Whole genome sequencing of OXA-232-producing wzi93-KL112-O1 carbapenem-resistant Klebsiella pneumoniae in human bloodstream infection co-harboring chromosomal ISEcp1-based $\text{bla}_\text{CTX-M-15}$ and one $\text{rmpA2}$-associated virulence plasmid

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**Objectives**: To characterize one OXA-232-producing wzi93-KL112-O1 carbapenem-resistant Klebsiella pneumoniae (CRKP) co-harboring chromosomal $\text{bla}_{\text{CTX-M-15}}$ and one $\text{rmpA2}$-associated virulence plasmid.

**Methods**: Minimum inhibitory concentrations (MICs) were measured via broth microdilution method. Conjugation, chemical transformation, string test and *Galleria mellonella* infection model experiments were also conducted. Whole-genome sequencing (WGS) was performed on the Illumina and Nanopore platforms. Antimicrobial resistance determinants were identified using ABRicate program with ResFinder database. Insertion sequences (ISs) were identified using ISfinder. Bacterial virulence factors were identified using virulence factor database (VFDB). Wzi, capsular polysaccharide (KL) and lipooligosaccharide (OCL) were analyzed using Kleborate with Kaptive. Phylogenetic analysis of 109 ST15 *K. pneumoniae* strains was performed using core genome multilocus sequence typing (cgMLST) on the Ridom SeqSphere+ server. MLST, replicons type, SNP strategies and another cgMLST analysis for 45 OXA-232-producing *K. pneumoniae* strains were further conducted using BacWGStdb server.
Results: *K. pneumoniae* KPTCM strain belongs to ST15 with wzi93, KL112 and O1. It possessed a multidrug-resistant (MDR) profile and was resistant to carbapenems (meropenem and ertapenem), ciprofloxacin and amikacin. Virulence assays demonstrated KPTCM strain possesses a low virulence phenotype. WGS revealed it contained one circular chromosome and nine plasmids. The carbapenemase-encoding gene bla\textsubscript{OXA-232} was located in a 6141-bp ColKP3-type non-conjugative plasmid and flanked by ΔS\textsubscript{Ecp1} and Δlys\textsubscript{R}-\textsubscript{DereA}. Interestingly, bla\textsubscript{CTX-M-15} was located in the chromosome mediated by S\textsubscript{Ecp1}-based transposon Tn2012. Importantly, it harbored a rmpA2-associated pLVPK-like virulence plasmid with iut\textsubscript{A}-iuc\textsubscript{ABCD} gene cluster and one IS26-mediated MDR fusion plasmid according to 8-bp (AGCTGCAC or GGCCTTTG) target site duplications (TSD). Based on the cgMLST and SNP analysis, data showed OXA-232-producing ST15 *K. pneumoniae* isolates were mainly isolated from China and have evolved in recent years.

Conclusions: Early detection of CRKP strains carrying chromosomal bla\textsubscript{CTX-M-15}, OXA-232 carbapenemase and pLVPK-like virulence plasmid is recommended to avoid the extensive spread of this high-risk clone.

KEYWORDS

CRKP, OXA-232, chromosomal bla\textsubscript{CTX-M-15}, pLVPK-like virulence plasmid, IS26, co-integration

Introduction

*Klebsiella pneumoniae* is an important opportunist pathogen associated with hospital- and community-acquired infections that has gained public attention due to its capability of acquiring resistance genes and plasmids (Kong et al., 2021a; Xu et al., 2021). Specifically, the emergence of carbapenem-resistant *K. pneumoniae* (CRKP) has limited effective therapies and poses a tremendous challenge in clinical settings (He et al., 2022). It is well documented that *K. pneumoniae* usually is classified into classical *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP) based on different virulence levels (Tian et al., 2021). In general, CRKP belong to the cKP group. When compared with cKP, hvKP mainly causes bloodstream infections (BSIs) and liver abscesses, both of which pose huge threat to patients (Wang et al., 2018; Wanford et al., 2021). Hypervirulent phenotype is mainly relevant to rmpA1/rmpA2, aerobactin (iuc\textsubscript{ABCD} and iut\textsubscript{A}) and salmochelin (iro\textsubscript{BCDN}). These virulence factors are usually on a pLVPK-like virulence plasmid. (Kong et al., 2021b). Of greater concern, the amount of carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) is increasing and infections caused by CR-hvKP strains generally lead to severe outcomes and mortality (Gu et al., 2018; Chen et al., 2021a).

OXA-232, a type of OXA-48-like enzyme, differs from OXA-48 and OXA-181 by five and one amino acid substitutions, respectively (Potron et al., 2013; Lahlaoui et al., 2017). OXA-232 could hydrolyze temocillin, penicillins, cefotaxime, cefepime and carbapenems with different hydrolytic activities (Potron et al., 2013). Catalytic activities of OXA-232 for all penicillins except temocillin (ten-fold lower) were two- to six-fold higher than OXA-181 and OXA-48, respectively (Potron et al., 2013; Oueslati et al., 2015). The catalytic activity of OXA-232 for imipenem was quite lower than OXA-181 and OXA-48. Hydrolysis abilities of meropenem and ertapenem were similar among OXA-232, OXA-48 and OXA-181 (Potron et al., 2013).

OXA-232-producing *K. pneumoniae* strains have been reported in Zhejiang and Shanghai, China (Shen et al., 2020b; Shi et al., 2020; Jia et al., 2021), but to our knowledge, no comprehensive phylogenetic analysis for ST15 *K. pneumoniae* isolates collected from various countries has been performed before. Moreover, a study by Zhu et al. in Yancheng, China reported clonal dissemination of ST15 OXA-232-producing *K. pneumoniae* strains (Zhu et al., 2021). Based on the short-read Illumina whole-genome sequencing (WGS), bla\textsubscript{CTX-M-15} was found in an IncFIK-type plasmid (Zhu et al., 2021). In addition, there are no further corresponding description about rmpA2-related virulence plasmid structure and virulence
assessment experiments in their ST15 OXA-232-producing \textit{K. pneumoniae} strains.

Here, one OXA-232-producing sequence type (ST) 15 CRKP in human bloodstream infection was isolated in January 2022 in China and the complete genetic characteristics were further investigated. This study provides a comprehensive description of the complete genomic features of a \textit{wzi93-KL112-O1} OXA-232-producing ST15 CRKP, which harbors a \textit{rmpA2}-related pLVPK-like virulence plasmid and the chromosomal \textit{bla\textsubscript{CTX-M-15}} mediated by \textit{ISEcp1}-based transposon Tn2012. Moreover, a multi-drug resistance plasmid may have evolved through IS26-mediated co-integration. This information will offer help to prevent and control the extensive spread of OXA-232-producing ST15 \textit{K. pneumoniae} isolates. Importantly, the combination of short-read Illumina and long-read MinION WGS was performed to provide complete insight into the genomic structure features of resistance and virulence plasmids of \textit{K. pneumoniae}.

Materials and methods

Flow chart illustrating the study process

A flow chart (Figure 1) was constructed to detail all experiments and procedures associated with this study using CmapTools v6.04 (https://cmap.ihmc.us) (Behzadi and Gajdacs, 2021). Based on different contents of experiments and bioinformatic analysis, this study was divided into two parts, consisting of Wet Lab and Dry Lab.

Bacterial isolation and identification

\textit{K. pneumoniae} strain (KPTCM) was isolated from one patient with BSIs and liver abscess during the routine diagnostic in the hospital in Hangzhou, China in January 2022. No history of travel was reported. Unfortunately, liver abscess developed rapidly and the outcome was death. According to the clinical symptoms of host, KPTCM may be a relatively hypervirulent strain and was chosen for further analysis. Isolate identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) and further confirmed by 16S rRNA gene-based PCR followed by sequencing (Huada, China).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) against multiple antimicrobial agents were determined using broth microdilution method and interpreted according to Clinical and Laboratory Standards Institute (CLSI) 2021 guidelines. Antibiotics, including imipenem (IPM), meropenem (MEM), ertapenem (ETP), amikacin (AMI), ciprofloxacin (CIP), colistin
(COL), tigecycline (TGC), ceftazidime/avibactam (CAZ-AVI), ceftazidime (CAZ), cefepime (FEP), meropenem-vaborbactam (M/V) and cefiderocol (CFD), were investigated in this study. *Escherichia coli* ATCC 25922 served as the quality control strain.

Conjugation and chemical transformation experiments

To determine the transferable ability of plasmid carrying bla<sub>OXA-232</sub>, conjugation experiments using *E. coli* J53 (sodium azide resistant) as the recipient strain were carried out using film mating method (Yang et al., 2021b). Transconjugants were screened on Mueller–Hinton agar plates containing sodium azide (100 mg/L) and meropenem (2 mg/L). Donor or recipient bacteria alone was used as the control culture (Liu et al., 2021). The identity of putative transconjugants was confirmed via PCR and MALDI-TOF MS. Experiments were performed in triplicate independently.

Chemical transformation was further performed when conjugation failed. Plasmid harboring bla<sub>OXA-232</sub> gene was transferred into *E. coli* DH5α via chemical transformation with imipenem (0.125 mg/L) for selection to yield *E. coli* DH5α:pOXA-232 (*E. coli* DH5α containing OXA-232 plasmid) (Froger and Hall, 2007). The MICs for carbapenems were measured and compared to the MICs values of *E. coli* DH5α.

Hypermucoviscous phenotype determination

String test assay was performed based on previous description (Shen et al., 2020a). Briefly, KPTCM strain was cultured on a sheep blood agar plate at 37°C for an overnight culture followed by streaking an inoculation loop through a colony in the next day. Formation of a viscous string > 5 mm in length was considered as a positive phenotype.

Galleria mellonella infection model

*Galleria mellonella* infection model was used to assess virulence as previously described (Menard et al., 2021; Zhao et al., 2021). In brief, log-phase bacteria were centrifuged, resuspended using phosphate-buffered saline (PBS) and then diluted 10-fold to yield 1 × 10<sup>8</sup> colony forming units/mL (CFU/mL). An aliquot of 10 μL (1 × 10<sup>6</sup> CFU) was injected into *G. mellonella* larvae (n = 10, 0.2–0.3 g; Yuejiayin, Tianjin, China) using a Hamilton syringe (Hamilton, USA). *G. mellonella* larvae were incubated at 37°C and survival was recorded every 12 h and monitored for 72 h in total. *K. pneumoniae* NTUH-2044 was used as a positive control, and one cKP isolate (*K. pneumoniae* ATCC700603) served as a virulence negative control (Liao et al., 2020). Experiments were carried out in triplicate independently.

Whole genome sequencing and bioinformatics analysis

Genomic DNA was extracted from KPTCM strain using a Qiaagen minikit (Qiagen, Hilden, Germany) upon the manufacturer’s recommendations with minor modifications. Whole genome was sequenced using both Illumina HiSeq platform (Illumina, San Diego, CA, USA) and Oxford Nanopore MinION platform (Nanopore, Oxford, UK). *De novo* assembly of the reads of Illumina and MinION was constructed using Unicycler v0.4.8 (Wick et al., 2021). Genome sequence annotation was performed using National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) (http://ncbi.nlm.nih.gov/genome/annotation_prok/) (Tatusova et al., 2016). Antimicrobial resistance determinants were identified using ABRicate program (https://github.com/tseemann/abricate) based on ResFinder database (http://www.genomicepidemiology.org/) (Zankari et al., 2012). Bacterial virulence factors were identified via virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/) (Liu et al., 2022). Insertion sequences (ISs) were identified using ISfinder (Siguitet et al., 2006). wzi, K and O antigen loci were analyzed using Kleborate with Kaptive (Lam et al., 2021; Lam et al., 2022). Plasmid structure was visualized using DNAplotter (https://www.sanger.ac.uk/tool/dnaplotter/). Plasmid comparison with p47733_OXA_181 (Genbank accession number: CP050368) (Chudejevo et al., 2021) was performed using Easyfig (Sullivan et al., 2011) and visualized with Adobe Illustrator. Phylogenetic analysis of 109 ST15 *K. pneumoniae* strains was performed using core genome multilocus sequence typing (cgMLST) on the Ridom SeqSphere+ server (Chen et al., 2021b). MLST, replicon type, single-nucleotide polymorphism (SNP) strategies, and further cgMLST analysis for 45 OXA-232-producing *K. pneumoniae* strains were conducted using BacWGSTdb server (Ruan et al., 2020; Feng et al., 2021). Generation tree file was visualized using the Interactive Tree of Life (iTOl, https://itol.embl.de/) (Letunic and Bork, 2021).

Statistical analysis

*G. mellonella* survival rates were evaluated through Kaplan–Meier survival curves. Statistical significance was analyzed using a log-rank (Mantel–Cox) test with GraphPad Prism 8.0.2
software (GraphPad Software, San Diego California USA). $P$ values of $< 0.05$ were considered statistically significant.

### Results

**MICs, antimicrobial resistance and virulence determinants**

Antimicrobial susceptibility testing revealed KPTCM strain possessed a multidrug-resistant (MDR) profile with meropenem and ertapenem MICs of 4 mg/L and 8 mg/L, respectively. Furthermore, KPTCM strain exhibited resistance to ceftazidime (> 32 mg/L), cefepime (> 64 mg/L), ciprofloxacin (> 32 mg/L) and amikacin (> 128 mg/L). However, it was still susceptible to imipenem (1 mg/L), meropenem-vaborbactam (2 mg/L), cefiderocol (0.25 mg/L), ceftazidime/avibactam (0.5 mg/L), colistin (0.5 mg/L) and tigecycline (1 mg/L).

Analysis of the genome of KPTCM strain revealed that, in addition to co-harboring chromosomal $\texttt{bla}_{\text{CTX-M-15}}$ and plasmid-mediated $\texttt{bla}_{\text{OXA-232}}$, a series of genes conferring resistance to $\beta$-lactams ($\texttt{bla}_{\text{SHV-106}}$, $\texttt{bla}_{\text{SHV-12}}$ and $\texttt{bla}_{\text{TEM-1B}}$), aminoglycosides ($\texttt{strAB}$ and $\texttt{rmtF}$), sulfonamides ($\texttt{sul2}$), trimethoprim/sulfamethoxazole ($\texttt{dfrA14}$), quinolones ($\texttt{qnrB1}$), rifampicin ($\texttt{arr-2}$) and chloramphenicol ($\texttt{catB}$) were also identified (Table 1).

Many virulence factors have been observed in KPTCM strain, including $\texttt{rmpA2}$, $\texttt{iucABCD}$ and $\texttt{iutA}$ gene cluster, which is responsible for encoding aerobactin. Furthermore, colibactin ($\texttt{clbF}$ and $\texttt{clbP}$), yersiniabactin ($\texttt{ybtAEPQSTUX}$, $\texttt{irp1}$, $\texttt{irp2}$ and $\texttt{fyuA}$), type 3 fimbriae ($\texttt{mrkABCDFHIJ}$) and genes encoding iron uptake function were further identified.

### Multilocus sequence typing, wzi type, KL and OCL

Based on the MLST scheme, KPTCM strain was typed into ST15. *Kaptive* showed Wzi, an outer membrane protein lectin, was classified as Wzi93. Furthermore, KPTCM strain contained O locus 1 (O1), matching the 98.51% nucleotide identity. The K locus in KPTCM strain was found to be KL112, to which it matches with an overall nucleotide identity of 98.65%.

### Transferability of plasmid harboring $\texttt{bla}_{\text{OXA-232}}$

Mating assays were performed to investigate the transferability of $\texttt{bla}_{\text{OXA-232}}$; however, $\texttt{bla}_{\text{OXA-232}}$ failed to transfer to the recipient strain through conjugation. Concerning chemical transformation, MICs of *E. coli* DH5α: $\texttt{pOXA-232}$ to imipenem, meropenem and ertapenem rose to 1 mg/L, 0.25 mg/L and 2 mg/L, respectively, which was higher than that of *E. coli* DH5α strain (imipenem 0.06 mg/L, meropenem 0.03 mg/L and ertapenem 0.03 mg/L, respectively).

### Virulence assessment of KPTCM strain

To explore the virulence level of KPTCM strain, virulence assays of string test and *G. mellonella* infection model were performed. As a result, negative phenotype was observed for string test experiment. In addition, *G. mellonella* infection model indicated virulence of KPTCM strain was significantly lower than standard hypervirulent isolate NTUH-2044 ($P = 0.0005$).

| Genome          | Replicon | Size (bp) | GC content | Resistance genes                           | Accession numbers |
|-----------------|----------|-----------|------------|--------------------------------------------|-------------------|
| chromosome      | ND       | 5,186,161 | 57.36%     | $\texttt{bla}_{\text{CTX-M-15}}$, $\texttt{bla}_{\text{SHV-106}}$, $\texttt{aopA6}$, $\texttt{aopB01}$, $\texttt{fouA6}$ | CP097385          |
| pKPTCM-1        | IncFIB,  | 167,179   | 50.59%     | ND                                         | CP097386          |
|                 | IncHI1B  |           |            | $\texttt{catB}$, $\texttt{arr-2}$, $\texttt{rmtF}$ | CP097387          |
| pKPTCM-2        | IncFIB   | 128,536   | 50.06%     | $\texttt{dfrA14}$, $\texttt{qnrB1}$        | CP097388          |
| pKPTCM-3        | IncFII   | 116,556   | 51.44%     | $\texttt{strAB}$, $\texttt{bla}_{\text{SHV-12}}$, $\texttt{sul2}$, $\texttt{bla}_{\text{TEM-11}}$ | CP097389          |
| pKPTCM-4        | IncFII,  | 50,463    | 50.86%     | ND                                         | CP097390          |
|                 | IncX3    |           |            | $\texttt{mrkABCDFHIJ}$                      | CP097391          |
| pKPTCM-5        | ColRNAI  | 9,730     | 53.22%     | ND                                         | CP097392          |
| pKPTCM-6        | ColKP3   | 6,141     | 52.19%     | $\texttt{bla}_{\text{OXA-232}}$            | CP097393          |
| pKPTCM-7        | ND       | 5,640     | 49.96%     | ND                                         | CP097392          |
| pKPTCM-8        | Col440I  | 4,510     | 44.70%     | ND                                         | CP097393          |
| pKPTCM-9        | ColRNAI  | 3,770     | 41.74%     | ND                                         | CP097394          |

ND, Not detected.
but slightly higher than K. pneumoniae ATCC700603, used as negative control (Figure 2). However, no statistical significance was observed between KPTCM and K. pneumoniae ATCC700603 strains (P > 0.05).

Genomic characterization of the chromosome

The hybrid assembly of both Illumina and MinION reads showed KPTCM strain contains a 5,186,161-bp circular chromosome with GC content of 57.36% (Table 1). The \( \text{bla}_{\text{CTX-M-15}} \) gene was embedded in the chromosome via \( \text{ISEcp1} \)-based transposon Tn2012. Moreover, two amino acid mutations of \( \text{gyrA} \) (S83F) and \( \text{parC} \) (S80I) were identified, which confer resistance to fluoroquinolones.

Plasmids in KPTCM strain

Nine plasmids were solved in KPTCM clinical strain, namely pKPTCM-1 to pKPTCM-9, with sizes from 3,770-bp to 167,179-bp and GC contents ranging from 41.74% to 53.22% (Table 1). pKPTCM-1 is a \( \text{rmpA2} \)-related pLVPK-like virulence plasmid with two replications (IncFIB and IncHI1B). pKPTCM-2, pKPTCM-3 and pKPTCM-4 plasmids were found to be MDR plasmids harboring various resistance genes. pKPTCM-6 is one ColKP3-type plasmid with one carbapenem resistance gene, \( \text{bla}_{\text{OXA-232}} \). However, no resistance genes were identified in pKPTCM-5, pKPTCM-7, pKPTCM-8 and pKPTCM-9.

Genetic features of pLVPK-like virulence plasmid pKPTCM-1

KPTCM clinical isolate harbored a 167,179-bp pLVPK-like virulence plasmid, namely pKPTCM-1, which was 99.65% identical to 177,790-bp pCR-HvKp1-VIR plasmid (GenBank accession number: CP040534) at 91% coverage (Figure 3A). pKPTCM-1 had an average GC content of 50.59% and consisted of four different regions, including virulence gene region, tellurium resistance region, silver/copper resistance region and mercury resistance region. \( \text{rmpA2} \) and \( \text{iucABCD-iutA} \) gene cluster were located in the virulence gene region. In addition, frameshift mutation was observed in \( \text{rmpA2} \) gene (Figure 3B and Figure S1).

IS26-mediated plasmids fusion of pKPTCM-4

pKPTCM-4 is an IncFII-IncX3-typed plasmid with GC content of 50.86% (Table 1). Five resistance genes and five IS26 genetic elements were identified in pKPTCM-4 plasmid (Figure 4A). In addition to IS26, \( \text{strAB} \), \( \text{bla}_{\text{SHV-12}} \), \( \text{sul2} \) and \( \text{bla}_{\text{TEM-1B}} \) were flanked by various mobile genetic elements. Moreover, 8-bp target site duplications ([TSD] AGCTGCAC) were observed upstream of all five IS26 genetic elements. The other 8-bp TSD (GGCCTTTG) were identified downstream of five IS26 in pKPTCM-4 (Figure 4B).
FIGURE 3
Circular map of pKPTCM-1 and comparison with pCR-HvKP1-VIR plasmid. (A) Circular map of pKPTCM-1 plasmid. Arrows show the direction of transcription of open reading frames (ORFs). Virulence genes region (red), tellurium resistance region (black), silver/copper resistance region (blue) and mercury resistance region (purple) are shown in different colors. ORFs of replicon, partition protein, mobile genetic elements (MGEs), replication, recombination and repair were further labeled. Light blue filled boxes represent other ORFs. (B) Structure of pKPTCM-1 compared with plasmid pCR-HvKP1-VIR (GenBank accession number: CP040534). The sequence of rmpA2 with the frameshift mutation was shown. Shades indicate regions with 90% to 100% identity.
FIGURE 4
Circular map of pKPTCM-4 and IS26 distribution. (A) Circular map of pKPTCM-4 plasmid. Arrows show the direction of transcription of ORFs. Red arrows indicate the antimicrobial resistance genes. Pink arrows indicate the replicons. ORFs of resistance genes, MGEs, copper resistance, recombination and repair were further labeled. (B) IS26 elements are shown as green filled boxes. 8-bp target site duplications (TSD) AGCTGCAC were labeled on the left side, and the other 8-bp TSD (GGCCTTTG) were labeled on the right side of the IS26. Resistance genes were labeled as red with black thin arrows indicating ORF directions.
Genetic features of ColKP3-typed pKPTCM-6 plasmid

*bla*OXA-232* gene was located in a small 6,141-bp plasmid, designated pKPTCM-6 with ColKP3-typed replicon (Table 1). The plasmid was composed of *repA*, *mob* related genetic elements, Δ*ise*cp1, *bla*OXA-232, ΔlysR and ΔereA (Figure 5A). A truncated Tn2013 transposon structure was identified with 5-bp TSD (ATATA) on the right side (Figure 5B). Moreover, another ColKP3-typed plasmid p47733_OXA_181 (a *bla*OXA-181-carrying plasmid) showed 51% coverage and 99.9% identity to pKPTCM-6. The genetic environment between *bla*OXA-232 and *bla*OXA-181 was identical and both flanked by Δ*ise*cp1 and ΔlysR-ΔereA (Figure 5B).

Comparative genomics analysis of 109 ST15 *K. pneumoniae* strains and 45 OXA-232-producing *K. pneumoniae* strains

To analyse the characteristics of ST15 *K. pneumoniae* strains with close genetic relationship, genomes in public databases were searched and downloaded based on 200 SNPs threshold using BacWGStdb server. To further study the characteristics of 109 ST15 *K. pneumoniae* strains from different countries, comparative genomics analysis was performed via Ridom SeqSphere+ server. All information of 109 ST15 *K. pneumoniae* strains is shown in Table S1. Based on the cgMLST tree, data showed ST15 *K. pneumoniae* isolates were mainly isolated from China (23.85%, 26/109), followed by...
Hungary (13.76%, 15/109), Spain (11.01%, 12/109) and United Kingdom (9.17%, 10/109) (Figure 6). ST15 K. pneumoniae strains collected from China and Hungary were shown in cluster 2 and 3, respectively. However, the number of strains isolated from other countries, such as Turkey, Nepal, Lebanon and Switzerland, was relatively small (Table S1).

Other 19 OXA-232-producing K. pneumoniae strains were further searched based on published reports, the features of all 45 OXA-232-producing K. pneumoniae strains were analyzed using BacWGSTdb server. Interestingly, all K. pneumoniae strains carrying OXA-232 plasmid belonged to ST15 in China. However, STs in several countries, such as India (mainly ST11, ST23 and ST231), South Korea (ST14) and Italy (ST16), were quite different (Figure 7).

Of note, our data revealed that the first ST15 K. pneumoniae strain was isolated in 2015 in China. Strains were collected from several different sources, including blood, sputum, fluid, swab, urine, rectum and other sites (Figure 7). Moreover, SNP analysis was performed for the 26 OXA-232-producing ST15 strains from China. SNP strategy showed 28-222 SNPs differences among 26 ST15 K. pneumoniae isolates.

Discussion

The presence of carbapenemase-producing K. pneumoniae has become dominant in several countries, and it is being increasingly considered a quite important nosocomial pathogen (Yang et al., 2021c). More importantly, BSIs caused by CRKP present critical problems in terms of clinical therapy (Xiao et al., 2020). Hypervirulent K. pneumoniae strains could also cause serious infections among healthy individuals (Sewunet et al., 2021). These hypervirulent K. pneumoniae strains are usually resistant to antimicrobials agents and become high-risk clones in the hospital (Sewunet et al., 2021).

Mobile genetic elements, including ISs, integrons, and transposons, play a particularly important role in the resistance gene transfer among different species (Gorbunova et al., 2021). One interesting finding from this study is that blaCTX-M-15 was located in the chromosome, which was mediated by ISEcp1-based transposon Tn2012. ISEcp1 transposase gene has 14-bp inverted repeats (IRs) and is more inclined to insert into targets with the 5-bp AT-rich sites. ISEcp1 transposase gene is characterized by its capacity to transfer DNA fragments, such as resistance genes. Shu et al. reported PE109-1-CTX plasmid was found to harbor the resistance gene blaCTX-M-15 in China (Shu et al., 2019). Thus, it could be concluded that chromosomal blaCTX-M-15 in our strain may be derived from other plasmids mediated by ISEcp1-based transposon Tn2012. Moreover, the genetic environment of arr-2 gene cassette was found in a complex class 1 integron. Integrons are other mobile genetic elements, which could capture gene cassettes via attL and attC, further contributing to the global resistance crisis (Ghaly et al., 2021). More importantly, IS26 plays a key role in the spread of resistance genes in Gram-negative bacteria (Harmer et al., 2014; Hua et al., 2020). A crucial feature of IS26 is its capability to form co-integrate molecules that consists of distinct DNA segments (Hua et al., 2020). Based on the analysis of ISs and TSD, it was hypothesized that pKPTCM-4 with IncFII-IncXIII replicons was formed by five IS26-mediated co-integration events. Consequently, the joint role of diverse mobile genetic elements enables bacteria to undergo frequent genetic transposition and further leads to the MDR phenotype (Yang et al., 2021a).

Note that KPTCM was found to carry rpmA2 and virulence-associated gene cluster (iucABCD-iutA), the virulence potential was further evaluated. Interestingly, hypermucoviscous phenotype was negative. Moreover, rpmA and rpmA2 genes have been identified to be strongly associated with the hypermucoviscous phenotype. Nevertheless, rpmA2 gene in KPTCM strain has indel mutations causing frameshifts, further rendering the gene product dysfunctional (Shen et al., 2020b). In the current study, the low virulence maybe is caused by frameshifts of rpmA2. Thus, screening for rpmA and rpmA2 genes may need to be followed by sequencing to ensure their integrity, then it could be associated with hypervirulent phenotype. Interestingly, the patient developed a liver abscess caused by KPTCM strain and the outcome was death in a short time. Based on its associated clinical disease and outcome, the KPTCM strain should be considered as a relatively hypervirulent strain. However, a huge difference was observed when compared with many phenotypic experiments. Consequently, our results manifested the complexity and difficulty with the current knowledge for the definition of hypervirulence in K. pneumoniae.

The OCL and KL gene clusters, which are responsible for the biosynthesis of the outer core of lipooligosaccharide and capsule, are potentially useful epidemiological markers and offer a key role and potential target for vaccine development (Wyres et al., 2020). In the current study, KL112 had a quite high identity but with few reports. Furthermore, analysis of the population structure of the strains with various STs showed transmission among diverse countries. Based on previous reports, various STs have been reported in OXA-232-producing K. pneumoniae strains in many countries, including UK (mainly ST14, ST147, and ST231) (Findlay et al., 2017), Italy (ST16) (Avolio et al., 2017), Switzerland (ST231) (Mancini et al., 2018) and Tunisia (ST147) (Lahlou et al., 2017). However, our data revealed CRKP isolates harboring OXA-232-type carbapenemase plasmid usually belong to ST15 in China. The ST15 clones in China seem to be different from those in other areas of the world in view of the allelic differences and are mainly disseminated between Zhejiang and Shanghai (two geographically adjacent regions in China) but with slight evolution based on the SNP differences.
Phylogenetic analysis of 109 ST15 K. pneumoniae strains. cgMLST was conducted based on the 2358 alleles using Ridom SeqSphere+ server with cluster alert distance set at 15. Strains collected from China, Hungary, Spain and United Kingdom were shown as different colors. The blue pentagram indicated the position of KPTCM strain.
However, some limitations in this study remain, including the use of an insect larvae model might not be the most suitable for this type of virulence assessment. Thus, a murine infection model would be more suitable. Additionally, at present, we are not able to confirm whether resistance plasmids in KPTCM strain could be transferred to other recipient bacteria via conjugation. Finally, virulence-associated studies are required to find the direct or indirect connection between the carriage of the pLVPK-like virulence plasmid and hypervirulent phenotype in *K. pneumoniae*.

**Conclusion**

This study provides a comprehensive description for the complete genome characteristics of a *wzi93-KL112-O1* OXA-232-producing ST15 CRKP, which harbors a *rmpA2*-associated pLVPK-like virulence plasmid and chromosomal *blaCTX-M-15* mediated by ISEcp1-based transposon Tn2012. Considering that ST15 CRKP strains possess the strong plasmid carrying capacity (9 plasmids in KPTCM strain), they still have the potential to become hypervirulent via acquiring another type of virulence.
plasmid. Therefore, early detection of \( K. \) pneumoniae strains carrying chromosomal \( \text{bla}_\text{CTX-M-15} \), OXA-232 carbapenemase and \( \text{plVPK} \)-like virulence plasmid is recommended to avoid the extensive spread of this high-risk clone in healthcare settings.

**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: Genbank, PRJNA838426.

**Ethics statement**

This study was approved by the local Ethics Committees of the Hospital with a waiver of informed consent due to this study mainly focused on bacterial genome and the retrospective nature of the study.

**Author contributions**

CT and MX designed the experiments, analyzed the data, and wrote the initial manuscript. CT, MX, YB, and YZ performed the majority of the experiments. MX collected the bacteria. XF, LF, and SW supervised this study and reviewed and edited the paper. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

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

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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.2022.984479/full#supplementary-material

**Supplementary Figure 1**

Sequence alignment of \( \text{rmpA2} \) genes. \( \text{rmpA2}^{*} \) indicates gene with frame-shifts in \( \text{pKPTCM-1} \) plasmid. \( \text{rmpA2} \) is the wild type in \( \text{pK2044} \) of \( K. \) pneumoniae NTUH-2044.

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