Outbreak of pulmonary infection caused by *Klebsiella pneumoniae* isolates harbouring *bla*<sub>IMP-4</sub> and *bla*DHA-1 in a neonatal intensive care unit in China

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Outbreaks caused by *Klebsiella pneumoniae* producing carbapenemases and other β-lactamases have been reported. Four neonates admitted to a neonatal intensive care unit (NICU) in a Chinese hospital developed respiratory infection while receiving intensive care. In all four cases, multidrug-resistant *K. pneumoniae* was isolated from multiple respiratory specimens, leading to additional characterization of these organisms and investigation of the local environment in the NICU. Multiple β-lactamase genes, including *bla*<sub>TEM-1</sub>, *bla*<sub>IMP-4</sub>, *bla*DHA-1 and *bla*<sub>CTX-M-14</sub>, as well as the quinolone resistance gene *qnrB4*, were harboured by transferable plasmids from all four clinical isolates. Furthermore, PFGE confirmed that three of the four clinical isolates from the patients and three *K. pneumoniae* isolates collected from the hands of healthcare workers and an incubator in the NICU belonged to the same PFGE cluster, indicating that an outbreak due to multidrug-resistant *K. pneumoniae* carrying *bla*<sub>IMP-4</sub> and *bla*DHA-1 occurred in this NICU. As far as is known, this is the first report of the co-existence of *bla*<sub>IMP-4</sub> and *bla*DHA-1 in the same *K. pneumoniae* isolate. These data suggest that additional precautions are needed to prevent outbreaks of infection caused by multidrug-resistant *K. pneumoniae* resulting from environmental exposure in NICUs.

INTRODUCTION

*Klebsiella pneumoniae* frequently exhibits resistance to extended-spectrum cephalosporins due to the production of extended-spectrum β-lactamases (ESBLs) (Falagas & Karageorgopoulos, 2009). Carbapenems or fluoroquinolones are often used for the treatment of clinical infections caused by this organism. However, resistance of *K. pneumoniae* to carbapenems has been increasing (Espedido et al., 2005; Gupta et al., 2011), and this is often due to the production of carbapenemases, particularly *K. pneumoniae* carbapenemases (KPCs) and class B metallo-β-lactamases (MBLs) (Gupta et al., 2011; Queenan & Bush, 2007; Robledo et al., 2011; Zioga et al., 2010). KPCs, especially KPC-2, have

**Abbreviations:** CLSI, Clinical and Laboratory Standards Institute; ESBL, extended-spectrum β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; MBL, class B metallo-β-lactamase; NICU, neonatal intensive care unit.
been found in members of the Enterobacteriaceae worldwide (Gupta et al., 2011; Nordmann et al., 2009). In addition to the family Enterobacteriaceae, KPCs have emerged in non-fermentative bacteria (Cuzon et al., 2011; Robledo et al., 2011; Villelas et al., 2007). In China, KPC-2 is a predominant carbapenemase and has been recognized in many members of the Enterobacteriaceae, such as K. pneumoniae, Serratia marcescens, Escherichia coli and Enterobacter cloacae (Cai et al., 2008; Wei et al., 2007; Wu et al., 2010). MBLs, including IMP and VIM, are commonly harboured by non-fermentative bacteria and have recently been identified in members of the Enterobacteriaceae worldwide (Maltezou, 2009; Queenan & Bush, 2007; Robledo et al., 2011). MBLs often confer high-level resistance to all β-lactams except aztreonam and are not inhibited by clavulanic acid, tazobactam or sulbactam. Although several IMP- and VIM-type MBLs have been described in China, most were found in non-fermentative Gram-negative bacilli (Walsh et al., 2005). Recent data indicate that IMP-4 confers reduced susceptibility to carbapenems for K. pneumoniae isolated from patients in China (Chen et al., 2009; Mendes et al., 2008; Wei et al., 2011). Two clinical strains of K. pneumoniae carrying plasmid-borne blaIMP-4, blaSIV-12 and armA were found at a paediatric centre in Shanghai, China (Liu et al., 2009). The blaIMP-4 gene was also found to co-exist with blaKPC-2 in K. pneumoniae isolates (Chen et al., 2009; Mendes et al., 2008; Wei et al., 2011). The K. pneumoniae isolates producing IMP-4 found in China were clonally unrelated and occurred sporadically. An outbreak caused by multi-drug-resistant K. pneumoniae harbouring blaIMP-4 has not been found previously in China. In this report, we describe an outbreak of multidrug-resistant K. pneumoniae infection in a neonatal intensive care unit (NICU) in China, as well as isolation of related organisms from environmental sources.

METHODS

Isolation and identification of bacterial strains. From October to December 2010, four neonates with asphyxia were admitted to the NICU of the 400-bed Shaoxing Municipal Women and Children Hospital in Shaoxing, eastern China. Of note, hospital admission for all four patients (designated patients 1–4) overlapped. Before admission to the NICU, pulmonary infections diagnosed by physical examination and new findings consistent with pneumonia on chest radiography were not found among the four patients. Bacterial isolates from sputum specimens growing over more than three-quarters of the plate by quantitative culture were considered to be responsible for the pulmonary infection. Bacterial isolates were identified by a Vitek-32 microbiology analyser (bioMérieux) according to the manufacturer’s instructions and additional biochemical tests. The initial K. pneumoniae isolates were screened for further investigation. After K. pneumoniae was isolated from patient 4, an outbreak control team was organized and infection control measures were implemented. Environmental samples were obtained for culture by rubbing sterile polyester-fibre-tipped swabs moistened with sterile saline repeatedly over designated sites in the immediate vicinity of the patients, including equipment used for their care and the fingers of medical staff caring for the patients.

Antimicrobial susceptibility testing. Antimicrobial susceptibilities were determined initially using GNS cards of the Vitek system (bioMérieux). Multidrug resistance profiles were then further evaluated by a disc diffusion test using commercial discs containing ceftazolin (30 μg), cefotaxime (30 μg), cefazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), cefoxitin (30 μg), imipenem (10 μg), meropenem (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), trimethoprim/sulphamethoxazole (1.25/23.75 μg), amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg) and levofloxacin (5 μg). An agar dilution method was used to determine MIC values according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). E. coli ATCC 25922 was used as a quality control strain for antimicrobial susceptibility testing.

Detection of β-lactamases. A modified Hodge test was performed to detect carbapenemases, as described previously (CLSI, 2011). A double-disc synergy test was designed to detect MBLs, as described by Peleg et al. (2005). All the isolates were studied for ESBL production by the CLSI-recommended confirmatory double-disc combination test (CLSI, 2011).

Detection of antimicrobial resistance determinants. Total DNA was extracted by boiling. Potential antimicrobial resistance determinants, which included carbapenemase genes, ESBL genes, plasmid-borne ampC genes and plasmid-borne quinolone resistance determinants, were investigated by PCR and nucleotide sequencing, using previously published primers (Bradford et al., 2004; Robicsek et al., 2006; Yu et al., 2007). The DNA sequences of the ompK35 and ompK36 genes were determined by PCR and DNA sequencing with primers ompK35-F (5’-ATGGATGAAGCGCAATATTCTGGCAGTGG-3’), ompK35-R (5’-TCGCCCTTGTCCGATCCGTC-3’), ompK36-F (5’-ATGAAGTAAAAGACTGTCCTC-3’) and ompK36-R (5’-GTCGTCGTTAGAGTACCGGC-3’). All amplicon sequences were compared with the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Transfer of carbapenem resistance determinants. In order to determine whether carbapenem resistance was transferable in K. pneumoniae isolates, a conjugation experiment was carried out in Luria–Bertani broth with E. coli J53 as the recipient, as described previously (Wang et al., 2003). Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 μg ml⁻¹) for counterselection and imipenem (0.5 μg ml⁻¹) for plasmid-mediated carbapenem resistance selection.

Determination of the flanking regions of the blaIMP-4 gene. Plasmid DNA of the transconjugants was extracted using a Plasmid Extraction Kit (Qiagen) according to the manufacturer’s instructions. Purified blaIMP-4-bearing conjugative plasmids extracted from the transconjugants were sequenced directly using a series of outward-directed primers specific for the locations next to the blaIMP-4 gene.

PFGE. Genomic DNA was prepared from all tested K. pneumoniae isolates and cleaved with 40 U XbaI. Electrophoresis was performed on 1% agarose gels in 0.5 M Tris/borate/EDTA buffer on a CHEF- Mapper XR PFGE system (Bio-Rad) for 24 h at 14 °C, with run conditions of 6 V cm⁻¹, a pulse angle of 120° and pulse times of 5–20 s. A λ DNA ladder (Amersham Biosciences) was used as a molecular mass marker and DNA bands were stained with ethidium bromide (0.5 μg ml⁻¹) prior to identification by photography under UV light. Band profiles were interpreted by the criteria of Tenover et al. (1995). Patterns with a difference of no more than three DNA bands were considered to belong to the same PFGE type.
RESULTS AND DISCUSSION

A suspected lung infection was diagnosed in patient 1 (aged 16 days), patient 2 (aged 17 days), patient 3 (aged 22 days) and patient 4 (aged 14 days). K. pneumoniae isolates designated KpSX1–KpSX4 were first recovered from sputum specimens of patients 1–4 following hospitalization for 22, 22, 32 and 16 days, respectively. Subsequently, K. pneumoniae isolates with identical antimicrobial resistance patterns were isolated from the sputum of each patient more than three times. The gestational ages of the four patients ranged from 26 to 29 weeks and their weights were only 1050–1370 g (Table 1); both of these are risk factors for the acquisition of hospital-associated infections. Three K. pneumoniae isolates designated KpE1, KpE2 and KpE3 were recovered from environmental samples, comprising two from the fingers of two different nurses and one from an incubator in the NICU.

Apart from multidrug-resistant K. pneumoniae, other multidrug-resistant pathogens, such as meticillin-resistant Staphylococcus aureus, ESBL-producing Enterobacteriaceae, vancomycin-resistant enterococci and multidrug-resistant Acinetobacter baumannii, were not isolated from the clinical specimens including stools of the four investigated patients. Each of the four clinical isolates and the three environmental isolates was susceptible to amikacin but resistant to cefazolin, cefazidime, cefotaxime, cefepime, aztreonam, cefoxitin, gentamicin, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole (Table 1) as determined by a disc diffusion test according to the criteria of the CLSI (2011). Apart from KpSX1, which exhibited no zones of inhibition, all tested isolates exhibited similar zones of inhibition and MIC values for IMP and MEM (Table 1). According to the interpretive standards for IMP and MEM for Enterobacteriaceae recommended by the CLSI (2011), all isolates were resistant to IMP and MEM. A modified Hodge test was positive for all tested isolates, indicating that these isolates produced carbapenemases. However, all tested isolates were negative for MBLs determined by a double-disc synergy test and negative for ESBLs determined by the CLSI-recommended double-disc test (CLSI, 2011). Prior to the recovery of multidrug-resistant K. pneumoniae isolates, all four patients were treated with intravenous mezlocillin (75 mg kg\(^{-1}\) every 12 h) plus cefmetazole (50 mg kg\(^{-1}\) every 12 h) for preventing infections, and patient 1 was treated additionally with intravenous panipenem (20 mg kg\(^{-1}\) every 12 h) after the suspected lung infection was found. Treatment with intravenous cephalosporins or carbapenems may facilitate increased colonization by resistant K. pneumoniae, which can subsequently cause infection. After multidrug-resistant K. pneumoniae isolates were identified, patient 1 was treated with intravenous amikacin (7.5 mg kg\(^{-1}\) every 24 h) and levofloxacin (10 mg kg\(^{-1}\) every 24 h) for 10 days according to the results of antimicrobial susceptibility testing. Thereafter, multidrug-resistant K. pneumoniae was not isolated and the symptoms of lung infection disappeared in patient 1, who left the hospital on day 46. Although the isolates KpSX2, KpSX3 and KpSX4 exhibited low-level resistance to imipenem, patients 2, 3 and 4 were treated with intravenous imipenem (20 mg kg\(^{-1}\) every 12 h) for 10–14 days and left the hospital on days 44, 47 and 40, respectively, after multidrug-resistant K. pneumoniae was no longer isolated and the symptoms of lung infection had disappeared.

All tested isolates harboured int1, bla\(_{SHV}\), bla\(_{TEM}\), bla\(_{IMP}\), bla\(_{DHA\_0}\), bla\(_{CTX\_M-1}\) and qnrB4 genes detected by PCR. Additional sequencing of the amplified PCR products revealed the presence of bla\(_{SHV\_11}\), bla\(_{TEM\_1}\), bla\(_{IMP\_4}\), bla\(_{DHA\_1}\), bla\(_{CTX\_M-14}\) and qnrB4 among these isolates. Therefore, coexistence of multiple β-lactamase genes within each individual isolate could explain the resistance of these isolates to all β-lactams tested. To the best of our knowledge, this is the first report of the co-existence of bla\(_{IMP\_4}\) and bla\(_{DHA\_1}\) in the same K. pneumoniae isolate.

Detection of multiple β-lactamases produced by members of the Enterobacteriaceae in the clinical laboratory is challenging. The lack of ESBLs in the present study could be attributable to the masking effect of co-production of ESBLs, AmpCs and carbapenemases. For example, co-production of KPCs and MBLs masked the results of EDTA- or boronic acid-based testing in one study (Zioga et al., 2010). Some carbapenemase-producing members of the Enterobacteriaceae also exhibit low-level resistance or even susceptibility to carbapenems (Espediel et al., 2005; Liu et al., 2009). The decreased susceptibility of our isolates to ciprofloxacin could be explained by the existence of qnrB4.

β-Lactam resistance could be transferred by conjugation from all K. pneumoniae isolates to their recipients. All transconjugants harbouring int1, bla\(_{TEM\_1}\), bla\(_{IMP\_4}\), bla\(_{DHA\_1}\), bla\(_{CTX\_M-14}\) and qnrB4 but not bla\(_{SHV\_11}\). The E. coli transconjugants also exhibited relatively low MICs for IMP and MEM that were insufficient to explain the carbapenem resistance exhibited by the parental isolates. Therefore, we sought to identify additional mechanisms for carbapenem resistance in these isolates.

IMP-4 MBL production combined with loss of outer-membrane proteins confers high-level resistance to carbapenems in Klebsiella oxytoca (Chen et al., 2009). Therefore, we sought to identify resistance determinants within the ompK35 and ompK36 genes for our K. pneumoniae isolates. The nucleotide sequences of the ompK35 genes of all seven isolates were identical to that of carbapenem-susceptible K. pneumoniae ATCC 13883. A C→T mutation was observed at nt 160 in the ompK36 gene for isolate KpSX1, resulting in initiation of a stop codon at position 54 (CAG→TAG) for this strain. An early termination of translation caused by the C→T mutation at nt 160 in the ompK36 gene might lead to the loss of OmpK36. These data indicated that high-level resistance of KpSX1 to IMP and MEM may be due to the combination of IMP-4 and deficiency of the porin.

We found that bla\(_{IMP\_4}\) was located within a class I integron whose order was int1–bla\(_{IMP\_4}–orfII–orfIII–qacE\_D1–sulI. 
Table 1. Phenotypic and genotypic characteristics of the *K. pneumoniae* clinical and environmental isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Patient/sample</th>
<th>BW (g)*</th>
<th>GA (weeks)†</th>
<th>Disc diffusion zone (mm)</th>
<th>MIC (mg l⁻¹)</th>
<th>Antimicrobial resistance‡</th>
<th>PFGE</th>
<th>Resistance gene</th>
<th>intl§</th>
<th>Antimicrobials for treatment‖</th>
</tr>
</thead>
<tbody>
<tr>
<td>KpSX1</td>
<td>Patient 1 1060</td>
<td>26</td>
<td></td>
<td></td>
<td>64</td>
<td>128</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>MEZ, CMZ, CAZ, AMC, PAP, AMK, LEV</td>
</tr>
<tr>
<td>KpSX2</td>
<td>Patient 2 1360</td>
<td>29</td>
<td></td>
<td></td>
<td>4</td>
<td>16</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>MEZ, CMZ, CAZ, AMC, PAP, IMP</td>
</tr>
<tr>
<td>KpSX3</td>
<td>Patient 3 1370</td>
<td>27</td>
<td></td>
<td></td>
<td>4</td>
<td>16</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>MEZ, CMZ, CAZ, IMP</td>
</tr>
<tr>
<td>KpSX4</td>
<td>Patient 4 1050</td>
<td>28</td>
<td></td>
<td></td>
<td>4</td>
<td>16</td>
<td>B</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>MEZ, CMZ, IMP</td>
</tr>
<tr>
<td>KpE1</td>
<td>Fingers 1 19</td>
<td>12</td>
<td></td>
<td></td>
<td>4</td>
<td>16</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>+</td>
</tr>
<tr>
<td>KpE2</td>
<td>Fingers 2 19</td>
<td>12</td>
<td></td>
<td></td>
<td>4</td>
<td>16</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>+</td>
</tr>
<tr>
<td>KpE3</td>
<td>Incubator 16</td>
<td>10</td>
<td></td>
<td></td>
<td>8</td>
<td>32</td>
<td>A</td>
<td>SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14</td>
<td>B4</td>
<td>+</td>
</tr>
</tbody>
</table>

*BW, Birth weight of patient.
†GA, Gestational age of patient.
‡ATM, Aztreonam; C, chloramphenicol; CAZ, ceftazidime; CEC, cefaclor; CTX, cefotaxime; CZ, cefazolin; FEP, cepfime; FOX, cefoxitin; GEN, gentamicin; IMP, imipenem; MEM, meropenem; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline.
§+, Positive.
‖AMC, Amoxicillin plus clavulanic acid; AMK, amikacin; CMZ, cefmetazole; LEV, levofloxacin; MEZ, mezlocillin; PAP, panipenem.
In conclusion, our data suggest that an outbreak due to multidrug-resistant \textit{K. pneumoniae} occurred in this NICU.

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