ST11, the dominant clone of KPC-producing Klebsiella pneumoniae in China

Yan Qi1,2, Zeqing Wei3, Shujuan Ji1, Xiaoxing Du1, Ping Shen3 and Yunsong Yu1*

1Department of Infectious Diseases, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, People’s Republic of China; 2Department of Clinical Laboratory, Hangzhou Hospital of Traditional Chinese Medicine, Hangzhou, Zhejiang, People’s Republic of China; 3State Key Laboratory for Diagnosis and Treatment of Infectious Disease, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, People’s Republic of China

*Corresponding author: Tel: +86 571 8723 6421; Fax: +86 571 8723 6423; E-mail: yvys119@163.com

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Objectives: Klebsiella pneumoniae carbapenemase (KPC)-producing Klebsiella pneumoniae has spread rapidly in China. In this study, we aimed to investigate the molecular epidemiology of KPC-producing K. pneumoniae isolates in China.

Methods: Ninety-five carbapenem-resistant K. pneumoniae isolates from 13 hospitals in nine cities covering five provinces in China were analysed. Antibiotic susceptibility was determined by the Etest. Multilocus sequence typing (MLST) and PFGE were used for epidemiological analysis. The genetic structure around blaKPC and the encoding genes of extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes were detected by PCR and sequencing. Plasmids were analysed by transformation, restriction and Southern blot.

Results: All isolates harboured the blaKPC-2 gene. Seven sequence types (STs) were obtained. The dominant clone was ST11 (61/95), which was identified in isolates from Zhejiang province (four hospitals in Hangzhou and one hospital in Ningbo), Jiangsu province (one hospital in Nanjing) and Anhui province (one hospital in Hefei). Isolates with ST11 showed >80% similarity in PFGE patterns. Plasmids from 14 selected transformants, their original isolates representing different STs and/or regions, had a diversity of HindIII restriction maps.

Conclusions: The dominant clone of KPC-producing K. pneumoniae in China is ST11, which is closely related to ST258, which has been reported worldwide.

Keywords: carbapenem resistance, MLST, PFGE, plasmid, transformation

Introduction

Klebsiella pneumoniae carbapenemase (KPC) has been emerging increasingly in recent years. This resistance mechanism is therefore a significant public health concern with regard to broad-spectrum activity of the enzyme and mobility of the gene. Until now, the enzyme has been found in multiple species of the Enterobacteriaceae and even in non-lactose-fermenting bacteria. There are reports that KPC-producing isolates have disseminated worldwide, including several countries across Asia, America and Europe.

Recently, it has been proved that ST258 is the most frequent clone contributing to the worldwide spread of KPC-producing K. pneumoniae, which has been identified in Poland, Norway, Sweden, Greece, Israel, Finland, Italy, Germany, Denmark, Hungary and especially the USA. The first report of KPC-producing K. pneumoniae in China was from Zhejiang province in 2007. However, little is known about the sequence type of KPC-producing K. pneumoniae that has spread in China. The aim of this study was to analyse the molecular epidemiology of KPC-producing K. pneumoniae circulating in China on the basis of sequence typing.

Materials and methods

Bacterial isolates

From March 2006 to December 2009, a total of 95 clinical isolates of K. pneumoniae with carbapenem resistance were obtained from 13 hospitals in nine cities covering five provinces in Mid-Eastern China, including Hangzhou (five hospitals, 44 isolates), Ningbo (one hospital, 26 isolates), Taizhou (one hospital, 6 isolates), Shaoxing (one hospital, 2 isolates), Shanghai (one hospital, 1 isolate), Wuhan (one hospital, 4 isolates), Hefei (one hospital, 1 isolate), Nanjing (one hospital, 9 isolates) and Zhengzhou (one hospital, 2 isolates). Hangzhou, Ningbo, Taizhou and Shaoxing are in Zhejiang province (Eastern China), Wuhan is in Hubei province (Middle China), Hefei is in Anhui province (Middle China), Nanjing is in Jiangsu province (East China), Taizhou is in Zhejiang province (East China), and Shaoxing is in Zhejiang province (East China).
Nanjing is in Jiangsu province (Eastern China) and Zhengzhou is in Henan province (Middle China) [see Figure S1, available as Supplementary data at JAC Online]. The bla\textsubscript{KPC} gene was detected by PCR and sequencing with primers KPC-A (TGTAAATGCGGTGCAGG), KPC-B (CCGAGCGCGGG CATAGTCAAT). PCR experiments were performed according to standard conditions with an annealing temperature of 58°C. All isolates were confirmed as KPC-2 carbapenemase producers by PCR and sequencing.

**Antimicrobial susceptibility testing**

The MICs of antimicrobial agents were determined by the Etest (bioMérieux, France) according to the manufacturer's instructions and were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI) update of June 2010 guidelines. The interpretive criterion used for tigecycline was based on FDA breakpoint values for Enterobacteriaceae, which define MIC ≤2 mg/L as susceptible. A colistin concentration of 4 mg/L was used as the breakpoint to designate resistant isolates. Escherichia coli ATCC 25922 and E. coli ATCC 35218 were used as quality controls.

**MLST with seven housekeeping genes**

MLST with seven housekeeping genes (\textit{gapA}, \textit{infB}, \textit{mdh}, \textit{pgi}, \textit{phoE}, \textit{rpoB} and \textit{tonB}) was performed on all isolates according to the protocol described on the \textit{K. pneumoniae} MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

**PFGE**

Isolates were typed by PFGE of XbaI-digested genomic DNA using the contour-clamped homogeneous electric field (CHEF) technique. Electrophoresis was run at 14°C and 6 V/cm and with alternating pulses at 120 V in a 2–40 s pulse time gradient for 22.5 h in 0.5 Tris-boric acid-EDTA (TBE) buffer with the CHEF apparatus (CHEF MAPPER XA; Bio-Rad, USA). Isolates were typed by PFGE of XbaI-digested genomic DNA using the \textit{K. pneumoniae} MLST protocol described on the \textit{K. pneumoniae} MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

**Plasmid manipulation and analysis**

Plasmids were extracted from original isolates using the Qiagen Midi Kit (Qiagen, Germany) and transformed into \textit{E. coli} DH5\textalpha. The transformants were selected on Mueller–Hinton agar containing meropenem (1 mg/L). The presence of \textit{bla\textsubscript{KPC}} in the transformed colonies was verified by PCR. The \textit{bla\textsubscript{KPC}}-harbouring plasmids DNA of the transformants were extracted with the Qiagen Midi Kit, digested with HindIII (Sangon, China) and sequenced with an ABI3730 sequencer (Applied Biosystems) and the sequences were compared with the reported sequences from GenBank (www.ncbi.nlm.nih.gov/blast/).

**Detection of extended-spectrum \(\beta\)-lactamase (ESBL) and plasmid-mediated AmpC \(\beta\)-lactamase genes**

All original isolates were screened by PCR with specific primers for \textit{bla\textsubscript{CTX-M}}, \textit{bla\textsubscript{TEM}}, \textit{bla\textsubscript{SHV}}, \textit{bla\textsubscript{VIM}} and six families of plasmid-mediated \textit{AmpC} \(\beta\)-lactamase genes (MOX, CIT, DHA, ACC, EBC, FOX). All of the positive PCR products were sequenced with an ABI3730 sequencer (Applied Biosystems) and the sequences were compared with the reported sequences from GenBank (www.ncbi.nlm.nih.gov/blast/).

**Genetic environment of \textit{bla\textsubscript{KPC}} gene**

A series of primers were designed based on sequences surrounding \textit{bla\textsubscript{KPC}} and PCR experiments were performed under previously reported conditions. The amplification products obtained were sequenced.

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Table 1. STs and antimicrobial susceptibility profiles of 95 KPC-2-producing isolates

<table>
<thead>
<tr>
<th>Origin city, province (hospital)</th>
<th>Isolate</th>
<th>ST</th>
<th>MLST profile</th>
<th>Genetic structure</th>
<th>MIC (mg/L)</th>
<th>Imipenem (IPM)</th>
<th>Meropenem (MEM)</th>
<th>Gentamicin (GAM)</th>
<th>Cefazolin (CAZ)</th>
<th>Ceftazidime (CFT)</th>
<th>Ciprofloxacin (CIP)</th>
<th>Amikacin (AMK)</th>
<th>Tazobactam/piperacillin (TZP)</th>
<th>Carba-penemase</th>
<th>CRST</th>
</tr>
</thead>
</table>
| Nanjing, Jiangsu (hospitals A, B, C, D) | 25 | 11 | 3-3-1-1-1-4 variant 1 and variant 2 | 16 to 32 | 32 | 4 to 32 | 64 to 256 | 32 to 256 | 32 | 128–256 | 128 to 256 | 256 to 256 | 0.5–1 | 0.5–2
| Hefei, Anhui (hospital E) | 1 | 11 | 3-3-1-1-1-4 variant 2 | 16 to 32 | 32 | 12 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Hangzhou, Zhejiang (hospital F) | 6 | 23 | 2-1-1-1-4-12 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Taizhou, Zhejiang | 6 | 23 | 2-1-1-1-4-12 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Shaoxing, Zhejiang | 2 | 23 | 2-1-1-1-4-12 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Hangzhou, Zhejiang (hospital G) | 4 | 43 | 1-3-1-1-1-1-1 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Zhengzhou, Henan | 2 | 43 | 1-3-1-1-1-1-1 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Wuhan, Hubei | 4 | 43 | 1-3-1-1-1-1-1 original structure | 32 | 32 | 2 | 2 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; FOX, cefoxitin; CIP, ciprofloxacin; AMK, amikacin; TZP, piperacillin/tazobactam; CRST, carbapenemase; CTX-M, CTX-M; MOX, MOX; CIT, CIT; DHA, DHA; ACC, ACC; EBC, EBC; FOX, FOX. |
Results

**Antimicrobial susceptibility testing**

The MICs of antimicrobial agents tested against all isolates are shown in Table 1. All isolates exhibited resistance to imipenem and/or meropenem, with MICs ranging from 3 to >32 mg/L. In addition, no susceptible isolates were detected when testing susceptibility to ceftazidime, cefoxitin, piperacillin/tazobactam and cefoperazone/sulbactam. These isolates demonstrated variable susceptibilities to ciprofloxacin and amikacin. Moreover, all isolates were susceptible to colistin (MIC ≤2 mg/L) and tigecycline (MIC ≤2 mg/L).

![Dendrogram](image-url)  
**Figure 1.** Dendrogram of XbaI-digested genomic DNA from KPC-producing K. pneumoniae identified in eight cities in China. Isolates representing the diversity of geographic location appear on the right of the figure. The Dice coefficient was used to calculate similarities, and the unweighted pair-group method with arithmetic mean (UPGMA) method was used for cluster analysis with BioNumerics software version 5.10 (Applied Maths, St-Martens-Latem, Belgium).

![Southern blot](image-url)  
**Figure 2.** (a) HindIII restriction digests of KPC-producing plasmid DNAs from E. coli transconjugants. M, λHindIII DNA ladder and 2000 kb DNA ladder; ST11, lanes 1, 2, 5 and 8 (Hangzhou, hospital A, B, C and D), lanes 3 and 4 (Nanjing), lane 7 (Ningbo), lane 9 (Hefei); ST15, lane 10 (Hangzhou, hospital A); ST23, lane 6, (Taizhou); ST349, lane 12 (Hangzhou, hospital E); ST351, lane 14 (Shanghai); ST438, lane 11 (Zhengzhou); ST439, lane 13 (Wuhan). (b) Southern blot hybridized with a bla_KPC-specific probe.
**PFGE and MLST analysis**

MLST showed seven sequence types (STs) based on the analysis of the seven housekeeping genes among the 95 clinical isolates of KPC-producing *K. pneumoniae*. According to the *K. pneumoniae* MLST database, the prevalent clone was ST11 (61/95, 64.2%), which was detected in isolates from Hangzhou (four hospitals, 25 isolates), Ningbo (one hospital, 26 isolates), Nanjing (one hospital, 9 isolates) and Hefei (one hospital, 1 isolate). ST15 was detected in 18 isolates only from one hospital in Hangzhou, implying that this strain is endemic in this institution. Furthermore, five types were identified: ST23 (eight isolates, Taizhou and Shaoxing), ST349 (one isolate, Hangzhou), ST351 (one isolate, Shanghai), ST438 (two isolates, Zhengzhou) and ST439 (four isolates, Wuhan) (Table 1).

To understand the significance of PFGE pattern similarities and differences, 14 KPC-2-producing *K. pneumoniae* isolates representing different STs and regions were selected for PFGE and plasmid analysis. Eight isolates were of the observed dominant clone ST11 and were collected from Zhejiang province (Hangzhou, Ningbo), Jiangsu province (Nanjing) and Anhui province (Hefei) to represent the diversity of geographic location. The remaining six isolates represented ST15, ST23, ST349, ST351, ST438 and ST439. The MLST data were in concordance with the results generated by PFGE and the isolates with ST11 shared >80% similarity in PFGE patterns (Figure 1).

**Plasmid manipulation and analysis**

All of the *bla*<sub>KPC</sub>-harbouring plasmids of the 14 isolates we selected were transferred successfully, with sizes of ~40–180 kb evaluated experimentally. Restriction analysis and hybridization experiments using plasmid DNA from transformants of 14 selected isolates representing different STs and regions showed a diversity of band patterns (Figure 2).

**PCR amplification and DNA sequencing of ESBL and plasmid-mediated AmpC β-lactamase genes**

Sequencing confirmed the *bla<sub>TEM</sub>* gene as *bla<sub>TEM-1</sub>* in 43 isolates and the *bla<sub>WEB</sub>* gene was not detected in any of the K. *pneumoniae* isolates. Among the 61 ST11 isolates, 38 contained ESBL genes (*bla<sub>CTX-M-3</sub>*, *bla<sub>CTX-M-14</sub>*, *bla<sub>SHV-12</sub>*), two contained an AmpC gene (*bla<sub>DHA-1</sub>*) and three isolates contained both ESBL and AmpC genes. Simultaneously, of the 32 isolates with other STs (ST15, ST23, ST349, ST351 and ST439), 9 contained ESBL genes (*bla<sub>CTX-M-3</sub>*, *bla<sub>CTX-M-14</sub>*, *bla<sub>SHV-12</sub>*), 13 contained an AmpC gene (*bla<sub>DHA-1</sub>*) and 4 contained both ESBL and AmpC genes. In the other two isolates with ST438, no ESBL and AmpC genes were detected. The distribution of the ESBL and plasmid-mediated AmpC genes in the 95 KPC-2-producing *K. pneumoniae* isolates with seven STs is shown in Table 2.

**Genetic environment of *bla*KPC gene**

As reported previously, the environment surrounding *bla*KPC-2 in plasmids from China is composed of a partial Tn4401 structure and a Tn3-based element with the gene order Tn3-transposase, Tn3-resolvase, ISKpn8, the *bla*KPC-2 gene and the ISKpn6-like element. In addition, there are two other variants. In our study, we observed that all the three structures as reported appeared in the 95 isolates, including 33 isolates with the original structure, 12 isolates with the structure of variant 1 and 50 isolates with the structure of variant 2 (Table 1).

**Discussion**

The emergence and dissemination of KPC-producing Enterobacteriaceae has become a serious problem worldwide. In China in 2007, plasmid-mediated KPC-2 in a *K. pneumoniae* isolate was first reported in Zhejiang province. Since then, KPC-2-producing Enterobacteriaceae isolates have spread widely and rapidly in this region. Recently, ST258 KPC-producing *K. pneumoniae* was reported worldwide as a dominant molecular epidemiology clone, especially in the USA. In this study, ST11 was demonstrated as a predominant clone of KPC-producing *K. pneumoniae* in China, which was detected in isolates from seven hospitals in Zhejiang, Jiangsu and Anhui province, while ST258 was not detected. However, ST11 is a single-locus variant (tonB) of ST258, indicating a close relationship between them. It is reported that ST11 and ST258 belong to the clone

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ST11 (61)</th>
<th>ST15 (18)</th>
<th>ST23 (8)</th>
<th>ST349 (1)</th>
<th>ST351 (1)</th>
<th>ST438 (4)</th>
<th>ST439 (2)</th>
<th>Total</th>
</tr>
</thead>
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<tr>
<td>CTX-M-3</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>CTX-M-3 + SHV-12</td>
<td>4</td>
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<td>1</td>
<td>1</td>
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<td></td>
<td></td>
<td>4</td>
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<tr>
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<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>CTX-M-3 + SHV-12 + DHA-1</td>
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<td>1</td>
<td>1</td>
<td>3</td>
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<td>69</td>
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</tbody>
</table>

Table 2. Distribution of ESBL and plasmid-mediated AmpC genes in 95 KPC-2-producing *K. pneumoniae* isolates

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complex CC258, which encompass ST11, ST258 and another five STs (ST270, ST340, ST379, ST407 and ST418). It seems that CC258 spread rapidly across the world in a similar fashion to CC17 of vancomycin-resistant Enterococcus faecium (VRE) and CC22 of carbapenem-resistant Acinetobacter baumannii (CRAB). A KPC-producing K. pneumoniae isolate in Korea has also been identified as ST11.22 Seemingly, ST11 may be another dominant clone of KPC-producing K. pneumoniae. From this, we speculated that some STs (such as ST11 and ST258) would be good colonizers to capture plasmids. These isolates are easily transmitted between patients.

In addition, ST15 is a single-locus variant (infB) of ST14 and probably represents regional dissemination in the Midwestern USA.13 ST15 in China was detected in just one hospital in Hangzhou, and it also spread regionally. ST15 and ST14 probably belong to another potential clone complex that might spread. Both ST11 and ST15 were submitted to the MLST database in 2005 and were described in Hungarian K. pneumoniae isolates producing ESBLs.23 In this report, the ESBL and/or the plasmid-mediated AmpC genes were detected in six STs besides ST438. Moreover, CTX-M type ESBLs and DHA-1 AmpC enzyme were the most common genotypes, which is consistent with the prevalence in China.24 However, it is hard to distinguish differences in the presence of resistant genes between the STs.

In our present study, four novel STs were detected: ST349 (Hangzhou), ST351 (Shanghai), ST438 (Zhengzhou) and ST439 (Wuhan). The KPC-2 encoded plasmid differs in different regions. We speculated that the sporadic spread of blaKPC among these KPC-producing K. pneumoniae is due to transmission of the plasmid or mobilization of genetic elements in plasmids, such as transposons and insertion sequences.

Within China, KPC-producing K. pneumoniae has been reported previously only in Zhejiang province, but now it is widespread in China. The spread of KPC-producing K. pneumoniae is worrying. KPC-producing bacteria may not be detected in routine antibiotic susceptibility testing. We should therefore pay careful attention to this problem from the public health point of view. It should be monitored closely and strict infection control measures should be adopted to control nosocomial infection.

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Transparency declarations
None to declare.

Supplementary data
Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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