elements carrying VIM-1 and KPC-2 carbapenemases in citrobacter freundii isolates in Madrid. *Int. J. Med. Microbiol.* 307, 340–345. doi: 10.1016/j.ijmm.2017.07.001

Yao, Y., Falgenhauer, L., Falgenhauer, J., Hauri, A. M., Heinmüller, P., Domann, E., et al. (2021). Carbapenem-resistant citrobacter spp. as an emerging concern in the hospital-setting: Results from a genome-based regional surveillance study. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/FCIMB.2021.744431

Yoon, Y. K., Suh, J. W., Kang, E. J., and Kim, J. Y. (2019). Efficacy and safety of fecal microbiota transplantation for decolonization of intestinal multidrug-resistant microorganism carriage: beyond clostridioides difficile infection. *Ann. Med.* 51, 379. doi: 10.1080/07853890.2019.1662477

Yousfi, H., Hadjadj, L., Dandachi, I., Lalaoui, R., Merah, A., Amoura, K., et al. (2019). Colistin- and carbapenem-resistant *Klebsiella pneumoniae* Clinical_Isolates: Algeria. *Microb. Drug Resist.* 25, 258–263. doi: 10.1089/mdr.2018.0147

Zimmerman, F. S., Assous, M. V., Bdolah-Abram, T., Lachish, T., Yinnon, A. M., and Wiener-Well, Y. (2013). Duration of carriage of carbapenem-resistant enterobacteriaceae following hospital discharge. *Am. J. Infect. Control* 41, 190–194. doi: 10.1016/J.AJIC.2012.09.020

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A comprehensive description of the TolC effect on the antimicrobial susceptibility profile in *Enterobacter bugandensis*

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Background: Enterobacter bugandensis is an emerging human pathogen in which multidrug resistant strains have been continuously isolated from various environments. Thus, this organism possesses the potential to pose challenges in human healthcare. However, the mechanisms, especially the efflux pumps, responsible for the multidrug resistance in E. bugandensis remain to be well elucidated.

Methods: The Enterobacter strain CMCC(B) 45301 was specifically identified using whole genome sequencing. The specific CMCC(B) 45301 homologues of the TolC dependent efflux-pump genes characterized in Escherichia coli were identified. The tolC deletion mutant in CMCC(B) 45301 was constructed and subjected to susceptibility tests using 26 different antimicrobial agents, along with the wild type strain. The synergistic effects combining the Bacillus crude extract (BCE) and several other TolC-affected compounds against CMCC(B) 45301 were assayed.

Results: We reclassified the Enterobacter CMCC(B) 45301 strain from species cloacae to bugandensis, on the basis of its whole genome sequence. We found that the CMCC(B) 45301 TolC, AcrAB, AcrD, AcrEF, MdtABC, EmrAB, and MacAB exhibit high similarity with their respective homologues in E. coli and Enterobacter cloacae. Our results for the susceptibility tests revealed that lacking tolC causes 4- to 256-fold decrease in the minimal inhibitory concentrations of piperacillin, gentamicin, kanamycin, tetracycline, norfloxacin, ciprofloxacin, chloramphenicol, and erythromycin against CMCC (B) 45301. In addition, the inhibition zones formed by cefuroxime, cefoperazone, amikacin, streptomycin, minocycline, doxycycline, levofloxacin, florfenicol, trimethoprim-sulfamethoxazole, azithromycin, lincomycin, and clindamycin for the tolC mutant were larger or more

obvious than that for the parent. Our data suggested the important role played by TolC in CMCC(B) 45301 susceptibility to common antibiotic families covering ß-lactam, aminoglycoside, tetracycline, fluoroquinolone, phenicol, folate pathway antagonist, macrolide, and lincosamide. Deletion for tolC also increased the susceptibility of CMCC(B) 45301 to berberine hydrochloride and BCE, two natural product-based agents. Finally, we found that erythromycin, norfloxacin, and ciprofloxacin can potentiate the antibacterial activity of BCE against CMCC(B) 45301.

Discussion: The present study elaborated the comprehensive TolC effect on the antimicrobial susceptibility profile in E. bugandensis, which might contribute to the development of more therapeutic options against this nosocomial pathogen.

KEYWORDS

TolC, efflux pumps, antimicrobial agents, susceptibility, synergistic effects, *Enterobacter bugandensis*

Introduction

Enterobacter spp. are gram-negative bacteria inhabiting a wide range of environmental niches with the human gastrointestinal tract being the most noticeable one (Mezzatesta et al., 2012). Some members of genus *Enterobacter* are well-known nosocomial pathogens, capable of causing infections in the blood streams, lungs, urinary tracts, and peritoneum of immunocompromised individuals, especially those accepting intensive cares in hospitals (Chow et al., 1991; Fernandez-Baca et al., 2001; Davin-Regli and Pages, 2015; Gravey et al., 2020). In the past decades, clinical isolates possessing multidrug resistance resulted from production of extended spectrum ß-lactamases (ESBLs) and carbapenemases, overexpression of AmpC, reduction in membrane permeability, and overexpression of efflux pumps are frequently isolated in *Enterobacter*, which poses a tremendous challenge to human healthcare, world-widely (Sanders and Sanders, 1997; Hilty et al., 2013; Chavda et al., 2016; Telke et al., 2017; Liu et al., 2021). *Enterobacter bugandensis* was identified as a novel species by Doijad and colleagues (Doijad et al., 2016), and subsequently expanded as the identification of strains isolated from the international space station (ISS), various nosocomial settings, and vegetables (Singh et al., 2018; Falgenhauer et al., 2019; Matteoli et al., 2020; Moon et al., 2021). As known, conventional typing methods based on 16S rDNA sequencing and phenotypic assays are not adequate for accurate identification of species within genus *Enterobacter* (Annavajhala et al., 2019). Recently, as the growing use of whole genome sequencing, the resolution of molecular typing of bacterial strains has been elevated to the genome level (Federhen et al., 2016; Ciufo et al., 2018). This facilitated the reclassification of some strains from *Enterobacter cloacae* to *E. bugandensis*, which was based on the average nucleotide identity (ANI) as compared their respective genomes against known *E. bugandensis* strains (Ciufo et al., 2018; Matteoli et al., 2020). Notably, the first identified *E. bugandensis* strains were neonatal-blood isolates, indicating their potential causing severe systemic infections in human beings (Doijad et al., 2016). The pathogenicity of *E. bugandensis* was evidenced by the results that *E. bugandensis* EB-247 establishes faster infections in *Galleria mellonella* and yields a higher survival rate grown in human serum, compared with *E. cloacae* ATCC 13047, a known pathogenic strain of *Enterobacter* (Pati et al., 2018). Thus, *E. bugandensis* has been considered as the most pathogenic species within genus *Enterobacter* (Pati et al., 2018; Matteoli et al., 2020). Besides, the multidrug resistant (MDR) strains of *E. bugandensis* have been continuously isolated (Doijad et al., 2016; Moon et al., 2021). Taken together, reclassification or specific identification of the *E. bugandensis* strains is exceedingly required, in order to provide references important for precise reaction combating this organism and effective genetic engineering for better understanding of this species.

Efflux pumps are important mechanisms for the emergence of multidrug resistance in gram-negative bacteria and comprise classes of RND, MF, ABC, MATE, SMR, and PACE, see reference Nikaido and Zgurskaya, 1999; Li et al., 2015 and Nishino et al., 2021 for reviews. The RND transporters are tripartite efflux pumps, among which AcrAB-TolC is the major one responsible for expelling many antimicrobial agents in *Enterobacter* (Perez et al., 2007; Guerin et al., 2016). It has been described that substrates of AcrAB-TolC encompass almost all the commonly-used antibiotic families, namely ß-lactam inhibitor, aminoglycoside, tetracycline, macrolide, lincosamide, fluoroquinolone, folate pathway antagonist, and phenicol, in

some *Enterobacter* strains (Perez et al., 2007; Guerin et al., 2016; Liu et al., 2018; Gravey et al., 2020). Alarmingly, AcrAB-TolC can confer, at least in part, the increased resistance to some effective pharmaceutical options against MDR strains, like the combination of ß-lactam/ß-lactamase inhibitor and the last-line drugs, carbapenems, polymyxins, and tigecycline, in *Enterobacter* spp. (Perez et al., 2007; Telke et al., 2017; Huang et al., 2019; Gravey et al., 2020; Senchyna et al., 2021). Efflux of antimicrobial natural products can also be performed by AcrAB-TolC in *Enterobacter* strains (Kuete et al., 2010). Furthermore, *Enterobacter* strains are able to gain spontaneous mutations in the *acrB* gene or genes encoding regulators of *acrAB* and *tolC*, for their adaptations to antibiotic stresses (Telke et al., 2017; Gravey et al., 2020; Senchyna et al., 2021). As an outer-membrane channel, TolC can form efflux pumps with some other RND-type transporters, MF transporters, and ABC transporters, overexpression of which is capable of restoring the resistance to one or more antibiotic agents in bacterial strains devoid of AcrB (Kobayashi et al., 2001; Nagakubo et al., 2002; Nishino et al., 2003; Li et al., 2015; Yousefian et al., 2021; Nishino et al., 2021). Thus, studies regarding the TolC-involved efflux pumps in gram-negative bacteria including *Enterobacter* spp. are imperative for developing novel therapeutic strategies, for instance, the combination of efflux-pumped antibiotic/efflux-pump inhibitor, against the MDR strains (Morita et al., 2016; Li et al., 2017; Grimsey et al., 2020; Li et al., 2021b).

In *E. bugandensis*, some isolates are tested positive for the resistance/reduced susceptibility to cephalosporins, imipenem, aminoglycosides, fluoroquinolones, and polymyxin B (Doijad et al., 2016; Moon et al., 2021; Sarangi et al., 2022). Studies revealed that the plasmid-borne resistance genes and the chromosomally integrated gene encoding imipenemase IMI-1 might contribute to the multidrug resistance in *E. bugandensis* EB-247 and the imipenem resistance in *E. bugandensis* S68-1, respectively (Pati et al., 2018; Moon et al., 2021). However, investigations of the multidrug resistance/susceptibility concerning efflux pumps in *E. bugandensis* are still lacking. To our knowledge, a comprehensive study about the correlation between TolC or TolC-related efflux pumps and multidrug resistance/susceptibility in *E. bugandensis* has not been reported. Here, we constructed a clean deletion mutant of *tolC* in the *E. bugandensis* CMCC(B) 45301 strain, and tested the changes in its susceptibility to a range of 26 antimicrobial agents, including antibiotics belonging to different families and natural products, as compared with the parent strain. In addition, certain combinations of the TolC-affected antibiotic and natural product were assessed for their synergistic effects against the wild-type strain, for exploring novel antibacterial options combating *E. bugandensis*. The present study mainly aimed to elucidate the effect of TolC on the antimicrobial susceptibility profile in *E. bugandensis*, in advance providing information assisting actions against this bacterium in the future.

Results

Whole genome sequencing-based reclassification of *E. cloacae* CMCC(B) 45301

In this study, we used *E. cloacae* CMCC(B) 45301, a strain collected in China National Center for Medical Culture Collections (CMCC; http://www.cmccb.org.cn/cmccbnew/) and initially identified as *E. cloacae*, as the experimental material. To obtain the comprehensive genetic information of this strain, we sequenced and assembled its complete genome using the combination of Pacbio and Illumina platforms, see materials and methods. Our results showed that the circular chromosome DNA of CMCC(B) 45301 (GenBank accession No. CP097255) possesses a size of 4,631,472 base pairs (bp) and a G+C content of 56.13%. In addition, strain CMCC(B) 45301 harbors an 81,691-bp plasmid (GenBank accession No. CP097254) with a G+C content of 47.35%. The chromosome and plasmid sequences contain a total of 4,499 genes annotated *via* the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Importantly, according to the result of the quality-control test that compared the genome sequence of CMCC(B) 45301 against the type-strain genomes throughout GenBank as previously described (Federhen et al., 2016), strain CMCC(B) 45301 should be identified more specifically as an *E. bugandensis* organism, given the 98.8% ANI comparing its genomic assembly (GenBank accession No. GCA_023374275.1) with the best-matching type-strain assembly (GenBank accession No. GCA_019046905.1) of *E. bugandensis* (Ciufo et al., 2018). Thus, the original *E. cloacae* CMCC(B) 45301 strain was reidentified and designated as *E. bugandensis* CMCC(B) 45301 (EBU45301).

Identification of the TolC dependent efflux-pump genes in EBU45301

As known, TolC can form efflux pumps with the RND-type transporters, AcrAB, AcrAD, AcrEF, MdtEF, and MdtABC, the MF-type transporters, EmrAB and EmrKY, and the ABC-type transporter MacAB in *Escherichia coli* (Nishino et al., 2003). Recently, effects of *acrD*, *acrEF*, *mdtABC*, and some other RND efflux-pump genes on the resistance/susceptibility to various antibiotics in the *E. cloacae* ATCC 13047 strain (ECL13047) were characterized, describing a landscape of TolC-dependent RND efflux pumps in *E. cloacae* highly similar to that in *E. coli* (Guerin et al., 2016). To locate the respective EBU45301 homologues of the TolC dependent efflux-pump genes in *E. coli* K-12 MG1655 (GenBank accession No. U00096.3), BLAST was performed comparing each of these *E. coli* genes against the whole genome of EBU45301, and the locus whose product displayed

distinguishable-high identities/positives with that of the corresponding *E. coli* gene was considered as a specific homologue. The identified EBU45301 efflux-pump genes were also subjected to comparisons with their respective homologues in ECL13047 (GenBank accession No. NC_014121), for further confirmation. Our results revealed that specific homologues of *tolC*, *acrAB*, *acrD*, *acrEF*, *mdtABC*, *emrAB*, and *macAB* are encoded in both EBU45301 and ECL13047, and yet such homologues for *mdtEF* and *emrKY* can be found throughout the genome of neither of these strains (Table 1). EBU45301 harbors genes encoding for some other RND efflux-pump components, whose specific homologues are either present or absent in ECL13047 (Tables S1, S2), exhibiting potential in interaction with TolC (Guerin et al., 2016). In addition, several MF efflux pump proteins showing partial homology with the respective EmrAB- and EmrKY-TolC components were identified in EBU45301 (Table S3), indicating that there are EmrAB/EmrKY-like efflux pumps involving TolC in this bacterium.

We also compared the *tolC* gene of EBU45301 with that of the type or recommended reference (by the NCBI Genome database) strains representing the species belonging to genus *Enterobacter*. A maximum-likelihood tree was constructed based on *tolC*, and it is shown that EBU45301 is clustered with *E. bugandensis* EB-247 in the same clade closely related to *Enterobacter chuandaensis* 090028 and *Enterobacter sichuanensis* WCHECL1597 (Figure 1A). Furthermore, the TolC amino-acid sequences from EBU45301, *E. bugandensis* EB-247, *Enterobacter chuandaensis* 090028, *Enterobacter sichuanensis* WCHECL1597, *Enterobacter cloacae* ATCC 13047, *Enterobacter asburiae* JCM 6051, *Enterobacter hormaechei* ATCC 49162, and *E. coli* K-12 MG1655 were aligned for a comparison, to investigate the homology of EBU45301 TolC with its respective homologues in the closely related species and species in which AcrAB-TolC has been reported as a powerful MDR mechanism (Li et al., 2015; Guerin et al., 2016; Telke et al., 2017; Gravey et al., 2020). The alignment result revealed that EBU45301 TolC is highly homologous with the other TolC sequences (Figure 1B), suggesting its function transporting multiple compounds. Taken together, we hypothesized that TolC plays a magnificent role in mediating the antimicrobial susceptibility profile of EBU45301.

TABLE 1 TolC related efflux-pump genes in EBU45301 located based on their homologies with the respective sequences in *E. cloacae* and *E. coli*.

Locus tag EBU45301	Locus tag ECL13047[a]	Gene *E. coli*	Identities/positives (%)[b] EBU45301 *versus* (vs.) ECL13047	Identities/positives (%) EBU45301 vs. *E. coli*
RND type				
M1V99_19125	ECL_RS21755	*tolC*	96/98	85/92
M1V99_05805	ECL_RS06015	*acrA*	95/97	87/93
M1V99_05800	ECL_RS06010	*acrB*	99/99	93/96
M1V99_16450	ECL_RS18730	*acrD*	97/98	91/96
M1V99_20240	ECL_RS23185	*acrE*	94/96	75/86
M1V99_20245	ECL_RS23190	*acrF*	96/98	84/92
ND[c]	ND	*mdtE*	NA[c]	NA
ND	ND	*mdtF*	NA	NA
M1V99_15070	ECL_RS16825	*mdtA*	96/98	85/91
M1V99_15075	ECL_RS16830	*mdtB*	97/99	90/96
M1V99_15080	ECL_RS16835	*mdtC*	98/99	92/97
MF type				
M1V99_17535	ECL_RS20065	*emrA*	98/99	86/93
M1V99_17540	ECL_RS20070	*emrB*	99/100	93/98
ND	ND	*emrK*	NA	NA
ND	ND	*emrY*	NA	NA
ABC type				
M1V99_07880	ECL_RS13615	*macA*	97/98	83/91
M1V99_07885	ECL_RS13610	*macB*	97/98	87/92

[a] Genes in ECL13047 has been designated with new locus tags, which are different from the ones described in reference Guerin et al., 2016.
[b] This result reveals the similarity of an EBU45301 amino acid sequence against its respective homologues in ECL13047 and *E. coli* K-12 MG1655. The query covers (%) of sequences used in alignments for each of the EBU45301 protein are all larger or equal to 98.
[c] ND, non-detectable; NA, non-applicable.

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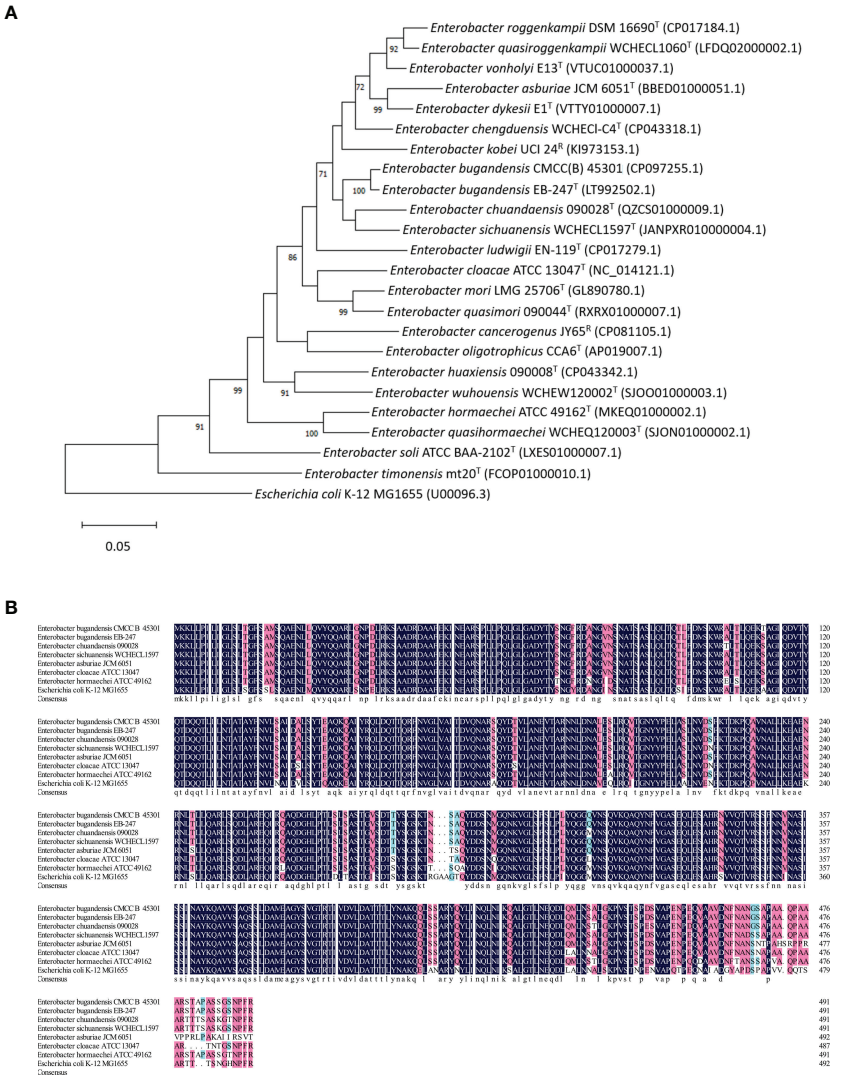

FIGURE 1
Comparative analyses of EBU45301 and the type/reference strains representing *Enterobacter* species based on the *tolC* gene. **(A)** A maximum-likelihood tree was constructed based on the *tolC* gene sequences retrieved from the genomes of the *Enterobacter* strains as indicated. The Genbank accession No. for each of the used genomic sequence was shown in parentheses. The shown values on certain branch nodes were bootstrap values >70%, based on 500 replications. The bar represents 0.05 substitutions per site. *E. coli* K-12 MG1655 was used to root the tree. T, type; R, reference. **(B)** An alignment of the amino acid sequences of TolC from EBU45301 and several other *Enterobacter* strains (as indicated) was performed. The homology levels were shaded as follows: ≥50%, light blue; ≥70%, pink; =100%, dark blue. The consensus sequence was shown at the bottom of this alignment in lower case.

Investigation of the TolC effect on the susceptibility of EBU45301 to different antimicrobial agents

To study the role of TolC in EBU45301 physiology, we constructed a clean deletion mutant for *tolC via* the pRE112 system (Edwards et al., 1998). It has been described that the TolC-dependent efflux pumps affect the susceptibility of *E. coli* and some *Enterobacter* species to a broad spectrum of antibiotics (see Table S4) and natural products like isobavachalcone and Diospyrone (Kuete et al., 2010). Here, we assayed the susceptibility of the *tolC* mutant (*tolC*) and the wild-type strain (WT) to a range of 26 antimicrobial agents, covering the extensively-applied antibiotic families, ß-lactam, aminoglycoside, tetracycline, fluoroquinolone, macrolide, lincosamide, phenicol, and folate pathway antagonist, and two other agents, berberine hydrochloride (BBH) and the *Bacillus* crude extract (BCE), see materials and methods for details.

Our results showed that the wild-type EBU45301 is intrinsically resistant to cefazolin and susceptible to piperacillin, cefuroxime,

ceftazidime, ceftriaxone, cefoperazone, imipenem, amikacin, gentamicin, kanamycin, streptomycin, tetracycline, minocycline, doxycycline, norfloxacin, ciprofloxacin, levofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole (Tables 2–4), according to the respective breakpoints recommended in the performance standards provided by CLSI, 2022. We also found that the *tolC* deletion causes the increased susceptibility of EBU45301 to all the tested antimicrobial agents, except for cefazolin, ceftazidime, ceftriaxone, and imipenem (Tables 3, 4). The minimal inhibitory concentration (MIC) of piperacillin for the *tolC* mutant was 8-16 times smaller than that for the parent (Table 3), in accordance with the disk-diffusion results for piperacillin (Table 4 and Figure S1). In terms of drugs classified as cephalosporins, the *tolC* deletion rendered EBU45301 more sensitive to cefuroxime and cefoperazone, different from that for cefazolin, ceftazidime, and ceftriaxone (Tables 3, 4, and Figure S1). In addition, loss of *tolC* resulted in a 4-fold decrease in the MICs of gentamicin, kanamycin, and tetracycline against EBU45301 (Table 3), which was consistent with the slightly larger inhibition zones for the *tolC* mutant than WT, revealed in the disk-diffusion tests for these drugs (Table 4 and Figure S1). Similarly, in the cases of the other two aminoglycosides, amikacin and streptomycin, the effect of TolC was observed but not very obvious (Table 4 and Figure S1). However, not like that for tetracycline, strain *tolC* appeared to be much more sensitive to minocycline and doxycycline, also belonging to family tetracycline, than WT, which might be due to the weaker activity of these two antibiotics against the wild-type EBU45301 (Table 4 and Figure S1). The TolC effect was found remarkable on the tested fluoroquinolones. The respective fold changes in MIC for norfloxacin and ciprofloxacin were 64 and 32, comparing *tolC* with WT (Table 3). These phenotypes in susceptibility observed using the broth-microdilution method were confirmed *via* the well-diffusion method, for norfloxacin and ciprofloxacin (Table 4 and Figure S1). Moreover, the inhibition zone using levofloxacin for strain *tolC* was obviously larger than strain WT, suggesting a TolC effect on levofloxacin efflux as strong as that for norfloxacin or ciprofloxacin (Table 4 and Figure S1). Moreover, compared with the wild type, the *tolC* mutant exhibited defects in resisting chloramphenicol and florfenicol, which were determined as the 8-fold decrease in MIC (Table 3) and the much larger inhibition zone as shown (Table 4 and Figure S2), respectively. Trimethoprim-sulfamethoxazole, a combination of folate pathway antagonists, showed higher activity combating *tolC* than WT, assayed through the disk-diffusion tests (Table 4 and Figure S2). We also tested the

TABLE 2 Antibiotic susceptibility profile of the wild-type EBU45301.

Antibiotic	Susceptibility	MIC (mg/L[a])	Diameter (mm[a])
ß-lactam			
Piperacillin	Susceptible	≤8	
Cefazolin	Resistant	≥8	
Cefuroxime	Susceptible		≥18
Ceftazidime	Susceptible	≤4	
Ceftriaxone	Susceptible		≥23
Cefoperazone	Susceptible		≥21
Imipenem	Susceptible	≤1	
Aminoglycoside			
Amikacin	Susceptible		≥17
Gentamicin	Susceptible	≤4	
Kanamycin	Susceptible	≤16	
Streptomycin	Susceptible		≥15
Tetracycline			
Tetracycline	Susceptible	≤4	
Minocycline	Susceptible		≥16
Doxycycline	Susceptible		≥14
Fluoroquinolone			
Norfloxacin	Susceptible	≤4	
Ciprofloxacin	Susceptible	≤0.25	
Levofloxacin	Susceptible		≥21
Phenicol			
Chloramphenicol	Susceptible	≤8	
Folate pathway antagonist			
Trimethoprim-sulfamethoxazole	Susceptible		≥16

[a] mg/L, milligram/liter; mm, millimeter.

TABLE 3 Phenotypes of the EBU45301 *tolC* mutant in drug susceptibility (microdilution method).

Antimicrobial	MIC (mg/L) for			
	WT	*tolC*	*tolC*/complementation[a]	*tolC*/vector[b]
ß-lactam				
Piperacillin	2-4	0.25	2	0.25
Cefazolin	64	64		
Ceftazidime	0.5	0.5		
Imipenem	0.125	0.125		
Aminoglycoside				
Gentamicin	2	0.5	1	0.5
Kanamycin	2	0.5	1	0.25-0.5
Tetracycline				
Tetracycline	2	0.5	2	0.5
Fluoroquinolone				
Norfloxacin	0.384	0.006	0.192	0.006
Ciprofloxacin	0.032	0.001	0.016	<0.001
Phenicol				
Chloramphenicol	8	1	8	1
Macrolide				
Erythromycin	1,536	6	768	6
Natural product				
BBH[c]	>1,024	512	>1,024	512
BCE[d]	0.031x original	0.016x original	0.031x original	0.016x original

[a] For tests with tetracycline, BBH, and BCE, the *tolC*/complementation strain used was *tolC*/pBYL030. For tests with chloramphenicol, the *tolC*/complementation strain used was *tolC*/pBYL032. For the rest cases, the *tolC*/complementation strain used was *tolC*/pBYL024.
[b] For tests with tetracycline, BBH, and BCE, the *tolC*/vector strain used was *tolC*/pBYL031. For tests with chloramphenicol, the *tolC*/vector strain used was *tolC*/pBYL033. For the rest cases, the *tolC*/vector strain used was *tolC*/pACYC184.
[c] The same amount of DMSO as that contained in 2,048 mg/L BBH could completely inhibit the growth of EBU45301. Thus, the activity of 2,048 mg/L BBH against WT could not be assessed. For WT, there was obvious turbidity in wells supplemented with 1,024 mg/L BBH, indicating a MIC of BBH larger than 1,024 mg/L, against this strain.
[d] Original, the original stock of BCE prepared from the YPD culture of bacteria (see materials and methods).

TolC effect on EBU45301 susceptibility to antibiotics that are not recommended for cases of *Enterobacteriaceae*, such as macrolides and lincosamides. Larger and clearer inhibition zones were observed for the *tolC* mutant than the WT strain, when the susceptibility to erythromycin, azithromycin, and clindamycin was tested (Table 4 and Figure S2). In assays using lincomycin, there were areas with fewer bacteria instead of clear zones around the disks for *tolC*, and yet none of these were found for WT (Table 4 and Figure S2). Note that there is a 256-fold decrease in the MIC of erythromycin against *tolC*, compared with WT (Table 3), indicating that the efflux of erythromycin is tightly related to TolC in EBU45301.

Berberine is known as a plant-isolated natural product exhibiting antibacterial activity against nosocomial pathogens like *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Also, berberine (hydrochloride) was considered as a substrate of the AcrB-homologous transporters in *A. baumannii* and *P. aeruginosa* (Morita et al., 2016; Li et al., 2021b). Here, we assessed the activity of BBH against the EBU45301 strains, and found that there is an at least 2-fold decrease in the MIC of BBH for *tolC*, compared with WT (Table 3). This result indicated that BBH displays intrinsic antibacterial activity for EBU45301 and can interact with one or more TolC-related efflux pumps in this bacterium.

It has been well characterized that *Bacillus* strains are able to synthesize various secondary metabolites, including fengycin, iturin, surfactin, difficidin, bacilysin, bacillibactin, bacillaene, amylocyclicin, subtilosin A, sublancin, and macrolactin, that possess antibacterial activity (Wilson et al., 1987; Scholz et al., 2014; Cavera et al., 2015; Liu et al., 2019; de Souza Freitas et al., 2020; Lv et al., 2020; Li et al., 2021a; Chakraborty et al., 2022; Erega et al., 2022; Zhou et al., 2022). There is a recent study showing that the transportation of bacilysin and bacillaene can be affected by RND-type transporters in *Campylobacter jejuni* (Erega et al., 2022). In the present study, we used the crude extract from strain GHZJ-1, which is a laboratory stock initially identified as *Bacillus* sp. and found exhibiting anti-yeast activity (data not shown), as one antimicrobial agent used for the susceptibility tests with the EBU45301 strains, see materials and methods for details. Obviously, comparing with the commercial antibiotic products, BCE used here was a crude agent and its exact concentration was difficult to determine. Thus, we first tested the MIC of BCE prepared from different batches with the EBU45301 strains. Our data revealed that BCE displays antibacterial activity against

TABLE 4 Phenotypes of the EBU45301 *tolC* mutant in drug susceptibility (disk/well-diffusion method).

Antimicrobial	Diameter of the inhibition zone (mm) for							
	WT	*tolC*	*tolC*/comp[a]	*tolC*/vect[b]	*p*-1[c]	*p*-2[c]	*p*-3[c]	*p*-4[c]
ß-lactam								
Piperacillin	28.5 ± 0.5	33.5 ± 1.0	28.3 ± 0.6	34.3 ± 1.6	0.0015	0.0015	0.0037	0.4883
Cefuroxime	22.8 ± 1.0	26.2 ± 1.5	22.2 ± 0.3	27.7 ± 1.3	0.0354	0.0112	0.0018	0.2595
Cefoperazone	30.7 ± 1.1	34.3 ± 1.2	29.2 ± 0.8	32.5 ± 0.5	0.0180	0.0030	0.0032	0.0651
Ceftriaxone	29.0 ± 2.0	28.8 ± 1.8			0.9189			
Aminoglycoside								
Gentamicin	19.5 ± 0.5	22.1 ± 1.2	19.6 ± 0.4	22.0 ± 0.5	0.0290	0.0270	0.0030	0.9126
Kanamycin	19.8 ± 0.3	22.8 ± 0.3	18.8 ± 0.8	21.7 ± 0.8	0.0002	0.0011	0.0105	0.0686
Amikacin	21.7 ± 0.6	24.3 ± 1.2	20.2 ± 0.3	23.8 ± 0.3	0.0232	0.0037	0.0001	0.5072
Streptomycin	16.2 ± 0.3	18.5 ± 0.5	17.0 ± 0.5	20.3 ± 0.6	0.0022	0.0213	0.0016	0.0142
Tetracycline								
Tetracycline	23.7 ± 0.6	27.5 ± 1.3	24.3 ± 0.6	27.5 ± 0.5	0.0100	0.0191	0.0020	1.0000
Minocycline	17.2 ± 0.8	24.3 ± 1.5	17.3 ± 0.3	22.8 ± 1.6	0.0019	0.0015	0.0043	0.3063
Doxycycline	16.3 ± 0.8	24.5 ± 1.0	18.3 ± 1.0	26.5 ± 0.5	0.0004	0.0018	0.0003	0.0363
Fluoroquinoline								
Ciprofloxacin[d]	26.2 ± 0.3	29.7 ± 1.2	25.7 ± 0.3	28.8 ± 0.8	0.0070	0.0043	0.0026	0.3560
Norfloxacin[d]	28.2 ± 0.6	31.2 ± 1.3	27.7 ± 0.8	29.7 ± 0.6	0.0199	0.0146	0.0224	0.1338
Levofloxacin	32.3 ± 0.8	37.2 ± 0.3	27.8 ± 2.8	36.8 ± 0.8	0.0005	0.0267	0.0055	0.5185
Phenicol								
Florfenicol	25.3 ± 1.5	35.5 ± 0.9	24.7 ± 1.3	35.3 ± 0.6	0.0006	0.0003	0.0002	0.7953
Folate pathway antagonist								
SXT[e]	25.0 ± 1.8	30.7 ± 0.8	24.7 ± 2.1	29.3 ± 1.9	0.0074	0.0094	0.0453	0.3211
Macrolide								
Erythromycin	ND[f]	17.8 ± 0.8	ND	17.7 ± 1.2	NA[f]	NA	NA	0.8450
Azithromycin	16.2 ± 0.8	19.7 ± 0.3	16.0 ± 0.4	20.0 ± 0.9	0.0018	0.0003	0.0020	0.5614
Lincosamide								
Lincomycin	None	Exist, ND	None	Exist, ND	NA	NA	NA	NA
Clindamycin	ND	18.0 ± 2.0	ND	16.7 ± 2.3	NA	NA	NA	0.4918
BCE	7.2 ± 0.2	10.4 ± 0.3	7.8 ± 0.1	11.0 ± 0.3	0.0001	0.0001	0.0001	0.0872

[a] For tests with tetracycline, doxycycline, and BCE, the *tolC*/comp (complementation) strain used was *tolC*/pBYL030. For the rest cases, the *tolC*/complementation strain used was *tolC*/pBYL024.
[b] For tests with tetracycline, doxycycline, and BCE, the *tolC*/vect (vector) strain used was *tolC*/pBYL031. For the rest cases, the *tolC*/vector strain used was *tolC*/pACYC184.
[c] *p*-1, the *p*-value comparing *tolC* with WT; *p*-2, the *p*-value comparing *tolC*/complementation with *tolC*; *p*-3, the *p*-value comparing *tolC*/vector with *tolC*/complementation; *p*-4, the *p*-value comparing *tolC*/vector with *tolC*.
[d] In the cases of ciprofloxacin or norfloxacin against the *tolC*$^+$ strains, an inhibition zone with complete none growth of bacteria (inner) and an inhibition zone with limited growth of bacteria (outer) were observed (Figure S1). Here, we considered both these areas as the inhibition zones for diameter determination.
[e] SXT, trimethoprim-sulfamethoxazole.
[f] ND, not determine-able: In the cases of erythromycin against the *tolC*$^+$ strains, the inhibition zones were not big enough for precise determination of diameters; In the cases of lincomycin against the *tolC*$^-$ strains and clindamycin against the *tolC*$^+$ strains, the inhibition zones were not regular enough for precise determination of diameters. NA, not applicable.

EBU45301 and that its MICs against WT and *tolC* are 0.031x original (BCE stock) and 0.016x original, respectively, no matter which batch of the BCE product was used (Table 3). Moreover, using the well-diffusion method, BCE formed larger inhibition zones for *tolC* than that for WT (10.4 mm vs. 7.2 mm; Table 4), and the variations among different batches were highly small (Figure S2), indicating that the antibacterial activity of BCE against EBU45301 is fairly stable. These results suggested the existence of the TolC pump-transported antibacterial metabolite (s) within BCE, like that observed in *C. jejuni* (Erega et al., 2022). Undoubtedly, identification of these specific metabolite(s) warrants more experiments.

The increased susceptibility to antimicrobial agents in the *tolC* mutant was complemented *via* introduction of the wild-type *tolC* (Tables 3, 4, Figures S1, Figure S2), which confirmed the TolC effect on the susceptibility profile in this organism. Note that some of the complementation performed using plasmid pBYL024 is not exactly complete. This is likely due to the slight growth defect in strains harboring the pACYC184-based plasmids (Figure S3), which was not observed for strains carrying the plasmids (pBYL030-033)

whose introduction was facilitated *via* integration into the chromosome (data not shown).

Synergistic effects combining BCE and other TolC-affected antibiotics

The altered susceptibility to BCE observed in the *tolC* mutant suggests that there are substances capable of traveling through one or more TolC-mediated efflux pumps in this extract (Tables 3, 4). Thus, BCE can be considered as a competitor of some other TolC-affected antibiotics for the capacity of efflux pumps regarding TolC. Besides, BCE is likely to contain lipopeptides, for instance, surfactin, that are able to change the cell-wall permeability and therefore potentiate the activity of other antibiotics against gram-negative bacteria (Liu et al., 2019). Taken together, we hypothesized that combinations of BCE and some TolC-affected antibiotics can confer synergistic effects against EBU45301. Here, we selected erythromycin, norfloxacin, and ciprofloxacin, whose MICs were decreased the most in EBU45301 strains devoid of *tolC* (Table 3), as the agents respectively used in association with BCE, for synergistic assays. As shown in Table 5, weak synergistic effects were observed for combinations of BCE/erythromycin: in the presence of 0.5x MIC BCE, the MIC of erythromycin against EBU45301 WT was 384 mg/L (0.25x MIC), and *vice versa*. However, such synergistic effects were not observed for combinations of BCE/norfloxacin or ciprofloxacin (Table 5). It has been characterized that the checkerboard method is unable to fully test synergistic effects due to its twofold dilution feature (Grimsey et al., 2020). Thus, we also performed synergistic assays using the well-diffusion method, as previously described (Grimsey et al., 2020). Our results showed that in the presence of 12 mg/L (0.008x MIC) erythromycin, the antibacterial activity of BCE against WT is increased, compared with that in the absence of antibiotics (9.9 mm vs. 7.4 mm; Figure 2). This increased phenotype in BCE activity was enhanced in the presence of 24 mg/L (0.016x MIC) erythromycin (11.6 mm vs. 7.4; Figure 2). Similarly, the BCE activity against WT was elevated in the presence of 0.006 mg/L (0.016x MIC) norfloxacin (9.9 mm vs. 7.4 mm) or 0.5 μg/L (0.016x MIC) ciprofloxacin (8.8 mm vs. 7.4 mm), and yet the existence of neither of these antibiotics at 0.008x MIC (0.003 mg/L or 0.25 μg/L) could potentiate the BCE activity (Figure 2). It appeared that 0.016x MIC norfloxacin increases the BCE activity slightly more than 0.016x MIC ciprofloxacin (Figure 2). These results revealed that at sub-MICs, erythromycin, norfloxacin, and ciprofloxacin can all enhance the BCE activity inhibiting EBU45301 with erythromycin being the most efficient. Our findings also supported that BCE used in this study can serve as a resource developing novel antibacterial agents or antibiotic adjuvants against *E. bugandensis*. Of course, analysis and isolation of the active substances within BCE is inevitable in future studies.

Discussion

E. bugandensis is an emerging pathogen in which MDR strains are frequently isolated, which poses serious problems in their treatments during nosocomial infections (Doijad et al., 2016; Pati et al., 2018; Singh et al., 2018; Falgenhauer et al., 2019; Matteoli et al., 2020; Moon et al., 2021). Genomic analyses have demonstrated that many efflux pump genes are included in genomes of *E. bugandensis* strains (Matteoli et al., 2020), and yet a comprehensive description of the relationship between efflux pumps and antibiotic susceptibility/resistance in this species has not been reported. Here, we studied the effect of TolC, an outer-membrane component of different efflux pumps, on the susceptibility to a range of 26 antimicrobial agents in a newly reclassified *E. bugandensis* strain, CMCC(B) 45301 (EBU45301). We also investigated whether particular combinations of the TolC-affected natural product/antibiotic exhibited synergistic effects. The goal of

TABLE 5 Synergistic tests using BCE in combination with certain TolC-affected antibiotics (checkerboard method).

Antibiotic	Concentration of BCE (Fraction of MIC[a])	MIC (mg/L) against WT
Erythromycin	None	1,536
	0.125x	1,536
	0.25x	768
	0.5x	384
Norfloxacin	None	0.384
	0.125x	0.384
	0.25x	0.384
	0.5x	0.384
Ciprofloxacin	None	0.032
	0.125x	0.032
	0.25x	0.032
	0.5x	0.032

[a] Note that the MIC of BCE against WT was 0.031x original (see Table 3).

this study is to elucidate the physiological role of TolC in the antimicrobial susceptibility profile of EBU45301, which might contribute to the development and application of new therapeutic options against (MDR) *E. bugandensis*.

In the present study, we sequenced the complete genome and plasmid harbored in the *Enterobacter* strain CMCC(B) 45301 (GenBank accession No. CP097254-CP097255), and reidentified this organism from *E. cloacae* to *E. bugandensis*, on the basis of the ANI of its genomic assembly against the best-matching type-strain assembly of *E. bugandensis* (Ciufo et al., 2018). This provides a full-detail reference of the EBU45301 genome, which is still scarce within species *bugandensis*. Using this reference sequence, we identified the specific EBU45301 homologues of the respective *tolC* dependent efflux-pump genes characterized in *E. coli* and *E. cloacae* (Table 1). In addition, some putative efflux-pump genes whose products display homology with components involved in the known TolC-related efflux pumps were located in the EBU45301 genome (Tables S1-3). The specific homologues for some of these genes were not found in the genome of *E. cloacae* ATCC 13047 (Tables S2, Table S3), indicating that they are not part of the core resistome in genus *Enterobacter*. All these comparative results suggested a significant role of TolC in EBU45301. Intriguingly, we noticed that genes of M1V99_14575, M1V99_14580 (Table S2), M1V99_14525, M1V99_14530, and M1V99_14535 (Table S3) are included within a ~90,000 bp sequence (Position 2864027-2954705) in the EBU45301 genome that is not highly identical to any sequences but one within the genome of *E. ludwigii* strain UW5 (Position 3536652-3627328), BLASTing it against the NCBI database. Moreover, several hypothetical MF transporters are encoded in this big-chunk DNA fragment, suggesting a unique armory of MDR mechanisms in EBU45301. More research regarding this fragment may provide insights into areas of how MDR genes are transferred horizontally among environmental strains.

We constructed a deletion mutant for *tolC* and subjected it to antimicrobial susceptibility tests, along with the wild type. It is found that, following the guidelines of breakpoints recommended for *Enterobacteriaceae*, the wild-type EBU45301 is resistant to cefazolin, weakly susceptible to streptomycin, doxycycline, minocycline, and chloramphenicol, susceptible to piperacillin, cefuroxime, ceftazidime, ceftriaxone, cefoperazone, imipenem, amikacin, gentamicin, kanamycin, tetracycline, norfloxacin, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole (Tables 2-4). Furthermore, the *tolC* deletion resulted in the increased susceptibility to all the assayed antimicrobial agents, except cefazolin, ceftazidime, ceftriaxone, and imipenem, in EBU45301 (Tables 2-4). However, it has been described that efflux pumps regarding TolC are likely to be capable of transporting cefazolin, ceftazidime, ceftriaxone, and imipenem in *E. coli* or certain *Enterobacter* species (Chang et al., 2007; Perez et al., 2007; Moosavian et al., 2021). The phenotype unresponsive to the *tolC* deletion for these four ß-lactams in EBU45301 indicated that the deletion phenotype is not obvious enough, as the overexpression phenotype, or that even similar efflux pumps identified in closely-related species vary in their specific substrates (Perez et al., 2007; Chang et al., 2007; Guerin et al., 2016). Despite of this, the effect of TolC-involved efflux pumps on other tested antibiotics belonging to ß-lactam, aminoglycoside, tetracycline, fluoroquinolone, phenicol, macrolide, lincosamide, and folate pathway antagonist observed in EBU45301 is similar to that found in *E. coli* or *Enterobacter*, as listed in Table S4. Moreover, the *tolC* strain exhibited increased susceptibility to the natural product-based agents, BBH and BCE (Tables 3, 4), in EBU45301, which to our best knowledge has not been characterized for *Enterobacter*.

Berberine or BBH can be used as an efflux-pump inhibitor in association with some efflux-pumped antibiotics against MDR *A. baumannii* and *P. aeruginosa* (Morita et al., 2016; Li et al., 2021b). However, there was no synergistic effects detected combining BBH and the respective TolC-affected antibiotics, erythromycin, norfloxacin, ciprofloxacin, chloramphenicol, and tetracycline, in EBU45301 (data not shown). This might be due to the just-constitutive expression of the efflux pump genes in EBU45301, different from the strains previously used for testing the activity of certain combinations of efflux-pumped antibiotic/efflux-pump inhibitor, which are either spontaneously or artificially MDR (Morita et al., 2016; Grimsey et al., 2020; Li et al., 2021b). Therefore, in our future studies, synergistic tests using BBH and other TolC-affected antibiotics will be performed with artificial MDR strains, such as the *ramR* mutant, in EBU45301. Given the fact that BCE includes substances TolC efflux pump-transported, we also assayed the synergistic effects using BCE in combination with erythromycin, norfloxacin, or ciprofloxacin, three antibiotics the susceptibility to which was affected the most in *tolC*. Our results demonstrated that erythromycin, norfloxacin, and ciprofloxacin can potentiate the activity of BCE against EBU45301, at their sub-MICs (Table 5 and Figure 2). Erythromycin exhibited the highest ability to potentiate the BCE activity against EBU45301, and norfloxacin seemed to be more efficient as an adjuvant for BCE than ciprofloxacin (Figure 2). It appeared that the more an antibiotic is affected by TolC, the better a synergistic compound for BCE against EBU45301 it is. To elucidate this, more experiments are required. In terms of BCE, we surely can consider it as a reservoir isolating for active substances as the antibacterial agents or antibiotic adjuvants against *E. bugandensis* in the future. However, we still have a long way to go. First, whole genome sequencing of the *Bacillus* strain used in this study is warranted for typing it to a specific species and providing a genetic reference including clusters encoding different secondary metabolites. Secondly, antibacterial substances, especially those with the efflux-pumped features, like

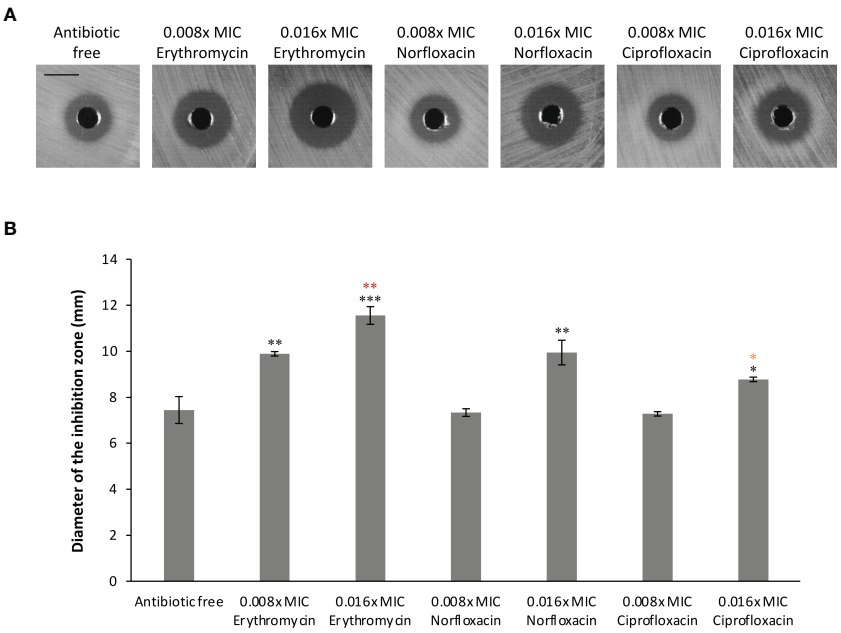

FIGURE 2
Synergistic effects combining BCE and erythromycin, norfloxacin, or ciprofloxacin against EBU45301. The susceptibility of WT to BCE was tested in the presence of different concentrations (fraction of MIC) of erythromycin, norfloxacin, or ciprofloxacin as indicated, using the well-diffusion method (see materials and methods). The inhibition zones formed by different BCE/antibiotic combinations against WT **(A)** were observed and determined for diameters **(B)**. The shown images are a representative of three independent experiments. All the black bars represent 10 mm. The diameter of inhibition zones was calculated as average ± SD of three independent experiments. * (black), $p \leq 0.05$; ** (black), $p \leq 0.01$; *** (black), $p \leq 0.001$ comparing the inhibition-zone diameter with that for BCE against WT, in the absence of antibiotic. ** (red), $p \leq 0.01$ comparing the inhibition-zone diameter for BCE in combination with 0.016x MIC erythromycin with that for BCE in combination with 0.008x MIC erythromycin. * (orange), $p \leq 0.05$ comparing the inhibition-zone diameter for BCE in combination with 0.016x MIC ciprofloxacin with that for BCE in combination with 0.016x MIC norfloxacin.

bacilysin and bacillaene in *C. jejuni* (Erega et al., 2022), and the antibiotic-adjuvant features, like surfactin in *E. coli* and subtilosin A in *Gardnerella vaginalis* (Cavera et al., 2015; Liu et al., 2019), are required to be identified and isolated for more specific studies. Finally, experiments about genetic modification or incubation conditions promoting the production of useful metabolites of our *Bacillus* strain are desired, for a more efficient application of this organism.

In summary, the present study revealed that TolC is involved in the efflux of a broad range of antimicrobial agents encompassing antibiotic families of ß-lactam, aminoglycoside, tetracycline, fluoroquinolone, phenicol, macrolide, lincosamide, and folate pathway antagonist, and natural product-based agents, like BBH and BCE, in EBU45301. In addition, certain combinations of the TolC-affected agents, namely BCE and erythromycin, norfloxacin, or ciprofloxacin, displayed synergistic effects combating EBU45301. This investigation provided a comprehensive description of the TolC effect on multidrug susceptibility/resistance in *E. bugandensis*, which can be used as a reference for the future emergence of MDR isolates belonging to this species and as research endorsing the development of more therapeutic strategies against this opportunistic pathogen.

Materials and methods

Bacterial strains and growth conditions

All the bacterial strains used in the present study are listed in Table 6. Except where indicated, the *E. coli* and EBU45301 strains were grown in LB broth or agar (Solarbio, Beijing, China) at 37°C aerobically. If required, 12.5 mg/L chloramphenicol (Solarbio) or 10 mg/L tetracycline (Solarbio), was used for the *E. coli* and EBU45301 strains.

Whole genome sequencing of EBU45301

In this study, the whole genome sequencing of EBU45301 was accomplished using a combination of the Pacbio and Illumina platforms. For sequencing performed by the Pacbio platform, the total DNA isolated from EBU45301 was subjected to construction of the 10Kb (kilo base) SMRTbell library, using the SMRTbell™ Template kit version 1.0. The constructed library was tested using Qubit and Agilent 2100 for its quality and fragment-size, respectively, followed by the sequencing conducted *via* PacBio

TABLE 6 Bacterial strains used in this study.

Strain/Plasmid	Genotypes[a]	Source
Strain		
EBU45301 (WT)	The *E. bugandensis* CMCC(B) 45301 strain	CMCC
S17-1 λpir/pBYL009	The *E. coli* S17-1 λpir strain transformed with pBYL009 (Cm^r)	This study
tolC	The clean deletion mutant for *tolC* in EBU45301 (mediated by pBYL009)	This study
DH5α/pBYL024	The *E. coli* DH5α strain transformed with pBYL024 (Cm^r)	This study
tolC/pBYL024	The *tolC* strain transformed with pBYL024 (Cm^r)	This study
tolC/pACYC184	The *tolC* strain transformed with pACYC184 (Cm^r, Tc^r)	This study
S17-1 λpir/pBYL030	The *E. coli* S17-1 λpir strain transformed with pBYL030 (Cm^r)	This study
S17-1 λpir/pBYL031	The *E. coli* S17-1 λpir strain transformed with pBYL031 (Cm^r)	This study
tolC/pBYL030	The *tolC* strain with pBYL030 integrated into the genome (Cm^r)	This study
tolC/pBYL031	The *tolC* strain with pBYL031 integrated into the genome (Cm^r)	This study
S17-1 λpir/pBYL032	The *E. coli* S17-1 λpir strain transformed with pBYL032 (Tc^r)	This study
S17-1 λpir/pBYL033	The *E. coli* S17-1 λpir strain transformed with pBYL033 (Tc^r)	This study
tolC/pBYL032	The *tolC* strain with pBYL032 integrated into the genome (Tc^r)	This study
tolC/pBYL033	The *tolC* strain with pBYL033 integrated into the genome (Tc^r)	This study
Bacillus sp. GHZJ-1	The *Bacillus* strain GHZJ-1 isolated from a waste-water pond of Panjin Guanghe Crab Industry Co., Ltd., Panjin, China	Lab stock
Plasmid		
pRE112	Cloning vector (Cm^r)	Edwards et al., 1998
pACYC184	Cloning vector (Cm^r, Tc^r)	Chang and Cohen, 1978
pDMS197	Cloning vector (Tc^r)	Edwards et al., 1998
pBYL009	The Δ*tolC* fragment cloned in pRE112 (Cm^r)	This study
pBYL024	The *tolC* fragment cloned in pACYC184 (Cm^r)	This study
pBYL030	The *tolC* fragment cloned in pRE112 (Cm^r)	This study
pBYL031	The $tolC^{UP}$ fragment cloned in pRE112 (Cm^r)	This study
pBYL032	The *tolC* fragment cloned in pDMS197 (Tc^r)	This study
pBYL033	The $tolC^{UP}$ fragment cloned in pDMS197 (Tc^r)	This study

[a] Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant.

Sequel. For sequencing performed by the Illumina platform, the total DNA was randomly broken into fragments of ~350 bp using the Covaris shearing instrument, and a library of these fragments was constructed according to the manufacturer's instructions of the NEBNext®Ultra™ DNA Library Prep Kit for Illumina (NEB, MA, USA). Similarly, the Illumina library was subjected to quality tests by Qubit and fragment-size checking by Agilent 2100, which was followed by the sequencing conducted *via* Illumina NovaSeq PE150. The original sequencing data were filtered for the elimination of low-quality reads and adaptors, and the obtained clean data were assembled using the software SMRT Link version 5.0.1, as previously described (Ardui et al., 2018; Reiner et al., 2018).

Construction of phylogenic tree based on the *tolC* gene sequences

The *tolC* gene sequences were retrieved from the genomic sequences of the type or recommended reference strains representing their species in *Enterobacter*, as indicated in Figure 1A. These *tolC* sequences were aligned by MUSCLE (Codons) in MEGA version 11, the result of which was subsequently saved and used for phylogenic-tree construction *via* the method of maximum-likelihood. The bootstrap values were determined based on 500 replications.

Alignment of the amino acid sequences of TolC from different *Enterobacter* strains

The alignment of the nucleotide sequences of *tolC* described above (for the phylogenic tree) was translated into amino acid sequences using the translating function for alignments in MEGA version 11. The resulted alignment of the amino acid sequences was saved as a FASTA file. This file was opened, edited for display settings, and finally exported as a figure using the software DNAMAN version 10.

Construction of strains for the *tolC* deletion and complementation

To construct a clean deletion for *tolC* in EBU45301, a pRE112-based system was used as previously described (Edwards et al., 1998). Generally, the upstream and downstream of the EBU45301 *tolC* gene were PCR amplified using the primer pairs $tolC^{UP}$-F: 5'-GAG CTC CCA GAT AGC TCA ACA CCG GT-3'/$tolC^{UP}$-R: 5'-GGC GTG ATA ACA CTC TTG CAT TCC TTG TTG TGA AG-3' and $tolC^{DOWN}$-F: 5'-CAA CAA GGA ATG CAA GAG TGT TAT CAC GCC CTC TC-3'/$tolC^{DOWN}$-R: 5'-GGT ACC CAT CAT GTA ACC TGC CAT TAA T-3', respectively. These two DNA fragments were subsequently spliced by overlap extension (SOE) PCR (Thornton, 2016), to obtain the DNA product of Δ*tolC*, flanked by the built-in sequences of Sac I and Kpn I. The Δ*tolC* fragment was subcloned into pRE112 between Sac I and Kpn I, resulting in plasmid pBYL009. pBYL009 was then transformed into *E. coli* S17-1 λpir, and the resultant strain was used along with the wild-type EBU45301 for conjugation performed at 30°C. The conjugants of EBU45301 were selected as chloramphenicol-resistant (Cm^r) and cefazolin-resistant (Cz^r) colonies in LB plates supplemented with 20 mg/L cefazolin (Apexbio, TX, USA) and 12.5 mg/L chloramphenicol. These conjugants were subsequently streaked on LB plates supplemented with 6% (w/v) sucrose (Solarbio) for separated single colonies, randomly picking and patching of which were conducted simultaneously on LB plates supplemented with or without chloramphenicol. The sucrose-resistant but chloramphenicol-sensitive colonies were further subjected to PCR tests using the primer pair $tolC^{UP}$-F/$tolC^{DOWN}$-R, and isolates of the *tolC* mutant were screened out as the ones from which a ~1100 bp product was amplified by this primer pair.

To complement the *tolC* deletion, fragment of the wild-type *tolC* gene was PCR amplified using the primer pair *tolC*-F1: 5'-CCC GTC CTG TGG ATC CTC GCC CTC TTC GAT CAT CC-3'/*tolC*-R1: 5'-CCG GCG TAG AGG ATC CAT CAT GTA ACC TGC CAT TAA T-3'. In this way, the *tolC* fragment was flanked by the sequences (underlined) homologous with those in plasmid pACYC184. This *tolC* fragment and the linear pACYC184, digested at site BamH I, were ligated according to the manufacturer's instructions of the ClonExpress Ultra One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China), and the resulted plasmid was designated as pBYL024. The *tolC* mutant was subsequently transformed with pBYL024, and the *tolC*/pBYL024 strains were isolated as Cm^r colonies. In the meanwhile, the *tolC* mutant was transformed with pACYC184, and the Cm^r transformants were isolated and used as a control strain for the empty vector. Given that the tetracycline-resistant (Tc^r) feature of pACYC184 is not suitable for the susceptibility tests using antibiotics like tetracycline and doxycycline, the fragment including gene *tolC* and its upstream and downstream was inserted in pRE112, to obtain an alternative complementing plasmid. Briefly, the *tolC* fragment was PCR amplified using the primer pair *tolC*-F2: 5'-TAT CGC ATG CGG TAC CTC GCC CTC TTC GAT CAT CC-3'/*tolC*-R2: 5'-TTC TTC TAG AGG TAC CAT CAT GTA ACC TGC CAT TAA T-3'. The sequences homologous with those in pRE112 have been underlined. This fragment was then subcloned in pRE112 at site Kpn I, in the same manner as that described for pBYL024, and the resulted plasmid was designated as pBYL030. As shown in Figure S4, the suicide plasmid pBYL030 is introduced and integrated into the genome of the *tolC* mutant, *via* the homologous recombination during the conjugation of S17-1 λpir/pBYL030 and *tolC*. The *tolC*/pBYL030 strain used in this study was selected as Cm^r and Cz^r colonies in which the homologous recombination was occurred within the upstream of *tolC* (Figure S4). To check this, PCR tests using the primer pair *tolC*-CF:5'-AGG GCG GTC AGG TAA ACT CT-3'/*tolC*-CR1: 5'-GCG TGT TAC GGT GAA AAC CT-3' were performed, as described in Figure S4. The vector-control strain, *tolC*/pBYL031, was constructed in the same manner as that described for *tolC*/pBYL030, except the different primer pair used for cloning of the upstream of *tolC* ($tolC^{UP}$; Figure S5). This primer pair was *tolC*-F2/*tolC*-R3: 5'-TTC TTC TAG AGG TAC TTG CAT TCC TTG TTG TGA AG-3'. In addition, to perform complementation suitable for the susceptibility tests using chloramphenicol, the other alternative complementing plasmid, pBYL032, and its vector-control, pBYL033, were obtained, in the same manner as that described for pBYL030 and pBYL031, except that pDMS197 was used instead of pRE112 for cloning. Strain *tolC*/pBYL032 and *tolC*/pBYL033 were selected as Tc^r and Cz^r colonies, *via* the same pipelines constructing *tolC*/pBYL030 and *tolC*/pBYL031 (Figures S4, S5). To check the desirable *tolC*/pBYL032 isolates, the primer pair used for PCR was *tolC*-CF/*tolC*-CR2: 5'-GAC AGC ATC GCC AGT CAC TA-3'.

Initial identification of the *Bacillus* sp. GHZJ-1 strain

The bacterial strain GHZJ-1 was isolated from a waste-water pond possessed by the Panjin Guanghe Crab Industry Co., Ltd and exhibited anti-yeast activity in other studies of our group (data not shown). To identify this bacterium, the partial region of its 16S rDNA gene was PCR amplified using the universal primer pair 27F/1492R. The PCR products were subjected to sanger sequencing, and the 16S rDNA sequence of GHZJ-1 was subsequently assembled (GenBank accession No. OP316901). BLASTing this sequence against the NCBI database, its homologues in strains identified as *Bacillus amyloliquefaciens* and *Bacillus velezensis* were shown with the highest score (bits), 100% identities and 100% query cover. Thus, GHZJ-1 was initially identified as a *Bacillus* strain.

Preparation of BCE

To prepare the crude extract of *Bacillus* sp. GHZJ-1, the strain was grown in YPD broth (Solarbio) at 28°C with aeration for 72 hours (h). Subsequently, the YPD culture of GHZJ-1 was subjected to 1.5-h sonication (every 15-second sonication process was followed by a 15-second pause) and centrifugation at 4°C, to obtain the supernatant. This supernatant was further sterilized *via* the 0.22-micrometer filters (Sangon Biotech, Shanghai, China) and finally used as the BCE agent (original) in susceptibility tests against EBU45301. In this manner, three independent batches of BCE were prepared for the susceptibility tests. The BCE stocks were preserved at -80°C and thawed on ice before use.

Antimicrobial susceptibility test

In this study, the MICs of piperacillin, cefazolin, ceftazidime, imipenem, gentamicin, kanamycin, tetracycline, norfloxacin, ciprofloxacin, chloramphenicol, and erythromycin were determined using the method of broth microdilution, according to the standards for the dilution methods provided by CLSI, 2018a. Briefly, the tested antibiotics at desired concentrations were prepared using cation-adjusted Mueller-Hinton broth (CAMHB; Solarbio) in a twofold-dilution manner and aliquoted in 96-well microtiter plates. The final cell density of the inoculum for each well was 5x 10^5 CFU/milliliter (mL). The microtiter plates were incubated at 37°C for 18 h in an ambient air incubator, and the MIC of antibiotics was measured as their lowest concentration that completely inhibit bacteria growth (CLSI, 2018a). The concentrations used for the tested antibiotics ranged as follows: piperacillin (Solarbio), 64-0.031 mg/L; cefazolin (Apexbio), 512-0.5 mg/L; ceftazidime (Solarbio), 64-0.031 mg/L; imipenem (Solarbio), 16-0.008 mg/L; gentamicin (Solarbio), 32-0.016 mg/L; kanamycin (Solarbio), 32-0.016 mg/L; tetracycline (Solarbio), 32-0.016 mg/L; norfloxacin (Solarbio), 6.144-0.0015 mg/L; ciprofloxacin (Solarbio), 0.512-0.00025 mg/L; chloramphenicol (Solarbio), 128-0.06 mg/L; erythromycin (Solarbio), 6,144-0.75 mg/L. Three independent experiments were performed to determine the MICs of these antibiotics, and in every independent experiment, the tests for each concentration used were conducted in triplicates. The disk-diffusion tests were performed according to the standards for the methods of disk diffusion provided by CLSI, 2018b, using the antibiotic disks (BKMAM, Hunan, China) as follows: piperacillin, 100 micrograms (μg); cefuroxime, 30 μg; ceftriaxone, 30 μg; cefoperazone, 75 μg; amikacin, 30 μg; gentamicin, 10 μg; kanamycin, 30 μg; streptomycin, 10 μg; tetracycline, 30 μg; minocycline, 30 μg; doxycycline, 30 μg; levofloxacin, 5 μg; florfenicol, 30 μg; trimethoprim-sulfamethoxazole, 1.25-23.75 μg; erythromycin, 15 μg; azithromycin, 15 μg; lincomycin, 2 μg; clindamycin, 2 μg. Briefly, the tested bacteria were seeded in the Mueller-Hinton agar (MHA; Solarbio) plates *via* evenly coating the plates with streaks of the inoculum (0.5 McFarland standard). The antibiotic disks were then placed properly in the inoculated MHA plates, which was followed by 18-h incubation at 37°C of these plates in an ambient air incubator (CLSI, 2018b). The well-diffusion tests were performed for ciprofloxacin and norfloxacin. Basically, the MHA plates were evenly coated with bacterial cultures, as that described for the disk-diffusion tests (CLSI, 2018b). Afterwards, cylinder wells with 6 mm-diameter base sides were drilled in the inoculated agar with a height of approximately 3 mm. 75 microliters (μL) of 0.16 mg/L ciprofloxacin or 1.92 mg/L norfloxacin (both diluted in CAMHB) was aliquoted into each well for the tests. Finally, all the treated agar plates were incubated at 37°C for 18 hours in an ambient air incubator, to observe the inhibition zones. 75 μL CAMHB was used as the negative control for each well. Diameter of the inhibition zone for well-diffusion tests was determined as the value subtracting the well diameter (6 mm) from the whole diameter for the inhibition circle. For each tested antibiotic in disk-diffusion and well-diffusion assays, diameter of the inhibition zone was measured as average ± SD based on three independent experiments. The comparative analyses of data were performed using student's *t* tests. The susceptibility/resistance of EBU45301 to piperacillin, cefazolin, ceftazidime, imipenem, gentamicin, kanamycin, tetracycline, norfloxacin, ciprofloxacin, and chloramphenicol was determined according to the MIC-based breakpoints provided by CLSI, 2022. The susceptibility/resistance of EBU45301 to cefuroxime, ceftriaxone, cefoperazone, amikacin, streptomycin, minocycline, doxycycline, levofloxacin, and trimethoprim-sulfamethoxazole was determined according to the breakpoints based on the inhibition-zone diameters, provided by CLSI, 2022. For the susceptibility tests the readings of which were used for susceptibility/resistance determination, the *E. coli* ATCC 25922 strain was used as a quality-control strain (CLSI, 2022).

To assess the susceptibility to BBH in the EBU45301 strains, 18 mg/mL BBH was prepared using DMSO as the solvent. The MICs of BBH against the tested strains were determined using the broth-microdilution method as described above. In the meanwhile, the antibacterial activity of DMSO at the concentrations same as that contained in the serially diluted BBH was tested independently, to rule out the interference of DMSO in inhibition of EBU45301 growth. The concentrations of BBH used for the tests ranged from 2,048 mg/L to 4 mg/L.

The susceptibility of the EBU45301 strains to BCE was tested using both the broth-microdilution and well-diffusion method, as described above. To determine the MIC of BCE against the EBU45301 strains, the concentrations ranged from 0.5x original (BCE) to 0.001x original (BCE) were prepared by performing serial twofold dilutions with CAMHB and used for the tests. In the meanwhile, the antibacterial activity of YPD broth at the concentrations same as that contained in the serially diluted BCE

was tested independently, to rule out the interference of YPD broth in inhibition of EBU45301 growth. Using the same batch of BCE (three batches in total), two independent experiments were performed for each of the EBU45301 strain, and in every independent experiment, the tests for each concentration used were conducted in triplicates. In the well-diffusion assays, 75 μL BCE (original) was aliquoted into each well, and 75 μL YPD broth was used as the negative control for each well. Three independent experiments were performed with each one using BCE from different batches for comparisons. To determine the diameter of the inhibition zones formed by BCE, the average reading for the three BCE batches used in the same independent experiment was calculated first, and the final value of diameter was determined as average ± SD based on the average readings for the three independent experiments. The comparative analyses of data were performed using student's t tests.

Synergistic assay

The synergistic assays testing BCE in association with erythromycin, norfloxacin, or ciprofloxacin against the EBU45301 WT were performed using both the checkerboard method and the well-diffusion method. The checkerboard arrangements for the tested combinations were in the same manner as previously described (MacNair et al., 2018). In the checkerboard assays, the fractional inhibitory concentrations of BCE, erythromycin, norfloxacin, and ciprofloxacin against WT were determined using the broth-microdilution protocols for these four antibiotics, respectively, as described above. To better assay the synergistic effects combining BCE and erythromycin, norfloxacin, or ciprofloxacin against WT, the well-diffusion protocol described for testing the antibacterial activity of BCE was used. The inhibition zones formed by BCE in the seeded MHA plates supplemented with 12 mg/L erythromycin, 24 mg/L erythromycin, 0.003 mg/L norfloxacin, 0.006 mg/L norfloxacin, 0.25 μg/L ciprofloxacin, 0.5 μg/L ciprofloxacin, or none antibiotics were observed and determined for diameters, as described above. The comparative analyses of data were performed using student's *t* tests.

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

BL and JZ conducted the experiments. BL, JZ, and XL analyzed the data. BL and XL drafted the manuscript on the basis of the analyzed data. BL and XL designed the whole project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author XL is employed by Panjin Guanghe Crab Industry Co., Ltd.

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/fcimb.2022.1036933/full#supplementary-material

References

Annavajhala, M. K., Gomez-Simmonds, A., and Uhlemann, A. C. (2019). Multidrug-resistant enterobacter cloacae complex emerging as a global, diversifying threat. *Front. Microbiol.* 10, 44. doi: 10.3389/fmicb.2019.00044

Ardui, S., Ameur, A., Vermeesch, J. R., and Hestand, M. S. (2018). Single molecule real-time (Smrt) sequencing comes of age: Applications and utilities for medical diagnostics. *Nucleic Acids Res.* 46, 2159–2168. doi: 10.1093/nar/gky066

Cavera, V. L., Volski, A., and Chikindas, M. L. (2015). The natural antimicrobial subtilosin a synergizes with lauramide arginine ethyl ester (Lae), epsilon-Poly-L-Lysine (Polylysine), clindamycin phosphate and metronidazole, against the vaginal pathogen gardnerella vaginalis. *Probiotics Antimicrob. Proteins* 7, 164–171. doi: 10.1007/s12602-014-9183-1

Chakraborty, K., Kizhakkekalam, V. K., Joy, M., and Chakraborty, R. D. (2022). Bacillibactin class of siderophore antibiotics from a marine symbiotic bacillus as promising antibacterial agents. *Appl. Microbiol. Biotechnol.* 106, 329–340. doi: 10.1007/s00253-021-11632-0

Chang, A. C., and Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy dna cloning vehicles derived from the P15a cryptic miniplasmid. *J. Bacteriol.* 134, 1141–1156. doi: 10.1128/jb.134.3.1141-1156.1978

Chang, T. M., Lu, P. L., Li, H. H., Chang, C. Y., Chen, T. C., and Chang, L. L. (2007). Characterization of fluoroquinolone resistance mechanisms and their correlation with the degree of resistance to clinically used fluoroquinolones among escherichia coli isolates. *J. Chemother.* 19, 488–494. doi: 10.1179/joc.2007.19.5.488

Chavda, K. D., Chen, L., Fouts, D. E., Sutton, G., Brinkac, L., Jenkins, S. G., et al. (2016). Comprehensive genome analysis of carbapenemase-producing enterobacter spp.: New insights into phylogeny, population structure, and resistance mechanisms. *Mbio* 7 (6), e02093–16. doi: 10.1128/mBio.02093-16

Chow, J. W., Fine, M. J., Shlaes, D. M., Quinn, J. P., Hooper, D. C., Johnson, M. P., et al. (1991). Enterobacter bacteremia: Clinical features and emergence of antibiotic resistance during therapy. *Ann. Intern. Med.* 115, 585–590. doi: 10.7326/0003-4819-115-8-585

Ciufo, S., Kannan, S., Sharma, S., Badretdin, A., Clark, K., Turner, S., et al. (2018). Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes At the ncbi. *Int. J. Syst. Evol. Microbiol.* 68, 2386–2392. doi: 10.1099/ijsem.0.002809

CLSI (2018a). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically CLSI standard M07. 11th ed* (Wayne, PA: CLSI).

CLSI (2018b). *Performance standards for antimicrobial disk susceptibility tests CLSI standard M02. 13th ed* (Wayne, PA: CLSI).

CLSI (2022). *Performance standards for antimicrobial susceptibility testing CLSI supplement M100. 32nd ed* (Wayne, PA: CLSI).

Davin-Regli, A., and Pages, J. M. (2015). Enterobacter aerogenes and enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment. *Front. Microbiol.* 6, 392. doi: 10.3389/fmicb.2015.00392

de Souza Freitas, F., de Assis Lage, T. C., Ayupe, B. A. L., de Paula Siqueira, T., De Barros, M., and Totola, M. R. (2020). Bacillus subtilis Tr47ii as a source of bioactive lipopeptides against gram-negative pathogens causing nosocomial infections. *3 Biotech.* 10, 474. doi: 10.1007/s13205-020-02459-z

Doijad, S., Imirzalioglu, C., Yao, Y., Pati, N. B., Falgenhauer, L., Hain, T., et al. (2016). Enterobacter bugandensis sp. nov., isolated from neonatal blood. *Int. J. Syst. Evol. Microbiol.* 66, 968–974. doi: 10.1099/ijsem.0.000821

Edwards, R. A., Keller, L. H., and Schifferli, D. M. (1998). Improved allelic exchange vectors and their use to analyze 987p fimbria gene expression. *Gene* 207, 149–157. doi: 10.1016/S0378-1119(97)00619-7

Erega, A., Stefanic, P., Danevcic, T., Smole Mozina, S., and Mandic Mulec, I. (2022). Impact of bacillus subtilis antibiotic bacilysin and campylobacter jejuni efflux pumps on pathogen survival in mixed biofilms. *Microbiol. Spectr.* 10 (4), e0215622. doi: 10.1128/spectrum.02156-22

Falgenhauer, J., Imirzalioglu, C., Falgenhauer, L., Yao, Y., Hauri, A. M., Erath, B., et al. (2019). Whole-genome sequences of clinical enterobacter bugandensis isolates from Germany. *Microbiol. Resour. Announc.* 8 (29), e00465–19. doi: 10.1128/MRA.00465-19

Federhen, S., Rosello-Mora, R., Klenk, H. P., Tindall, B. J., Konstantinidis, K. T., Whitman, W. B., et al. (2016). Meeting report: Genbank microbial genomic taxonomy workshop (12–13 may 2015). *Stand Genom. Sci.* 11, 15. doi: 10.1186/s40793-016-0134-1

Fernandez-Baca, V., Ballesteros, F., Hervas, J. A., Villalon, P., Dominguez, M. A., Benedi, V. J., et al. (2001). Molecular epidemiological typing of enterobacter cloacae isolates from a neonatal intensive care unit: Three-year prospective study. *J. Hosp. Infect.* 49, 173–182. doi: 10.1053/jhin.2001.1053

Gravey, F., Cattoir, V., Ethuin, F., Fabre, L., Beyrouthy, R., Bonnet, R., et al. (2020). Ramr deletion in an enterobacter hormaechei isolate as a consequence of therapeutic failure of key antibiotics in a long-term hospitalized patient. *Antimicrob. Agents Chemother.* 64 (10), e00962–20. doi: 10.1128/AAC.00962-20

Grimsey, E. M., Fais, C., Marshall, R. L., Ricci, V., Ciusa, M. L., Stone, J. W., et al. (2020). Chlorpromazine and amitriptyline are substrates and inhibitors of the acrb multidrug efflux pump. *Mbio* 11 (3), e00465–20. doi: 10.1128/mBio.00465-20

Guerin, F., Lallement, C., Isnard, C., Dhalluin, A., Cattoir, V., and Giard, J. C. (2016). Landscape of resistance-Nodulation-Cell division (Rnd)-type efflux pumps in enterobacter cloacae complex. *Antimicrob. Agents Chemother.* 60, 2373–2382. doi: 10.1128/AAC.02840-15

Hilty, M., Sendi, P., Seiffert, S. N., Droz, S., Perreten, V., Hujer, A. M., et al. (2013). Characterisation and clinical features of enterobacter cloacae bloodstream infections occurring At a tertiary care university hospital in Switzerland: Is cefepime adequate therapy? *Int. J. Antimicrob. Agents* 41, 236–249. doi: 10.1016/j.ijantimicag.2012.10.022

Huang, L., Feng, Y., and Zong, Z. (2019). Heterogeneous resistance to colistin in enterobacter cloacae complex due to a new small transmembrane protein. *J. Antimicrob. Chemother.* 74, 2551–2558. doi: 10.1093/jac/dkz236

Kobayashi, N., Nishino, K., and Yamaguchi, A. (2001). Novel macrolide-specific abc-type efflux transporter in escherichia coli. *J. Bacteriol.* 183, 5639–5644. doi: 10.1128/JB.183.19.5639-5644.2001

Kuete, V., Ngameni, B., Tangmouo, J. G., Bolla, J. M., Alibert-Franco, S., Ngadjui, B. T., et al. (2010). Efflux pumps are involved in the defense of gram-negative bacteria against the natural products isobavachalcone and diospyrone. *Antimicrob. Agents Chemother.* 54, 1749–1752. doi: 10.1128/AAC.01533-09

Li, J., Chen, J., Yang, G., and Tao, L. (2021a). Sublancin protects against methicillin-resistant staphylococcus aureus infection by the combined modulation of innate immune response and microbiota. *Peptides* 141, 170533. doi: 10.1016/j.peptides.2021.170533

Li, Y., Huang, J., Li, L., and Liu, L. (2017). Synergistic activity of berberine with azithromycin against pseudomonas aeruginosa isolated from patients with cystic fibrosis of lung *In vitro* and *In vivo*. *Cell Physiol. Biochem.* 42, 1657–1669. doi: 10.1159/000479411

Li, X. Z., Plesiat, P., and Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria. *Clin. Microbiol. Rev.* 28, 337–418. doi: 10.1128/CMR.00117-14

Li, X., Song, Y., Wang, L., Kang, G., Wang, P., Yin, H., et al. (2021b). A potential combination therapy of berberine hydrochloride with antibiotics against multidrug-resistant acinetobacter baumannii. *Front. Cell Infect. Microbiol.* 11, 660431. doi: 10.3389/fcimb.2021.660431

Liu, S., Fang, R., Zhang, Y., Chen, L., Huang, N., Yu, K., et al. (2021). Characterization of resistance mechanisms of enterobacter cloacae complex Co-resistant to carbapenem and colistin. *BMC Microbiol.* 21, 208. doi: 10.1186/s12866-021-02250-x

Liu, J., Wang, X., Shi, W., Qian, Z., and Wang, Y. (2019). Sensitization of avian pathogenic escherichia coli to amoxicillin *In vitro* and *In vivo* in the presence of surfactin. *PloS One* 14, E0222413. doi: 10.1371/journal.pone.0222413

Liu, L., Yu, J., Tang, M., and Liu, J. (2018). Mechanisms of resistance in clinical isolates of enterobacter cloacae that are less susceptible to cefepime than to ceftazidime. *Ann. Clin. Lab. Sci.* 48, 355–362.

Lv, J., Da, R., Cheng, Y., Tuo, X., Wei, J., Jiang, K., et al. (2020). Mechanism of antibacterial activity of bacillus amyloliquefaciens c-1 lipopeptide toward anaerobic clostridium difficile. *BioMed. Res. Int.* 2020, 3104613. doi: 10.1155/2020/3104613

MacNair, C. R., Stokes, J. M., Carfrae, L. A., Fiebig-Comyn, A. A., Coombes, B. K., Mulvey, M. R., et al. (2018). Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. *Nat. Commun.* 9, 458. doi: 10.1038/s41467-018-02875-z

Matteoli, F. P., Passarelli-Araujo, H., Pedrosa-Silva, F., Olivares, F. L., and Venancio, T. M. (2020). Population structure and pangenome analysis of enterobacter bugandensis uncover the presence of blactx-M-55, blandm-5 and blaimi-1, along with sophisticated iron acquisition strategies. *Genomics* 112, 1182–1191. doi: 10.1016/j.ygeno.2019.07.003

Mezzatesta, M. L., Gona, F., and Stefani, S. (2012). Enterobacter cloacae complex: Clinical impact and emerging antibiotic resistance. *Future Microbiol.* 7, 887–902. doi: 10.2217/fmb.12.61

Moon, S. H., Udaondo, Z., Li, X., Yang, X., Jun, S. R., and Huang, E. (2021). Isolation and characterisation of carbapenemase-producing and polymyxin b-resistant enterobacter bugandensis from a vegetable. *J. Glob. Antimicrob. Resist.* 26, 264–265. doi: 10.1016/j.jgar.2021.07.013

Moosavian, M., Sima, M. K., Khosravi, N. A., and Montazeri, E. A. (2021). Detection of oqxab efflux pumps, a multidrug-resistant agent in bacterial infection in patients referring to teaching hospitals in ahvaz, southwest of Iran. *Int. J. Microbiol.* 2021, 2145176. doi: 10.1155/2021/2145176

Morita, Y., Nakashima, K., Nishino, K., Kotani, K., Tomida, J., Inoue, M., et al. (2016). Berberine is a novel type efflux inhibitor which attenuates the mexxy-mediated aminoglycoside resistance in pseudomonas aeruginosa. *Front. Microbiol.* 7, 1223. doi: 10.3389/fmicb.2016.01223

Nagakubo, S., Nishino, K., Hirata, T., and Yamaguchi, A. (2002). The putative response regulator baer stimulates multidrug resistance of escherichia coli *Via* a novel multidrug exporter system, mdtabc. *J. Bacteriol.* 184, 4161–4167. doi: 10.1128/JB.184.15.4161-4167.2002

Nikaido, H., and Zgurskaya, H. I. (1999). Antibiotic efflux mechanisms. *Curr. Opin. Infect. Dis.* 12, 529–536. doi: 10.1097/00001432-199912000-00001

Nishino, K., Yamada, J., Hirakawa, H., Hirata, T., and Yamaguchi, A. (2003). Roles of tolc-dependent multidrug transporters of escherichia coli in resistance to beta-lactams. *Antimicrob. Agents Chemother.* 47, 3030–3033. doi: 10.1128/AAC.47.9.3030-3033.2003

Nishino, K., Yamasaki, S., Nakashima, R., Zwama, M., and Hayashi-Nishino, M. (2021). Function and inhibitory mechanisms of multidrug efflux pumps. *Front. Microbiol.* 12, 737288. doi: 10.3389/fmicb.2021.737288

Pati, N. B., Doijad, S. P., Schultze, T., Mannala, G. K., Yao, Y., Jaiswal, S., et al. (2018). Enterobacter bugandensis: A novel enterobacterial species associated with severe clinical infection. *Sci. Rep.* 8, 5392. doi: 10.1038/s41598-018-23069-z

Perez, A., Canle, D., Latasa, C., Poza, M., Beceiro, A., Tomas Mdel, M., et al. (2007). Cloning, nucleotide sequencing, and analysis of the acrab-tolc efflux pump of enterobacter cloacae and determination of its involvement in antibiotic resistance in a clinical isolate. *Antimicrob. Agents Chemother.* 51, 3247–3253. doi: 10.1128/AAC.00072-07

Reiner, J., Pisani, L., Qiao, W., Singh, R., Yang, Y., Shi, L., et al. (2018). Cytogenomic identification and long-read single molecule real-time (Smrt) sequencing of a bardet-biedl syndrome 9 (Bbs9) deletion. *NPJ Genom. Med.* 3, 3. doi: 10.1038/s41525-017-0042-3

Sanders, W. E.Jr., and Sanders, C. C. (1997). Enterobacter spp.: Pathogens poised to flourish At the turn of the century. *Clin. Microbiol. Rev.* 10, 220–241. doi: 10.1128/CMR.10.2.220

Sarangi, J., Matsuo, N., Nonogaki, R., Hayashi, M., Kawamura, K., Suzuki, M., et al. (2022). Molecular epidemiology of enterobacter cloacae complex isolates with reduced carbapenem susceptibility recovered by blood culture. *Jpn J. Infect. Dis.* 75, 41–48. doi: 10.7883/yoken.JJID.2021.141

Scholz, R., Vater, J., Budiharjo, A., Wang, Z., He, Y., Dietel, K., et al. (2014). Amylocyclicin, a novel circular bacteriocin produced by bacillus amyloliquefaciens Fzb42. *J. Bacteriol.* 196, 1842–1852. doi: 10.1128/JB.01474-14

Senchyna, F., Tamburini, F. B., Murugesan, K., Watz, N., Bhatt, A. S., and Banaei, N. (2021). Comparative genomics of enterobacter cloacae complex before and after acquired clinical resistance to ceftazidime-avibactam. *Diagn. Microbiol. Infect. Dis.* 101, 115511. doi: 10.1016/j.diagmicrobio.2021.115511

Singh, N. K., Bezdan, D., Checinska Sielaff, A., Wheeler, K., Mason, C. E., and Venkateswaran, K. (2018). Multi-drug resistant enterobacter bugandensis species isolated from the international space station and comparative genomic analyses with human pathogenic strains. *BMC Microbiol.* 18, 175. doi: 10.1186/s12866-018-1325-2

Telke, A. A., Olaitan, A. O., Morand, S., and Rolain, J. M. (2017). Soxrs induces colistin hetero-resistance in enterobacter asburiae and enterobacter cloacae by regulating the acrab-tolc efflux pump. *J. Antimicrob. Chemother.* 72, 2715–2721. doi: 10.1093/jac/dkx215

Thornton, J. A. (2016). Splicing by overlap extension pcr to obtain hybrid dna products. *Methods Mol. Biol.* 1373, 43–49. doi: 10.1007/7651_2014_182

Wilson, K. E., Flor, J. E., Schwartz, R. E., Joshua, H., Smith, J. L., Pelak, B. A., et al. (1987). Difficidin and oxydifficidin: Novel broad spectrum antibacterial antibiotics produced by bacillus subtilis. ii. isolation and physico-chemical characterization. *J. Antibiot. (Tokyo)* 40, 1682–1691. doi: 10.7164/antibiotics.40.1682

Yousefian, N., Ornik-Cha, A., Poussard, S., Decossas, M., Berbon, M., Daury, L., et al. (2021). Structural characterization of the emrab-tolc efflux complex from e. coli. *Biochim. Biophys. Acta Biomembr.* 1863, 183488. doi: 10.1016/j.bbamem.2020.183488

Zhou, P., Chen, W., Zhu, Z., Zhou, K., Luo, S., Hu, S., et al. (2022). Comparative study of bacillus amyloliquefaciens X030 on the intestinal flora and antibacterial activity against aeromonas of grass carp. *Front. Cell Infect. Microbiol.* 12, 815436. doi: 10.3389/fcimb.2022.815436

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Development of a novel loop-mediated isothermal amplification assay for ß-lactamase gene identification using clinical isolates of Gram-negative bacteria

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Rapid evaluation of antimicrobial susceptibility is important in the treatment of nosocomial infections by Gram-negative bacteria, which increasingly carry carbapenemases and metallo-β-lactamases. We developed loop-mediated isothermal amplification (LAMP)-based assays for four β-lactamase genes (bla_{KPC}, bla_{NDM-1}, bla_{IMP-1} group, and bla_{VIM}). The assays were evaluated using eight reference bacterial strains (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter bereziniae*) harboring six β-lactamase genes. A total of 55 Gram-negative bacterial strains, including 47 clinical *P. aeruginosa* isolates, fully characterized by next-generation sequencing (NGS), were used to evaluate the LAMP assays. The results were compared to those of conventional PCR. The LAMP assays were able to detect as few as 10 to 100 copies of a gene, compared to 10 to 10^4 copies for conventional PCR. The LAMP assay detected four β-lactamase genes with a sensitivity similar to that using purified DNA as the template in DNA-spiked

urine, sputum, and blood specimens. By contrast, the sensitivity of PCR was 1- to 100-fold lower with DNA-spiked clinical specimens. Therefore, the LAMP assays were proved to be an appropriate tool for the detection of four β-lactamases.

KEYWORDS

loop-mediated isothermal amplification, ß-lactamase gene, Gram-negative bacteria, *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{IMP-1}$ group, *bla*$_{VIM}$

Introduction

Antimicrobial resistance (AMR) is a serious public health problem globally (Laxminarayan et al., 2016). Increased use of antimicrobial drugs promotes the emergence of antimicrobial-resistant bacterial strains and related infections. Some of those bacteria have become resistant to several antibiotics, including carbapenems and third-generation cephalosporins (Arumugham et al., 2022; Palacios-Baena et al., 2021)—the best-available antibiotics for treating infections by multidrug-resistant bacteria. In 2017, the World Health Organization (WHO) published a global priority list of antibiotic-resistant bacteria to guide research, discovery, and the development of new antibiotics (WHO, 2017). These critical multidrug-resistant bacteria include carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, and carbapenem-resistant/third-generation cephalosporin-resistant *Enterobacteriaceae* (including *Klebsiella pneumoniae, Escherichia coli*, and *Enterobacter* spp.). These have marked effects on mortality and healthcare.

Carbapenemases are categorized into Ambler class A, B, and D β-lactamases. Ambler classes A and D are serine proteases while class B enzymes are metallo-β-lactamases (MBL). *Klebsiella pneumoniae* carbapenemase (*bla*$_{KPC}$) is plasmid-encoded and is the most prevalent class A carbapenemase worldwide, including in the United States (Chen et al., 2014). New Delhi metallo-β-lactamase (*bla*$_{NDM}$), imipenem-resistant *Pseudomonas* type carbapenemase (*bla*$_{IMP}$), and Verona integron-encoded metallo-β-lactamase (*bla*$_{VIM}$) are class B carbapenemases that can be transmitted among different strains of the same bacterial species and among different bacterial species or genera (WHO, 2020b). The transmission of resistance can occur by transformation (bacteria take up DNA from the environment), transduction (DNA transfer between bacterial cells by bacteriophages), conjugation (plasmids harboring mobile elements), and the transfer of plasmids between bacterial cell *via* outer membrane vesicles. Thus, in a hospital, several bacterial species may have the same resistance determinants which were transferred by mobile genetic elements.

AMR, especially in Gram-negative bacteria, is complex, has a variety of underlying mechanisms and links to diseases in humans and other animals (WHO, 2017). The resistance of common bacteria, including *P. aeruginosa* and *Enterobacteriaceae*, is rendering treatments for common infections ineffective, worsening outcomes and increasing healthcare costs (WHO, 2020a). Global laboratory-based surveillance for AMR is now critical to understand the spread of multidrug resistant organisms as well as evaluate the impact of interventions designed to reduce the clinical and community burden of these dangerous pathogens.

Rapid, simple, and reliable identification of AMR is essential to ensure that antibiotic use is appropriate, and for surveillance in low- or middle-income countries. Carbapenem resistance is examined by calculating the minimum inhibitory concentration (MIC) using the disc diffusion test, gradient method, or synergy test (double-disc test), but these do not clarify the mechanism of drug resistances. Molecular assays are the standard tests for the identification of carbapenemase-encoding genes (Solanki et al., 2014).

Genotype-based methods (*i.e.*, PCR, real-time PCR, and microarrays) can subgroup carbapenemases and monitor the transmission of resistance. Conventional PCR-based assays can detect β-lactamase genes but require well-equipped laboratories. However, the equipment required for conventional PCR or real-time PCR assays is expensive, and these techniques are complex, hampering their adoption by laboratories with limited experience in molecular testing, particularly in low- or middle-income countries (WHO, 2019).

The loop-mediated isothermal amplification (LAMP) assay overcomes the limitations of phenotyping and PCR methods (Mori and Notomi, 2019). This method uses a unique priming mechanism that yields specific DNA products more rapidly than PCR. It does not require expensive equipment or a sophisticated laboratory. LAMP is convenient in terms of point-of-care testing (POCT). For surveillance, it is appropriate for laboratories lacking experience with molecular testing. We developed LAMP assays for four β-lactamase genes (*bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{IMP-1}$ group, and *bla*$_{VIM}$) and evaluated each using clinical strains isolated at diverse geographical locations (Kos et al., 2015). There are several previous reports mentioning the LAMP assay for the four β-lactamase genes identification (Moreno-Morales et al., 2020; Feng et al., 2021). While these reports used

isolates from a limited number of regions, this is the first report to use clinical strains isolated at diverse geographical locations. In addition, phylogenetical analysis verified the extent of its efficacy. This is in stark contrast to previous reports that simply referred to effectiveness.

Materials and methods

Bacterial strains

A total of 63 bacterial strains, 8 reference and 55 clinical were used to develop the LAMP assays. The reference strains harboring six β-lactamase genes (*bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$) were three *K. pneumoniae*, one *E. coli*, three *P. aeruginosa*, and one *Acinetobacter bereziniae* strains; all were provided by AstraZeneca (Waltham, MA) (Table 1).

Preparation of chromosomal DNA

Genomic DNA was extracted using a Maxwell 16-Cell DNA Purification kit (Promega, Madison, WI). The concentration of purified genomic DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The genomic DNA copy number was calculated based on genome size (5.4 Mbp for *K. pneumoniae* [Kp52.154, GenBank accession number: FO834906.1], 5.2 Mbp for *E. coli* [CFT073, AE014075.1], 6.5 Mbp for *P. aeruginosa* [PB369, CP025049.1], 4.5 Mbp for *A. bereziniae* [XH901, NZ_CP018259.1], and 4.0 Mbp for *A. baumannii* [ATCC19606, CP059040.1]).

The eight standard reference strains including seven β-lactamase genes (Table 1) were used to evaluate the specificity of the LAMP assay for each targeted β-lactamase gene. The specificity of each LAMP reactions was examined using 10^5 copies of reference genomic DNA per reaction. A serial 10- times dilution of reference genomic DNA was used to test the sensitivity of the LAMP assays in comparison to that of PCR.

Clinical Gram-negative bacterial strains

Fifty-five clinical Gram-negative isolates harboring β-lactamase genes were used to evaluate the LAMP assays (Table 2). Among them, 47 clinical *P. aeruginosa* strains were randomly selected from 388 strains of known genotypes and phenotypes (Kos et al., 2015) isolated at diverse geographical locations (Colombia, India, Spain, France, Greece, Germany, Argentina, Croatia, China, Brazil, Mexico, Romania, and the Philippines) between 2003 and 2012. Originally, those isolates were obtained from the International Health Management Association (IHMA). Whole-genome sequences were analyzed on the HiSeq 2000 or MiSeq platform (Illumina, San Diego, CA, United States) (Kos et al., 2015). Susceptibility to meropenem was explored using frozen Trek-Sensititre custom plates (Thermo Fisher Scientific Inc.) following the 2012 guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2012). The MICs for meropenem are listed in Table 2. Other eight clinical Gram-negative isolates including three *A. baumannii*, two *E. coli*, two *K. pneumoniae*, and one *E. cloacae* were obtained from AstraZeneca culture collection (Table 2).

LAMP primer design

To design primers for *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$, we compared the sequences of variants using Clustal X v. 2.0 (Larkin et al., 2007) to identify consensus regions and to select

TABLE 1 Reactivities and specificities of LAMP assays.

Strain ID	Species	Genotype	LAMP Assays [a]			
			bla$_{KPC}$	*bla*$_{NDM-1}$	*bla*$_{IMP-1}$	*bla*$_{VIM}$
ARC2945	*K. pneumoniae*	KPC-2	(+) [b]	(-)	(-)	(-)
ARC2929	*K. pneumoniae*	KPC-3	(+)	(-)	(-)	(-)
ARC3600	*E. coli*	NDM-1	(-)	(+)	(-)	(-)
ARC3802	*K. pneumoniae*	NDM-1	(-)	(+)	(-)	(-)
ARC2780	*A. bereziniae*	IMP-1	(-)	(-)	(+)	(-)
ARC3471	*P. aeruginosa*	VIM-2	(-)	(-)	(-)	(+)
ARC3475	*P. aeruginosa*	OXA-48	(-)	(-)	(-)	(-)
ARC3917	*P. aeruginosa*	GES-1	(-)	(-)	(-)	(-)

[a]LAMP results determined via Loopamp real-time turbidimetry and the naked eye.
[b](-), negative; (+), positive.

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TABLE 2 Clinical isolates evaluated.

Strain ID	Species	Origin of isolate		Genotype	Meropenem		LAMP Assays			
		Country	Anatomical site		MIC (mg/L)	Susceptibility	*bla*$_{KPC}$	*bla*$_{NDM-1}$	*bla*$_{IMP-1\ group}$	*bla*$_{VIM}$
KPC, NDM-1, IMP-1 group, and VIM ß-lactamase-producing strains										
AZPAE14719	*P. aeruginosa*	Colombia	RTI[a]	KPC-2	>32	(R)[d]	(+)	(-)	(-)	(-)
AZPAE14720	*P. aeruginosa*	Colombia	UTI	KPC-2, OXA-2	>32	(R)	(+)	(-)	(-)	(-)
AZECO 3801	*E. coli*	–	–	NDM-1, OXA-1, TEM-1, CTX-M-15	>8	(R)	(-)	(+)	(-)	(-)
AZKPN 4770	*K. pneumoniae*	–	–	NDM-1, OXA-1, TEM-1, CTX-M-15	>8	(R)	(-)	(+)	(-)	(-)
AZABA 5986	*A. baumannii*	–	–	NDM-1,OXA-10, OXA-23, OXA-69	>8	(R)	(-)	(+)	(-)	(-)
AZECL 5127	*E. cloacae*	–	–	IMP-1, OXA-10, OXA-48, TEM-1	>8	(R)	(-)	(-)	(+)	(-)
AZPAE14702	*P. aeruginosa*	Philippines	RTI	IMP-4, OXA-10	>32	(R)	(-)	(-)	(+)	(-)
AZPAE14703	*P. aeruginosa*	Philippines	IAI	VIM-1, OXA-10	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14811	*P. aeruginosa*	India	RTI	VIM-2, OXA-4	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14922	*P. aeruginosa*	France	RTI	VIM-2, OXA-1	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14929	*P. aeruginosa*	Germany	UTI	VIM-2, OXA-4	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14958	*P. aeruginosa*	India	IAI	VIM-2, OXA-10	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14984	*P. aeruginosa*	France	UTI	VIM-2, OXA-4	>32	(R)	(-)	(-)	(-)	(+)
AZPAE15029	*P. aeruginosa*	France	RTI	VIM-2, OXA-4	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14706	*P. aeruginosa*	Greece	IAI	PSE-1, VIM-4, OXA-35	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14865	*P. aeruginosa*	India	RTI	VEB-like, VIM-5, OXA-10	>32	(R)	(-)	(-)	(-)	(+)
AZPAE14900	*P. aeruginosa*	India	IAI	VEB-like, VIM-5, OXA-10	16	(R)	(-)	(-)	(-)	(+)
AZPAE13879	*P. aeruginosa*	Argentina	–	VIM-11, OXA-17	16	(R)	(-)	(-)	(-)	(+)
Other 37 ß-lactamase-producing strains including IMP-13, 15 and 18, and VIM-7										
AZPAE14862	*P. aeruginosa*	India	UTI	IMP-13	2	(S)	(-)	(-)	(-)	(-)

(Continued)

TABLE 2 Continued

Strain ID	Species	Origin of isolate		Genotype	Meropenem		LAMP Assays			
		Country	Anatomical site		MIC (mg/L)	Susceptibility	bla_{KPC}	bla_{NDM-1}	$bla_{IMP-1\ group}$	bla_{VIM}
AZPAE13872	*P. aeruginosa*	Mexico	–	IMP-15	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14688	*P. aeruginosa*	Mexico	–	IMP-18	>32	(R)	(-)	(-)	(-)	(-)
AZPAE 3936	*P. aeruginosa*	–	–	VIM-7	–	–	(-)	(-)	(-)	(-)
AZPAE13848	*P. aeruginosa*	India	–	GES-9	0.25	(S)	(-)	(-)	(-)	(-)
AZPAE13856	*P. aeruginosa*	India	–	GES-7	0.5	(S)	(-)	(-)	(-)	(-)
AZPAE13880	*P. aeruginosa*	Mexico	–	OXA-2, GES-19	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14694	*P. aeruginosa*	Romania	UTI	PER-1, OXA-2, OXA-74	16	(R)	(-)	(-)	(-)	(-)
AZPAE14708	*P. aeruginosa*	Greece	IAI	OXA-19	16	(R)	(-)	(-)	(-)	(-)
AZPAE14819	*P. aeruginosa*	Brazil	UTI	SPM-1, OXA-56	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14821	*P. aeruginosa*	Brazil	UTI	SPM-1, OXA-56	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14822	*P. aeruginosa*	Brazil	IAI	OXA-56	8	(R)	(-)	(-)	(-)	(-)
AZPAE14831	*P. aeruginosa*	Argentina	RTI	GES-1	0.5	(S)	(-)	(-)	(-)	(-)
AZPAE14834	*P. aeruginosa*	Argentina	UTI	OXA-2	8	(R)	(-)	(-)	(-)	(-)
AZPAE14838	*P. aeruginosa*	China	RTI	OXA-10	8	(R)	(-)	(-)	(-)	(-)
AZPAE14846	*P. aeruginosa*	France	RTI	PSE-1	32	(R)	(-)	(-)	(-)	(-)
AZPAE14852	*P. aeruginosa*	Brazil	RTI	OXA-17	32	(R)	(-)	(-)	(-)	(-)
AZPAE14853	*P. aeruginosa*	Brazil	RTI	SPM-1, OXA-56	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14886	*P. aeruginosa*	Croatia	UTI	PSE-1	16	(R)	(-)	(-)	(-)	(-)
AZPAE14887	*P. aeruginosa*	Croatia	IAI	OXA-2	32	(R)	(-)	(-)	(-)	(-)
AZPAE14912	*P. aeruginosa*	Croatia	RTI	OXA-2	32	(R)	(-)	(-)	(-)	(-)
AZPAE14923	*P. aeruginosa*	Brazil	RTI	OXA-56, SPM-1	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14926	*P. aeruginosa*	Brazil	UTI	AER-like	16	(R)	(-)	(-)	(-)	(-)

(Continued)

TABLE 2 Continued

Strain ID	Species	Origin of isolate		Genotype	Meropenem		LAMP Assays			
		Country	Anatomical site		MIC (mg/L)	Susceptibility	bla_{KPC}	bla_{NDM-1}	$bla_{IMP-1\ group}$	bla_{VIM}
AZPAE14933	*P. aeruginosa*	France	UTI	OXA-9, PSE-1	0.25	(S)	(-)	(-)	(-)	(-)
AZPAE14948	*P. aeruginosa*	Argentina	IAI	GES-5	>32	(R)	(-)	(-)	(-)	(-)
AZPAE14956	*P. aeruginosa*	Germany	IAI	VEB-1, OXA-10	16	(R)	(-)	(-)	(-)	(-)
AZPAE14976	*P. aeruginosa*	China	RTI	PSE-1	32	(R)	(-)	(-)	(-)	(-)
AZPAE14983	*P. aeruginosa*	Croatia	RTI	PSE-1	16	(R)	(-)	(-)	(-)	(-)
AZPAE15000	*P. aeruginosa*	Spain	UTI	OXA-2	16	(R)	(-)	(-)	(-)	(-)
AZPAE15002	*P. aeruginosa*	Spain	IAI	OXA-46, OXA-101	>32	(R)	(-)	(-)	(-)	(-)
AZPAE15047	*P. aeruginosa*	Argentina	RTI	TEM-1	>32	(R)	(-)	(-)	(-)	(-)
AZPAE15054	*P. aeruginosa*	Colombia	UTI	OXA-2	8	(R)	(-)	(-)	(-)	(-)
AZPAE15063	*P. aeruginosa*	Brazil	RTI	CTX-M-2, OXA-129	1	(S)	(-)	(-)	(-)	(-)
AZABA 2675	*A. baumannii*	–	–	OXA-113	–	–	(-)	(-)	(-)	(-)
AZABA 2782	*A. baumannii*	–	–	OXA-23, OXA-66, TEM-1, PER-1	–	–	(-)	(-)	(-)	(-)
AZECO 4089	*E. coli*	–	–	OXA-48	<=1	(S)	(-)	(-)	(-)	(-)
AZKPN 4593	*K. pneumoniae*	–	–	OXA-48	>8	(R)	(-)	(-)	(-)	(-)

[a]RTI, Respiratory tract infections; UTI, Urinary tract infections; IAI, Intra-abdominal infections.
[b](S), susceptible; (R), resistant.

the target region. The GenBank accession numbers and names of the bla_{KPC}, bla_{NDM}, bla_{IMP}, and bla_{VIM} sequences are listed in Supplementary Table 1. The alignments of the four target genes are shown in Supplementary Figure 1.

LAMP primers were designed using Primer Explorer v. 5 software according to the sequences of bla_{KPC} (GenBank accession number NG_049253.1), bla_{NDM-1} (FN396876.1), bla_{IMP-1} group (GU831546.1), and bla_{VIM} (GQ853417.1) (Supplementary Figure 2). Nucleotide 22 of the backward inner primer (BIP) for bla_{VIM} was changed from G to C to increase the sensitivity and prevent primer dimer formation (Table 3).

Based on the primer sequences, we aligned primers' binding sites of variants of each β-lactamase gene, also phylogenetic trees were conducted by the neighbor-joining method using MEGA v. 11 (Tamura et al., 2021) (Supplementary Figure 3). According to an *in silico* analysis (FastPCR (PrimerDigital, 2022)), variants in the blue square in Supplementary Figure 3 were expected to be detected by the LAMP assays.

LAMP reaction

The LAMP reaction mixture (25 μL) contained 1.6 μM FIP and BIP, 0.2 μM F3 and B3, 0.8 μM LF and LB, 8 U of *Bst* Polymerase (large fragment) (New England Biolabs, Ipswich, MA), 1.4 mM four deoxynucleoside triphosphates, 0.8 M betaine (Sigma, St. Louis, MO), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% (v/v) Tween 20, and template DNA (2 μL) (Takano et al., 2019). Reactions were incubated at 63-

67°C for 60 min (Table 3) and heated at 80°C for 2 min to terminate the reaction.

Analysis of LAMP products

The LAMP reaction was monitored by measuring turbidity in real time using Loopamp® Real-Time Turbidimeters (LA-500 and LA-200; Eiken Chemical Co., Tokyo, Japan) at a wavelength of 650 nm (OD_{650}) at 6 s intervals. The reaction time was recorded when the turbidity level exceeded 0.1 in accordance with the manufacturer's protocol. Amplified products were also evaluated visually. Amplified products were sequenced at the Akita Prefectural University Biotechnology Center using a BigDye® Terminator v. 3.1 Cycle Sequencing Kit and 3130xL Genetic Analyzer (Applied Biosystems, Foster City, CA). The F2 and B2 primers used to sequence the target regions are shown in Supplementary Table 2.

TABLE 3 LAMP primer sequences in this study.

Primer name	LAMP primer Sequence (Sequence 5′-3′)	Length (base pairs)	Gene/Genbank no.	Reaction temperature
KPC_F3	TGT ACG CGA TGG ATA CCG G	19	*bla*$_{KPC/}$ NG_049253	65°C
KPC_B3	CAC CGT CAT GCC TGT TGT	18		
KPC_FIP	CAG CAC AGC GGC AGC AAG AAA TGT AAG TTA CCG CGC TGA GG	41		
KPC_BIP	GGC TTG CTG GAC ACA CCC ATT TTC CGA GAT GGG TGA CCA C	40		
KPC_LF	GCC CTT GAA TGA GCT	15		
KPC_LB	CGT TAC GGC AAA AAT GCG C	19		
NDM-1_F3	TGC ATG CCC GGT GAA ATC C	19	*bla*$_{NDM-1}$/ FN396876	63°C
NDM-1_B3	TCA TCG GTC CAG GCG GTA T	19		
NDM-1_FIP	GAG CTG GCG GAA AAC CAG ATC GAC GAT TGG CCA GCA AAT	39		
NDM-1_BIP	ATG TCT GGC AGC ACA CTT CCG CCA TCC CTG ACG ATC AAA C	40		
NDM-1_LF	AAC CGT TGG TCG CCA GTT T	19		
NDM-1_LB	TTT CGG GGC AGT CGC TT	17		
IMP-1_F3	CCG GGA CAC ACT CCA GAT	18	*bla*$_{IMP-1}$/ GU831546	65°C
IMP-1_B3	GTT TCA AGA GTG ATG CGT CTC C	22		
IMP-1_FIP	CAC CCA AAT TGC CTA AAC CGT CGT AGT GGT TTG GTT GCC TG	41		
IMP-1_BIP	AGA AGC TTG GCC AAA GTC CGT GGA ACA ACC AGT TTT GCC TTA	42		
IMP-1_LF	CCA CCG AAT AAT ATT TTC CT	20		
IMP-1_LB	CCA AAT TAT TAA AGT CCA AAT ATG G	25		
VIM_F3	CGT GAT GGT GAT GAG TTG CT	20	*bla*$_{VIM-2}$/ GQ853417	67°C
VIM_B3	TCG TTC CCC TCT ACC TCG	18		
VIM_FIP	CGC GTT ACA GGA AGT CCA AGG GTG CGA AAA ACA CAG C	37		
VIM_BIP	CTC CAC GCA CTT TCA TGA CGG C[a]TG ATG CGT ACG TTG CC	38		
VIM_LF	TTT GCT TCT CAA TCT CCG	18		
VIM_LB	TTG ATG TCC TTC GGG C	16		

[a]original sequence was G (to avoid formation of primer dimer and nonspecific reactions).

PCR

The PCR primers and conditions for the β-lactamases (Supplementary Table 3) were described previously (Senda et al., 1996; Monteiro et al., 2012). PCR assays were performed in a 25 μL reaction mixture containing 0.2 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 2.5 mM $MgCl_2$, 0.4 μM each primer, 2.5 U of Ex Taq DNA Polymerase (Takara, Shiga, Japan), and template DNA (2 μL). Reactions were performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems). The PCR program was 5 min at 94°C; followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 55°C for 30 s, and extension at 72°C; and a final extension at 72°C for 15 min, followed by storage at 4°C. The PCR products were subjected to agarose gel electrophoresis and visualized with Midori Green Advance (NIPPON Genetics, Tokyo, Japan).

DNA-spiked specimens

To analyze the effects of biological substances on the established LAMP assays, the detection limits of the LAMP assays were determined using DNA-spiked clinical samples. Urine and blood specimens were collected from five healthy volunteers at Nihon University School of Medicine. The blood specimens were heparinized and stored at -80°C. Sputum specimens were obtained from seven patients at Ageo Central General Hospital and frozen at -80°C to inactivate bacteria. After approval by the Biosafety Committee of Nihon University, the specimens were handled using the risk group 2 protocol of the Laboratory Biosafety Manual of the WHO, Geneva, 2004. Urine specimens were boiled at 95°C for 5 min and centrifuged at 1500 rpm. DNA was extracted from blood and sputum samples using a Loopamp™ PURE DNA Extraction Kit (Eiken Chemical Co.) according to the manufacturer's instructions. Purified β-lactamase DNA (bla_{KPC}, ARC2945; bla_{NDM-1}, ARC3600; bla_{IMP-1} group, ARC2780; and bla_{VIM}, ARC3471) was added to the specimens and used to determine the detection limits of the PCR and LAMP assays.

Results

Analytical reactivity and specificity of the β-lactamase LAMP assay

The LAMP assays amplified the target sequences, as confirmed by turbidity in the reaction tube and by real-time turbidimetry (Table 1 and Figure 1). Of the eight strains including 7 β-lactamase genes, each assay detected only the target gene. In contrast, no other genes were not amplified in this assay (Table 1). The LAMP reactions reached the detection threshold (turbidity level of 0.1) within 35 min, whereas no turbidity rise was detected for 60 min in negative reaction. The sequences of the LAMP products were identical to those expected (Supplementary Figures 2, 4).

Analytical sensitivity of the β-lactamase LAMP assay

Serial 10-fold-diluted DNA samples (adjusted at 10^4, 10^3, 10^2, 10, and 1, and 0 genome copies per reaction in 25 μL) were amplified by LAMP and the results were compared to those of PCR. The detection limits of the LAMP assays were 10^2 copies per reaction for bla_{KPC}, 10^2 copies for bla_{NDM-1}, 10^2 copies for bla_{IMP-1}, and 10 copies for bla_{VIM}. The detection limits for PCR

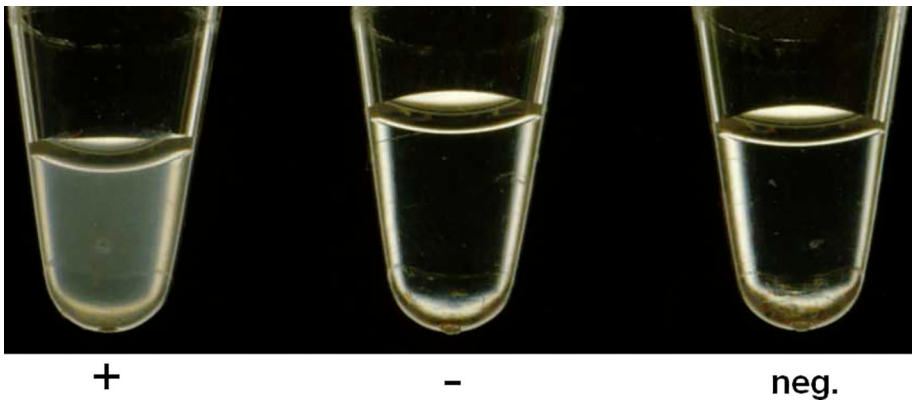

FIGURE 1
bla_{KPC}-LAMP assay detecting Visual detection of a white precipitate in positive samples. The figure shows a positive (bla_{KPC-2}) and a negative (non-bla_{KPC}, bla_{NDM-1}) sample, together with a negative control (neg.).

were 10^4 copies for bla_{KPC}, 10^3 copies for bla_{NDM-1}, 10^2 copies for bla_{IMP}, and ten copies for bla_{VIM} (Table 4). Therefore, the LAMP assays were up to 100-fold more analytically sensitive than PCR. Although the detection limits of the LAMP assays for bla_{IMP-1} and bla_{VIM} were identical to those of PCR, the LAMP reaction was more rapid, and the results could be confirmed visually.

DNA-spiked specimens

The detection limit of the LAMP assays using clinical specimens was determined using DNA-spiked urine, sputum, and blood samples. The detection limit of the LAMP assays was 10^2 copies per reaction for bla_{KPC}, 10^2 copies for bla_{NDM-1}, 10^2 copies for bla_{IMP-1}, and 10 copies for bla_{VIM} in DNA-spiked urine, sputum, and blood specimens. These values were identical to those obtained using purified DNA as the template (Table 4).

The detection limit of PCR for bla_{KPC} in DNA-spiked urine, sputum, and blood specimens was 10^4 copies per reaction, identical to that using purified DNA as the template. The detection limit of PCR for bla_{NDM-1} was 10^4 copies per reaction in DNA-spiked urine, sputum, and blood specimens, which was 10-fold lower than when purified DNA was used as the template. The detection limit of PCR for bla_{IMP} in DNA-spiked urine and sputum specimens was 10^2 copies, identical to that using purified DNA as a template. However, the sensitivity decreased 100-fold (10^4 copies per reaction) for the DNA-spiked blood specimens compared to using purified DNA as the template. Finally, the detection limit of PCR for bla_{VIM} in DNA-spiked urine and sputum specimens was 10-fold less sensitive (10^2 copies per reaction) and that in DNA-spiked blood specimens was 100-fold less sensitive (10^3 copies per reaction) compared to the use of purified DNA as the template (10 copies per reaction). The sensitivity of LAMP for DNA-spiked blood specimens was up to 100-fold higher than that of PCR.

Evaluation of LAMP assays for clinical Gram-negative bacterial strains

The LAMP assays for four β-lactamases were validated using 55 clinical Gram-negative bacterial strains isolated at diverse geographical locations (Table 2). The LAMP assay for bla_{KPC} specifically amplified the target bla_{KPC} segments (bla_{KPC-2}) and no other genotype. The LAMP assay for bla_{NDM-1} specifically amplified three isolates with the target gene bla_{NDM-1}, and no other genotype. The LAMP assay for bla_{IMP-1} amplified two isolates with bla_{IMP-1} and bla_{IMP-4}, but not three isolates with bla_{IMP-13}, bla_{IMP-15}, and bla_{IMP-18} (Table 2) or any other genotype. The LAMP assay for bla_{VIM} amplified 11 isolates with bla_{VIM-1}, bla_{VIM-2}, bla_{VIM-4}, bla_{VIM-5}, and bla_{VIM-11}, but not an isolate with bla_{VIM-7} (Table 2) or any other genotype. The results of the LAMP assays for bla_{KPC}, bla_{NDM-1}, bla_{IMP-1}, and bla_{VIM} were thus identical to those simulated *in silico* (blue squares in Supplementary Figure 3).

Disscussion

We developed LAMP assays for four β-lactamase genes (bla_{KPC}, bla_{NDM-1}, bla_{IMP-1} group, and bla_{VIM}). Standard reference strains and 55 β-lactamase-carrying clinical isolates were correctly identified, although most Metallo β-lactamase genes have sequence variations and 47 clinical *P. aeruginosa* isolates were from diverse geographical locations. Theoretically, the capacity of detection will not change if the sequence of the genes is the same; however, the genome size of *P. aeruginosa*, co-existing drug-resistant genes, and the phenotype of the isolates are different depending on geographic distribution (Kos et al., 2015). Thus, the investigation using diverse geographic locations is important to assess the quality of the assay.

The results were in agreement with those of our in silico simulations. The sensitivity of the LAMP assays was comparable

TABLE 4 Detection limits of the PCR and LAMP assays used to detect bla_{KPC}, bla_{NDM-1}, bla_{IMP-1} and bla_{VIM} in DNA-spiked specimens.

	Detection limit							
	bla_{KPC}		bla_{NDM-1}		bla_{IMP-1}		bla_{VIM}	
	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP
Purified DNA	10^4 copies[a]	10^2	10^3	10^2	10^2	10^2	10	10
DNA spiked specimens								
Urine[b]	10^4	10^2	10^4	10^2	10^2	10^2	10^2	10
Sputum[c]	10^4	10^2	10^4	10^2	10^2	10^2	10^2	10
Blood[c]	10^4	10^2	10^4	10^2	10^4	10^2	10^3	10

[a]Amount of DNA per reaction;
[b]Supernatant data obtained after boiling and centrifugation;
[c]Samples prepared via Loopamp™ PURE DNA extraction kit (Eiken Chemical Co.).

between purified DNA and DNA-spiked clinical specimens. The LAMP reactions were not inhibited by contaminants in DNA-spiked samples. By contrast, PCR assays for three β-lactamase genes (bla_{NDM-1}, bla_{IMP-1} group, and bla_{VIM}) are inhibited by the contaminants. The LAMP assays were up to 100-fold more sensitive than PCR. Because of their robustness to biological substances, the LAMP assays detected DNA added to blood specimens with up to 100-fold higher sensitivity than PCR.

PCR assays are affected by, for example, heparin (Satsangi et al., 1994), heme, leukocyte DNA, and IgG (Al-Soud et al., 2000; Al-Soud and Radstrom, 2001). Therefore, it is not appropriate to use a simple DNA extraction method such as the Loopamp™ PURE DNA Extraction Kit (Eiken Chemical Co.), which is a contamination-resistant kit that does not require a centrifuge or refrigerator. Because of their robustness to biological substances, the LAMP assays can be performed at the bedside or resource-limited settings. The LAMP assays will promote the control of β-lactam resistance because rapid and accurate POCT for drug resistance is essential and will facilitate research on AMR and the effectiveness of the *One Health* approach to limit the spread of resistance (WHO, 2017).

In this study, we struggled to detect sequence variations for each of the MBL genotypes. Most MBL genes have sequence variations. Indeed, 93 variants of bla_{KPC}, 40 of bla_{NDM}, 87 of bla_{IMP}, and 77 variants of bla_{VIM} have been reported (Supplementary Table 1). To select the target region, we compared the sequences of the four genotypes, and identified a consensus region of each genotype (Supplementary Figure 1). The 93 bla_{KPC} variants do not differ markedly and all were expected to be detected by the LAMP assay (Supplementary Figures 1, 3). The 40 bla_{NDM} variants also do not differ markedly and all were expected to be detected by the LAMP assay (Supplementary Figures 1, 3). bla_{IMP} has 87 highly different variants, which prevented identification of a consensus region. Therefore, we focused on the bla_{IMP-1} group, which is main group among the variants of bla_{IMP} (Supplementary Figures 1, 3). Among the 77 bla_{VIM} variants, we identified a consensus region for 73; however, 4 variants (bla_{VIM-7}, bla_{VIM-18}, bla_{VIM-61}, and bla_{VIM-69}) were considerably different (Supplementary Figures 1, 3). Therefore, we focused on the 73 bla_{VIM} variants as targets for the LAMP assays.

We designed LAMP primers for four target genes (nucleotides 141 to 359 of bla_{KPC}, 76 to 287 of bla_{NDM-1}, 463 to 676 of bla_{IMP-1}, and 223 to 446 of bla_{VIM}) (Supplementary Figure 2). The results agreed with those of *in silico* simulations (blue squares in Supplementary Figure 3). Using the LAMP primers for the bla_{IMP-1} group, bla_{IMP-1} and bla_{IMP-4} (blue square in Supplementary Figure 3) were detected but bla_{IMP-13}, bla_{IMP-15}, bla_{IMP-18} (without the blue square in Supplementary Figure 3) were not. The LAMP primers for bla_{VIM} detected $bla_{VIM-1, 2, 4, 5, and 11}$ (blue square in Supplementary Figure 3), but not bla_{VIM-7} (without the blue square in Supplementary Figure 3). The LAMP primers for bla_{KPC} detected bla_{KPC-2} and bla_{KPC-3} (blue square in Supplementary Figure 3), and that for bla_{NDM-1} detected bla_{NDM-1} (blue square in Supplementary Figure 3).

Visual inspection of LAMP assay is sufficient to confirm positive results. For more complex diagnostic assays, such as arrays and whole-genome sequencing, complex analyses are needed to interpret the raw data. Indeed, LAMP-based diagnostic tests for extended-spectrum β-lactamases and carbapenemases are suitable for widespread use, including in low- or middle-income countries.

This study had a several limitations. The specificity of LAMP assays for bla_{IMP} and bla_{VIM} does not cover all of the genotypes. If MBL is suspected clinically but negative results are obtained, additional qualification should be considered, e.g., sequencing. Additional work using clinical specimens from patients is required.

In conclusion, we developed novel LAMP assays for four β-lactamase genes (bla_{KPC}, bla_{NDM-1}, bla_{IMP-1} group, and bla_{VIM}). Further evaluation of these assays is required using additional clinical specimens. Low-complexity and low-cost tests may be suitable for most types of laboratories, whereas high-complexity and high-cost tests may be suitable only for established national reference laboratories with a sufficient budget. Laboratories with no prior experience in molecular testing may consider low-complexity, low-cost tests such as LAMP-based assays. Further development of additional LAMP primer sets for bla_{VIM} and bla_{IMP} is now ongoing.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committees of Ageo Central General Hospital (approval # 434) and the IRB of Nihon University School of Medicine (approval # 28-9-0). Using the IRB-approved protocol, seven patient sputum specimens (Ageo Central General Hospital) were collected in accordance with the recommendations of the Japan Society of Clinical Examination Medicine. This guidance enables access to specimens when it is difficult to obtain consent, the sample is anonymized, and if the IRB has approved the study protocol. The requirement for written consent was waived because specimens were anonymized samples discarded by the Hospital's clinical laboratory. We also used urine and blood specimens from five healthy volunteers at Nihon University School of Medicine. The study protocol was reviewed and approved by the Institutional Review Board (IRB) of the Nihon University School of Medicine

(# 28-9-0). Written informed consent was obtained from the five healthy volunteers. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HG, RM, PK, SH, TH, DK, and MS contributed the conception of this study. EK, DK and MS designed the experiments. EK, JL, YY, DL, YB, TI, and MS performed the experiments. HG, RM, CT, SH and PK acquired the samples. EK, JL, JS, CT and MS analyzed the data. EK, JS, TI, DK, AN, TO, TH, and MS performed the phylogenetic and in silico analyses. EK, JS, MS, DK, and SH interpreted the data, drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

HG was employed by Harbour Biomed. It did not influence to the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

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/fcimb.2022.1000445/full#supplementary-material

References

Al-Soud, W. A., Jonsson, L. J., and Radstrom, P. (2000). Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J. Clin. Microbiol.* 38 (1), 345–350. doi: 10.1128/JCM.38.1.345-350.2000

Al-Soud, W. A., and Radstrom, P. (2001). Purification and characterization of PCR-inhibitory components in blood cells. *J. Clin. Microbiol.* 39 (2), 485–493. doi: 10.1128/JCM.39.2.485-493.2001

Arumugham, V. B., Gujarathi, R., and Cascella, M. (2022). Third generation cephalosporins, in *StatPearls* (Treasure Island (FL):StatPearls Publishing).

Chen, L., Mathema, B., Chavda, K. D., DeLeo, F. R., Bonomo, R. A., and Kreiswirth, B. N. (2014). Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. *Trends Microbiol.* 22 (12), 686–696. doi: 10.1016/j.tim.2014.09.003

Clinical and Laboratory Standards Institute (2012). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard M07-A9* (Wayne, PA: Clinical and Laboratory Standards Institute).

Feng, W., Niu, S., Chang, Y., Jia, X., Huang, S., and Yang, P. (2021). Design of rapid detection system for five major carbapenemase families (*bla* $_{KPC}$, *bla* $_{NDM}$, *bla* $_{VIM}$, *bla* $_{IMP}$ and *bla* $_{OXA-48-Like}$) by colorimetric loop-mediated isothermal amplification. *Infect. Drug Resist.* 14, 1865–1874. doi: 10.2147/IDR.S301757

Kos, V. N., Deraspe, M., McLaughlin, R. E., Whiteaker, J. D., Roy, P. H., Alm, R. A., et al. (2015). The resistome of pseudomonas aeruginosa in relationship to phenotypic susceptibility. *Antimicrob. Agents Chemother.* 59 (1), 427–436. doi: 10.1128/AAC.03954-14

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and clustal X version 2.0. *Bioinformatics* 23 (21), 2947–2948. doi: 10.1093/bioinformatics/btm404

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Rottingen, J. A., Klugman, K., et al. (2016). Access to effective antimicrobials: a worldwide challenge. *Lancet* 387 (10014), 168–175. doi: 10.1016/S0140-6736(15)00474-2

Monteiro, J., Widen, R. H., Pignatari, A. C., Kubasek, C., and Silbert, S. (2012). Rapid detection of carbapenemase genes by multiplex real-time PCR. *J. Antimicrob. Chemother.* 67 (4), 906–909. doi: 10.1093/jac/dkr563

Moreno-Morales, J., Vergara, A., Kostyanev, T., Rodriguez-Bano, J., Goossens, H., and Vila, J. (2020). Evaluation of a loop-mediated isothermal amplification assay to detect carbapenemases directly from bronchoalveolar lavage fluid spiked with acinetobacter spp. *Front. Microbiol.* 11, 597684. doi: 10.3389/fmicb.2020.597684

Mori, Y., and Notomi, T. (2019). Loop-mediated isothermal amplification (LAMP): Expansion of its practical application as a tool to achieve universal health coverage. *J. Infect. Chemother.* 26 (1), 13–17. doi: 10.1016/j.jiac.2019.07.020

Palacios-Baena, Z. R., Giannella, M., Manissero, D., Rodriguez-Bano, J., Viale, P., Lopes, S., et al. (2021). Risk factors for carbapenem-resistant gram-negative bacterial infections: A systematic review. *Clin. Microbiol. Infect.* 27(2), 228–235. doi: 10.1016/j.cmi.2020.10.016

PrimerDigital. (2022) FastPCR is an integrated tool for PCR primers or probe design, in silico PCR, oligonucleotide assembly and analyses, alignment and repeat searching. Available at: https://primerdigital.com/fastpcr.html.

Satsangi, J., Jewell, D. P., Welsh, K., Bunce, M., and Bell, J. I. (1994). Effect of heparin on polymerase chain reaction. *Lancet* 343 (8911), 1509–1510. doi: 10.1016/s0140-6736(94)92622-0

Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., et al. (1996). PCR detection of metallo-beta-lactamase gene (blaIMP) in gram-negative rods resistant to broad-spectrum beta-lactams. *J. Clin. Microbiol.* 34 (12), 2909–2913. doi: 10.1128/JCM.34.12.2909-2913.1996

Solanki, R., Vanjari, L., Subramanian, S., Aparna, B., Nagapriyanka, E., and Lakshmi, V. (2014). Comparative evaluation of multiplex PCR and routine laboratory phenotypic methods for detection of carbapenemases among gram negative bacilli. *J. Clin. Diagn. Res.* 8 (12), DC23–DC26. doi: 10.7860/JCDR/2014/10794.5322

Takano, C., Seki, M., Kim, D. W., Gardner, H., McLaughlin, R. E., Kilgore, P. E., et al. (2019). Development of a novel loop-mediated isothermal amplification method to detect Guiana extended-spectrum (GES) beta-lactamase genes in pseudomonas aeruginosa. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00025

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* 38 (7), 3022–3027. doi: 10.1093/molbev/msab120

WHO. (2017). Global priority list of antibiotic-resistant bacteria to guid research, discovery, and development of new antibiotics. (Geneva: WHO).

WHO. (2019). Molecular methods for antimicrobial resistance (AMR) diagnostics to enhance the global antimicrobial resistance surveillance system. (Geneva: WHO).

WHO. (2020a). A financial model for an impact investment fund for the development of antibacterial treatments and diagnostics: a user guide. (Geneva: WHO).

WHO. (2020b). GLASS whole-genome sequencing for surveillance of antimicrobial resistance. (Geneva: WHO).

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Klebsiella pneumoniae bacteremia mortality: a systematic review and meta-analysis

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Objective: To analyze the mortality rate of patients with *Klebsiella pneumoniae* bacteremia (KPB) and the impact of extended spectrum beta-lactamase (ESBL) producing or carbapenem-resistance (CR) KP on the mortality rate among patients with bacteremia.

Methods: EMbase, Web of Science, PubMed, and The Cochrane Library were searched up to September 18th, 2022. Two reviewers independently extracted data and evaluated risk of bias of included studies by ROBINS-I tool. A meta-regression analysis was conducted using a mixed-effects model to explore possible sources of heterogeneity. A random-effects model was used for pooled analysis in case of significant heterogeneity (I^2>50%). Otherwise, the fixed-effects model was performed.

Results: A total of 157 studies (37,915 enrolled patients) were included in the meta-analysis. The pooled death proportions of KPB were 17% (95% CI=0.14-0.20) at 7-day, 24% (95% CI=0.21-0.28) at 14-day, 29% (95% CI=0.26-0.31) at 30-day, 34% (95% CI=0.26-0.42) at 90-day, and 29% (95% CI=0.26-0.33) in hospital, respectively. Heterogeneity was found from the intensive care unit (ICU), hospital-acquired (HA), CRKP, and ESBL-KP in the meta-regression analysis. More than 50% of ICU, HA, CRKP, and ESBL-KP were associated with a significant higher 30-day mortality rates. The pooled mortality odds ratios (ORs) of CRKP *vs*. non-CRKP were 3.22 (95% CI 1.18-8.76) at 7-day, 5.66 (95% CI 4.31-7.42) at 14-day, 3.87 (95% CI 3.01-3.49) at 28- or 30-day, and 4.05 (95% CI 3.38-4.85) in hospital, respectively.

Conclusions: This meta-analysis indicated that patients with KPB in ICU, HA-KPB, CRKP, and ESBL-KP bacteremia were associated with a higher mortality rate. The high mortality rate caused by CRKP bacteremia has increased over time, challenging the public health.

KEYWORDS

***Klebsiella pneumoniae*, bacteremia, mortality, meta-analysis, carbapenem-resistant**

Introduction

Klebsiella pneumoniae (KP) is well known as an opportunistic pathogen which can cause invasive human infections such as bacteremia. Also, KP is the second most common cause of gram-negative bacteremia, following *Escherichia coli* (E. coli) (Cubero et al., 2018; Hyun et al., 2018). The estimated incidence rate of *Klebsiella pneumoniae* bacteremia (KPB) was increased from 10.2 to 18.7 per 100 000 inhabitants in a region of Canada (Pépin et al., 2010), and the reported mortality rate of KPB varied widely from 11% to 81% in most studies (Chang et al., 2015; Imai et al., 2019; Reid et al., 2019; Falcone et al., 2020; Balkan et al., 2021; Meng et al., 2022). It is important to accurately estimate the mortality rate of KPB to best define the infectious disease, and it can accurately convey prognosis and improve the guidance to control this disease.

Antibiotic resistance has become a major challenge for public health globally, which is associated with nearly 5 million deaths and killed at least 1.27 million people worldwide in 2019 alone (CDC, 2022). In addition, the cost to treat infections caused by multidrug-resistant pathogens is high, making a big healthcare burden to society (Zhen et al., 2021). ESBL-producing germs cost the highest for community-onset and hospital-onset infections, with an estimated more than $1.2 billion (Nelson et al., 2021). Antibiotic resistance can develop in KP isolates, especially producing extended-spectrum β-lactamases (ESBL) and carbapenemases. Although many studies (including guidelines) were adopted to maintain the progress in combating antimicrobial resistance, the prevalence of ESBL- and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has increased dramatically (Gao et al., 2020). According to the 20 Years of the SENTRY Antimicrobial Surveillance Program (Castanheira et al., 2019), a significant increase in CRKP strains was noted over time. Most studies reported patients with ESBL-producing or CRKP bacteremia were associated with higher mortality rate when compared with those who had non-ESBL or non-CRKP bacteremia (Lee et al., 2012; Li et al., 2014; Li and Huang, 2017; Xiao et al., 2018; Lee et al., 2019; Shen et al., 2020; Meng et al., 2022). However, some other researchers reported contrary or similar mortality rates (Lee et al., 2011; Pau et al., 2015; Kim et al., 2019; Lee et al., 2021). Therefore, conducting accurate estimation of the mortality gap between CRKP and non-CRKP bloodstream infections is needed.

Although many individual studies reported the incidence rates of KPB, there is a lack of precise estimations due to limitations, such as biased data collection from single medical center, relatively small sample size, and the different endpoints of the disease. Besides, the impact of ESBL-KP or CRKP on mortality rates among patients with bacteremia should also be studied since 2018 (Ramos-Castaneda et al., 2018). Thus, a systematic review was performed to analyze the mortality rate in KPB as well as the impact of ESBL-KP or CRKP on the mortality rate of patients with bacteremia.

Abbreviations: CRKP, carbapenem-resistant Klebsiella pneumoniae; CRE, carbapenem-resistant Enterobacteriaceae; CSKP, carbapenem-susceptible Klebsiella pneumoniae; E. coli, Escherichia coli; ESBL, extended-spectrum β-Lactamase-positive; HA, hospital-acquired; ICU, intensive care unit; KP, Klebsiella pneumoniae; KPB, Klebsiella pneumoniae bacteremia; KPC, Klebsiella pneumoniae carbapenemase; ORs, odds ratios; PRISMA, Preferred Reporting Items for Systematic reviews and Meta-Analyses; ROBINS-I, Nonrandomized Studies of Interventions tool.

Methods

Reporting guideline

The study was reported according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021). The PRISMA 2020 checklist is presented in Supplementary Materials 1 Table S1.

Database search

Two reviewers independently searched EMbase, Web of Science, PubMed, and The Cochrane Library, up to September 18th, 2022. The search was performed using both Medical Subject Headings (MeSH) and keywords: "Klebsiella pneumoniae", "bacteremia or bloodstream infection", and "mortality or death or survival or outcome" with no date or language restrictions. The search strategy is shown in Supplementary Material 1 Text S1. In addition, manual search was performed to find relevant studies from the references of the found studies.

Study criteria and definitions

Inclusion criteria were as follows: i) Observational studies; ii) Studies reported with more than fifty patients with bacteremia caused by KP; iii) Eligible studies were included with no language restrictions. Exclusion criteria were as follows: i) *In vitro* studies, case reports or case series (n < 50), reviews, conference abstracts, study protocols, trial registrations, and duplicate publications; ii) Studies that included KPB patients as a sub-group or included only a sub-population of KPB patients based on infectious foci or patient characteristics, for the reason that these studies in most cases cannot derive the number of patients who died from KPB or provide sufficient information to evaluate the risk of bias.

The definitions were based on articles included in the study. KPB was defined as the isolation of KP in a blood culture specimen. CRKP was defined as resistant to at least one carbapenem or produced a carbapenemase. Hospital-acquired (HA) bacteremia was defined as a positive blood culture from a patient 48 hours after admission and no signs of infection had been noted at admission. Appropriate antibiotic therapy was defined as treatment regimen included at least one antimicrobial agent active *in vitro* against KP.

Data extraction

Two authors independently extracted and then cross-checked the studies. The following data of the studies was extracted: the first

author's name, published year, study year(s), location, study design, patient characteristics (i.e., age, sex, and number of patients), and outcomes (7-day,14-day, 28- or 30-day, 90-day, and in hospital mortality rates if reported).

Risk of bias assessment

The Risk of Bias in Nonrandomized Studies of Interventions (ROBINS-I) tool (Sterne JAC and Elbers, 2016) was used to evaluate the risk of bias of the included studies. This tool covers seven domains and refer to a hypothetical randomized trial as a "target" randomized trial. The seven domains were as follows: bias due to confounding, bias in selection of participants, bias in classification of interventions, bias due to deviations from intended interventions, bias due to missing data, bias in measurement of outcomes, and bias in selection of the reported result. Two investigators independently answered the signaling questions to judge for each bias domain. The overall risk of bias is categorized as 'Low', 'Moderate', 'Serious' or 'Critical'. Any disagreement was resolved by a third researcher.

Statistical analysis

The primary outcome was the 30-day mortality rate of single proportion after diagnosis of KPB; besides, the 7-, 14-, 90-day, and in hospital mortality rates were also analyzed. If two studies have same data while the endpoints were different, both studies would be included. Statistical heterogeneity among studies was determined by I^2 statistics (degree of heterogeneity) and the Cochran Q test ($p<0.05$ indicated significant heterogeneity among studies). A random-effects model was used for pooled analysis in case of significant heterogeneity ($I^2>50\%$). Otherwise, the fixed-effects model was performed. Sensitivity analyses was performed by excluding one study in each turn to evaluate the influence of the individual trial on the overall pooled effects. The publication bias was assessed by visual inspection of the funnel plot and Peter's test (Bai et al., 2022). If publication bias exsists, trim and filled model will be used to adjust for the funnel plot asymmetry.

In order to explore possible sources of heterogeneity (single proportion of 30-day mortality), a meta-regression analyse was conducted using a mixed-effects model. The variables were as follows: proportion of male, study design (classified into prospective or retrospective, cohort or case control or cross sectional), country (Asia, Europe, North America), study period (2006–2010, 2011–2015, 2016–2020, 2021–2022), mean or median age, proportion of ICU patients, proportion of patients used appropriate empirical antibiotic therapy, proportion of patients with CRKP bacteremia, proportion of patients with ESBL-KP bacteremia, and proportion of patients with hospital-acquired (HA) KPB. Subgroup analyses would be prespecified if significant factors were found in the meraregression model. Besides, pooled odds ratios (ORs) and 95% CI were further performed to compare mortality rates in patients with ESBL-KP or CRKP with those in patients with non-ESBL-KP or non-CRKP bacteremia.

The meta-analysis was conducted by R (version 4.0.3) package meta (Schwarzer, 2022).

Results

Study characteristics

Of the 7,040 retrieved studies, 157 papers (37,915 enrolled patients) were included in the review (Figure 1). The references and studies with excluding reasons are listed in Supplementary Materials 1 Text S2 and Table S2, respectively. The characteristics of the included 157 articles are described in Supplementary Materials 1 Table S3, consisting of 18 prospective and 139 retrospective observational studies. Out of the 157 studies, 130 studies reported data from single centers and 27 studies reported data from multiple centers. These studies were published between 2001 and 2022, and the sample size varied from 50 to 5,712. The top five countries where studies were performed were China (65 studies), Italy (14 studies), South Korea (12 studies), Greece (10 studies), and Turkey (7 studies).

Risk of bias assessment

The Robvis tool (McGuinness and Higgins, 2021) was used to assess publication quality by the risk of bias. The results for each study and each domain are presented in Supplementary Materials 2 Figure S1. Since ROBINS-I tool emulates a hypothetical randomized trial to evaluate risk of bias in each domain, it is rare that an observational study would be judged as at low risk of bias in confounding domain. Therefore, most studies were judged as at least moderate overall risk of bias. In addition, for the reason that the primary study object was mortality rate, which was an

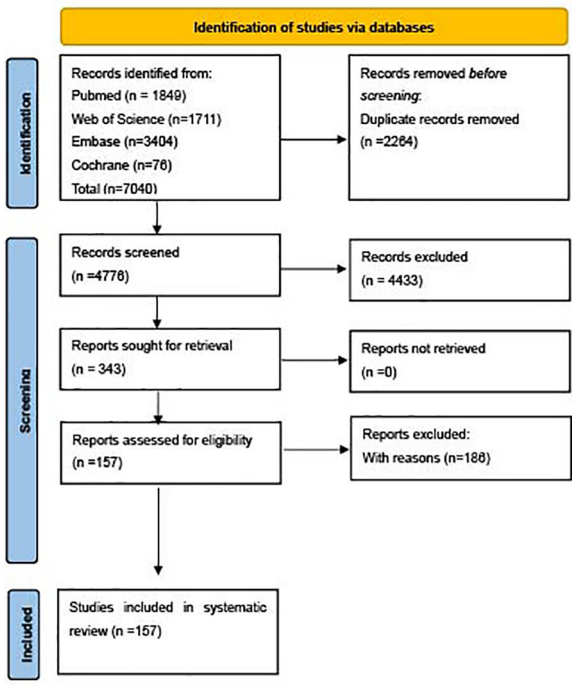

FIGURE 1
Flow of diagram of meta-analysis generated by PRISMA2020.

objective measure, it was unlikely to be manipulated, almost all of studies included (except two studies) in the meta-analysis were judged as low risk of bias in measurement of outcomes domain. 149 (94.9%) studies were judged as serious or critical overall risk of bias, and the left 8 (5.1%) studies were at moderate overall risk of bias.

Estimates of single proportion mortality rates in KPB

A total of 101 studies reported 30-day mortality with a total of 25,800 patients, yielding a proportion of 29% (95% CI=0.26-0.31) (Figure 2). In addition, the pooled 7-, 14-, 90-day, and in hospital mortality rates for KPB are listed in Supplementary Materials 2 Figures S2-S5, and the corresponding pooled death proportions were 17% (95% CI=0.14-0.20), 24% (95% CI=0.21-0.28), 34% (95% CI=0.26-0.42), and 29% (95% CI=0.26-0.33), respectively. Heterogeneities were observed high among all endpoints.

Comparison of ESBL-KP and non-ESBL-KP bacteremia

The pooled mortality rates at different time points (14-, 28- or 30-day, and in hospital) with ESBL-KP *vs.* non-ESBL-KP are presented in Supplementary Materials 2 Figures S6-S8. Overall, ESBL-KP bacteremia was associated with a higher mortality than non-ESBL-KP bacteremia. The pooled ORs at various time points (14-, 28- or 30-day, and in hospital) were 1.82 (95% CI 1.16-2.86), 1.57 (95% CI 1.07-2.32), and 1.57 (95% CI 1.23-2.02), respectively, with significant differences.

Comparison of CRKP and non-CRKP bacteremia

A total of 26 studies assessed 30-day mortality for CRKP *vs.* non-CRKP bacteremia producing an OR of 3.87 (95% CI 3.01-3.49) (Figure 3). Overall, CRKP bacteremia was associated with a significant higher mortality than non-CRKP bacteremia. The forest plots of different endpoints (7-, 14-day, and in hospital) can be found in Supplementary Materials 2 Figures S9-S11, and the corresponding pooled ORs at various endpoints (7-, 14-, in hospital) were 3.22 (95% CI 1.18-8.76), 5.66 (95% CI 4.31-7.42), and 4.05 (95% CI 3.38-4.85), respectively.

Sensitivity analysis and publication bias

Sensitivity analysis was performed to evaluate the influence of an individual trial on the overall pooled effects. The Forest plots of sensitivity test for all KPB are presented in Supplementary Materials 2 Figures S12-S23. In brief, almost all sensitivity analyses showed

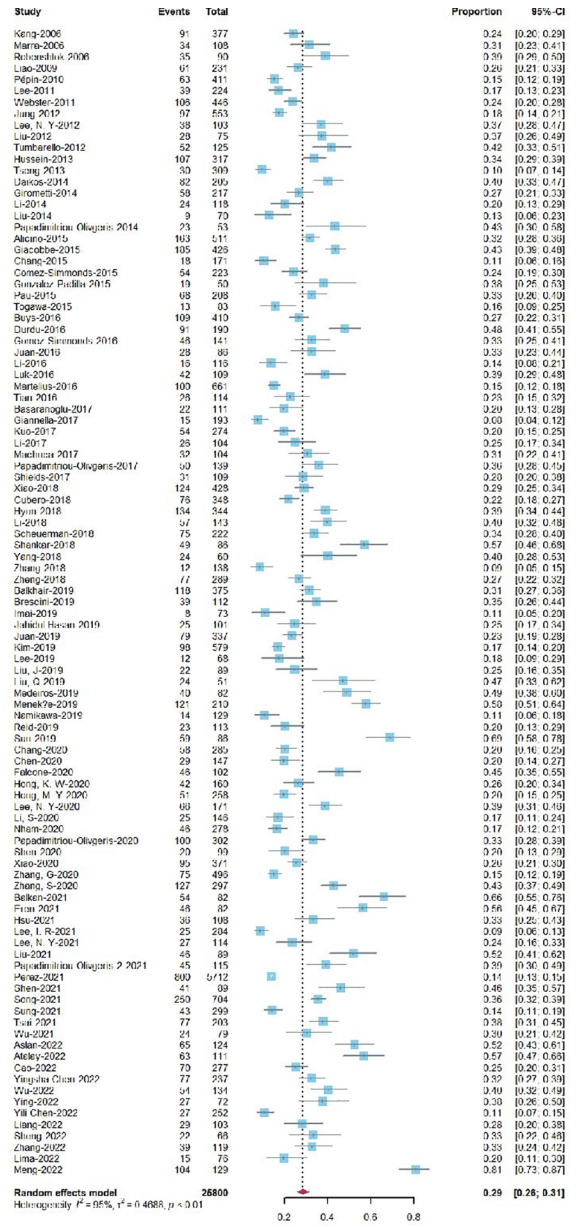

FIGURE 2
Forest plot of single proportion 30-day mortality for KPB.

that exclusion of any individual study did not affect the overall pooled effects of mortality. In terms of publication bias, no significant asymmetry was observed in single proportion 7-day mortality, mortality (14-, 30-day and in-hospital) for ESBL *vs.* non-ESBL KPB, and mortality (7-, 14-, 30-day and in-hospital) for CRKP *vs.* non-CRKP KPB. A significant asymmetry was found in funnel plots for single proportion 14-, 30-day, and in-hospital mortality. The funnel plots and Peter's tests are presented in Supplementary Materials 2 Figures S24-S35. Trim and filled model was further used to adjust the funnel plot asymmetry in single proportion 14-, 30-day and in-hospital mortality. The adjusted funnel plots are presented in Supplementary Materials 2 Figures S36-S38.

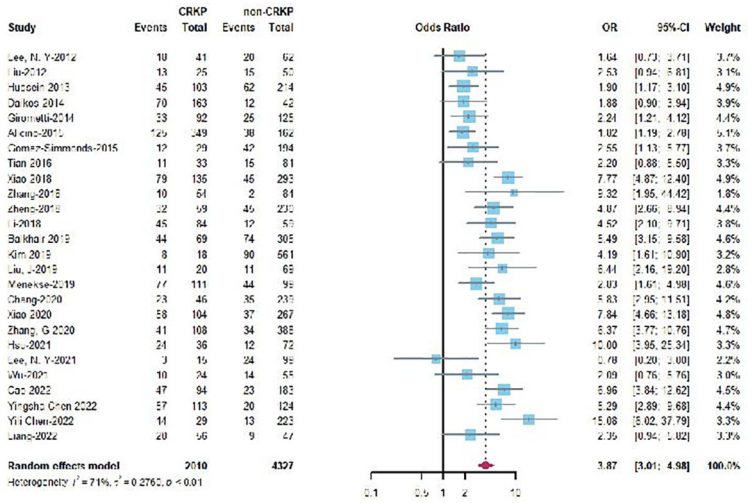

FIGURE 3
Forest plot of 30-day mortality for CRKP vs non-CRKP KPB.

Meta-regression and subgroup analysis of single proportion of 30-day mortality for KPB

Meta-regression showed that the potential sources of heterogeneity include the proportion of intensive care unit (ICU) patients, the proportion of HA patients, the proportion of patients with CRKP bacteremia, and the proportion of patients with ESBL-KP bacteremia (Table 1). Heterogeneity was not seen for factors including age, study period, study design, location, and the proportion of male, and appropriate empirical antibiotic therapy (Table 1). Subgroup analyses were further conducted to analyze the impact of the proportion of ICU, HA, CRKP, ESBL-KP on the 30-day mortality for KPB (Table 2). More than 50% of ICU, HA, CRKP, ESBL-KP were associated with a significantly higher 30-day mortality rates in patients with KPB. Partial heterogeneity was explained by these four factors

Discussion

In the systematic review of 157 studies on KPB, the pooled overall mortality rates were 17% at 7-day, 24% at 14-day, 29% at 30-day, 34% at 90-day, and 29% in hospital. It showed that more than 50% of ICU, HA, CRKP, ESBL-KP increased the 30-day mortality rates in patients with KPB.

KP belongs to *Enterobacteriaceae*, is one of the most life-threatening pathogens which can cause invasive infections (Zhang et al., 2022). Carbapenem-resistant Enterobacteriaceae (CRE) has been listed as "urgent threats" by the Centers for Disease Control and Prevention (CDC) (Chen et al., 2022). CRKP is one of the CRE species which the public most frequently encounter, accounting for 64-87.7% (Grundmann et al., 2017; Zhang et al., 2017; Li et al., 2021). However, with antimicrobial resistance increased annually causing a great health threat to the public, the accurate estimates of mortality rates for KPB have been insufficient. To our knowledge, this study provided the most up-to-date and comprehensive evidence of accurate estimation of KPB mortality rates. During the last decade, only one systematic review and meta-analysis (Kohler et al., 2017) evaluated the mortality rate of KPB including only 15 studies comparing CRKP *vs.* carbapenem-sensitive *Klebsiella pneumoniae* (CSKP). In the current study, the estimates of mortality rates for KPB were 17% at 7-day, 24% at 14-day, and 29% at 30-day, which were significantly higher than *E. coli* bacteremia (12.4%) reported by the systematic review (Bonten et al., 2021). The reasons for this scenario are not well-understood. However, there are several critical factors which might contribute to this disparity, for example patients with KPB are more likely to have a HA bacteremia, cardiovascular, or renal disease, and more likely to be in the ICU at the time of bacteremia diagnosis (Scheuerman et al., 2018). Additionally, to explore potential sources of heterogeneity, a meta-regression analyse was performed. Heterogeneity factors were found in the proportions of ICU patients, HA patients, and patients with CRKP or ESBL-KP bacteremia. However, when subgroup analyses were performed, few heterogeneities would be explained. It suggests that some other factors exist among these studies, such as the severity of illness, or the source of bacteremia. Due to the lack of data and different reporting scheme across studies, our analysis did not include these modulators. In addition, the results found a significantly higher 30-day mortality in patients with HA bacteremia. The same scenario happened for *Staphylococcus aureus* bacteremia reported in a systematic review and meta-analysis conducted by Bai and his colleagles (Bai et al., 2022). Previous studies (Tsay et al., 2002; Kang et al., 2006; Juan et al., 2019) revealed that nosocomial KPB was usually associated with a significantly higher mortality rate than community- acquired KPB. The current study re-emphasized the severity of nosocomial KPB.

In the present study, a significantly higher mortality rate in patients with CRKP bacteremia compared to those with non-CRKP bacteremia was found (Figure 3). The OR in each endpoint calculated by this study was higher than the previous study published in 2017 (Kohler et al., 2017). Alarmingly, although countermeasures have been taken to suppress these pathogens, the mortality rate of CRKP bacteremia in

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TABLE 1 Meta-regression results of single proportion of 30-day mortality for KPB.

Moderators		Number of studies	Proportion 95%-CI	Transformation	intrcpt	Test of Moderators P	heterogeneity		
							I^2 (%)	tau^2	p
Overall effect		101	0.28 (0.26; 0.31)	Logit	–	–	94.6	0.472	<0.001
Age (mean or median)	overall	92	0.30 (0.27; 0.33)	Logit	–	–	94.8	0.460	<0.001
	regression	–	–	–	-0.91	0.854	95.1	0.460	<0.001
Male (proportion)	overall	101	0.28 (0.26; 0.31)	Logit	–	–	94.6	0.472	<0.001
	regression	–	–	–	-1.96	0.111	95.1	0.459	<0.001
ICU (proportion)	overall	61	0.29 (0.26; 0.33)	Free-Tukey Double arcsine	–	–	94.6	0.023	<0.001
	regression	–	–		0.44	<0.001	93.2	0.016	<0.001
HA (proportion)	overall	48	0.27 (0.24; 0.30)	Logit	–	–	89.8	0.265	<0.001
	regression	–	–	–	-1.65	<0.001	88.4	0.201	<0.001
CRKP (proportion)	overall	69	0.32 (0.29; 0.36)	Logit	–	–	92.7	0.473	<0.001
	regression	–	–	–	-1.37	<0.001	91.1	0.289	<0.001
ESBL (proportion)	overall	24	0.21 (0.18; 0.25)	Logit	–	–	87.5	0.202	<0.001
	regression	–	–	–	-1.53	0.040	84.8	0.167	<0.001
Appropriate empirical antibiotic (proportion)	overall	52	0.31 (0.27; 0.35)	Free-Tukey Double arcsine	–	–	94.2	0.024	<0.001
	regression	–	–	–	0.66	0.245	94.5	0.024	<0.001
Location	overall	101	0.28 (0.26; 0.31)	Logit	–	–	94.6	0.472	<0.001
	regression	–	–	–	-0.94	0.405	94.5	0.457	<0.001
Design	overall	101	0.28 (0.26; 0.31)	Logit	–	–	94.6	0.472	<0.001
	regression	–	–	–	-1.53	0.585	94.8	0.440	<0.001
Period	overall	101	0.28 (0.26; 0.31)	Logit	–	–	94.6	0.472	<0.001
	regression	–	–	–	-1.04	0.265	94.8	0.453	<0.001

TABLE 2 Subgroup analyses on single proportion of 30-day mortality.

Moderators (proportion)	Number of studies	Proportion, 95%-CI	Transformation	P between Subgroups	Heterogeneity		
					Tau^2	P heterogeneity	I^2 (%)
ICU (overall)	61	0.29 (0.26; 0.33)	Free-Tukey Double arcsine	< 0.001	0.023	< 0.001	94.6
<50	39	0.24 (0.21; 0.27)	–	–	0.014	< 0.001	92.7
>=50	22	0.37 (0.34; 0.46)	–	–	0.021	< 0.001	91.9
HA (overall)	48	0.27 (0.24; 0.30)	Logit	0.003	0.265	< 0.001	89.8
<50	13	0.21 (0.18; 0.25)	–	–	0.129	< 0.001	84.4
>=50	35	0.30 (0.26; 0.34)	–	–	0.261	< 0.001	90.1

(Continued)

TABLE 2 Continued

Moderators (proportion)	Number of studies	Proportion, 95%-CI	Transformation	P between Sub-groups	Heterogeneity		
					Tau^2	P heterogeneity	I^2 (%)
CRKP (overall)	69	0.32 (0.29; 0.36)	Logit	< 0.001	0.473	< 0.001	92.7
<50	33	0.24 (0.21; 0.28)	–	–	0.243	< 0.001	89.6
>=50	36	0.41 (0.36; 0.46)	–	–	0.400	< 0.001	89.6
ESBL (overall)	24	0.21 (0.18; 0.25)	Logit	0.007	0.202	< 0.001	87.5
<50	18	0.19 (0.16; 0.23)	–	–	0.186	< 0.001	88.0
>=50	6	0.28 (0.23; 0.34)	–	–	0.076	< 0.001	71.0

patients was obviously raised with the increased of CRKP. Most recently, clinicians have faced an even greater challenge that CRKP coexists with high virulence. The scenario has led lethal outbreaks in several hospitals (Gu et al., 2018; Mohammad Ali Tabrizi et al., 2018; Zhao et al., 2019). It is concerning that these pathogens would spread rapidly if effective measurements were not applied.

The study has several limitations. First, all the included studies were observational studies, and most were retrospective. The confounding domains (age, male, severity of disease, healthcare use and so on) did not overcome or not fully overcome in most studies, therefore, most included studies were judged as serious or critical risk of bias in confounding domains producing serious or critical overall risk of bias. Second, the heterogeneities were high at most endpoints. Although a meta-regression model was used to analyze the potential sources of heterogeneity factors, due to the lack of data and different reporting scheme across studies, the study did not include some significant modulators. Third, the definitions of CRKP are somewhat variable and the microbiological breakpoints changed over time. Additionally, the definitions of HA and appropriate antibiotic therapy are somewhat different as well.

In summary, the study provides the most up-to-date and comprehensive evidence of accurate estimation if KPB mortality rate. In addition, the proportion of ICU, HA, CRKP, ESBL-KP more than 50% increased the 30-day mortality rates in patients with KPB. It is concerning that the mortality rates of patients with CRKP bacteremia may raise with the increase of CRKP. Effective countermeasures should be taken to curb this scenario.

Author contributions

JZ designed and supervised the study. DL and XH wrote the manuscript and revised the project. YL and HY performed the literature search. HR and SL checked the data. All authors approved the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1157010/full#supplementary-material

References

Bai, A. D., Lo, C. K., Komorowski, A. S., Suresh, M., Guo, K., Garg, A., et al. (2022). Staphylococcus aureus bacteremia mortality: a systematic review and meta-analysis. *Clin. Microbiol. Infect.* 28 (8), 1076–1084. doi: 10.1016/j.cmi.2022.03.015

Balkan, I. I., Alkan, M., Aygun, G., Kuskucu, M., Ankarali, H., Karagoz, A., et al. (2021). Colistin resistance increases 28-day mortality in bloodstream infections due to carbapenem-resistant klebsiella pneumoniae. *Eur. J. Clin. Microbiol. Infect. Dis.* 40 (10), 2161–2170. doi: 10.1007/s10096-020-04124-y

Bonten, M., Johnson, J. R., van den Biggelaar, A. H. J., Georgalis, L., Geurtsen, J., de Palacios, P. I., et al. (2021). Epidemiology of escherichia coli bacteremia: a systematic literature review. *Clin. Infect. Dis.* 72 (7), 1211–1219. doi: 10.1093/cid/ciaa210

Castanheira, M., Deshpande, L. M., Mendes, R. E., Canton, R., Sader, H. S., and Jones, R. N. (2019). Variations in the occurrence of resistance phenotypes and carbapenemase genes among isolates in 20 years of the SENTRY antimicrobial surveillance program. *Open Forum Infect. Dis.* 6 (Suppl 1), S23–S33. doi: 10.1093/ofid/ofy347

CDC (2022). Available at: https://www.cdc.gov/drugresistance/national-estimates.html.

Chang, E. K., Kao, K. L., Tsai, M. S., Yang, C. J., Huang, Y. T., Liu, C. Y., et al. (2015). Occult klebsiella pneumoniae bacteremia at emergency department: a single center experience. *J. microbiol. immunol. infection = Wei mian yu gan ran za zhi* 48 (6), 684–691. doi: 10.1016/j.jmii.2015.08.010

Chen, Y., Huang, H.-B., Peng, J.-M., Weng, L., and Du, B. (2022). Efficacy and safety of ceftazidime-avibactam for the treatment of carbapenem-resistant enterobacterales bloodstream infection: a systematic review and meta-analysis. *Microbiol. Spectr.* 10 (2), e0260321. doi: 10.1128/spectrum.02603-21

Cubero, M., Grau, I., Tubau, F., Pallarés, R., Domínguez, M., Liñares, J., et al. (2018). Molecular epidemiology of klebsiella pneumoniae strains causing bloodstream infections in adults. *Microb. Drug Resist.* 24 (7), 949–957. doi: 10.1089/mdr.2017.0107

Falcone, M., Bassetti, M., Tiseo, G., Giordano, C., Nencini, E., Russo, A., et al. (2020). Time to appropriate antibiotic therapy is a predictor of outcome in patients with bloodstream infection caused by KPC-producing klebsiella pneumoniae. *Crit. Care (London England)* 24 (1), 29. doi: 10.1186/s13054-020-2742-9

Gao, L., Lv, Y., and Li, Y. (2020). Analysis of the drug resistance of carbapenem-resistant klebsiella pneumoniae in the China antimicrobial resistance surveillance trial program, 2007-2018. *Microb. Drug Resist.* 26 (8), 944–950. doi: 10.1089/mdr.2019.0299

Grundmann, H., Glasner, C., Albiger, B., Aanensen, D. M., Tomlinson, C. T., Andrasević, A. T., et al. (2017). Occurrence of carbapenemase-producing klebsiella pneumoniae and escherichia coli in the European survey of carbapenemase-producing enterobacteriaceae (EuSCAPE): a prospective, multinational study. *Lancet Infect. Dis.* 17 (2), 153–163. doi: 10.1016/S1473-3099(16)30257-2

Gu, D., Dong, N., Zheng, Z., Lin, D., Huang, M., Wang, L., et al. (2018). A fatal outbreak of ST11 carbapenem-resistant hypervirulent klebsiella pneumoniae in a Chinese hospital: a molecular epidemiological study. *Lancet Infect. Dis.* 18 (1), 37–46. doi: 10.1016/S1473-3099(17)30489-9

Hyun, M., Noh, C. I., Ryu, S. Y., and Kim, H. A. (2018). Changing trends in clinical characteristics and antibiotic susceptibility of klebsiella pneumoniae bacteremia. *Korean J. Internal Med.* 33 (3), 595–603. doi: 10.3904/kjim.2015.257

Imai, K., Ishibashi, N., Kodana, M., Tarumoto, N., Sakai, J., Kawamura, T., et al. (2019). Clinical characteristics in blood stream infections caused by klebsiella pneumoniae, klebsiella variicola, and klebsiella quasipneumoniae: a comparative study, Japan, 2014-2017. *BMC Infect. Dis.* 19 (1), 946. doi: 10.1186/s12879-019-4498-x

Juan, C. H., Chuang, C., Chen, C. H., Li, L., and Lin, Y. T. (2019). Clinical characteristics, antimicrobial resistance and capsular types of community-acquired, healthcare-associated, and nosocomial klebsiella pneumoniae bacteremia. *Antimicrob. Resist. Infect. Control* 8, 1. doi: 10.1186/s13756-018-0426-x

Kang, C. I., Kim, S. H., Bang, J. W., Kim, H. B., Kim, N. J., Kim, E. C., et al. (2006). Community-acquired versus nosocomial klebsiella pneumoniae bacteremia: clinical features, treatment outcomes, and clinical implication of antimicrobial resistance. *J. Korean Med. Sci.* 21 (5), 816–822. doi: 10.3346/jkms.2006.21.5.816

Kim, D., Park, B. Y., Choi, M. H., Yoon, E. J., Lee, H., Lee, K. J., et al. (2019). Antimicrobial resistance and virulence factors of klebsiella pneumoniae affecting 30 day mortality in patients with bloodstream infection. *J. Antimicrob. Chemother.* 74 (1), 190–199. doi: 10.1093/jac/dky397

Kohler, P. P., Volling, C., Green, K., Uleryk, E. M., Shah, P. S., and McGeer, A. (2017). Carbapenem resistance, initial antibiotic therapy, and mortality in klebsiella pneumoniae bacteremia: a systematic review and meta-analysis. *Infection control Hosp. Epidemiol.* 38 (11), 1319–1328. doi: 10.1017/ice.2017.197

Lee, J. S., Choi, J. Y., Chung, E. S., Peck, K. R., and Ko, K. S. (2019). Variation in the formation of persister cells against meropenem in klebsiella pneumoniae bacteremia and analysis of its clinical features. *Diagn. Microbiol. Infect. Dis.* 95 (3), 114853. doi: 10.1016/j.diagmicrobio.2019.06.005

Lee, J. A., Kang, C. I., Joo, E. J., Ha, Y. E., Kang, S. J., Park, S. Y., et al. (2011). Epidemiology and clinical features of community-onset bacteremia caused by extended-spectrum β-lactamase-producing klebsiella pneumoniae. *Microb. Drug Resist.* 17 (2), 267–273. doi: 10.1089/mdr.2010.0134

Lee, N. Y., Lo, C. L., Chen, P. L., Syue, L. S., Li, C. W., Li, M. C., et al. (2021). Clinical impact of cefepime breakpoint in patients with carbapenem-resistant klebsiella pneumoniae bacteraemia. *Int. J. Antimicrob. Agents* 57 (2), 106250. doi: 10.1016/j.ijantimicag.2020.106250

Lee, N. Y., Wu, J. J., Lin, S. H., Ko, W. C., Tsai, L. H., and Yan, J. (2012). Characterization of carbapenem-nonsusceptible klebsiella pneumoniae bloodstream isolates at a Taiwanese hospital: clinical impacts of lowered breakpoints for carbapenems. *Eur. J. Clin. Microbiol. Infect. Dis.* 31 (8), 1941–1950. doi: 10.1007/s10096-011-1525-2

Li, D., Chen, Y., Zhang, W., Zheng, S., Zhang, Q., Bai, C., et al. (2014). Risk factors for hospital-acquired bloodstream infections caused by extended-spectrum β-lactamase klebsiella pneumoniae among cancer patients. *Irish J. Med. Sci.* 183 (3), 463–469. doi: 10.1007/s11845-013-1043-6

Li, L., and Huang, H. (2017). Risk factors of mortality in bloodstream infections caused by klebsiella pneumonia. *A single-center retrospective study in China. Medicine (Baltimore).* 96 (35):e7924. doi: 10.1097/MD.0000000000007924

Li, J., Huang, Z., Tang, M., Min, C., Xia, F., Hu, Y., et al. (2021). Clonal dissemination of multiple carbapenemase genes in carbapenem-resistant enterobacterales mediated by multiple plasmids in China. *Infection Drug resistance* 14, 3287–3295. doi: 10.2147/IDR.S327273

McGuinness, L. A., and Higgins, J. P. T. (2021). Risk-of-bias VISualization (robvis): An R package and shiny web app for visualizing risk-of-bias assessments. *Res. Synthesis Methods.* 12 (1):55–61. doi: 10.1002/jrsm.1411

Meng, H., Han, L., Niu, M., Xu, L., Xu, M., An, Q., et al. (2022). Risk factors for mortality and outcomes in hematological malignancy patients with carbapenem-resistant klebsiella pneumoniae bloodstream infections. *Drug Resistance* 15, 4241–4251. doi: 10.2147/IDR.S374904

Mohammad Ali Tabrizi, A., Badmasti, F., Shahcheraghi, F., and Azizi, O. (2018). Outbreak of hypervirulent klebsiella pneumoniae harbouring bla(VIM-2) among mechanically-ventilated drug-poisoning patients with high mortality rate in Iran. *J. Glob Antimicrob. Resist.* 15, 93–98. doi: 10.1016/j.jgar.2018.06.020

Nelson, R. E., Hatfield, K. M., Wolford, H., Samore, M. H., Scott, R. D., Reddy, S. C., et al. (2021). National estimates of healthcare costs associated with multidrug-resistant bacterial infections among hospitalized patients in the united states. *Clin. Infect. Dis.* 72 (Suppl 1), S17–S26. doi: 10.1093/cid/ciaa1581

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., et al. (2021)The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 372, n71. doi: 10.1136/bmj.n71

Pau, C. K., Ma, F. F., Ip, M., and You, J. H. (2015). Characteristics and outcomes of klebsiella pneumoniae bacteraemia in Hong Kong. *Infect. Dis. (Lond)* 47 (5), 283–288. doi: 10.3109/00365548.2014.985710

Pépin, J., Yared, N., Alarie, I., Lanthier, L., Vanasse, A., Tessier, P., et al. (2010). Klebsiella pneumoniae bacteraemia in a region of Canada. *Clin. Microbiol. Infect.* 16 (2), 141–146. doi: 10.1111/j.1469-0691.2009.02912.x

Ramos-Castaneda, J. A., Ruano-Ravina, A., Barbosa-Lorenzo, R., Paillier-Gonzalez, J. E., Saldana-Campos, J. C., Salinas, D. F., et al. (2018). Mortality due to KPC carbapenemase-producing klebsiella pneumoniae infections: systematic review and meta-analysis mortality due to KPC klebsiella pneumoniae infections. *J. Infection* 76 (5), 438–448. doi: 10.1016/j.jinf.2018.02.007

Reid, C. B., Steele, L., Pasquill, K., Parfitt, E. C., and Laupland, K. B. (2019). Occurrence and determinants of klebsiella species bloodstream infection in the western interior of British Columbia, Canada. *BMC Infect. Dis.* 19 (1), 1070. doi: 10.1186/s12879-019-4706-8

Scheuerman, O., Schechner, V., Carmeli, Y., Gutierrez-Gutierrez, B., Calbo, E., Almirante, B., et al. (2018). Comparison of predictors and mortality between bloodstream infections caused by ESBL-producing escherichia coli and ESBL-producing klebsiella pneumoniae. *Infection control Hosp. Epidemiol.* 39 (6), 660–667. doi: 10.1017/ice.2018.63

Schwarzer, G. (2022) *General package for meta-analysis*. Available at: https://link.springer.com/book/10.1007/978-3-319-21416-0.

Shen, Y. F., Chen, L. Y., and He, F. (2020). A study on clinical outcomes and death risk factors in adult patients with klebsiella pneumoniae septicemia. *Acta Med. Mediterr.* 36 (5), 3167–3171. doi: 10.19193/0393-6384_2020_5_489

Sterne JAC, H. J., and Elbers, R. G. (2016) *Reeves BC and the development group for ROBINS-I, risk of bias in non-randomized studies of interventions (ROBINS-i): detailed guidance, updated 12 October 2016*. Available at: http://www.riskofbias.info.

Tsay, R. W., Siu, L. K., Fung, C. P., and Chang, F. Y. (2002). Characteristics of bacteremia between community-acquired and nosocomial klebsiella pneumoniae infection: risk factor for mortality and the impact of capsular serotypes as a herald for community-acquired infection. *Arch. Intern. Med.* 162 (9), 1021–1027. doi: 10.1001/archinte.162.9.1021

Xiao, T., Yu, W., Niu, T., Huang, C., and Xiao, Y. (2018). A retrospective, comparative analysis of risk factors and outcomes in carbapenem-susceptible and carbapenem-nonsusceptible klebsiella pneumoniae bloodstream infections: tigecycline significantly increases the mortality. *Infection Drug resistance* 11, 595–606. doi: 10.2147/IDR.S153246

Zhang, R., Chan, E. W.-C., Zhou, H., and Chen, S. (2017). Prevalence and genetic characteristics of carbapenem-resistant enterobacteriaceae strains in China. *Lancet Infect. Dis.* 17 (3), 256–257. doi: 10.1016/S1473-3099(17)30072-5

Zhang, J., Li, D., Huang, X., Long, S., and Yu, H. (2022). The distribution of in different specimen sources and its antibiotic resistance trends in sichuan, China from 2017 to 2020. *Front. In Med.* 9, 759214. doi: 10.3389/fmed.2022.759214

Zhao, Y., Zhang, X., Torres, V. V. L., Liu, H., Rocker, A., Zhang, Y., et al. (2019). An outbreak of carbapenem-resistant and hypervirulent klebsiella pneumoniae in an intensive care unit of a major teaching hospital in wenzhou, China. *Front. Public Health* 7, 229. doi: 10.3389/fpubh.2019.00229

Zhen, X., Stålsby Lundborg, C., Sun, X., Zhu, N., Gu, S., and Dong, H. (2021). Economic burden of antibiotic resistance in China: a national level estimate for inpatients. *Antimicrobial. resistance infection control* 10 (1), 5. doi: 10.1186/s13756-020-00872-w

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Efficacy and safety of piperacillin–tazobactam compared with meropenem in treating complicated urinary tract infections including acute pyelonephritis due to extended-spectrum β-lactamase-producing *Enterobacteriaceae*

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Introduction: Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae pose a huge threat to human health, especially in the context of complicated urinary tract infections (cUTIs). Carbapenems and piperacillin–tazobactam (PTZ) are two antimicrobial agents commonly used to treat cUTIs.

Methods: A monocentric retrospective cohort study focused on the treatment of cUTIs in adults was conducted from January 2019 to November 2021. Patients with a positive urine culture strain yielding ≥ 103 colony-forming units per milliliter (CFU/mL), and sensitive to PTZ and carbapenems, were included. The primary endpoint was clinical success after antibiotic therapy. The secondary endpoint included rehospitalization and 90-day recurrence of cUTIs caused by ESBL-producing Enterobacteriaceae.

Results: Of the 195 patients included in this study, 110 were treated with PTZ while 85 were administered meropenem. The rate of clinical cure was similar between the PTZ and meropenem groups (80% vs. 78.8%, $p = 0.84$). However, the PTZ group had a lower duration of total antibiotic use (6 vs. 9; $p < 0.01$), lower

duration of effective antibiotic therapy (6 vs. 8; $p < 0.01$), and lower duration of hospitalization (16 vs. 22; $p < 0.01$).

Discussion: In terms of adverse events, the safety of PTZ was higher than that of meropenem in the treatment of cUTIs.

KEYWORDS

complicated urinary tract infections, piperacillin-tazobactam, ESBL, antibiotic treatment, meropenem

1 Introduction

Complicated urinary tract infections (cUTIs) are defined by the presence of systemic symptoms or by the susceptibility of the host to a complicated disease course (Geerlings et al., 2013). Systemic symptoms include fever, febrile UTI, and other symptoms indicative of tissue infections like pyelonephritis, prostatitis, or urosepsis syndrome (Geerlings et al., 2013). cUTIs occur in patients having a structural or functional abnormality in their genitourinary tract or through infection of urinary catheters (Melekos and Naber, 2000; Kongnakorn et al., 2019). Most of the challenging cUTIs are caused by Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, that can resist multiple antimicrobial agents by producing extended-spectrum β-lactamase (ESBL) enzymes, such as CTX-M enzymes, AmpC β-lactamases, and carbapenemases (Chatterjee et al., 2021; Syed, 2021; Jean et al., 2022; López Montesinos et al., 2022; Wald-Dickler et al., 2022; Zilberberg et al., 2022). By prolonging hospitalization, cUTIs have imposed a serious burden on healthcare systems (Food and Drug Administration, 2018; Vallejo-Torres et al., 2018; Zilberberg et al., 2021).

The treatment options for cUTIs are distinct from those for uncomplicated UTIs. For decades, carbapenems—members of the broad-spectrum β-lactam family—have been identified as the gold standard antibiotic therapy for cUTIs caused by ESBL-producing *Enterobacteriaceae*. They potently inhibit cell wall biosynthesis by preventing transpeptidation in many Gram-positive, Gram-negative, and anaerobic bacteria (Zhanel et al., 2007; Bush and Bradford, 2016; Armstrong et al., 2021). Meropenem is an antibacterial agent from the carbapenem family that is commonly used in empirical therapy for serious infections before the causative organism has been identified (Wiseman et al., 1995; Baldwin et al., 2008).

Piperacillin is a β-lactam antibiotic that is commonly used to boost the antibacterial activity of tazobactam (Drawz and Bonomo, 2010). ESBL-producing *Enterobacteriaceae* are susceptible to β-lactam/β-lactamase inhibitor combinations, such as piperacillin–tazobactam (PTZ), which can inhibit ESBL enzymes that confer antibiotic resistance.

Meropenem and PTZ constitute a considerable part of the therapeutic regimens employed in clinical practice at Xijing Hospital. The real-world setting for the treatment of cUTIs involves complex medical complications and variable physical conditions, which make the choice of the optimum antibiotic regimen a challenging one. Retrospective studies with real-world data can help clinical practitioners deliver precise prescriptions in the future.

This study aimed to utilize real-world data assessing the efficacy and safety of β-lactam antibiotics (PTZ) versus carbapenems (meropenem) for the effective treatment of cUTIs caused by ESBL-producing *Enterobacteriaceae*.

2 Methods

2.1 Objectives

A monocentric and retrospective cohort study was conducted involving patients treated for cUTIs between January 2019 and November 2021. The objective of this study was to compare the efficacy and safety of PTZ and meropenem for treating cUTIs caused by ESBL-producing *Enterobacteriaceae*.

2.2 Study design

Adult patients were eligible if they were diagnosed with cUTIs caused by ESBL-producing *Enterobacteriaceae* that were non-susceptible to ceftriaxone or cefotaxime, but susceptible to PTZ and meropenem. Some patients may suffer from acute cystitis and acute pyelonephritis due to the administered interventions. Patients were treated with PTZ or meropenem for more than 72 h. General practitioners adapted the antibiotic dosage, administration route, and treatment duration for each individual based on their renal function as measured by their creatinine clearance rate.

2.3 Inclusion/exclusion criteria

The local research team screened patients manually using inclusion and exclusion criteria, and data were selected from patient medical charts using standardized case report forms.

Abbreviations: ESBL, extended-spectrum β-lactamase; cUTIs, complicated urinary tract infections; BLBLIs, β-lactam/β-lactamase inhibitors; AEs, adverse events; IQR, interquartile range; OR, odds ratio; CI, confidence intervals; ICU, intensive care unit; SIRS, systemic inflammatory response syndrome; CCI, Charlson comorbidity index; Amino-R, aminoglycosides resistant; MIC, minimum inhibitory concentration; RCTs, randomized clinical trials.

2.3.1 Inclusion criteria

Patients who met all the following criteria were included in this study:

- Age ≥ 18 years
- Documented cUTIs, with a positive urine culture strain ≥ 10^3 colony-forming units per milliliter (CFU/ml) that is sensitive to PTZ and carbapenems. The cUTIs must be accompanied by at least two of the following signs and symptoms (Food and Drug Administration, 2018):
- chills or fever (temperature > 38°C)
- nausea or vomiting
- flank or pelvic pain
- dysuria, urinary frequency, or urgency
- costovertebral angle tenderness on physical examination (Delory et al., 2021)

2.3.2 Exclusion criteria

Patients who met any of the following criteria were excluded from this study:

- Antibiotic treatment for less than 72 h
- Urine culture positive for Gram-positive bacteria at a colony count ≥ 10^5 CFU/ml
- Urine culture positive for fungi at a colony count > 10^3 CFU/ml
- Repeated exposure to the drugs used in this study

2.4 Study procedures

The experimental procedures included baseline urine collection for quantitative culture. The signs and symptoms of cUTIs and adverse events were routinely monitored. Prespecified laboratory data, including chemistry panels and urine cultures, were collected.

Baseline urine cultures at a colony count ≥ 10^5 CFU/ml were sent to the central laboratory (Department of Laboratory Medicine, Institute of Clinical Laboratory Medicine of PLA, Xijing Hospital, Air Force Military Medical University) for identification, quantification, susceptibility testing, and further characterization of the organism(s). The minimum inhibitory concentrations (MICs) of PTZ and meropenem were determined using the agar dilution reference method, following the laboratory procedures and MIC breakpoints provided by CLSI (2021).

2.5 Study endpoints

The primary efficacy endpoint of this study was the clinical and microbiological response in patients with cUTIs at the end of the initial antibiotic regimens. The clinical response was defined by the Food and Drug Administration (FDA) as follows:

- Resolution of all core cUTI symptoms, including fever (temperature > 38°C), dysuria, urinary frequency, urinary urgency, suprapubic pain, and flank pain
- No appearance of new cUTI symptoms
- No further use of antibiotics with microbiological cure (urine culture at a colony count < 10^3 CFU/ml, negative blood culture)

According to the FDA guidelines, the microbiological response was defined as a colony count of the bacterial pathogen, identified at trail entry, falling below 10^3 CFU/ml in the urine (Food and Drug Administration, 2018).

Clinical failure was defined as the non-resolution of fever (temperature > 38°C) and cUTI symptoms, the development of new symptoms, or all-cause mortality of the patient. Microbiological failure was defined as the pathogen at a colony count exceeding or equalling 10^3 CFU/ml during or after the treatment. Clinical uncertainty was defined as the loss of urine samples after baseline therapy.

The secondary efficacy endpoint pertained to the determination of sustained microbiological success and all clinical cures. It was measured in terms of the rates of rehospitalization and recurrent cUTIs due to the same ESBL-producing *Enterobacteriaceae* within 3 months of the initial antibiotic treatment.

The safety endpoints included the incidence of *Clostridium difficile* infections, rehospitalization, the loss of intervention and follow-up, and all-cause mortality within 3 months of the first day of effective treatment.

2.6 Statistical analysis

The International Business Machines Corporation SPSS Statistics for Windows Version 22.0 and Microsoft Excel (MS Office 2019) were used for data analysis. The analysis population, which included any patients receiving the correct antibiotics, was used to compare the efficacy and safety of the treatment group (PTZ) with that of the control group (meropenem), in terms of the primary and secondary outcomes. The study outcomes were presented using descriptive statistics, such as frequencies and percentages, medians, and interquartile ranges. Medians and interquartile ranges were reported instead of means and standard deviations because the continuous variables were not normally distributed. Nonparametric tests were used in this data analysis. The primary analysis included a chi-squared test to compare the proportion of patients with cUTIs that showed clinical success in the PTZ and meropenem groups. The continuous variables were compared using chi-squared or Fisher's exact test. For all tests and results, a p-value less than 0.05 was considered to denote a statistically significant difference. The strength of association with primary and secondary endpoints was computed using conditional logistic regression adjusted on the study arm. Associations were reported as odds ratios with 95% confidence intervals. Data curation and statistical analyses were performed using Review Manager 5.3.

3 Results

3.1 Demographic and clinical characteristics of patients

From a total of 323 patients with cUTIs, 128 were excluded: 16 received antibiotics for less than 72 h, 59 carried Gram-positive organisms in their urine, 18 tested positive for fungi, and 35 had been repeatedly exposed to the studied antibiotics (Figure 1). Of the 195 patients eligible for this study, 110 constituted the PTZ group and 85 formed the meropenem group (Table 1). Between these two groups, 48 patients (25%) suffered from chronic liver diseases, and 32 (16%) took at least 20 mg of oral corticosteroids daily.

Overall, the baseline characteristics of the two groups were similar (Table 1), although the average age and the proportion of patients with chronic liver diseases were both higher in the meropenem group (71 years vs. 65 years, 33% vs. 18%,

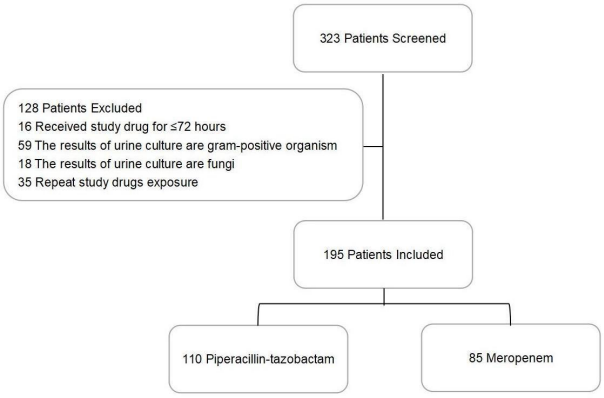

FIGURE 1
Patient selection procedures.

TABLE 1 Patients' and cUTI characteristics at baseline [a].

	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
Patients' characteristics				
Sex, male (%)	39 (35.5%)	41 (48.2%)	80 (41.0%)	0.072
Age [b]	65	71	67	**0.033**
Charlson score [b,c]	7	7	7	0.276
Creatinine clearance, eGFR formula [d]	68 (51–97)	70 (49–98)	68 (50–98)	0.829
SIRS [e]	55 (50.0%)	46 (54.1%)	101 (51.8%)	0.568
Confounding factors				
Immunocompromised	3 (2.7%)	3 (3.5%)	6 (3%)	0.748
Hematological malignancy	3 (2.7%)	3 (3.5%)	6 (3%)	0.748
Kidney transplant recipient	2 (1.8%)	0	2 (1%)	0.211
Other solid organ transplant recipient	1 (0.9%)	1 (1.2%)	2 (1%)	0.854
Diabetes mellitus	46 (42%)	29 (34%)	75 (38%)	0.273
Chronic kidney disease	41 (37%)	26 (31%)	67 (34%)	0.330
Chronic respiratory disease	8 (7.3%)	9 (11%)	17 (8.7%)	0.416
History of malignancy	20 (18%)	17 (20%)	37 (19%)	0.748
Chronic liver disease	20 (18%)	28 (33%)	48 (25%)	**0.018**
Risk factors for ESBL-producing Enterobacteriaceae				
Chemotherapy within 3 months	11 (10%)	4 (4.7%)	15 (7.7%)	0.169
Daily dose of oral corticosteroids ≥ 20 mg	24 (22%)	8 (9.4%)	32 (16%)	**0.020**
History of UTI	31 (28%)	31 (36%)	62 (32%)	0.218
Recurrent UTI [f]	25 (23%)	28 (33%)	53 (27%)	0.112
Hospitalization within 3 months	57 (52%)	53 (62%)	110 (56%)	0.141
Invasive procedure within 3 months	19 (17%)	24 (28%)	43 (22%)	0.067
Antibiotics within 6 months	51 (46%)	49 (58%)	100 (51%)	0.118
Fluoroquinolones	19 (17%)	11 (13%)	30 (15%)	0.406

(Continued)

TABLE 1 Continued

	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
Carbapenems	8 (7.3%)	15 (18%)	23 (12%)	**0.026**
Other β-lactams	24 (22%)	26 (31%)	50 (26%)	0.164
Unknown	15 (14%)	9 (11%)	24 (12%)	0.521
cUTI characteristics				
Signs and diagnosis	85 (77%)	56 (66%)	141 (72%)	0.078
Appropriate antibiotic therapy [g]	73 (66%)	44 (52%)	117 (60%)	**0.039**
Healthcare associated	57 (52%)	40 (47%)	97 (50%)	0.510
Fever (>38°C)	38 (35%)	44 (52%)	82 (42%)	**0.016**
Chill	8 (7.3%)	12 (14%)	20 (10%)	0.118
Bacteremia	12 (11%)	12 (14%)	24 (12%)	0.499
Required urinary catheterization	33 (30%)	48 (56%)	81 (42%)	**0.000**
Required double-J stent	2 (1.8%)	0	2 (1%)	0.211
ICU admission	11 (10%)	20 (24%)	31 (16%)	**0.010**

ICU, intensive care unit; IQR, interquartile range; SIRS, systemic inflammatory response syndrome.
[a]Data are expressed as No. (%) of participants unless otherwise indicated.
[b]Average.
[c]Charlson comorbidity index (CCI) is a health tool based on the CCI model that assesses the comorbidity risk associated with a series of conditions that offer medical specialists an informed decision-making process, specific screenings, or medical procedures.
[d]Median.
[e]SIRS, systemic inflammatory response syndrome.
[f]Recurrent UTI: Defined as the occurrence of ≥2 UTIs within 6 months, or ≥3 UTIs within a year.
[g]Appropriateness of antibiotic therapy was defined as an antibiotic therapy active on the strain involved in the current UTI, based on the local procedure for susceptibility testing.
The bold values means there is significant differences in this group of people.

respectively). In addition, the proportion of patients who received ≥ 20 mg of oral corticosteroids was 22% and 9.4% in the PTZ and meropenem groups, respectively. Before hospitalization and treatment, a larger proportion of patients in the PTZ group were receiving appropriate antibiotic treatments (66% vs. 52%). Moreover, 18% of patients took carbapenem antibiotics in the meropenem group, compared with 7.3% in the PTZ group. More patients from the meropenem group suffered from fever, urethral catheterization, and intensive care unit treatment (52% vs. 35%, 56% vs. 30%, and 24% vs. 10%, respectively). The etiological agents causing cUTIs were similarly distributed between the two groups (Table 2): a total of 82 patients (42%) were infected with *E. coli*, 35

TABLE 2 Microbiological characteristics [a].

	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
Gram-negative Enterobacteriaceae				
Escherichia coli	60 (55%)	22 (26%)	82 (42%)	**0.000**
Klebsiella pneumoniae	20 (18%)	15 (18%)	35 (18%)	0.923
Enterobacter guspura	3 (2.7%)	1 (1.2%)	4 (2%)	0.449
Serratia marcescens	2 (1.8%)	1 (1.2%)	3 (1.5%)	0.718
else	0	1 (1.2%)	1 (0.5%)	0.254
Gram-negative aerobes other than Enterobacteriaceae				
Pseudomonas aeruginosa	8 (7.3%)	3 (4.3%)	11 (5.8%)	0.261
Enterobacter aerogenes	1 (0.9%)	2 (2.9%)	3 (1.6%)	0.417
Proteus mirabilis	2 (1.8%)	1 (1.4%)	3 (1.6%)	0.718
Citrobacter koseri	1 (0.9%)	1 (1.4%)	2 (1.1%)	0.854
Drug resistance				

(Continued)

TABLE 2 Continued

	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
ESBL				
Escherichia coli	37 (33.6%)	12 (14.1%)	49 (25.1%)	**0.002**
Klebsiella pneumoniae	13 (11.8%)	9 (11%)	22 (11.3%)	0.788
Enterobacter guspura	3 (2.7%)	0	3 (1.5%)	-
Serratia marcescens	2 (1.8%)	1 (1.2%)	3 (1.5%)	0.718
Pseudomonas aeruginosa	4 (3.6%)	1 (1.2%)	5 (2.6%)	0.281
Enterobacter aerogenes	1 (0.9%)	2 (2.4%)	3 (1.5%)	0.417
Proteus mirabilis	1 (0.9%)	0	1 (0.5%)	-
Amino-R				
Escherichia coli	24 (21.8%)	6 (7.1%)	30 (15.4%)	**0.005**
Klebsiella pneumoniae	4 (3.6%)	4 (4.7%)	8 (4.1%)	0.709
Enterobacter aerogenes	1 (0.9%)	0	1 (0.5%)	-

ESBL, extended-spectrum β-lactamase; Amino-R, aminoglycosides resistant; MIC, minimum inhibitory concentration.
Using MICs from an accompanying antibiotic panel or agar dilution supplemented with glucose 6-phosphate for PTZ/Meropenem, urine isolates were identified to assess patients and microbiologic outcomes. The following definitions were used for this assessment-ESBL: ≥2 μg/ml MIC for aztreonam, ceftazidime, or ceftriaxone; Amino-R: gentamicin ≥8 μg/ml or amikacin ≥32 μg/ml. Patients could have more than 1 isolate from urine sources, and all organisms are presented for completeness. Patients with multiple organisms were counted only once per resistance grouping. If the same species was identified from a different source, the isolate was counted once for the microbiological outcomes.
[a]Data are expressed as No. (%) of participants unless otherwise indicated.
The bold values means there is significant differences in this group of people.

(18%) with *K. pneumoniae*, and 11 with *Pseudomonas aeruginosa* (5.6%).

3.2 Microbiology

E. coli and *K. pneumoniae* were considered the most common infection-causing pathogens in this study cohort. For the treatment of *E. coli* infections, 55% (60/110) of the patients received PTZ while 26% (22/85) chose meropenem. In contrast, 18% of the patients from both the groups (20/110, 15/85) chose the respective drugs to treat *K. pneumoniae* infections. ESBL-producing and aminoglycosides-resistant (amino-R) *E. coli* were identified as the most resistant pathogens. To combat the former, 33.6% (37/110) and 14.1% (12/85) of the patients used PTZ and meropenem, respectively. For the latter, 21.8% (24/110) and 7.1% (6/110) of the patients chose PTZ and meropenem, respectively. Overall, a higher proportion of patients in the PTZ group tested positive for three different types of *Enterobacteriaceae* in their urine: *E. coli*, ESBL-producing *E. coli*, and amino-R *E. coli* (p = 0.000, 0.002, and 0.005, respectively) (Table 2).

3.3 Treatment

3.3.1 Safety endpoints

The overall duration of antibiotic therapy (9 vs. 6), the effective duration of antibiotic therapy (8 vs. 6), the length of hospital stay (22 vs. 16), and all-cause mortality (18.8% vs. 7.3%) were all higher in the meropenem group than in the PTZ group (Table 3). However, no

TABLE 3 Several occurrences of safety endpoints [a].

Endpoints	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
Overall duration of antibiotic therapy [c]	6 (4–9)	9 (6–14.5)	7 (5–11)	**0.000**
Effective antibiotic therapy duration [b, c]	6 (4–9)	8 (5–13)	7 (5–10)	**0.000**
Length of hospital stay [c]	16 (11–30)	22 (15–41)	18 (13–34)	**0.010**
All-cause death	8 (7.3%)	16 (18.8%)	24 (12.3%)	**0.015**
Relapse of UTI	8 (7.3%)	6 (7.1%)	14 (7.2%)	0.954
Re-hospitalization	91 (82.7%)	63 (74.1%)	154 (79%)	0.143
Loss to follow-up	11 (10%)	7 (8.2%)	18 (9.2%)	0.673

[a]Data are expressed as No. (%) of participants unless otherwise indicated.
[b]Duration of effective antibiotic treatment: More than 3 consecutive days of therapy was considered as an effective treatment; if not, only continuous time was counted.
[c]Median.
The bold values means there is significant differences in this group of people.

significant difference was observed between the two groups in the relapse of cUTIs, rehospitalization, and loss to follow-up.

3.3.2 Efficacy endpoints

In terms of clinical cure and microbial eradication, the clinical success of both the groups was comparable. The overall success in the two groups was 80% and 78.8% (treatment difference, 1.2%; 95% confidence interval, 10.3–12.6) (Table 4, Figure 2).

3.3.3 Microbiological endpoints

Subgroup analyses focused on ESBL-producing and amino-R *E. coli* were conducted. Although clinical cure rates were high and did not differ significantly between the two groups, clinical and microbiological endpoint success was more significant in the PTZ group than in the meropenem group (Tables 5–7).

3.4 Safety

The incidence of adverse events was significantly lower in the PTZ group (4.5% vs. 15.3%, $p = 0.01$). In the meropenem group, the most common adverse event was abnormal liver function, afflicting 5.9% of the patients. In the PTZ group, the most common adverse event was anaphylaxis, affecting 2.7% of the patients (Table 8).

4 Discussion

According to the FDA (US Food & Drug Administration, 2018) and the Association of the British Pharmaceutical Industry, real-world evidence is defined as the analysis of real-world clinical data to evaluate what is happening in normal clinical practice. Following appeals from many agencies, studies focusing on real-world evidence have received much attention. Different from randomized clinical trials, clinical studies in the real-world setting deal with larger populations with complex situations, physical functions, and variable treatment combinations. Such studies represent the general population, provide deeper insights into clinical practice, and help discover real-life problems (Kim et al., 2018).

However, clinical retrospective studies in the real world suffer from some unavoidable drawbacks. The healthcare practitioners directly decide the drugs to be administered after analyzing multi-indicators. Changing the medicines for finishing the clinical study without considering clinical ethics is illegal.

This study aimed to compare the efficacy and safety of PTZ and meropenem in treating cUTIs in the real world. In this monocentric and case–control study, patients diagnosed with cUTIs received antibiotic therapy, and the clinical cure rate was high (79.5%) at the end of the treatment. The clinical characteristics of patients who took PTZ and meropenem were similar, and their clinical response

TABLE 4 Occurrence of primary and secondary endpoints [a].

Endpoints	PTZ (n = 110)	Meropenem (n = 85)	Total (n = 195)	p-value	Treatment difference (95% CI)
Primary efficacy endpoints					
Success	88 (80%)	67 (78.8%)	155 (79.5%)	0.840	1.2 (10.3–12.6)
Failure	10 (9.1%)	12 (14.1%)	22 (11.3%)		
Indeterminate	12 (10.9%)	6 (7.1%)	18 (9.2%)		
Secondary efficacy endpoint—clinical endpoint response					
Success	66 (60%)	48 (56.5%)	114 (58.5%)	0.620	3.5 (10.4–17.5)
Failure	19 (17.3%)	17 (20%)	36 (18.5%)		
Indeterminate	25 (22.7%)	20 (23.5%)	45 (23.1%)		
Secondary efficacy endpoint—microbiological endpoint response					
Success	66 (60%)	48 (56.5%)	114 (58.5%)	0.620	4.7 (9.3–18.7)
Failure	19 (17.3%)	16 (18.8%)	35 (17.9%)		
Indeterminate	25 (22.7%)	21 (24.7%)	46 (23.6%)		

[a]Data are expressed as No. (%) of participants unless otherwise indicated.

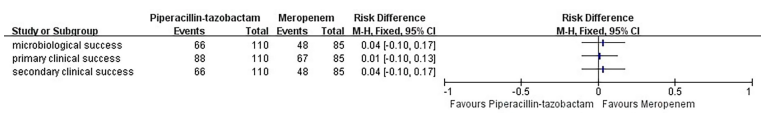

FIGURE 2
Primary efficacy endpoints.

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TABLE 5 Clinical and microbiologic outcomes among patients with baseline ESBL-producing *Escherichia coli* characteristics (occurrence of primary and secondary endpoints) [a].

Endpoints	PTZ (*n* = 37)	Meropenem (*n* = 12)	Total (*n* = 49)	*p*-value
Primary efficacy endpoints				
Success	27 (73%)	9 (75%)	36 (73.5%)	0.890
Failure	3 (8.1%)	1 (8.3%)	4 (8.2%)	
Indeterminate	7 (18.9%)	2 (16.7%)	9 (18.4%)	
Secondary efficacy endpoint—clinical endpoint response				
Success	19 (51.4%)	3 (25%)	22 (44.9%)	0.066
Failure	6 (16.2%)	3 (25%)	9 (18.4%)	
Indeterminate	12 (32.4%)	6 (50%)	18 (36.7%)	
Secondary efficacy endpoint—microbiological endpoint response				
Success	22 (59.5%)	4 (33.3%)	26 (53.1%)	0.115
Failure	6 (16.2%)	1 (8.3%)	7 (12.3%)	
Indeterminate	9 (24.3%)	7 (58.3%)	16 (32.7%)	

[a]Data are expressed as No. (%) of participants unless otherwise indicated.

rates were 80% and 78.8%, respectively. Furthermore, the clinical response did not differ significantly among patients with several health conditions, including immunocompromised patients and those with hematological malignancies, kidney and other solid organ transplantations, diabetes, chronic kidney disease, chronic respiratory disease, and other malignancies.

The higher clinical response of PTZ (80%) was not related to the microbiological or clinical characteristics of the patients. PTZ was used at a standard dosage of 4.5 g q8h, or adjusted according to creatinine clearance for pathogenic infections with a minimum inhibitory concentration ≤ 8 mg/L. In this study, the most common pathogens were *E. coli* (42%) and *K. pneumoniae* (18%), and one-third of the patients were diagnosed with diabetes mellitus or chronic kidney diseases.

The clinical efficacy and safety of PTZ and meropenem were similar in treating cUTIs. However, the increased use of carbapenems may lead to the selection of carbapenem resistance in Gram-negative bacilli and the spread of carbapenemase-encoding antibiotic resistance genes (Mendes et al., 2018). To avoid serious outcomes and achieve appropriate prescriptions,

TABLE 6 Clinical and microbiologic outcomes among patients with baseline amino-R *Escherichia coli* characteristics (occurrence of primary and secondary endpoints)[a].

Endpoints	PTZ (*n* = 24)	Meropenem (*n* = 6)	Total (*n* = 30)	*p*-value
Primary efficacy endpoints				
Success	20 (83.3%)	6 (100%)	26 (86.7%)	0.283
Failure	0	0	0	
Indeterminate	4 (16.7%)	0	4 (13.3%)	
Secondary efficacy endpoint—clinical endpoint response				
Success	18 (75%)	3 (50%)	21 (70%)	0.232
Failure	0	2 (33.3%)	2 (6.7%)	
Indeterminate	6 (25%)	1 (16.7%)	7 (23.3%)	
Secondary efficacy endpoint—microbiological endpoint response				
Success	18 (75%)	4 (66.7%)	22 (73.3%)	0.680
Failure	0	1 (16.7%)	1 (3.3%)	
Indeterminate	6 (25%)	1 (16.7%)	7 (23.3%)	

[a]Data are expressed as No. (%) of participants unless otherwise indicated.

TABLE 7 Clinical and microbiologic outcomes among patients with baseline ESBL-producing and amino-R *Escherichia coli* characteristics (occurrence of primary and secondary endpoints)[a].

Endpoints	PTZ (*n* = 61)	Meropenem (*n* = 18)	Total (*n* = 79)	*p*-value
Primary efficacy endpoints				
Success	47 (77%)	15 (83.3%)	62 (78.5%)	0.569
Failure	3 (4.9%)	1 (5.6%)	4 (5.1%)	
Indeterminate	11 (18%)	2 (11.1%)	13 (16.5%)	
Secondary efficacy endpoint—clinical endpoint response				
Success	37 (60.7%)	6 (33.3%)	43 (54.4%)	**0.041**
Failure	6 (9.8%)	5 (27.8%)	11 (13.9%)	
Indeterminate	18 (29.5%)	7 (38.9%)	25 (31.6%)	
Secondary efficacy endpoint—microbiological endpoint response				
Success	40 (65.6%)	8 (44.4%)	48 (60.8%)	0.107
Failure	6 (9.8%)	2 (11.1%)	8 (10.1%)	
Indeterminate	15 (24.6%)	8 (44.4%)	23 (29.1%)	

[a]Data are expressed as No. (%) of participants unless otherwise indicated.
The bold values means there is significant differences in this group of people.

TABLE 8 Adverse patient events [a].

	PTZ (*n* = 110)	Meropenem (*n* = 85)	Total (*n* = 195)	*p*-value
No. of all adverse events (%)	5 (4.5%)	13 (15.3%)	1 (0.5%)	**0.01**
Diarrhea	0	1 (1.2%)	1 (0.5%)	–
No. with thrombocytosis [b] (%)	0	4 (4.7%)	4 (2.1%)	–
No. with thrombocytopenia [c] (%)	2 (1.8%)	1 (1.2%)	3 (1.5%)	0.718
No. with bone marrow suppression [d](%)	0	1 (1.2%)	1 (0.5%)	–
No. with hepatic insufficiency (%)	0	5 (5.9%)	5 (2.6%)	–
No. with rash (%)	3 (2.7%)	1 (1.2%)	4 (2.1%)	0.449

[a]Data are expressed as No. (%) of participants unless otherwise indicated.
[b]Thrombocytosis = blood cell count of ≥450 cells × 10^9/L.
[c]Thrombocytopenia = blood cell count of <150 cells × 10^9/L.
[d]Bone marrow suppression = with leukopenia count (3.0–3.9) × 10^9/L, hemoglobin count 95–100 g/L, blood cell count (75–99) × 10^9/L.
The bold values means there is significant differences in this group of people.

many studies have compared carbapenems with other antibacterials (Chen et al., 2018; Sternbach et al., 2018; Zhong et al., 2018; Ezure et al., 2022). In these meta-analyses, carbapenems present similar clinical efficacies and possibly better microbiological responses than other antibacterial agents. When using antibiotics, the possibility and consequences of resistance and related health economics must be considered.

The efficacy of drugs varies with the patients' physical conditions, complications, and history of taking other drugs. We, as clinical practitioners, found that there is a lack of therapeutic advice on drugs that share similar indications or targeting the same pathogens, to help physicians deliver more effective, precise, and cost-effective treatments to different patients. More clinical real-world data need to be collected and analyzed to construct detailed guidelines for drug administration. These studies should be designed based on the standard format of medical records between multiple centers and should overcome limitations as much as possible.

5 Limitations

This study had several limitations. Firstly, this monocenter and retrospective study was limited by an insufficient sample size and a small number of case events, which led to restricted statistical power to manage confounding factors. Secondly, the extensive use of PTZ in the first line resulted in a population selection and biased measurement of point estimates. Only randomization can prevent such biases. Lastly, other antibiotics in clinical practice may influence the clinical efficacy and incidence of adverse effects.

6 Conclusions

PTZ is an effective, safe, and definite treatment option for cUTIs due to the presence of ESBL-producing and amino-R *Enterobacteriaceae*. Its efficacy is consistent with that of immunocompromised patients, including those with hematological malignancies, kidney and other solid organ transplant recipients, diabetes mellitus, chronic kidney disease, chronic respiratory disease, and a history of malignancies. However, the risk of emerging resistance stresses the need for close monitoring. A randomized comparison with carbapenems is warranted.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Hospital Personal Information. Requests to access these datasets should be directed to 1358359@qq.com.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the First Affiliated Hospital of the Air Force Medical University. The patients/participants provided written informed consent to participate in the study. 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

J-WW designed the study and approved the final version of the manuscript. WZ, C-YY, T-TF, YG, and S-RL performed the study. WZ and C-YY analyzed the data and wrote the manuscript. S-SC, BC, M-YL, B-YF, BJ, LiW, FC, JC, and LeW were involved in conceptualization, methodology, and analysis of the study. BC and T-TF provided statistical analysis assistance and helped to revise the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

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

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References

Armstrong, T., Fenn, S. J., and Hardie, K. R. (2021). JMM profile: Carbapenems: a broad-spectrum antibiotic. *J. Med. Microbiol.* 70 (12). doi: 10.1099/jmm.0.001462

US Food & Drug Administration (2018). *Framework for FDA's real-world evidence program* Available at: https://www.fda.gov/media/120060/download.

Baldwin, C. M., Lyseng-Williamson, K. A., and Keam, S. J. (2008). Meropenem: a review of its use in the treatment of serious bacterial infections. *Drugs* 68 (6), 803–838. doi: 10.2165/00003495-200868060-00006

Bush, K., and Bradford, P. A. (2016). β-lactams and β-lactamase inhibitors: An overview. *Cold Spring Harb. Perspect. Med.* 6 (8). doi: 10.1101/cshperspect.a025247

Chatterjee, S., Chakraborty, D. S., Choudhury, S., and Lahiry, S. (2021). Cefiderocol-a new antimicrobial for complicated urinary tract infection (CUTI) caused by carbapenem resistant enterobacteriaceae (CRE). *Curr. Drug Res. Rev.* 14 (1), 20–23. doi: 10.2174/2589977513666211206100749

Chen, M., Zhang, M., Huang, P., Lin, Q., Sun, C., Zeng, H., et al. (2018). Novel β-lactam/β-lactamase inhibitors versus alternative antibiotics for the treatment of complicated intra-abdominal infection and complicated urinary tract infection: A meta-analysis of randomized controlled trials. *Expert Rev. Anti Infect. Ther.* 16 (2), 111–120. doi: 10.1080/14787210.2018.1429912

CLSI (2021). *Performance standards for antimicrobial susceptibility testing, M100. 31st ed* (Wayne, PA: Clin Lab Stand Institute).

Delory, T., Gravier, S., Le Pluart, D., Gaube, G., Simeon, S., Davido, B., et al. (2021). Temocillin versus carbapenems for urinary tract infection due to ESBL-producing enterobacteriaceae: A multicenter matched case-control study. *Int. J. Antimicrob. Agents.* 58 (1), 106361. doi: 10.1016/j.ijantimicag.2021.106361

Drawz, S. M., and Bonomo, R. A. (2010). Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.* 23 (1), 160–201. doi: 10.1128/CMR.00037-09

Ezure, Y., Rico, V., Paterson, D. L., Hall, L., Harris, P. N. A., Soriano, A., et al. (2022). Efficacy and safety of carbapenems vs new antibiotics for treatment of adult patients with complicated urinary tract infections: A systematic review and meta-analysis. *Open Forum Infect. Dis.* 9 (5), ofaa480. doi: 10.1093/ofid/ofaa480

Food and Drug Administration. (2018). Complicated urinary tract infections: Developing drugs for treatment guidance for industry.

Geerlings, S. E., Van Nieuwkoop, C., Van Haarst, E., van Buren, M., Knotterus, B. J., Stobberingh, E. E., et al. (2013). *SWAB guidelines for antimicrobial therapy of complicated urinary tract infections in adults* (The Netherlands Institute for Health Services Research: SWAB Guidel).

Jean, S.-S., Lu, M.-C., Ho, M.-W., Ko, W.-C., and Hsueh, P.-R. (2022). Non-susceptibilities to antibiotics against important gram-negative bacteria, and imipenem-relebactam, meropenem-vaborbactam against carbapenem non-susceptible enterobacterales and pseudomonas aeruginosa isolates implicated in complicated intra-abdominal. *Int. J. Antimicrob. Agents*, 106521. doi: 10.1016/j.ijantimicag.2022.106521

Kim, H.-S., Lee, S., and Kim, J. H. (2018). Real-world evidence versus randomized controlled trial: Clinical research based on electronic medical records. *J. Korean Med. Sci.* 33 (34), e213. doi: 10.3346/jkms.2018.33.e213

Kongnakorn, T., Wagenlehner, F., Falcone, M., Tichy, E., Di Virgilio, R., Baillon-Plot, N., et al. (2019). Cost-effectiveness analysis of ceftazidime/avibactam compared to imipenem as empirical treatment for complicated urinary tract infections. *Int. J. Antimicrob. Agents.* 54 (5), 633–641. doi: 10.1016/j.ijantimicag.2019.06.008

López Montesinos, I., Gómez-Zorrilla, S., Palacios-Baena, Z. R., Prim, N., Echeverria-Esnal, D., Gracia, M. P., et al. (2022). Aminoglycoside or polymyxin monotherapy for treating complicated urinary tract infections caused by extensively drug-resistant pseudomonas aeruginosa: A propensity score-adjusted and matched cohort study. *Infect. Dis. Ther.* 11 (1), 335–350. doi: 10.1007/s40121-021-00570-z

Melekos, M. D., and Naber, K. G. (2000). Complicated urinary tract infections. *Int. J. Antimicrob. Agents.* 15 (4), 247–256. doi: 10.1016/S0924-8579(00)00168-0

Mendes, R. E., Castanheira, M., Woosley, L. N., Stone, G. G., Bradford, P. A., and Flamm, R. K. (2018). Molecular β-lactamase characterization of gram-negative pathogens recovered from patients enrolled in the ceftazidime-avibactam phase 3 trials (RECAPTURE 1 and 2) for complicated urinary tract infections: Efficacies analysed against susceptible and resis. *Int. J. Antimicrob. Agents.* 52 (2), 287–292. doi: 10.1016/j.ijantimicag.2018.04.001

Sternbach, N., Leibovici Weissman, Y., Avni, T., and Yahav, D. (2018). Efficacy and safety of ceftazidime/avibactam: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* 73 (8), 2021–2029. doi: 10.1093/jac/dky124

Syed, Y. Y. (2021). Cefiderocol: A review in serious gram-negative bacterial infections. *Drugs.* 81 (13), 1559–1571. doi: 10.1007/s40265-021-01580-4

Vallejo-Torres, L., Pujol, M., Shaw, E., Wiegand, I., Vigo, J. M., Stoddart, M., et al. (2018). Cost of hospitalised patients due to complicated urinary tract infections: A retrospective observational study in countries with high prevalence of multidrug-resistant gram-negative bacteria: The COMBACTE-MAGNET, RESCUING study. *BMJ Open* 8 (4), e020251. doi: 10.1136/bmjopen-2017-020251

US Food & Drug Administration. (2018). Framework for FDA's real-world evidence program. Available at: https://www.fda.gov/media/120060/download.

Wald-Dickler, N., Lee, T. C., Tangpraphaphorn, S., Butler-Wu, S. M., Wang, N., Degener, T., et al. (2022). Fosfomycin vs ertapenem for outpatient treatment of complicated urinary tract infections: A multicenter, retrospective cohort study. *Open Forum Infect. Dis.* 9 (1), ofab620. doi: 10.1093/ofid/ofab620

Wiseman, L. R., Wagstaff, A. J., Brogden, R. N., and Bryson, H. M. (1995). Meropenem. a review of its antibacterial activity, pharmacokinetic properties and clinical efficacy. *Drugs.* 50 (1), 73–101. doi: 10.2165/00003495-199550010-00007

Zhanel, G. G., Wiebe, R., Dilay, L., Thomson, K., Rubinstein, E., Hoban, D. J., et al. (2007). Comparative review of the carbapenems. *Drugs.* 67 (7), 1027–1052. doi: 10.2165/00003495-200767070-00006

Zhong, H., Zhao, X. Y., Zhang, Z. L., Gu, Z. C., Zhang, C., Gao, Y., et al. (2018). Evaluation of the efficacy and safety of ceftazidime/avibactam in the treatment of gram-negative bacterial infections: A systematic review and meta-analysis. *Int. J. Antimicrob. Agents.* 52 (4), 443–450. doi: 10.1016/j.ijantimicag.2018.07.004

Zilberberg, M. D., Nathanson, B. H., Sulham, K., and Shorr, A. F. (2021). Multiple antimicrobial resistance and outcomes among hospitalized patients with complicated urinary tract infections in the US, 2013-2018: A retrospective cohort study. *BMC Infect. Dis.* 21 (1), 159. doi: 10.1186/s12879-021-05842-0

Zilberberg, M. D., Nathanson, B. H., Sulham, K., and Shorr, A. F. (2022). Descriptive epidemiology and outcomes of hospitalizations with complicated urinary tract infections in the united states, 2018. *Open Forum Infect. Dis.* 9 (1), ofab591. doi: 10.1093/ofid/ofab591

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Optimal treatment of ceftazidime-avibactam and aztreonam-avibactam against bloodstream infections or lower respiratory tract infections caused by extensively drug-resistant or pan drug-resistant (XDR/PDR) *Pseudomonas aeruginosa*

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Objective: To evaluate the efficacy of ceftazidime-avibactam (CZA) and aztreonam-avibactam (AZA) against bloodstream infections (BSIs) or lower respiratory tract infections (LRTIs) – caused by extensive drug-resistant or pan drug-resistant (XDR/PDR) *Pseudomonas aeruginosa*.

Method: The two-fold dilution method was used to determine the minimum inhibitory concentrations (MICs) of CZA/AZA against XDR/PDR *P. aeruginosa*. Whole-genome sequencing was used to analyze the resistance determinants of each isolate. Monte Carlo simulations (MCSs) were used to evaluate the probability of target attainment (PTA) and the cumulative fraction of response (CFR) of each CZA/AZA dosing regimen *via* traditional infusion (TI)/optimized two-step-administration therapy (OTAT).

Results: We found that XDR/PDR P. aeruginosa may carry some rare MBLs (e.g.: IND-6, SLB-1, THIN-B). *P. aeruginosa* isolates producing IMP-45, VIM-1, or VIM-2 were inhibited by AZA at a concentration of 2 to 8 mg/L. All isolates producing IND-6 plus other serine β-lactamases were high-level resistant to CZA/AZA (MICs >64 mg/L). All simulated dosing regimens of CZA/AZA against BSIs-causing XDR/PDR *P. aeruginosa* achieved 100% PTA when the MIC was ≤32 mg/L.

Conclusion: AZA has been considered as an option for the treatment of infections caused by XDR/PDR *P. aeruginosa* producing IMP-45, VIM-1, or VIM-2. OTAT with sufficient pharmacodynamic exposure may be an optimal treatment option for XDR/PDR *P. aeruginosa* with a high-level MIC of CZA/AZA.

KEYWORDS

extensively drug-resistant *Pseudomonas aeruginosa*, pan drug-resistant *Pseudomonas aeruginosa*, ceftazidime-avibactam, 173 aztreonam-avibactam, whole-genome sequencing

1 Introduction

In the last decade, *P. aeruginosa* has spread widely throughout the world, posing a significant burden to the daily work of physicians and a serious threat to the lives of patients. *P. aeruginosa* displays resistance to various antibiotics, making treatment challenging (Horcajada et al., 2019). The Centers for Disease Control and Prevention (CDC) have defined multidrug resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* as a serious threat level (Centers for Disease Control and Prevention).

Ceftazidime/avibactam (CZA) is a novel β-lactam/β-lactamase inhibitor (BL/BLIs). And it was approved by the Chinese National Medical Products Administration (CNMPA) in 2019 for the treatment of complicated intra-abdominal infections (cIAIs), hospital-acquired pneumonia (HAP), and ventilator-associated pneumonia (VAP), and in adult patients with limited treatment options for infections caused by the following gram-negative bacteria sensitive to this product: *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*, *Acinetobacter chimaerae*, and *P. aeruginosa* (Product information a). CZA has good safety and was regarded as a vital treatment option for *P. aeruginosa* infections. CZA showed good capabilities against *P. aeruginosa* with a sensitivity rate ranging from 76.2% to 97.8% (Sader et al., 2017a). In terms of clinical efficacy, the clinical success rate of CZA on *P. aeruginosa* infections ranged from 64.3% to 90.6% (Mazuski et al., 2016; Torres et al., 2018).

Aztreonam (ATM) was the first monobactam antibiotic to be used in clinical therapy. It was approved by the U.S. Food and Drug Administration (FDA) in 1986 for treatment of various infections caused by sensitive aerobic gram-negative bacteria. ATM was stable to hydrolysis by Ambler class B Metallo-β-lactamases (MBLs) (Yong et al., 2009). However, MBLs-producing bacteria also produce other types of *β*-lactamases ((i.e., ESBLs, AmpC enzymes) against which ATM is ineffective. Avibactam (AVI) is a *β*-lactamase inhibitor with a wide enzyme inhibition spectrum, including Ambler class A (KPC, TEM), class C (AmpC), and class D (OXA-48 type) *β*-lactamase. The combination of ATM and AVI can potentially inhibit MBL-producing bacteria (Bhatnagar et al., 2021).

We performed antimicrobial susceptibility testing (AST) on XDR/PDR *P. aeruginosa* in this study. Whole-genome sequencing and bioinformatic analysis were used to identify resistance genes of each isolate. Besides, we combine the population pharmacokinetic parameters (PPKs) of CZA/AZA with minimum inhibitory concentration (MIC) distribution of XDR/PDR *P. aeruginosa* to evaluate the efficacy of various dosing regimens. Therefore, the objectives of our work are as follows. Firstly, our work aims to compare the *in vitro* activity of CZA and AZA against XDR/PDR *P. aeruginosa*. Secondly, our team wants to evaluate the relationship between resistance genes and CZA/AZA sensitivity rates of XDR/PDR *P. aeruginosa*. Thirdly, we assess the efficacy of CZA and AZA for the treatment of critically ill patients with BSIs/LRTIs caused by XDR/PDR *P. aeruginosa*.

2 Materials and methods

2.1 Bacterial strains and antimicrobial agents

We collected 67 *P. aeruginosa* from critically ill patients admitted to the First Medical Centre of Chinese PLA General Hospital from January 2016 to November 2021. A total of 10 *P. aeruginosa* strains were categorized as XDR according to CLSI criteria. Moreover, 57 *P. aeruginosa* strains were categorized as PDR (Abbey and Deak, 2019). All *P. aeruginosa* were identified by VITEK®2 system (bioMérieux, Marcy-l'Étoile, France). Ceftazidime, avibactam, and aztreonam standards were purchased from MedChemExpress. The resistance rates of XDR/PDR *Pseudomonas aeruginosa* to cefoperazone-sulbactam, imipenem, ciprofloxacin, piperacillin-tazobactam, ceftazidime, levofloxacin, meropenem, tobramycin, amikacin and gentamicin were 89.6%, 100%, 89.6%, 100%, 97%, 89.6%, 100%, 97.1%, 100%, 100%, respectively.

2.2 Antimicrobial susceptibility tests

We used the broth two-fold dilution method to determine the minimum inhibitory concentrations (MICs) of CZA/AZA against XDR/PDR *P. aeruginosa*. A fixed concentration of AVI at 4 mg/L, 8mg/L, and 16mg/L combined with 2-fold diluted CAZ and ATM were used in ASTs. The quality control strains of our tests were *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. The MICs are defined as the lowest concentration of antibiotics that inhibits the growth of bacteria. The definition of MIC_{50} is a drug concentration

that inhibits the growth of bacteria by 50%. Similarly, MIC_{90} is a drug concentration inhibiting 90% of bacterial growth. MIC distributions of CZA/AZA against *P. aeruginosa* were represented by cumulative inhibition rates (CIRs). Besides, all experiments were conducted three times following Clinical and Laboratory Standards Institute (CLSI) standards.

2.3 Whole-genome sequencing

67 *P. aeruginosa* were subjected to whole-genome sequencing (WGS) using Illumina MiSeq short-read sequencing (Illumina, San Diego, CA, USA). Sequenced isolates were evaluated using FASTQC, version 0.11.6, and MultiQC, version 1.6. Trimmomatic, version 0.39, removed adapters and trimmed low-quality paired end reads. Comprehensive Antibiotic Resistance Database v.1.2.0 (McMaster University, Hamilton, Ontario) was used to identify drug resistance genes in the strains.

2.4 Pharmacokinetic/pharmacodynamics modeling

Population pharmacokinetic (PPK) parameters of CZA and AZA were obtained from previously published articles (Vinks et al., 2007; Stein et al., 2019; Cornely et al., 2020). CAZ is a time-dependent antibiotic, $\%50fT > MIC$ is the best indicator for assessing BSIs. Besides, $\%50fT > 5 \times MIC$ is the best indicator for assessing LRTIs. When combined with CAZ, $\%50fT > CT$ of 1 mg/L was considered the *Pharmacokinetic (PK)/Pharmacodynamics (PD)* target of avibactam. PK/PD targets of ATM for BSIs was $\%60fT > MIC$. And PK/PD targets of ATM for LRTIs were $\%60fT > 5 \times MIC$. As for avibactam, $\%50fT > CT$ of 2.5 mg/L was considered appropriate for guiding dosage selection for AZA (Nichols et al., 2018). Optimized two-step administration therapy (OTAT) refers to a rapid injection (0.5h) of a loading dose in the first step and a continuous infusion (2h) in the second step to maintain adequate drug exposure. The $\%fT > n \times MIC$ equation was based on previous studies (Eguchi et al., 2010; Schaumburg et al., 2019; Song et al., 2019).

2.5 Monte carlo simulations

We conducted 10000-patient Monte Carlo simulations (MCSs) using Oracle Crystal Ball version.11.1.24. PK parameters (V_d, CL, $t_{1/2}$) followed a log-normal distribution. All simulated dosing regimens *via* traditional infusion (TI)/optimized two-step-administration therapy (OTAT) were listed in Table 1. The definition of probability of target attainment (PTA) was the probability of reaching the PK/PD target at different MICs. The equation for cumulative fraction of response (CFR) is $CFR = \sum_{i=1}^{n} PTA(MICi) \times p(MICi)$. *MICi* means each MIC value. *p(MICi)* means the percentage of each MIC value. A CFR ≥ 90% is adequate PD exposure for this dosing regimen.

3 Results

3.1 Resistance genes

From the predicted results, antibiotic efflux accounted for approximately 75%, previous studies have also shown that efflux pumps are a mechanism for the acquisition of drug resistance in this organism (Horcajada et al., 2019), and furthermore the presence of antibiotic inactivation, such as OXA beta-lactamase. This study further discusses the clinical potential of beta-lactamase inhibitors in combination with other drugs for the treatment of this bacterial infection.

3.2 Sensitivity tests

Figure 1 shows the cumulative inhibition ratios (CIRs) of ceftazidime (CAZ) and aztreonam (ATM) with increased avibactam concentration against XDR/PDR *P. aeruginosa*. As the concentration of avibactam increased, the CIRs of XDR/PDR *P. aeruginosa* by ceftazidime and aztreonam increased. As shown in Table 1, the susceptibility rate of CZA against 57 PDR *P. aeruginosa* and 10 XDR *P. aeruginosa* was 19.3% and 40%, respectively. Besides, the MIC_{50} and MIC_{90} of CZA against PDR *P. aeruginosa* were 32mg/L and >64mg/L, respectively. The MIC_{50} and MIC_{90} of CZA against XDR *P. aeruginosa* were 16mg/L and >64mg/L, respectively. CLSI has not published the breakpoint of AZA. When the concentration of avibactam was 4mg/L, AST showed that the MIC_{50} and MIC_{90} of AZA against PDR *P. aeruginosa* were 64mg/L and >64mg/L, respectively. Similarly, when the concentration of avibactam was 4mg/L, the MIC_{50} and MIC_{90} of AZA against XDR *P. aeruginosa* were 16mg/L and >64mg/L, respectively.

3.3 Comparative MICs of CZA and AZA

The comparative MICs (mg/L) of CZA and AZA against 67 XDR/PDR *P. aeruginosa* positive for the OXA gene (with or without other β-lactamase enzymes) were listed in Table 2. Most XDR/PDR

TABLE 1 The susceptibility rate of CZA against XDR/PDR *P. aeruginosa* and the MIC50 and MIC90 of AZA against XDR/PDR *P. aeruginosa*.

Isolates	CZA				AZA		
	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	S (%)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
XDR-PA (10)	16	>64	4–>64	40.0	16	64	2–>64
PDR-PA (57)	32	>64	4–>64	19.3	>64	>64	8–>64

Since the concentration of avibactam is fixed at 4 mg/L in clinical practice, only the MIC of avibactam at 4 mg/L is indicated in this table.

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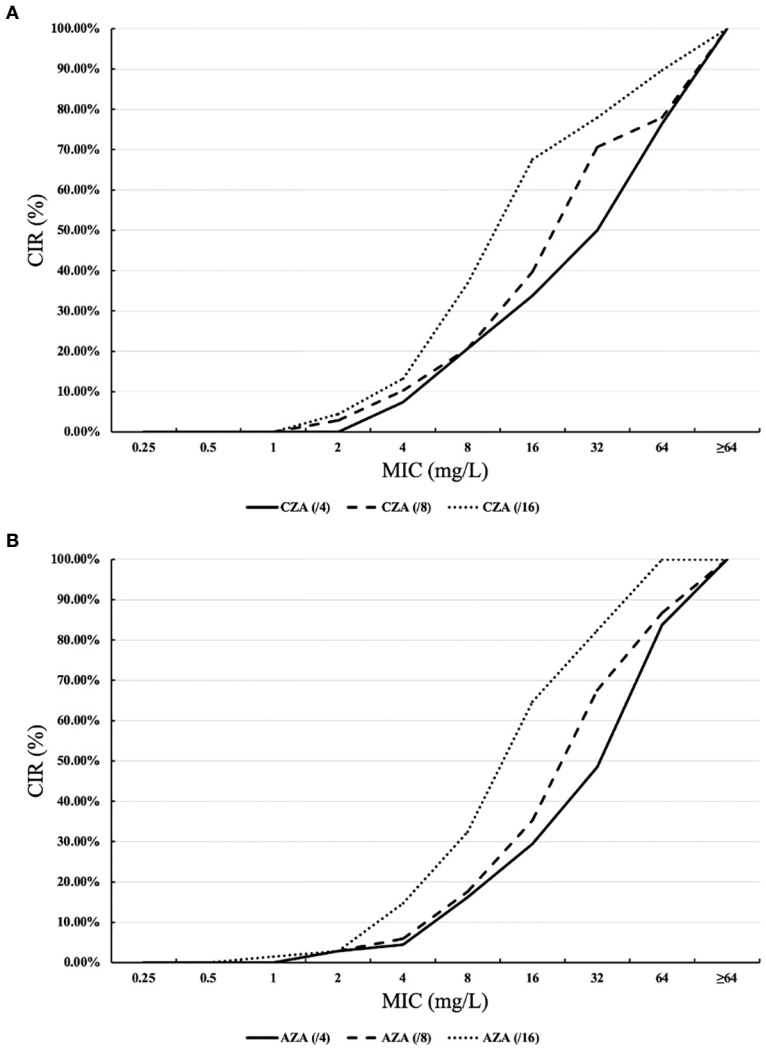

FIGURE 1
Cumulative inhibition ratios (CIRs) of ceftazidime-avibactam (CAZ) and aztreonam (ATM) against XDR/PDR *P. aeruginosa*. **(A)** ceftazidime +avibactam, **(B)** aztreonam +avibactam. MIC: minimum inhibitory concentration. The horizontal axis of (Part label **A**) represents the MICs of ceftazidime combined with avibactam against XDR/PDR *P. aeruginosa* at avibactam concentrations of 4 mg/L, 8 mg/L, and 16 mg/L. The vertical axis of (Part label **A**) shows the CIRs of ceftazidime in combination with avibactam against XDR/PDR *P. aeruginosa* at avibactam concentrations of 4 mg/L, 8 mg/L, and 16 mg/L. Similarly, the horizontal axis of (Part label **B**) represents the MICs of aztreonam in combination with avibactam against XDR/PDR *P. aeruginosa* at avibactam concentrations of 4 mg/L, 8 mg/L, and 16 mg/L. The vertical axis of (Part label **B**) shows the CIRs of aztreonam in combination with avibactam against XDR/PDR *P. aeruginosa* at avibactam concentrations of 4 mg/L, 8 mg/L, and 16 mg/L.

P. aeruginosa with an OXA-101 also produced OXA-850. CZA was as effective as AZA against these isolates. For isolates with an OXA gene plus IMP-45, VIM-1, or VIM-2, AZA was much more potent than CZA against these isolates, with all isolates being inhibited by a concentration of 8 mg/L. For isolates with an IND-6 plus more serine β-lactamases, all isolates produced CZA/AZA MICs of >64 mg/L. For XDR/PDR *P. aeruginosa* with other genotypes, the efficacy of CZA against these isolates did not differ significantly from that of AZA.

3.4 Probability of target attainment

3.4.1 Probability of target attainment of BSIs

The probability of target attainment (PTA) of each simulated dosing regimen for BSIs was listed in Table 3A. All dosing regimens of CZA/AZA achieved a PTA of 100% when the MIC was ≤32 mg/L. When the MIC was 64 mg/L, CZA 2.5g q8h declined to a PTA of 88.6%. When the MIC was >64 mg/L, CZA 2.5g q8h, 2.5g q6h, 4g q8h, 4g q6h, 1.25g (0.5h) +1.25g (2h) q8h, 1.25g (0.5h) +1.25g (2h) q6h, 0.675g (0.5h) +0.675g (2h) q8h, 0.675g (0.5h) +0.675g (2h) q8h, 0.675g (0.5h) +0.675g (2h) q6h achieved a PTA of 0, 5.48%, 2.78%, 88.85%, 93.21%, 96.05%, 80.34% and 80.03%, respectively. When the MIC was 64 mg/L, AZA 2.5g q8h, 0.675g (0.5h) +0.675g (2h) q8h and 0.675g (0.5h) +0.675g (2h) q6h achieved a PTA of 89.49%, 93.77% and 99.83%, respectively. When the MIC was >64 mg/L, AZA 2.5g q8h, 2.5g q6h, 4g q8h, 4g q6h, 1.25g (0.5h) +1.25g (2h) q8h, 1.25g (0.5h) +1.25g (2h) q6h, 0.675g (0.5h) +0.675g (2h) q8h, 0.675g (0.5h) +0.675g (2h) q8h, 0.675g (0.5h) +0.675g (2h) q6h achieved a PTA of 0, 75.75%, 14.15%, 91.78%, 94.35%, 99.77%, 7.43%, 14.67%, respectively.

TABLE 2 Comparative MICs (mg/L) range for CZA and AZA against 67 XDR/PDR *P. aeruginosa* positive for OXA gene alone and one or more additional β-lactamase genes.

Group (n)	MICs (mg/L) range for	
	CZA	AZA
OXA-1 + OXA-50 + IMP-45 (2)	>64	2–16
OXA-1 + OXA-488 + OXA-573 + VIM-1 + IMP-45 (1)	>64	2
OXA-7 + OXA-50 + OXA-101 (1)	4	8
OXA-10 + OXA-50 + VIM-2 (1)	>64	8
OXA-17 + OXA-129 + OXA-488 (2)	32–64	32–64
OXA-50 (5)	4–64	4–32
OXA-50 + OXA-101 + OXA-573 (1)	64	64
OXA-50 + OXA-246 + PEDO-3 (1)	64	64
OXA-50 + OXA-573 (2)	8–16	16–32
OXA-101 + OXA-246 + OXA-573 + OXA-846 (1)	64	64
OXA-101 + OXA-488 + OXA-573 (1)	8	16
OXA-101 + OXA-573 + OXA-850 + THIN-B (1)	16	64
OXA-101 + OXA-573 + OXA-850 (1)	16	32
OXA-101 + OXA-850 (22)	8–>64	16–64
OXA-101 + OXA-850 + IND-6 (8)	>64	>64
OXA-101 + OXA-850 + SLB-1 (1)	64	>64
OXA-129 + OXA-488 (1)	64	64
OXA-246 + OXA-486 + OXA-573 + KPC-2 (3)	8–16	8–16
OXA-246 + OXA-573 + OXA-846 (2)	64–>64	64–>64
OXA-488 + OXA-573 (1)	4	16
OXA-488 (2)	8	16
OXA-573 + OXA-846 (1)	16	32
OXA-846 (2)	4–16	8–32
OXA-850 (4)	8–64	8–64

3.4.2 Probability of target attainment of LRTIs

The PTA of each simulated dosing regimen for LRTIs was listed in Table 3B. All dosing regimens of CZA/AZA achieved a PTA of 100% when the MIC was ≤8 mg/L. When the MIC was 16 mg/L, CZA 2.5g q8h declined a PTA of 12.31%, and AZA 2.5g q8h declined a PTA of 31.95%. All OATA dosing regimens of CZA achieved a PTA of 100% when the MIC was 32 mg/L. When the MIC was 32 mg/L, AZA 2.5g q8h, 2.5g q6h, 4g q8h, 4g q6h, 1.25g (0.5h) +1.25g (2h) q8h, 1.25g (0.5h) +1.25g (2h) q6h, 2g (0.5h) +2g (2h) q8h, 2g (0.5h) +2g (2h) q8h, 2g (0.5h) +2g (2h) q6h achieved a PTA of 0, 0, 0.96%, 99.72%, 89.14%, 98.66%, 100% and 100%, respectively. When the MIC was 64 mg/L, CZA 1.25g (0.5h) +1.25g (2h) q8h, 1.25g (0.5h) +1.25g (2h) q6h, 2g (0.5h) +2g (2h) q8h, 2g (0.5h) +2g (2h) q6h achieved a PTA of 40.85%, 45.54%, 100% and 100%, respectively. When the MIC was 64 mg/L, AZA 2g (0.5h) +2g (2h) q8h, 2g (0.5h) +2g (2h) q6h achieved a PTA of 90.95% and 98.6%, respectively. When the MIC was >64 mg/L, CZA 2g (0.5h) +2g (2h) q8h, 2g (0.5h) +2g (2h) q6h achieved a PTA of 41.33% and 46.32%, respectively.

3.5 Cumulative fraction of response

3.5.1 Cumulative fraction of response of BSIs

The cumulative fraction of response (CFR) of each simulated dosing regimen for BSIs was listed in Table 3A. If CZA was administered *via* traditional infusion, the CFR was 95.63% for 4g q6h. If CZA was administered *via* OTAT, the CFR was 96.67% for 1.25g (0.5h) +1.25g (2h) q8h, 97.35% for 1.25g (0.5h) +1.25g (2h) q6h, 93.60% for 0.675g (0.5h) +0.675g (2h) q8h and 93.53% for 0.675g (0.5h) +0.675g (2h) q8h. If AZA was administered *via* traditional infusion, the CFR was 92.50% for 2.5g q6h and 96.36% for 4g q6h. If AZA was administered *via* OTAT, the CFR was 96.95% for 1.25g (0.5h) +1.25g (2h) q8h, 98.25% for 1.25g (0.5h) +1.25g (2h) q6h. The above dosing regimens are considered to provide adequate PD exposures for the treatment of CZA/AZA against *XDR/PDR P. aeruginosa* BSIs.

3.5.2 Cumulative fraction of response of LRTIs

Table 3B showed CFRs of CZA/AZA against LRTIs caused by XDR/PDR *P. aeruginosa*. CFRs were less than 90% for all simulated dosing regimens (i.e., 2.5 g [e.g., 1.25 g (0.5h) + 1.25 g (2h)] q6h, 2.5 g [e.g., 1.25 g (0.5h) + 1.25 g (2h)] q8h, 4g [e.g., 2 g (0.5h) + 2 g (2h)] q6h, 4g [e.g., 2 g (0.5h) + 2 g (2h)] q8h).

4 Discussion

In recent years, *P. aeruginosa* has spread widely worldwide and the treatment of BSIs or LRTIs caused by XDR/PDR *P. aeruginosa* has become a tough problem (Horcajada et al., 2019). The International Network for Optimal Resistance Monitoring Program (INORMP) in the United States (2012-2015) showed the prevalence of MDR and XDR *P. aeruginosa*, with rates of 15.4% and 9.4%, respectively (Sader et al., 2017b). Nowadays, CZA and AZA are considered treatment options for MDR/XDR *P. aeruginosa* infections (Horcajada et al., 2019). Besides, optimizing the use of antimicrobials that are currently available can be considered as a solution to this dilemma.

In this study, we collected 67 XDR/PDR *P. aeruginosa* isolates from a 3000-bed teaching hospital in northern China. Firstly, the MICs of CZA/AZA for XDR/PDR *P. aeruginosa* isolates were evaluated using the doubling dilution method. Secondly, we conducted WGS and performed bioinformatics analysis to determine the resistance genes of each isolate. Finally, we used MCS to analyze the PTA and CFR of different CZA/AZA dosing regimens.

TABLE 3A Probability of target attainment (PTA) and cumulative fraction of response (CFR) of CZA/AZA against bloodstream infections (BSIs) caused by XDR/PDR *P. aeruginosa.*

Antibiotics	Dosing regimens	PTA of different MICs										CFR (%)
		0.25	0.5	1	2	4	8	16	32	64	>64	
CZA	2.5g q8h	100	100	100	100	100	100	100	100	88.6	0	71.36
	2.5g q6h	100	100	100	100	100	100	100	100	100	5.48	75.72
	4g q8h	100	100	100	100	100	100	100	100	100	2.78	75.08
	4g q6h	100	100	100	100	100	100	100	100	100	88.85	**95.63**
	1.25g (0.5h) +1.25g (2h) q8h	100	100	100	100	100	100	100	100	100	**93.21**	**96.67**
	1.25g (0.5h) +1.25g (2h) q6h	100	100	100	100	100	100	100	100	100	**96.05**	**97.35**
	0.675g (0.5h) +0.675g (2h) q8h	100	100	100	100	100	100	100	100	100	80.34	**93.60**
	0.675g (0.5h) +0.675g (2h) q6h	100	100	100	100	100	100	100	100	100	80.03	**93.53**
AZA	2.5g q8h	100	100	100	100	100	100	100	100	89.49	0	71.59
	2.5g q6h	100	100	100	100	100	100	100	100	100	75.75	**92.50**
	4g q8h	100	100	100	100	100	100	100	100	100	14.15	77.79
	4g q6h	100	100	100	100	100	100	100	100	100	**91.78**	**96.36**
	1.25g (0.5h) +1.25g (2h) q8h	100	100	100	100	100	100	100	100	100	**94.35**	**96.95**
	1.25g (0.5h) +1.25g (2h) q6h	100	100	100	100	100	100	100	100	100	**99.77**	**98.25**
	0.675g (0.5h) +0.675g (2h) q8h	100	100	100	100	100	100	100	100	93.77	7.43	74.51
	0.675g (0.5h) +0.675g (2h) q6h	100	100	100	100	100	100	100	100	99.83	14.67	77.87

TABLE 3B Probability of target attainment (PTA) and cumulative fraction of response (CFR) of CZA/AZA against lower respiratory tract infections (LRTIs) caused by XDR/PDR *P. aeruginosa.*

Antibiotics	Dosing regimens	PTA of different MICs										CFR (%)
		0.25	0.5	1	2	4	8	16	32	64	>64	
CZA	2.5g q8h	100	100	100	100	100	100	12.31	0	0	0	19.38
	2.5g q6h	100	100	100	100	100	100	100	0	0	0	31.16
	4g q8h	100	100	100	100	100	100	100	0	0	0	31.16
	4g q6h	100	100	100	100	100	100	100	0.13	0	0	44.61
	1.25g (0.5h) +1.25g (2h) q8h	100	100	100	100	100	100	100	100	40.85	0	58.53
	1.25g (0.5h) +1.25g (2h) q6h	100	100	100	100	100	100	100	100	45.54	0	59.79
	2g (0.5h) +2g (2h) q8h	100	100	100	100	100	100	100	100	100	41.33	84.29
	2g (0.5h) +2g (2h) q6h	100	100	100	100	100	100	100	100	100	46.32	85.48
AZA	2.5g q8h	100	100	100	100	100	100	31.95	0	0	0	23.68
	2.5g q6h	100	100	100	100	100	100	100	0	0	0	32.82
	4g q8h	100	100	100	100	100	100	100	0.96	0	0	33.00
	4g q6h	100	100	100	100	100	100	100	99.72	0	0	52.16
	1.25g (0.5h) +1.25g (2h) q8h	100	100	100	100	100	100	100	89.14	0	0	50.11
	1.25g (0.5h) +1.25g (2h) q6h	100	100	100	100	100	100	100	98.66	0	0	51.96
	2g (0.5h) +2g (2h) q8h	100	100	100	100	100	100	100	100	90.95	0	84.78
	2g (0.5h) +2g (2h) q6h	100	100	100	100	100	100	100	100	98.60	0	87.51

We found that the resistance rate of CZA against XDR *P. aeruginosa* was 60%. *Schaumburg et al.* found that the resistance rate of CZA against XDR *P. aeruginosa* was 50.9% (Schaumburg et al., 2019). However, INORMP in the United States (2012-2015) showed that the sensitivity rate of CZA against XDR *P. aeruginosa* was 75.8% (Sader et al., 2017b). The different sensitivity rates of CZA against XDR *P. aeruginosa* may be related to the different sources and resistance genes of strains. In our study, all isolates were collected from northern China and most of the isolates produced OXA- β-lactamases. AVI has been shown in previous studies to be effective against OXA-48-producing isolates, but its effect on other OXA-β-lactams is unknown (Bhatnagar et al., 2021). This may explain the high resistance rate of CZA against the strains we collected.

Our work found that AZA was more effective than CZA for the treatment of MBL-producing XDR/PDR *P. aeruginosa* (e.g.: IMP-45, VIM-1, VIM-2). AVI is ineffective against MBL-producing isolates. However, the combination of AVI and ATM was a treatment option for MBL-producing isolates. *Lee et al.* also found that the combination of ATM and CZA may be a treatment option for VIM-2-producing *P. aeruginosa* (Lee et al., 2021). Our study found that CZA with avibactam at 8 and 16 mg/L was inactive against MBL-positive isolates. We also found that the *in vitro* activity of CZA/AZA against XDR/PDR *P. aeruginosa* could be improved with increasing AVI concentration. Nevertheless, *Yu et al.* found that CZA with avibactam at 8 and 16 mg/L was active against MBL-positive isolates (Yu et al., 2021). This may be because our collected strains also produced OXA-β- lactamases. Therefore, more exploration is needed in the future to figure out the resistance mechanism of XDR/PDR *P. aeruginosa*.

Our study had several interesting findings. Using whole genome sequencing, we found that XDR/PDR *P. aeruginosa* may carry some rare MBLs (e.g.: IND-6, SLB-1, THIN-B). IND-6 is a highly divergent IND-type MBL. It was first isolated from *Chryseobacterium indologenes* strain 597 in Burkina Faso (Zeba et al., 2009). SLB-1 was first identified from *Shewanella livingstonensis* in 2005 (Poirel et al., 2005). Besides, THIN-B was first identified from *Janthinobacterium lividum* (Rossolini et al., 2001). The resistance mechanism of XDR/PDR *P. aeruginosa* carrying IND-6, SLB-1, or THIN-B is needed to explore in the future.

The treatment of XDR/PDR *P. aeruginosa* infections was difficult, especially in immunocompromised patients (i.e.: patients who received hematopoietic stem cell transplantation, patients with nephrotic syndrome, patients with various malignant tumors) (Poole, 2011). Besides, *P. aeruginosa* has a huge intrinsic resistome and can be resistant to antibiotics through chromosomal mutations (Lister et al., 2009). Mobile genetic elements can be shared between *P. aeruginosa*. These elements produce carbapenemase enzymes, which makes *P. aeruginosa* resistant to carbapenems. These reasons have led researchers to develop novel antibiotics and methods to improve the therapeutic effect of XDR/PDR *P. aeruginosa* infections (Subedi et al., 2018). Our work found that OTAT could improve the PTA and CFR of CZA/AZA monotherapy for the treatment of critically ill patients with BSIs caused by XDR/PDR *P. aeruginosa*. From a pharmacoeconomic point of view, OTAT can reduce the financial burden of critically ill patients. The study by *Eguchi et al.* also confirmed that CFRs of OTAT with sufficient pharmacokinetic exposures were higher than traditional infusion (Eguchi et al., 2010). Besides, both OTAT and TI dosing regimens had poor efficacy against XDR *P. aeruginosa* LRTIs. This may be related to the lower penetration of ceftazidime-avibactam in the epithelial lining fluid compared to the blood (Nicolau et al., 2015).

Our study had several limitations that should be noted. Firstly, the collection of *P. aeruginosa* was confined to a small sample size and northern China. Secondly, our study only focused on partial beta-lactamases (class B β-lactamases and class D β-lactamases). Therefore, large-scale animal or clinical trials are needed in the future to confirm the efficacy of CZA/AZA against BSIs caused by XDR/PDR *P. aeruginosa*.

The main resistance mechanisms in *P. aeruginosa* are intrinsic, mutational, and horizontally acquired resistomes (Horcajada et al., 2019). We found that the efflux pump is indeed what makes *P. aeruginosa* drug resistant. The efflux pump was also considered in the study of this article (see the whole genome sequencing results in the supplementary file for details), but the efflux pump is not the focus of this article.

In conclusion, our work has the following results. Firstly, AZA was considered as an option for the treatment of XDR/PDR *P. aeruginosa* harbouring IMP-45, VIM-1, or VIM-2. Secondly, OTAT with sufficient PD exposure may be an optimal treatment option for BSI caused by XDR/PDR *P. aeruginosa* with a high-level MIC of CZA/AZA.

Data availability statement

The original contributions presented in the study are publicly available. This data has been deposited into the NCBI repository under accession: PRJNA967114.

Ethics statement

Ethical approval was granted by the Chinese People's Liberation Army General Hospital.

Author contributions

YK and JC designed and managed the project. YK performed all the experiments and wrote the manuscript. LX analyzed bioinformatics analysis. JY provided technical guidance. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1023948/full#supplementary-material

References

Abbey, T. C., and Deak, E. (2019). What's new from the CLSI subcommittee on antimicrobial susceptibility testing M100, 29th edition. *Clin. Microbiol. Newslett.* 41 (23), 203–209. doi: 10.1016/j.clinmicnews.2019.11.002

Bhatnagar, A., Ransom, E. M., Machado, M. J., Boyd, S., Reese, N., Anderson, K., et al. (2021). Assessing the *in vitro* impact of ceftazidime on aztreonam/avibactam susceptibility testing for highly resistant MBL-producing enterobacterales. *J. Antimicrob. Chemother.* 76 (4), 979–983. doi: 10.1093/jac/dkaa531

Centers for Disease Control and Prevention *Bacteria and fungi listed in the 2019 AR threats report*. Available at: https://www.cdc.gov/drugresistance/pdf/threats-report/pseudomonas-aeruginosa-508.pdf.

Cornely, O. A., Cisneros, J. M., Torre-Cisneros, J., Rodríguez-Hernández, M. J., Tallón-Aguilar, L., Calbo, E., et al. (2020). Pharmacokinetics and safety of aztreonam/avibactam for the treatment of complicated intra-abdominal infections in hospitalized adults: results from the REJUVENATE study. *J. Antimicrob. Chemother.* 75 (3), 618–627. doi: 10.1093/jac/dkz497

Eguchi, K., Kanazawa, K., Shimizudani, T., Kanemitsu, K., and Kaku, M. (2010). Experimental verification of the efficacy of optimized two-step infusion therapy with meropenem using an *in vitro* pharmacodynamic model and Monte Carlo simulation. *J. Infect. Chemother.* 16 (1), 1–9. doi: 10.1007/s10156-009-0001-8

Horcajada, J. P., Montero, M., Oliver, A., Sorli, L., Luque, S., Gomez-Zorrilla, S., et al. (2019). Epidemiology and treatment of multidrug-resistant and extensively drug-resistant pseudomonas aeruginosa infections. *Clin. Microbiol. Rev.* 32 (4). doi: 10.1128/CMR.00031-19

Lee, M., Abbey, T., Biagi, M., and Wenzler, E. (2021). Activity of aztreonam in combination with ceftazidime-avibactam against serine- and metallo-β-lactamase-producing pseudomonas aeruginosa. *Diagn. Microbiol. Infect. Dis.* 99 (1), 115227. doi: 10.1016/j.diagmicrobio.2020.115227

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22 (4), 582–610. doi: 10.1128/CMR.00040-09

Mazuski, J. E., Gasink, L. B., Jon, A., Helen, B., Stone, G. G., Douglas, R., et al. (2016). Efficacy and safety of ceftazidime-avibactam plus metronidazole versus meropenem in the treatment of complicated intra-abdominal infection: Results from a randomized, controlled, double-blind, phase 3 program. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. America* 11), 1380–1389. doi: 10.1093/cid/ciw133

Nichols, W. W., Newell, P., Critchley, I. A., Riccobene, T., and Das, S. (2018). Avibactam Pharmacokinetic/Pharmacodynamic targets. *Antimicrob. Agents Chemother.* 62 (6). doi: 10.1128/AAC.02446-17

Nicolau, D. P., Siew, L., Armstrong, J., Li, J., Edeki, T., Learoyd, M., et al. (2015). Phase 1 study assessing the steady-state concentration of ceftazidime and avibactam in plasma and epithelial lining fluid following two dosing regimens. *J. Antimicrob. Chemother.* 70 (10), 2862–2869. doi: 10.1093/jac/dkv170

Poirel, L., Heritier, C., and Nordmann, P. (2005). Genetic and biochemical characterization of the chromosome-encoded class b beta-lactamases from shewanella livingstonensis (SLB-1) and shewanella frigidimarina (SFB-1). *J. Antimicrob. Chemother.* 55 (5), 680–685. doi: 10.1093/jac/dki065

Poole, K. (2011). Pseudomonas aeruginosa: resistance to the max. *Front. Microbiol.* 2. doi: 10.3389/fmicb.2011.00065

Product information (a). *Ceftazidime and avibactam sodium for injection*. Available at: https://reference.medscape.com/drug/avycaz-ceftazidime-avibactam-999985.

Product Information (b). *Aztreonam for injection*. Available at: https://packageinserts.bms.com/pi/pi_azactam.pdf.

Rossolini, G. M., Condemi, M. A., Pantanella, F., Docquier, J. D., Amicosante, G., and Thaller, M. C. (2001). Metallo-beta-lactamase producers in environmental microbiota: new molecular class b enzyme in janthinobacterium lividum. *Antimicrob. Agents Chemother.* 45 (3), 837–844. doi: 10.1128/AAC.45.3.837-844.2001

Sader, H. S., Castanheira, M., and Flamm, R. K. (2017a). Antimicrobial activity of ceftazidime-avibactam against gram-negative bacteria isolated from patients hospitalized with pneumonia in U.S. medical centers 2011 to 2015. *Antimicrob. Agents Chemother.* 61 (4). doi: 10.1128/aac.02083-16

Sader, H. S., Huband, M. D., Castanheira, M., and Flamm, R. K. (2017b). Pseudomonas aeruginosa antimicrobial susceptibility results from four years, (2012 to 2015) of the international network for optimal resistance monitoring program in the united states. *Antimicrob. Agents Chemother.* 61 (3). doi: 10.1128/AAC.02252-16

Schaumburg, F., Bletz, S., Mellmann, A., Becker, K., and Idelevich, E. A. (2019). Comparison of methods to analyse susceptibility of German MDR/XDR pseudomonas aeruginosa to ceftazidime/avibactam. *Int. J. Antimicrob. Agents* 54 (2), 255–260. doi: 10.1016/j.ijantimicag.2019.05.001

Song, X., Wu, Y., Cao, L., Yao, D., and Long, M. (2019). Is meropenem as a monotherapy truly incompetent for meropenem-nonsusceptible bacterial strains? a Pharmacokinetic/Pharmacodynamic modeling with Monte Carlo simulation. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02777

Stein, G. E., Smith, C. L., Scharmen, A., Kidd, J. M., Cooper, C., Kuti, J., et al. (2019). Pharmacokinetic and pharmacodynamic analysis of Ceftazidime/Avibactam in critically ill patients. *Surg. Infect. (Larchmt)* 20 (1), 55–61. doi: 10.1089/sur.2018.141

Subedi, D., Vijay, A. K., and Willcox, M. (2018). Overview of mechanisms of antibiotic resistance in pseudomonas aeruginosa: an ocular perspective. *Clin. Exp. Optom.* 101 (2), 162–171. doi: 10.1111/cxo.12621

Torres, A., Zhong, N., Pachl, J., Timsit, J.-F., Kollef, M., Chen, Z., et al. (2018). Ceftazidime-avibactam versus meropenem in nosocomial pneumonia, including ventilator-associated pneumonia (REPROVE): a randomised, double-blind, phase 3 non-inferiority trial. *Lancet Infect. Dis.* 18 (3), 285–295. doi: 10.1016/s1473-3099(17)30747-8

Vinks, A. A., van Rossem, R. N., Mathôt, R. A., Heijerman, H. G., and Mouton, J. W. (2007). Pharmacokinetics of aztreonam in healthy subjects and patients with cystic fibrosis and evaluation of dose-exposure relationships using monte carlo simulation. *Antimicrob. Agents Chemother.* 51 (9), 3049–3055. doi: 10.1128/aac.01522-06

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., et al. (2009). Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in klebsiella pneumoniae sequence type 14 from India. *Antimicrob. Agents Chemother.* 53 (12), 5046–5054. doi: 10.1128/aac.00774-09

Yu, W., Xiong, L., Luo, Q., Chen, Y., Ji, J., Ying, C., et al. (2021). *In vitro* activity comparison of ceftazidime–avibactam and aztreonam–avibactam against bloodstream infections with carbapenem-resistant organisms in China. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.780365

Zeba, B., De Luca, F., Dubus, A., Delmarcelle, M., Simpore, J., Nacoulma, O. G., et al. (2009). IND-6, a highly divergent IND-type metallo-beta-lactamase from chryseobacterium indologenes strain 597 isolated in Burkina Faso. *Antimicrob. Agents Chemother.* 53 (10), 4320–4326. doi: 10.1128/AAC.01607-08

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Colonization of extended-spectrum β-lactamase-producing Enterobacteriaceae does not affect subsequent infection and liver transplant outcomes: a retrospective observational cohort study

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Objective: To investigate the colonization rate of extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E), subsequent infections by ESBL-E and ESBL-producing gram-negative bacilli (ESBL-GNB), and the effect of ESBL-E colonization on clinical outcomes in liver transplantation (LT) recipients.

Methods: This is a retrospective cohort study that included patients who underwent LT at Shanghai Renji Hospital between July 2016 and December 2017. Rectal swabs from LT patients at the postoperative ICU enrollment were screened anonymously for ESBL-E carriage. Demographics data, laboratory indexes, operative complications, and clinical course information were also obtained. The extent of ESBL-E colonization, the subsequent infection rates of ESBL-E and ESBL-GNB, and the clinical outcomes were compared between ESBL-E colonized and non-colonized patients.

Results: In total, 496 liver transplant recipients (387 males) were included in this study. ESBL-E colonization was detected in 240 patients (48.4%). There was no significant difference between the rates of ESBL-E infection (5.8 vs. 3.1%, $p = 0.143$), Ischemia-reperfusion ≥ 3 (27.9 vs. 24.6%, $p = 0.403$), acute kidney injury (39.6 vs. 38.7%, $p = 0.835$), acute rejection (2.1 vs. 1.6%, $p = 0.664$), graft versus host reaction (1.3 vs. 1.2%, $p = 0.937$), duration of hospitalization (22 vs. 23 days, $p = 0.568$), 90-day mortality (7.1 vs. 4.7%, $p = 0.262$) and 1-year mortality (12.9 vs. 9.3%, $p = 0.265$) in patients with and without ESBL-E colonization. Though the ESBL-GNB infection rate was higher in ESBL-E colonized patients (12.1 vs. 6.6%, $p = 0.037$), multivariate analysis showed that ESBL-E colonization did not increase the risk of ESBL-GNB infection (Model 1: aOR 1.755, 95% CI: 0.911–3.380, $p = 0.093$; Model 2: aOR 1.556, 95% CI: 0.761–3.181, $p = 0.226$). The ESBL-producing bacteria spectrum of colonization was significantly different from that of infections occurring after LT, with only three colonization events leading to infection by the same pathogen identified.

Conclusion: ESBL-E colonization in liver transplant patients is not associated with ESBL-E infection, nor is it a risk factor for post-transplant ESBL-GNB infection. Additionally, ESBL-E colonization does not lead to worse prognoses when compared with non-colonized patients.

Clinical trial registration: Chinese Clinical Trial Registry, Identifier [ChiCTR2100043034].

KEYWORDS

liver transplant, extended-spectrum β-lactamase-producing Enterobacteriaceae, extended-spectrum β-lactamase-producing gram-negative bacilli, colonization, infection

Introduction

Since the first human orthotopic liver transplantation was carried out in 1963, liver transplantation (LT) has become the most effective choice for the treatment of end-stage liver disease and acute liver failure (1). Enterobacteriaceae that produce extended-spectrum β-lactamases (enzymes that are able to hydrolyze β-lactam antibiotics) have emerged as a significant threat to LT candidates and recipients, as its presence has been linked to increased mortality and morbidity rates (2).

Analysis with regard to extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) colonization in LT patients has mainly been conducted in Western countries (3), rather than regions such as Southeast Asia, where ESBL-E prevalence is higher (4). Given the significant heterogeneity of ESBL-E reported in different parts of the world and the high volume of transplantation activity globally, the lack of ESBL-E data from regions, such as Southeast Asia, should be underscored (4, 5).

Previous literature has found that almost 40% of ESBL-E colonized LT patients developed an ESBL-E infection during the post-transplant period, compared with 3.5% of non-colonized patients (6), and ESBL-E colonization has been associated with a 12 times greater risk of such infections (7). However, other studies have questioned the benefit of screening for ESBL-E. In Swedish patients with fecal ESBL-E colonization, the risk of ESBL-E bacteremia was estimated to be very low (0.7%) (8), and in general ICU settings, only 10–25% of ESBL-E carriers developed an ESBL-E-related infection (9, 10).

Enterobacteriaceae that produce ESBL are predominantly found in *Escherichia coli (E.coli)* and *Klebsiella pneumoniae (K. pneumoniae)*, though they may also be present in other types of gram-negative bacilli (GNB), including *Pseudomonas* spp., *Burkholderia* spp., and *Acinetobacter* spp. (11–13). The multidrug-resistant nature of ESBL-E may be explained by the production of plasmid-encoded enzymes, which carry multi-resistance genes via plasmids, transposons, and integrons (14). *E. coli* can also transfer plasmids carrying antibiotic resistance genes between co-existing bacteria that are part of the commensal intestinal flora, especially under antibiotic pressure (15, 16); however, these bacteria are not necessarily the same species (14). The extent of the changes in ESBL-carrying bacterial species and the capacity for the possible transfer of ESBL-carrying plasmids in GNB isolates in LT patients remains, to our knowledge, largely unexplored.

In this study, we sought to examine the ESBL-E colonization burden of LT patients, to evaluate the link between ESBL-E colonization and subsequent infections of ESBL-E and ESBL-producing gram-negative bacilli (ESBL-GNB), and to explore the clinical outcomes. To our knowledge, this is the first study to consider ESBL-E epidemiology in LT cases in China.

Materials and methods

Study population

This is a retrospective observational cohort study. Patients included in the current study were from the Department of Liver Surgery and Liver Transplantation Center of Renji Hospital, Shanghai Jiao Tong University (Shanghai, China), between July 2016 and December 2017. Patients $\geq$ 18 years old who had undergone a rectal active surveillance culture (ASC) following a postoperative intensive care unit admission were eligible for inclusion in this study. The exclusion criteria were as follows: death within 72 h of transplantation or retransplant within 90 days of the original operation. If a retransplant was performed at $\geq$ 90 days after the first LT, the case was included as a separate event. This study was approved by the ethics committee of Renji Hospital, Shanghai, China (Ethics Number: KY2021-019).

Study design and definitions

Data gathered for this study included patients' baseline anthropometric measurements, laboratory indexes, operative complications, clinical course and survival status via the inpatient and outpatient information collection system.

A rectal swab to screen for ESBL-E was collected anonymously in liver transplant recipients at the time of their postoperative ICU enrollment. ChromID ESBL screening agar plates (bioMérieux, Marcy-l'Etoile, France) and mass spectrometric analysis were used for ESBL-E identification. Post-transplant bacterial infection was defined according to the NHSN/CDC guidelines, factoring in clinical symptoms and culture results as previously described (17). Clinical samples of the cultures were collected when an infection was suspected, and each case was adjudicated independently by two clinicians.

Ceftriaxone lasting for 5 to 7 days was used as standard perioperative antibiotic prophylaxis. In some instances, the surgeon may have modified a patient's prophylactic regimen according to their history of infectious disease. Selective digestive decolonization was not performed. Infection control policy in our center included protective gown and glove usage associated with adequate hand washing, routine screening of ESBL-E, written antibiotic treatment protocol, and continuous surveillance of nosocomial infections.

Three induction immunosuppression (IS) regimens were carried out by our institution, including a standard triple IS regimen, a basiliximab regimen, and a steroid-free regimen. The standard triple IS regimen consisted of a steroid, tacrolimus (TAC), and mycophenolate mofetil (MMF); the basiliximab regimen used basiliximab, steroids, and MMF with delayed introduction of calcineurin inhibitor (CNI); and the steroid-free regimen used basiliximab, TAC, and MMF. The maintenance IS regimen includes combination or separate use of steroids, CNI, sirolimus, and MMF. Following discharge, patients were monitored at the outpatient clinic as previously described (18).

Clinically significant ESBL-E and ESBL-GNB infections at 6 months post-transplant were the primary outcomes identified. Secondary endpoints included Ischemia-reperfusion $\geq$ 3, acute kidney injury (AKI), hospitalization days, episodes of acute rejection, graft versus host reaction (GvHD), and mortality at 90 days and 1-year post-transplant. Ischemia reperfusion injury (IRI) was defined by measuring aspartate aminotransferase (AST) levels during the first 3 days post-transplant, according to previously published criteria (Group 1: <600 IU/L; Group 2: 601–2,500 IU/L; Group 3: 2,501–5,000 IU/L) (19).

Statistical methods

Anthropometric data and laboratory measurements were analyzed using statistical software (SPSS 22.0, SPSS Inc., Chicago, IL, USA). Continuous data were analyzed by *t*-tests or Mann-Whitney U test. Categorical variables were compared using the $\chi 2$ test or Fisher's exact test. The 90-day and 1-year survival rates were calculated using the Kaplan-Meier method. Multivariable analysis was carried out using logistic regression. All values are expressed as mean ± standard deviation (SD), median (interquartile range, IQR), number, and percent (%), as appropriate. Two-sided *p*-values < 0.05 were considered statistically significant.

Result

Characteristics of the cohort

During the study period, a total of 509 adults who underwent LT at our center during an 18-month period were retrospectively reviewed, of whom six patients died within 48 h after operation, three patients did not have at least one rectal ASC, three patients were missing data >10%, and one patient committed suicide. These 13 patients were excluded from the study. Our final cohort, therefore, included 496 LT recipients with a mean age of 50.1 ± 10.3 years, and 387 (78%) of whom were male. The median MELD score was 13 (IQR 9–20). Prevalence of ESBL-E colonization at the time of transplant was 48.4% (240/496). In the colonized group, 14 patients developed ESBL-E infections and 29 patients developed ESBL-GNB infections, while in the non-colonized group, eight patients developed ESBL-E infections and 17 patients developed ESBL-GNB infections post-transplant. The flow chart of patient selection is shown in Figure 1.

Anthropometric and perioperative measurements according to ESBL-E colonization

Anthropometric and perioperative measurements of the enrolled patients are summarized in Table 1. The median MELD score was higher in patients colonized with ESBL-E at baseline (14, IQR 10–23) compared with those without colonization (12, IQR 9–18, $p = 0.027$). Anhepatic phase time was longer in ESBL-E colonized patients (40 min, IQR 38–45 vs. 40 min, IQR 35–42, $p = 0.018$). ESBL-E colonization did not vary significantly by age, gender, etiology of liver disease, transplantation reason, previous transplant, PNI, NLR, MLR, or PLR, renal insufficiency, diabetes mellitus, preoperative antibiotic use, pre-transplantation hospitalization days, transfused red blood cells, blood loss, postoperative ICU stay >72 h, postoperative intubation time >72 h, introduction regime, early reoperation rate (All $p > 0.05$).

Impact of ESBL-E colonization on predefined endpoints

The primary and secondary endpoints of ESBL-E colonization are summarized in Table 2. ESBL-E colonization was associated with a higher rate of clinically significant ESBL-GNB infection (12.1 vs. 6.6%, $p = 0.037$) but not with ESBL-E infection (5.8 vs. 3.1%, $p = 0.143$). ESBL-E colonization was also not associated with increased risk of Ischemia-reperfusion $\geq$ 3, acute kidney injury, prolonged hospital-stay, acute rejection, GvHD, 90-day mortality, or 1-year mortality (all $p > 0.05$).

Impact of ESBL-E colonization on ESBL-GNB infection

Multivariate analysis aimed at identifying independent variables associated with ESBL-GNB infection at 6 months post-transplant was performed (Table 3). After adjustment for ESBL-E colonization, MELD score, age, diabetes mellitus in model 1, ESBL-E colonization did not increase the risk of ESBL-GBN infection (aOR 1.755, 95% CI: 0.911–3.380; $p = 0.093$). After adjustment for ESBL-E colonization, MELD score, age, diabetes mellitus, pre-transplant antibiotic use, pre-transplant hospitalization days and anhepatic phase time in model 2, ESBL-E colonization still had no significant favorable results for ESBL-GBN infection (aOR 1.556, 95% CI: 0.761–3.181; $p = 0.226$). However, MELD score >25 was associated with an increased risk of ESBL-GNB infection for both models (Model 1: aOR 3.200,

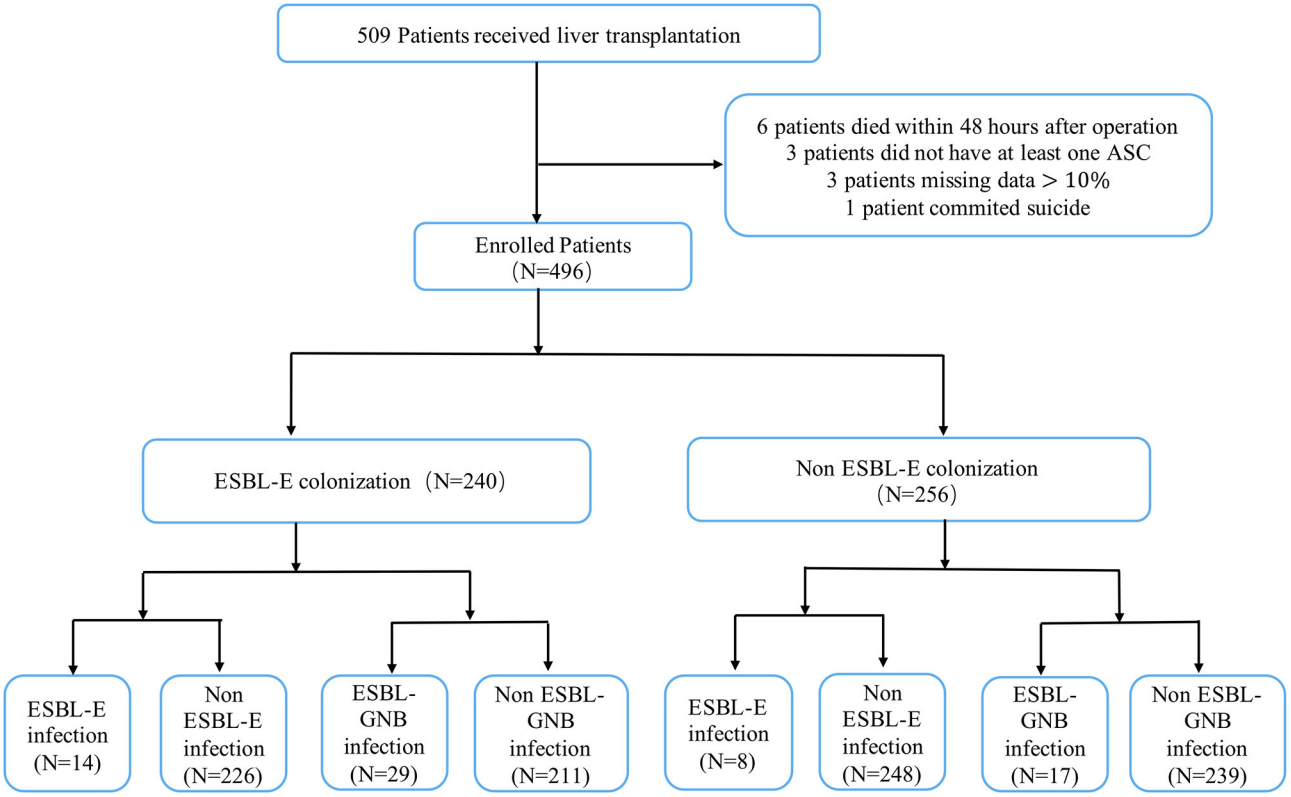

FIGURE 1
Flowchart of adult patients who underwent liver transplantation in Renji Hospital during the period July 2016 to December 2017.

95% CI 1.547–6.619, $p = 0.002$ and Model 2: aOR 3.583, 95% CI 1.568–8.188, $p = 0.002$).

ESBL-E and ESBL-GNB infections after liver transplantation

During the study period, 44 episodes of ESBL-E infection developed in 22 patients (4.4%) within 6 months following LT (range, 1 to 5 infections per subject). The median time from LT to ESBL-E infection was 16 days (IQR 5–29 days). ESBL-E infection was the first post-transplant infection to occur in 20 out of 22 patients, and the sites of ESBL-E infection were as follows: multi-site (n = 11), bloodstream (n = 6), intra-abdominal (n = 3), and respiratory tract ($n = 2$).

Eighty-eight episodes of ESBL-GNB infection occurred in 46 patients (9.2%) within 6 months following LT (range: 1 to 6 infections per subject). The median time from LT to ESBL-GNB infection was 13 days (IQR 5–23 days). ESBL-GNB infection was the first post-transplant infection to occur in 45 out of 46 patients. The sites of ESBL-GNB infection were as follows: multi-site (n = 18), respiratory tract (n = 10), bloodstream (n = 9), and intraabdominal ($n = 9$).

ESBL-producing bacterial distribution of colonization and infection

Of the 240 patients with ESBL-E rectal carriage, 246 strains were isolated; six patients had two distinct ESBL-E isolates. The species distribution of the 246 isolates was as follows: *E. coli* (n = 211, 85.8%), *K.pneumoniae* (n = 26, 10.6%), *Proteus mirabilis* ($n = 8$, 3.3%), and *Enterobacter aerogenes* ($n = 1$, 0.4%) (Figure 2A).

Of the 22 patients with ESBL-E post-transplant infections, 25 strains were isolated; three patients had two distinct ESBL-E isolates. The species distribution of the 25 pathogen isolates was as follows: *K.pneumoniae* (n = 17, 68%), *E. coli* (n = 4, 16%), *Enterobacter cloacae* ($n = 2$, 8%), and *Enterobacter aerogenes* (n = 2, 8%) (Figure 2B). Three colonized patients developed infections of the same strain (two patients with *E.coli*; one with *K.pneumoniae*).

Of the 46 patients with ESBL-GNB post-transplant infections, 58 strains were isolated; two patients had three distinct ESBL-GNB isolates; eight patients had two distinct ESBL-GNB isolates. The species distribution of the 58 pathogen isolates was as follows: *Acinetobacter baumannii* (n = 25, 43.1%), *K.pneumoniae* (n = 17, 29.3%), *E. coli* (n = 4, 6.9%), *Enterobacter cloacae* (n = 2, 3.4%), *Enterobacter aerogenes* (n = 2, 3.4%), and other types of GNB (n = 8, 13.8%) (Figure 2C).

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TABLE 1 Cohort characteristics and demographics.

Variables	ESBL-E colonized ($n = 240$)	Non ESBL-E colonized ($n = 256$)	p-value
Preoperative data			
Mean age (SD)	50.1 ± 10.3	51.0 ± 10.4	0.971
Male sex	195 (81.3%)	192 (75.0%)	0.093
MELD score (IQR)	14 (10, 23)	12 (9, 18)	0.027
MELD score group			0.006
≤15	134 (56.0%)	174 (68.0%)	
16–25	53 (22.0%)	49 (19.1%)	
>25	53 (22.0%)	33 (12.9%)	
Etiology of liver disease			0.110
Viral	162 (67.5%)	180 (70.3%)	
Autoimmune	25 (10.4%)	22 (8.6%)	
Alcohol	14 (5.8%)	5 (2.0%)	
Others	39 (16.3%)	49 (19.1%)	
Reason of transplantation			0.204
Carcinoma	112 (46.7%)	131 (51.2%)	
Decompensated cirrhosis	90 (37.5%)	98 (38.3%)	
Acute liver failure	38 (15.8%)	27 (10.5%)	
Previous transplant	4 (1.7%)	4 (1.6%)	0.927
PNI (IQR)	40.5 (36.6, 46.9)	40.8 (36.3, 46.5)	0.911
NLR (IQR)	3.21 (1.96, 5.70)	3.02 (1.82, 5.07)	0.270
MLR (IQR)	0.49 (0.33, 0.73)	0.45 (0.30, 0.70)	0.233
PLR (IQR)	88.2 (56.2, 137.2)	88.2 (54.7, 136.7)	0.870
Renal insufficiency	20 (8.3%)	17 (6.6%)	0.473
Diabetes mellitus	44 (18.3%)	52 (20.3%)	0.577
Preoperative antibiotic use	63 (26.3%)	57 (22.3%)	0.300
Pre-transplantation hospitalization, days (IQR)	3 (1, 8)	3 (1, 9)	0.725
Intraoperative data			
Anhepatic phase time, min (IQR)	40 (38, 45)	40 (35, 42)	0.018
Red blood cells transfused, units (IQR)	4 (0, 7)	4 (0, 6)	0.673
Blood loss, ml (IQR)	500 (400, 1,000)	500 (400, 800)	0.968
Postoperative data			
Postoperative ICU stay > 72 h	10 (4.2%)	5 (2.0%)	0.150
Postoperative intubation time > 72 h	8 (3.3%)	4 (1.6%)	0.200
Introduction			0.107
Standard triple induction	15 (6.3%)	29 (11.3%)	
Steroid-free induction	7 (2.9%)	10 (3.9%)	
Basiliximab induction	218 (90.8%)	217 (84.8%)	
Early reoperation	13 (5.4%)	16 (6.3%)	0.693

Data represent no. (%) of patients unless otherwise specified; SD, standard deviation; IQR, interquartile range; MELD, model of end-stage liver disease; PNI, prognostic nutritional index, calculated as [10 × serum albumin (g/dL)] + [0.005 × peripheral lymphocyte count (per mm3)]; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; Early reoperation, reoperation within 1 month after liver transplantation (20).

TABLE 2 Cohort clinical outcomes.

Variables	ESBL-E colonized ($n = 240$)	Non ESBL-E colonized ($n = 256$)	p-value
Ischemia-reperfusion			
Ischemia-reperfusion ≥ 3	67 (27.9%)	63 (24.6%)	0.403
Kidney function			
Acute kidney injury	95 (39.6%)	99 (38.7%)	0.835
Infections			
ESBL-E infection	14 (5.8%)	8 (3.1%)	0.143
ESBL-GNB infection	29 (12.1%)	17 (6.6%)	0.037
Hospital stay (IQR)	22 (17, 31)	23 (17, 52)	0.568
Acute rejection	5 (2.1%)	4 (1.6%)	0.664
GvHD	3 (1.3%)	3 (1.2%)	0.937
Mortality			
90-day mortality	17 (7.1%)	12 (4.7%)	0.262
1-year mortality	31 (12.9%)	24 (9.3%)	0.265

Data represent no. (%) of patients unless otherwise specified. GvHD, graft versus host reaction.

Discussion

One important finding from this study was the high prevalence of ESBL-E gut colonization identified at the time of liver transplantation, which accounted for 48.4% of cases in the study, a figure considerably higher than what has been reported in previous literature (13.3∼17%) (6, 21). This could also suggest the presence of a particularly heavy ESBL-E burden in our country; as mentioned earlier, the rate of ESBL-E carriers varies greatly across geographic regions. Certain regions in the world, such as Southeast Asia, are known to face a higher ESBL-E burden than others (4, 22).

TABLE 3 Multivariate analysis of risk factors for ESBL-GNB infection at 6 months.

Variable	Model 1		Model 2	
	aOR (95% CI)	*p*	aOR (95% CI)	*p*
ESBL-E colonization				
No	1.00 (ref)	/	1.00 (ref)	/
Yes	1.755 (0.911–3.380)	0.093	1.556 (0.761–3.181)	0.226
MELD score		0.006		0.007
≤15	1.00 (ref)	/	1.00 (ref)	/
16–25	1.185 (0.502–2.800)	0.698	1.234 (0.466–3.267)	0.672
>25	3.200 (1.547–6.619)	0.002	3.583 (1.568–8.188)	0.002

aOR, adjusted odds ratio; 95% CI, 95% confidence interval; Model 1 was adjusted for ESBL-E colonization, MELD score, Age and Diabetes mellitus; Model 2 was adjusted for ESBL-E colonization, MELD score, Age, Diabetes mellitus, Pre-transplant antibiotic use, Pre-transplant hospitalization days and Anhepatic phase time.

There are relatively few studies investigating ESBL-E colonization in LT recipients and these studies are mainly focusing on Europe (21), and as a result, there is currently no data available that explains the interregional differences affecting the LT population while taking ESBL-E into account. The absence of such research suggests that more comparative studies are needed to assess the ESBL-E colonization rate in endemic areas and in high-risk groups, including LT patients. Our study addresses these gaps in knowledge pertaining to the ESBL-E colonization rate in LT patients in China.

In addition to different epidemiologic features, some aspects of the ESBL-E colonization rate may also be related to the method used to define carriers. For example, in our study, rectal swabs were used to identify carriers, whereas in other studies on LT recipients, positive fecal cultures were used to identify a patient as an ESBL-E carrier (7, 23).

Our data show that patients colonized with ESBL-E had higher median MELD scores at the time of LT ($p = 0.027$). This could be related to the fact that bacteria colonization is more likely to be detected in patients who are sicker; these patients are frequently critically ill, and experience prolonged-hospital stays involving invasive devices and anti-infective agents for both therapeutic and prophylactic purposes, and these factors may also increase the risk of acquiring ESBL-E (7, 24). The median anhepatic phase time was longer in ESBL-E colonized patients ($p = 0.018$). The prolonged anhepatic phase time may also reflect the severity of illness and complexity of the surgery.

Of the 496 transplant patients, 4.4% (22/496) developed ESBL-E infections within 6 months of surgery, an incidence rate comparable with a prior study conducted in one of the largest LT centers in France in which 5.5% of patients developed an ESBL-E infection after LT (7). Our study demonstrates that ESBL-E infections are not often preceded by ESBL-E gastrointestinal (GI) colonization, and the likelihood of ESBL-E carriers developing ESBL-E infections was only 5.8% (14/240) in our LT population, with no significant differences between colonized and non-colonized patients. The infection incidence in colonized patients was lower than that in previous studies, where 44.8 and 39% of patients colonized with ESBL-E developed a subsequent infection (6, 7). Additional risk factor analysis carried out by Logre et al. (6) found that *K. pneumoniae* carriage (compared with other types of ESBL-E) was among the most important predictors for post-LT ESBL-E infection, regardless of the type of infection. In our LT patient cohort, *E. coli* (85.8%) rather than *K. pneumoniae* (10.6%) was the dominant type of colonization, which may partially explain the low rate of subsequent ESBL-E infection. Additionally, many variables can impact the occurrence of infection after LT, especially severity of illness and/or surgery and its complications, and a single rectal swab is unlikely to play a decisive role (25, 26).

To our knowledge, the role of ESBL-E colonization in the development of post-LT ESBL-GNB infections had not been previously demonstrated. This study determined that GI colonization of ESBL-E conferred no increased risk of developing an ESBL-GNB infection after LT; rather, a patient's severity of illness (MELD score > 25) had a greater impact. Although, the impact of MELD score on post-transplant ESBL-GNB infection is revealed for the first time, previous reports indicated that increased pre-transplant MELD score (per 10-point change: HR = 1.59) is a risk factor of developing any infection within 1 year of transplant (27). In prior studies that included both LDLT and DDLT, MELD > 20 was associated with early bacterial infection (28) as well as post-LT septic shock (29).

We herein found that the pathogen spectrum of ESBL-E colonization was significantly different from that of infection after liver transplantation with only three colonization events leading to infection by the same pathogen (all were multiple-site infections). *E.coli* (85.8%, 211/246) was the most prevalent ESBL-producing Enterobacteriaceae for colonization; however, the most common infection causing ESBL-E was *K.pneumoniae* (68.0%, 17/25), rather than *E.coli* (16.0%, 4/25). The most common infection causing ESBL-GNB was *Acinetobacter baumannii* (*A.baumannii,* 43.1%, 25/58), followed by *K.pneumoniae* (29.3%, 17/58). Recent years have witnessed increasing rates of antimicrobial resistance in *K.pneumoniae* and *A.baumannii* in solid organ transplant recipients (30, 31). Our results are consistent with the previous studies wherein *A.baumannii* and *K.pneumonia* were the primary GNB identified in liver transplant recipients (32, 33).

Few prior studies on outcomes of bacterial colonization in liver transplant recipients have been conducted, and these studies reported conflicting results. According to a study conducted in 2008, liver transplant candidates and recipients with VRE colonization had an increased risk of death, whereas those with MRSA colonization had no increased risk of death (34). More recent studies have shown that colonization with MDRO is not associated with increased mortality in short-term follow-ups with LT recipients (35), and the infection free-survival rate following LT does not differ for ESBL-E carriage groups (23). Our results support these more recent findings; that is, colonization of ESBL-E does not impact 90-day or 1-year mortality. This result can be explained by the fact that ESBL-E colonization was not associated with post-LT ESBL-E infection, and neither was it a risk factor for ESBL-GNB infection in our patient cohort.

This study has several limitations. First, the retrospective nature and the single center design was the limitation of the study. Second, the precision of a single rectal swab for detecting colonization may be limited and could have led to the misclassification of colonized

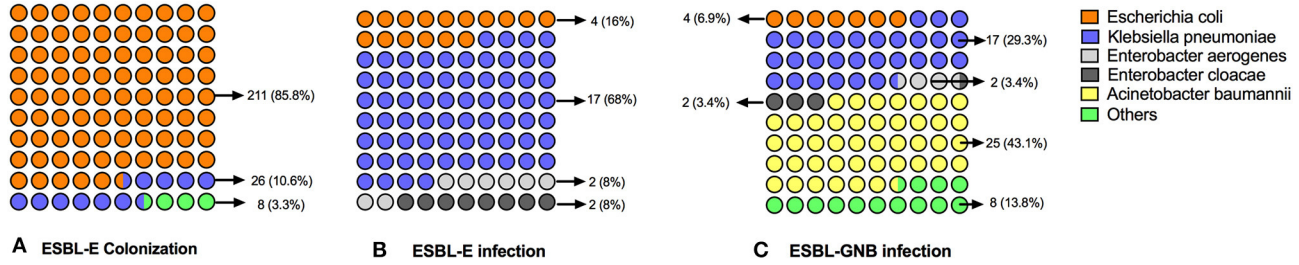

FIGURE 2
Proportional distribution chart of ESBL-producing bacteria for colonization and infection. A distribution chart showing proportions (with numbers in parentheses) of the predominant ESBL-producing organisms (ESBL-E and ESBL-GNB) annotated by colonization and infection. **(A)** The most predominant ESBL-producing Enterobacteriaceae for colonization was *Escherichia coli* (85.8%); **(B)** the most predominant ESBL-producing Enterobacteriaceae for infection was *Klebsiella pneumoniae* (68.0%); **(C)** the most predominant organism for ESBL-GNB infection were *Acinetobacter baumannii* (43.1%), followed by *Klebsiella pneumoniae* (29.3%).

patients. However, this would bias the results toward the null hypothesis–that ESBL-E colonization status has a limited impact on the risk of developing subsequent ESBL-producing bacterial infections affecting prognoses. In this article, we found that ESBL-E colonization was not significantly associated with post-LT ESBL-E infection, nor was it a risk factor for ESBL-GNB infection. Additionally, ESBL-E gut colonization did not lead to a worse prognosis compared with non-colonized patients in our study, and the pathogen spectrum of colonization was found to differ significantly from that of bacterial infections. A policy of universal ASC generates both a massive workload for laboratory staffs and substantial expenditures for the hospital system. With the available evidence, we argue against the usefulness of active screening of ESBL-E in liver transplant ICUs where ESBL-E colonization burden is heavy and ESBL- producing *E. coli* predominate. More future researches are needed to verify the clinical value of ESBL-E colonization screening in LT patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Renji Hospital, Shanghai, China. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CS and YD conceived and designed the study. CS and RY wrote the manuscript. YY and HZ collected and analyzed the data. JZ and QX interpreted the results. YG and YD revised the paper. 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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References

1. Meirelles Junior RF, Salvalaggio P, Rezende MB, Evangelista AS, Guardia BD, Matielo CE, et al. Liver transplantation: history, outcomes and perspectives. *Einstein*. (2015) 13:149–52. doi: 10.1590/S1679-45082015RW3164

2. Hand J, and Patel G. Multidrug-resistant organisms in liver transplant: mitigating risk and managing infections. *Liver Transpl*. (2016) 22:1143–53. doi: 10.1002/lt.24486

3. Massa E, Michailidou E, Agapakis D, Papadopoulos S, Tholioti T, Aleuroudis I, et al. Colonization and infection with extensively drug resistant gram-negative bacteria in liver transplant recipients. *Transplant Proc*. (2019) 51:454–6. doi: 10.1016/j.transproceed.2019.01.076

4. Jean SS, Hsueh PR. High burden of antimicrobial resistance in Asia. *Int J Antimicrob Agents*. (2011) 37:291–5. doi: 10.1016/j.ijantimicag.2011.01.009

5. White SL, Hirth R, Mahillo B, Dominguez-Gil B, Delmonico FL, Noel L, et al. The global diffusion of organ transplantation: trends, drivers and policy implications. *Bull World Health Organ*. (2014) 92:826–35. doi: 10.2471/BLT.14.137653

6. Logre E, Bert F, Khoy-Ear L, Janny S, Giabicani M, Grigoresco B, et al. Risk factors and impact of perioperative prophylaxis on the risk of extended-spectrum beta-lactamase-producing enterobacteriaceae-related

infection among carriers following liver transplantation. *Transplantation*. (2021) 105:338–45. doi: 10.1097/TP.0000000000003231

7. Bert F, Larroque B, Paugam-Burtz C, Dondero F, Durand F, Marcon E, et al. Pretransplant fecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae and infection after liver transplant, France. *Emerg Infect Dis.* (2012) 18:908–16. doi: 10.3201/eid1806.110139

8. Lindblom A, Karami N, Magnusson T, Ahren C. Subsequent infection with extended-spectrum beta-lactamase-producing enterobacteriaceae in patients with prior infection or fecal colonization. *Eur J Clin Microbiol Infect Dis.* (2018) 37:1491–7. doi: 10.1007/s10096-018-3275-x

9. De Jong A, Molinari N, Sebbane M, Prades A, Futier E, Jung B, et al. Feasibility and effectiveness of prone position in morbidly obese patients with ARDS: a case-control clinical study. *Chest.* (2013) 143:1554–61. doi: 10.1378/chest.12-2115

10. Carbonne H, Le Dorze M, Bourrel AS, Poupet H, Poyart C, Cambau E, et al. Relation between presence of extended-spectrum beta-lactamase-producing Enterobacteriaceae in systematic rectal swabs and respiratory tract specimens in ICU patients. *Ann Intensive Care.* (2017) 7:13. doi: 10.1186/s13613-017-0237-x

11. Canton R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, et al. Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe. *Clin Microbiol Infect.* (2008) 14:144–53. doi: 10.1111/j.1469-0691.2007.01850.x

12. Abayneh M, Worku T. Prevalence of multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacilli: a meta-analysis report in Ethiopia. *Drug Target Insights.* (2020) 14:16–25. doi: 10.33393/dti.2020.2170

13. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clin *Microbiol Rev*. (2005) 18:657–86. doi: 10.1128/CMR.18.4.657-686.2005

14. Diriba K, Awulachew E, Gemede A, Anja A. The magnitude of extended-spectrum beta-lactamase- producing Enterobacteriaceae from clinical samples in Ethiopia: a systematic review and meta-analysis. *Access Microbiol.* (2021) 3:000195. doi: 10.1099/acmi.0.000195

15. Porse A, Gumpert H, Kubicek-Sutherland JZ, Karami N, Adlerberth I, Wold AE, et al. Genome dynamics of escherichia coli during antibiotic treatment: transfer, loss, and persistence of genetic elements in situ of the infant gut. *Front Cell Infect Microbiol.* (2017) 7:126. doi: 10.3389/fcimb.2017.00126

16. Lindblom A, Sriram KK, Muller V, Oz R, Sandstrom H, Ahren C, et al. Interspecies plasmid transfer appears rare in sequential infections with extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae. *Diagn Microbiol Infect Dis.* (2019) 93:380–5. doi: 10.1016/j.diagmicrobio.2018.10.014

17. Chao JS, Zhao SL, Ou-Yang SW, Qian YB, Liu AQ, Tang HM, et al. Post-transplant infection improves outcome of hepatocellular carcinoma patients after orthotopic liver transplantation. *World J Gastroenterol.* (2019) 25:5630–40. doi: 10.3748/wjg.v25.i37.5630

18. Sun H, Teng M, Liu J, Jin D, Wu J, Yan D, et al. FOXM1 expression predicts the prognosis in hepatocellular carcinoma patients after orthotopic liver transplantation combined with the Milan criteria. *Cancer Lett.* (2011) 306:214–22. doi: 10.1016/j.canlet.2011.03.009

19. Chiang D, Dingle TC, Belga S, Kabbani D, Bhanji RA, Walter J, et al. Association between gut colonization of vancomycin-resistant enterococci and liver transplant outcomes. *Transpl Infect Dis.* (2022) 24:e13821. doi: 10.1111/tid.13821

20. Elsabbagh AM, Girlanda R, Hawksworth J, Pichert MD, Williams C, Pozzi A, et al. Impact of early reoperation on graft survival after liver transplantation: univariate and multivariate analysis. *Clin Transplant.* (2018) 32:e13228. doi: 10.1111/ctr.13228

21. Alevizakos M, Kallias A, Flokas ME, Mylonakis E. Colonization with extended-spectrum beta-lactamase-producing Enterobacteriaceae in solid organ transplantation: a meta-analysis and review. *Transpl Infect Dis.* (2017) 19:e12718. doi: 10.1111/tid.12718

22. Alevizakos M, Mylonakis E. Colonization and infection with extended-spectrum beta-lactamase producing Enterobacteriaceae in patients with malignancy. *Expert Rev Anti Infect Ther.* (2017) 15:653–61. doi: 10.1080/14787210.2017.1348895

23. Magro B, Mazzola A, Munteanu M, Goumard C, Martinez V, Bernard D, et al. Consequences of extended spectrum beta-lactamase-producing enterobacteriaceae and methicillin-resistant staphylococcus aureus carriage in awaiting liver transplant patients. *Liver Transpl.* (2021) 27:43–54. doi: 10.1002/lt.25897

24. Thiebaut AC, Arlet G, Andremont A, Papy E, Sollet JP, Bernede-Bauduin C, et al. Variability of intestinal colonization with third-generation cephalosporin-resistant Enterobacteriaceae and antibiotic use in intensive care units. *J Antimicrob Chemother.* (2012) 67:1525–36. doi: 10.1093/jac/dks072

25. He Q, Liu P, Li X, Su K, Peng D, Zhang Z, et al. Risk factors of bloodstream infections in recipients after liver transplantation: a meta-analysis. *Infection.* (2019) 47:77–85. doi: 10.1007/s15010-018-1230-5

26. Pereira MR, Scully BF, Pouch SM, Uhlemann AC, Goudie S, Emond JE, et al. Risk factors and outcomes of carbapenem-resistant Klebsiella pneumoniae infections in liver transplant recipients. *Liver Transpl.* (2015) 21:1511–9. doi: 10.1002/lt.24207

27. Abad CL, Lahr BD, Razonable RR. Epidemiology and risk factors for infection after living donor liver transplantation. *Liver Transpl.* (2017) 23:465–77. doi: 10.1002/lt.24739

28. Avkan-Oguz V, Ozkardesler S, Unek T, Ozbilgin M, Akan M, Firuzan E, et al. Risk factors for early bacterial infections in liver transplantation. *Transplant Proc.* (2013) 45:993–7. doi: 10.1016/j.transproceed.2013.02.067

29. Hung HC, Lee JC, Wang YC, Cheng CH, Wu TH, Wu TJ, et al. Living-donor liver transplantation for hepatocellular carcinoma: impact of the MELD score and predictive value of NLR on survival. *Curr Oncol.* (2022) 29:3881–93. doi: 10.3390/curroncol29060310

30. Cervera C, van Delden C, Gavalda J, Welte T, Akova M, Carratala J, et al. Multidrug-resistant bacteria in solid organ transplant recipients. *Clin Microbiol Infect.* (2014) 20:49–73. doi: 10.1111/1469-0691.12687

31. Bartoletti M, Giannella M, Tedeschi S, Viale P. Multidrug-resistant bacterial infections in solid organ transplant candidates and recipients. *Infect Dis Clin North Am.* (2018) 32:551–80. doi: 10.1016/j.idc.2018.04.004

32. Tu Z, Xiang P, Xu X, Zhou L, Zhuang L, Wu J, et al. DCD liver transplant infection: experience from a single centre in China. *Int J Clin Pract.* (2016) 70:3–10. doi: 10.1111/ijcp.12810

33. Freire MP, Soares Oshiro IC, Bonazzi PR, Guimaraes T, Ramos Figueira ER, Bacchella T, et al. Surgical site infections in liver transplant recipients in the model for end-stage liver disease era: an analysis of the epidemiology, risk factors, and outcomes. *Liver Transpl.* (2013) 19:1011–9. doi: 10.1002/lt.23682

34. Russell DL, Flood A, Zaroda TE, Acosta C, Riley MM, Busuttil RW, et al. Outcomes of colonization with MRSA and VRE among liver transplant candidates and recipients. *Am J Transplant.* (2008) 8:1737–43. doi: 10.1111/j.1600-6143.2008.02304.x

35. Ferstl PG, Filmann N, Heilgenthal EM, Schnitzbauer AA, Bechstein WO, Kempf VAJ, et al. Colonization with multidrug-resistant organisms is associated with in increased mortality in liver transplant candidates. *PLoS ONE.* (2021) 16:e0245091. doi: 10.1371/journal.pone.0245091

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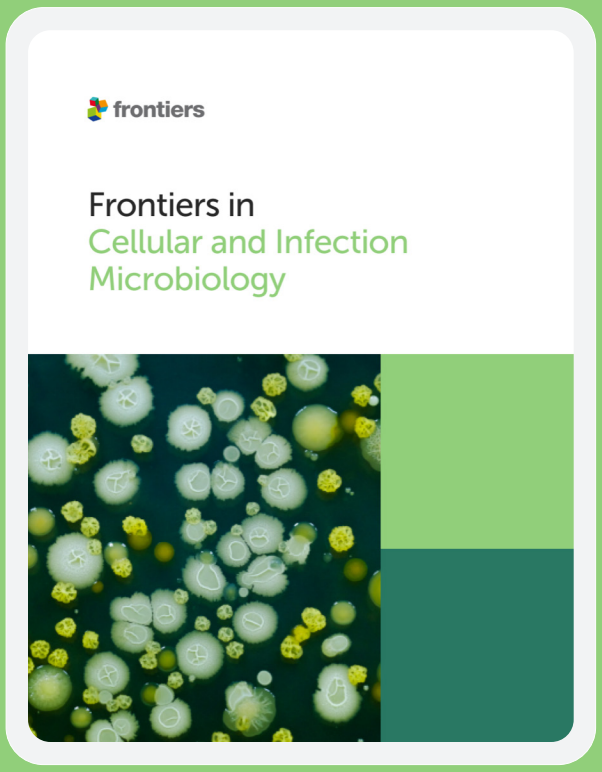

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