**An Outbreak of Infections Caused by a Klebsiella pneumoniae ST11 Clone Coproducing Klebsiella pneumoniae Carbapenemase-2 and RmtB in a Chinese Teaching Hospital**

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**Abstract**

**Background:** *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* bacteria, which cause serious disease outbreaks worldwide, was rarely detected in Xiangya Hospital, prior to an outbreak that occurred from August 4, 2014, to March 17, 2015. The aim of this study was to analyze the epidemiology and molecular characteristics of the *K. pneumoniae* strains isolated during the outbreak.

**Methods:** Nonduplicate carbapenem-resistant *K. pneumoniae* isolates were screened for *bla*<sub>KPC-2</sub> and multiple other resistance determinants using polymerase chain reaction. Subsequent studies included pulsed-field gel electrophoresis (PFGE), multilocus sequence typing, analysis of plasmids, and genetic organization of *bla*<sub>KPC-2</sub> locus.

**Results:** Seventeen *bla*<sub>KPC-2</sub>-positive *K. pneumoniae* were identified. A wide range of resistant determinants was detected. Most isolates (88.2%) coharbored *bla*<sub>KPC-2</sub> and *rmtB* in addition to other resistance genes, including *bla*<sub>SHV-1</sub>, *bla*<sub>TEM-1</sub>, and *aac(3)-IIa*. The *bla*<sub>KPC-2</sub> and *rmtB* genes were located on the conjugative IncFIB-type plasmid. Genetic organization of *bla*<sub>KPC-2</sub> locus in most strains was consistent with that of the plasmid pKP048. Four types (A1, A2, A3, and B) were detected by PFGE, and Type A1, an ST11, was the predominant PFGE type. A novel *K. pneumoniae* sequence type (ST1883) related to ST11 was discovered.

**Conclusions:** These isolates in our study appeared to be clonal and ST11 *K. pneumoniae* was the predominant clone attributed to the outbreak. Co harboring of *bla*<sub>KPC-2</sub> and *rmtB*, which were located on a transferable plasmid, in clinical *K. pneumoniae* isolates may lead to the emergence of a new pattern of drug resistance.

**Key words:** Carbapenem; *Klebsiella pneumoniae*; *Klebsiella pneumoniae* Carbapenemase; Outbreak; *RmtB*

**Introduction**

During the last decade, carbapenem resistance has been increasingly reported in *Klebsiella pneumoniae* strains and is largely attributed to the production of carbapenem-hydrolyzing enzymes. Carbanapen-hydrolyzing β-lactamases belonging to Ambler classes A, B, and D have been reported worldwide among *Enterobacteriaceae*. The most clinically significant types are the *K. pneumoniae* carbapenemase (KPC)-type (Class A), the imipenem (IMP) and Verona integron-encoded metallo-β-lactamase types (Class B), and the OXA-48 type (oxacillin-hydrolyzing, Class D), mostly identified in *K. pneumoniae* as a source of nosocomial outbreaks. In China, production of KPC is the main cause of carbapenem resistance in *K. pneumoniae* and ST11 is the dominant clone of KPC-producing *K. pneumoniae* (KPC-KP).

In our previous studies, from 2011 to 2013, the prevalence of carbapenem-resistant *K. pneumoniae* (CR-KP) isolates was very low (about 3%) in our hospital. However, the number of CR-KP isolates increased dramatically (9.1%) in 2014...
as an outbreak of CR-KP isolates was detected (data not shown). Between 2010 and 2011, Jian et al. reported that strains producing the IMP-type carbapenemase were the main cause for the carbapenem resistance in K. pneumoniae in our hospital.[11] However, in our previous studies, CR-KP isolates collected during the outbreak were all shown to produce KPC and to be resistant to all antimicrobial agents tested except for colistin and cotrimoxazole. Thus, these isolates were found to be extensively drug-resistant strains with limited treatment options for infections due to these strains. Taken together, these data prompted us to investigate the epidemiology and molecular characteristics of these KPC-KP isolates.

**Methods**

**Bacterial strains**

Nonduplicate CR-KP isolates were recovered from August 4, 2014, to March 17, 2015, from a 3500-bed teaching hospital in China. The isolates were screened for the blaKPC gene and the positive isolates were re-identified by the VITEK 2 microbial identification system (BioMérieux, France). Salmonella enterica strain H9812 was used as the molecular size marker for pulsed-field gel electrophoresis (PFGE) and was obtained from the respiratory laboratory of infectious diseases in China. The outbreak was defined as the occurrence of congener’s infection more than three cases during a short time in medical institutions or departments.

**Antimicrobial susceptibility testing**

The minimal inhibitory concentration (MIC) of ten antibiotics, including aztreonam, IMP, ertapenem, ceftazidime, cefepime, piperacillin, ciprofloxacin, amikacin, piperacillin-tazobactam, and cotrimoxazole, were measured using the Agar dilution method according to the Clinical Laboratory Standards Institute (CLSI, M100-S24) and by concurrent testing of two quality control strains, Escherichia coli ATCC25922, and Pseudomonas aeruginosa ATCC27853. The MIC results were analyzed and interpreted according to CLSI. Colistin MICs were determined by Etest strips (BioMérieux). Following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/), isolates with a colistin MIC of ≤2 μg/ml were categorized as susceptible, while those with MICs of >2 μg/ml were considered to be resistant.

**Detection of genes encoding antimicrobial resistance determinants**

To amplify genes encoding antimicrobial resistance determinants (RDs) in a polymerase chain reaction (PCR), the genomic DNA template was obtained by incubating bacterial suspensions at 95°C for 10 min followed by centrifugation for 10 min at 12,000 ×g to remove cellular debris. Genes encoding carbapenem β-lactamases (blaKPC, blaSIM, blaGES, blaIMP, blaVIM, blaGIM, blaSIM1, blaSPM, blaNDM1, and blaOXA-48), extended-spectrum β-lactamas (ESBL) (blaCTX-M, blaTEM1, and blaSHV1), AmpC β-lactamases (blaMOX, blaFOX, blaDHA, and blaCTX), and aminoglycoside-modifying enzymes (aac(3)-IIa and ant(2′)-Ia), and 16S-RMTases (armA, npmA, rmtA, rmtB, rmtC, rmtD, and rmtE) were detected by PCR using previously described primers.[12-16] All amplified DNA fragments were sequenced and then analyzed using the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST).

**Molecular typing**

The GenePath System from Bio-Rad Laboratories (Hercules, CA, USA) was used for PFGE analysis, and the genomic DNA was prepared according to the manufacturer’s instructions. In brief, the genomic DNA was prepared in agarose blocks, digested with the restriction enzyme XbaI (Promega, USA) for 3 h, and embedded into a 1% PFGE agarose gel. The PFGE was performed for 18.5 h at 5.5 V/cm and 12°C, with a pulse angle of 120°, and a switch time from 6 to 36 s. Genomic DNA from S. enterica strain H9812 was used as the molecular size marker. The gel was stained with ethidium bromide for 20 min and photographed with the Gel Doc 2000 Imaging System from Bio-Rad. The band patterns were analyzed by the BioNumerics software platform (Applied Math, Sint-Maten-Latem, Belgium) using the dice similarity coefficient. Isolates were classed in the same PFGE group if they possessed ≥85% genetic similarity.[17]

Multilocus sequence typing (MLST) was performed with primers listed in the online database for K. pneumoniae (http://bigd.db.web.pasteur.fr/klebsiella/klebsiella.html). The resultant PCR products were purified and sequenced. Sequence types (STs) were assigned using online database tools (http://pubmlst.org/). To determine the clonal relatedness of the novel ST1883, the eBURST software eBURSTv3 (http://ebrurst.mlst.net/) was used to compare the ST1883 strain with the K. pneumoniae ST11 isolates and K. pneumoniae ST strains with five or more of the same housekeeping genes as that of the ST1883 strain type.

**Conjugation and plasmid analysis**

Conjugation assays were performed as previously described.[18] In brief, the 17 isolates were used as donors and an azide-resistant E. coli J53 strain was used as the recipient. Sodium azide (150 mg/L from Sigma Chemical Co., St. Louis, MO, USA) and meropenem (12.5 mg/L from Oxoid) resistance were used to select for E. coli transconjugant strains.

Plasmid DNA was extracted using an E.Z.N.A. BAC/PAC DNA Kit (OMEGA, USA) according to the manufacturer’s instructions. The plasmid DNA was then used in a PCR-based replicon typing method with previously described primers and conditions[19,20] to determine the plasmid incompatibility groups including F, FIA, FIB, FIC, H11, HI2, 11-1c, L/M, N, P, W, T, A/C, K, B/O, X, Y, and FII.

**Genetic organization of blaKPC gene locus**

PCR mapping was used to analyze the genetic organization of the blaKPC locus. Amplifications were performed using...
previously described primers and conditions. All amplification products were sequenced.

**Results**

**Clinical features of Klebsiella pneumoniae carbapenemase-producing Klebsiella pneumoniae isolates**

In this study, strains were defined as nonduplicated strains only if they were isolated from different patients or from different tissues within the same patient. In total, 17 nonduplicated KPC-KP strains were isolated from 15 patients. Ten of these patients were localized in the Intensive Care Unit (ICU) and the remaining patients were localized in other wards: Gastrointestinal surgery (GS) \( n = 2 \), epilepsy specialist (ES) \( n = 1 \), cardiothoracic surgery (CS) \( n = 1 \), and integrative medicine \( n = 1 \). The type of patient specimen from which the isolates were collected and the number of strains collected from each specimen were as follows: Wound secretions \( n = 4 \), lower respiratory tract \( n = 4 \), drainage of pleural fluid or biliary drainage \( n = 3 \), blood \( n = 2 \), cerebrospinal fluid (CF) \( n = 2 \), ascites \( n = 1 \), and urine \( n = 1 \) [Figure 1].

**Outbreak description**

Strain number 1 was the first KPC-KP isolate identified and was isolated from a drainage sample on August 4, 2014, from a male patient (patient 1) that was admitted to a 10-bed ICU 22 days after admission into the hospital. The KPC-KP isolate was assumed to have been transmitted within the ICU environment. On the same day, the second KPC-KP strain (strain number 4) was isolated from the CF of the same patient from which strain number 1 was isolated. Thereafter, patients infected with a KPC-KP strain from other wards were at some point during their hospitalization transferred to the ICU or were hospitalized during overlapping periods of time in the ICU with the index patient. Infection control measures were improved to include the reinforcement of diligent hand hygiene prior to and after patient handling, the use of disposable gloves, and the disinfection of inanimate surfaces related to the patients in question. Despite these precautionary measures, a KPC-KP outbreak occurred again on February 7, 2015. All clinical isolates were isolated 15–26 days following the admission of the patient and were, therefore, characterized as a hospital-acquired isolate.

**Antimicrobial susceptibilities**

Antimicrobial susceptibility testing showed that the isolates were resistant to all antimicrobial agents tested including the third and fourth generation cephalosporins, quinolones, aminoglycosides, and carbapenem. In addition, all the isolates were only sensitive to cotrimoxazole and colistin [Table 1].

**Prevalence of resistant determinants**

A wide range of resistant genes was detected and four or more genes were coharbored in all KPC-KP isolates. Among the 17 KPC-KP strains, 15 carried \textit{rmtB} and 17 produced the ESBLs TEM-1 and SHV-1. Two genes, \textit{aac(3)-IIa} and \textit{ant(2”)Ia}, encoding two types of aminoglycoside-modifying enzymes

![Figure 1: Clinical features, molecular characterization, and clonal relatedness of the 17 Klebsiella pneumoniae carbapenemase-producing Klebsiella pneumoniae isolates. The dendrogram was developed using the BioNumerics software platform. Key: Klebsiella pneumoniae 1-17; P1-P15: Patient number; IM: Integrative medicine; ES: Epilepsy specialist; GS: Gastrointestinal surgery; CS: Cardiothoracic surgery; WS: Wound secretion; CF: Cerebrospinal fluid; LRT: Lower respiratory tract; Ss: Specimens; RDs: Resistance determinants; PFGE: The pulsed-field gel electrophoresis Types A and B and Subtypes A1–A3; ST: Sequence types ST11 and ST1883, which is a novel sequence type.](image-url)
were detected in 11.8% and 88.2% of KPC-KP isolates, respectively. Figure 1 lists the antibiotic resistance genes detected in each isolate.

**Pulsed-field gel electrophoresis and multilocus sequence typing analysis**

Using PFGE, the 17 KPC-KP strains were divided into two types, Type A and Type B [Figure 1]. Type A strains predominated and accounted for 94.1% (16/17) of the strains. Type A included three Subtypes, A1, A2, and A3, while Type B was a nonsubtype. Subtype A1 accounted for 70.5% (12/17) of the strains and was found in the ICU, as well as other hospital wards, including GS, ES, and CS. Two strains (1 and 4) were isolated from the same patient but belonged to two different subtypes. Strain number 1 belonged to Subtype A3, while strain number 4 belonged to Subtype A1.

To characterize the STs of the 17 KPC-KP strains, MLST was performed. The PFGE Subtype A1, Subtype A2, and Type B strains all grouped with *K. pneumoniae* ST11 and account for 94.1% (16/17) of the KPC-KP strains. Notably, the Subtype A3 strain (strain number 1), on the other hand, formed a novel *K. pneumoniae* ST, ST1883. The relationship of the novel ST1883 strain to the predominant ST11 hospital isolates and to *K. pneumoniae* ST strains with five or more of the same housekeeping genes as that of the novel ST1883 strain type were further analyzed using eBURST. ST11 was shown to be the founding genotype and ST1883 had a single locus variant to ST11. Only the *rpoB* housekeeping gene was different between ST11 and ST1883. Thus, ST11 and ST1883 belong to the same clone complex.

**Conjugation and plasmid analysis**

The *bla*KPC-2 and *rmtB* genes were transferred by conjugation from the 17 isolates into a recipient *E. coli* J53 strain indicating that these resistance genes are located on a transferable plasmid. For all strains, only an IncFIB type plasmid was detected.

**Genetic organization of *bla*KPC-2 gene locus**

The genes flanking the *bla*KPC-2 gene in most KPC-KP isolates were consistent with the genetic organization of the *bla*KPC-2 gene locus on the plasmid pKP048. For strain 6 (Type B), a 245-bp insertion in a truncated *bla*TEM was detected upstream of the *bla*KPC-2 gene [Figure 2].

**DISCUSSION**

The first KPC-KP isolate was reported from a North Carolina Hospital in the United States in 2001. Since then, KPCs have spread internationally among Gram-negative bacteria, especially *K. pneumoniae*. Now, KPC-KP isolates are major hospital pathogens worldwide.[13,16,22-25] In China, a KPC-KP isolate was first identified in 2004 from a 75-year-old ICU patient in Zhejiang Province. Thereafter, KPC-KP isolates have been reported in numerous hospitals.[8,9,21] KPC-2 has become the most common carbapenemase in China, and *K. pneumoniae* is the predominant host species. Before 2014, KPC-KP isolates were rarely detected in our hospital. However, an outbreak of KPC-KP isolates, which also displayed extensive drug resistance, was detected from August 4, 2014, to March 17, 2015. Importantly, the KPC-KP isolates were resistant to aminoglycosides agents, which were previously effective against KPC-KP isolates,[26] leaving few choices for the treatment of KPC-KP infections.

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**Table 1: Antibiotic susceptibilities of the 17 KPC-KP isolates**

| Isolate | ATM | IMP | ETP | CST | CAZ | FEP | PRL | CIP | AMK | TZP | CTZ |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1       | ≥64 | ≥16 | ≥4  | 0.25| ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 2       | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 3       | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 4       | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 5       | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 6       | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 7       | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 8       | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 9       | ≥64 | ≥16 | ≥4  | 0.25| ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 10      | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 11      | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 12      | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 13      | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 14      | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 15      | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 16      | ≥64 | ≥16 | ≥4  | 0.5 | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |
| 17      | ≥64 | ≥16 | ≥4  | 1   | ≥64 | ≥32 | ≥256| ≥4  | ≥64 | ≥32 | ≤20 |

1–17: Number of isolate; ATM: Aztreonam; IPM: Imipenem; ETP: Ertapenem; CST: Colistin; CAZ: Ceftazidime; FEP: Cefepime; PRL: Pipercillin; CIP: Ciprofloxacin; AMK: Amikacin; TZP: Pipercillin/tazobactam; CTZ: Cotrimoxazole; KPC: *Klebsiella pneumoniae* carbapenemase; KP: *Klebsiella pneumoniae*; MIC: Minimal inhibitory concentration.
Therefore, in this study, the molecular characterization and clonal relatedness of the clinical KPC-KP isolates were investigated. Recently, coproduction of KPC and 16S-RMTase, including KPC-ArmA,[27] KPC-RmtB,[28] KPC-RmtD,[29] and KPC-RmtG,[29] was detected in Enterobacteriaceae isolates and most of the genes encoding these RDS were located on plasmids. In China, isolates coproducing KPC and ArmA/RmtB have been detected and the genes encoding these resistance factors are located on a single plasmid.[30] In addition, a KPC-2-RmtB-positive K. pneumoniae displayed more severe resistance phenotype showing sensitivity to only colistin and tigecycline than strains that produced only the KPC-2 carbapenemase. KPC-2-RmtB-positive K. pneumoniae strains are widespread and have replaced KPC-KP strains in some hospitals.[30,31] Similarly, coproduction of bla*KPC* and rmtB was detected in most of the 17 KPC-KP isolates in our study, and these isolates displayed resistance to all antimicrobial agents tested except for colistin and cotrimoxazole. Furthermore, bla*KPC* and rmtB were located on a transferable plasmid and were transferred by conjugation into a recipient E. coli J53 strain, indicating that these genes may be mobilized into bacterial strains that lack these genes. These data indicate that coproduction of bla*KPC* and rmtB in K. pneumoniae may lead to the emergence of a new pattern of drug resistance.

The genetic relatedness of the KPC-KP isolates revealed that most isolates were of the same ST type. In addition, most of the isolates harboring the bla*KPC* and rmtB genes belonged to ST11, which is the prevalent ST type in KPC-producing K. pneumoniae in China[10,31,32] and which is partially similar to the clonal complex 258 (ST258, ST512, and ST101) found in Europe and the USA.[33,34] Notably, the MLST analysis showed that the Subtype A3 strain is a novel ST, ST1883.

Transposon elements are considered to be responsible for the rapid spread of bla*KPC*. Tn440I is regarded as the origin of bla*KPC*-like gene acquisition and dissemination.[35] Until now, seven isoforms of Tn440I have been identified (a through f, with two isoforms called Tn440Ia and Tn440Id).[30,40] In China, a distinct genetic organization of the bla*KPC* locus was detected by Shen et al. The genetic locus contains Tn3, ISKpn8, and an ISKpn6-like element and is located on the plasmid pKP048.[21] Further studies have shown that the genetic organization of bla*KPC* locus is similar in other isolates (ISKpn8, bla*KPC*-like gene, and ISKpn6-like element) but is found on different plasmids.[27,41,42] In the present study, the genes flanking the bla*KPC*-2 gene in most of the isolates were consistent with that of plasmid pKP048, further suggesting that the bla*KPC*-2 gene in China has a common origin and that the spread of the bla*KPC*-2 gene between K. pneumoniae strains may be due to horizontal transmission. Nevertheless, recently, kinds of variants of the surrounding of bla*KPC*-2 gene were detected, and most of which were mainly due to the insertion of a truncated bla*TEM* gene sequence between ISKpn8 and bla*KPC* gene with different sizes.[22] A novel variant was also detected in our study, strain 6 (Type B) with a 245-bp insertion in a truncated bla*TEM* at the upstream of the bla*KPC*-2 gene, indicating that the new variants are emerging.

In conclusion, we have reported that the KPC-KP isolates in this study appear to be clonal and that the K. pneumoniae ST11 was the predominant clone attributed to the outbreak. Most of the KPC-KP isolates were further shown to coharbor bla*KPC* and rmtB, which were located on a transferable plasmid that may facilitate the spread of these resistance genes to other bacteria. These observations, together with the fact that few therapeutic options are available for these infections, compelled us to take urgent actions to control this outbreak and the persistent spread of KPC-RmtB co-producing K. pneumoniae in our hospital setting.

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