Outbreak of pulmonary infection caused by *Klebsiella pneumoniae* isolates harbouring *bla*<sub>IMP-4</sub> and *bla*<sub>DHA-1</sub> 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*<sub>DHA-1</sub> 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*<sub>DHA-1</sub> 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*<sub>DHA-1</sub> 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.
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; Villegas 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 *bla*<sub>IMP-4</sub> and *armA* were found at a paediatric centre in Shanghai, China (Liu et al., 2009). The *bla*<sub>IMP-4</sub> gene was also found to co-exist with *bla*KPC-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 multidrug-resistant *K. pneumoniae* harbouring *bla*<sub>IMP-4</sub> 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 DNA bands were considered to belong to the same PFGE type.

**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 cefazolin (30 μg), cefotaxime (30 μg), cefazidime (30 μg), cepofeime (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'-ATGATGAAGCGCAATATTCTGGCAGTGG-3') and *ompK35*-R (5'-TCGCGAATTTGCGCATTGCCTCA-3'), *ompK36*-F (5'-ATGAAAGTTAAAGTACTGTCCCTC-3') and *ompK36*-R (5'-CTGTCGGTAGAGATCCGCCG-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<sup>−1</sup>) for counterselection and imipenem (0.5 μg ml<sup>−1</sup>) for plasmid-mediated carbapenem resistance selection.

**Determination of the flanking regions of the *bla*<sub>IMP-4</sub> gene.** Plasmid DNA of the transconjugants was extracted using a Plasmid Miniprep Kit (Qiagen) according to the manufacturer’s instructions. Purified *bla*<sub>IMP-4</sub>-bearing conjugative plasmids extracted from the transconjugants were sequenced directly using a series of outward-directed primers specific for the locations next to the *bla*<sub>IMP-4</sub> 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<sup>−1</sup>, 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<sup>−1</sup>) prior to identifiication 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, ceftazidime, 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 *intI1*, *bla*\(_{SHV}\), *bla*\(_{TEM}\), *bla*\(_{IMP}\), *bla*\(_{DHA}\), *bla*\(_{CTX-M}\) and *qnrB4* genes detected by PCR. Additional sequencing of the amplified PCR products revealed the presence of *bla*\(_{SHV}\), *bla*\(_{TEM}\), *bla*\(_{IMP}\)–*4*, *bla*\(_{DHA}\), *bla*\(_{CTX-M}\)–*14* and *qnrB4* among these isolates. Therefore, coexistence of multiple \(\beta\)-lactamase genes within each individual isolate could explain the resistance of these isolates to all \(\beta\)-lactams tested. To the best of our knowledge, this is the first report of the co-existence of *bla*\(_{IMP}\)–*4* and *bla*\(_{DHA}\) in the same *K. pneumoniae* isolate.

Detection of multiple \(\beta\)-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 (Espedido et al., 2005; Liu et al., 2009). The decreased susceptibility of our isolates to ciprofloxacin could be explained by the existence of *qnrB4*.

\(\beta\)-Lactam resistance could be transferred by conjugation from all *K. pneumoniae* isolates to their recipients. All transconjugants harbouring *intI1*, *bla*\(_{TEM}\), *bla*\(_{IMP}\)–*4*, *bla*\(_{DHA}\)–*1*, *bla*\(_{CTX-M}\)–*14* and *qnrB4* but not *bla*\(_{SHV}\). 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\rightarrowTAG\) mutation was observed at nt 160 in the *ompK36* gene for isolate KpSX1, resulting in initiation of a stop codon at position 54 (CAG\rightarrowTAG) for this strain. An early termination of translation caused by the C\rightarrowT 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 *intI1-bla*\(_{IMP}\)–*4*-orfII-orfIII-qacE\(_{A1}\)-sul1.
Table 1. Phenotypic and genotypic characteristics of the *K. pneumoniae* clinical and environmental isolates

| Strain | Patient/s sample | BW (g)* | GA (weeks)† | Disc diffusion zone (mm) | MIC (mg l⁻¹) | Antimicrobial resistance‡ | PFGE | Resistance gene | int$§$ | Antimicrobials for treatment||
|--------|-----------------|---------|------------|--------------------------|-------------|---------------------------|------|----------------|--------|-----------------------------|
|        |                 | IMP     | MEM        | IMP                      | MEM         | CTX, CAZ, C, SXT, TE, ATM, CZ, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + MEZ, CMZ, CAZ, AMC, PAP, AMK, LEV |
| KpSX1  | Patient 1       | 1060    | 26         | 0                        | 0           | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + MEZ, CMZ, CAZ, AMC, PAP, IMP |
| KpSX2  | Patient 2       | 1360    | 29         | 19                       | 10          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + MEZ, CMZ, CAZ, AMC, PAP, IMP |
| KpSX3  | Patient 3       | 1370    | 27         | 19                       | 12          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + MEZ, CMZ, CAZ, IMP |
| KpSX4  | Patient 4       | 1050    | 28         | 19                       | 12          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | B    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + MEZ, CMZ, IMP |
| KpE1   | Fingers 1       | 19      | 12         | 19                       | 12          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + |
| KpE2   | Fingers 2       | 19      | 12         | 19                       | 12          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + |
| KpE3   | Incubator       | 16      | 10         | 16                       | 10          | CTX, CAZ, C, SXT, TE, ATM, CEC, GEN, FEP, FOX, IMP, MEM | A    | SHV-11, TEM-1, IMP-4, DHA-1, CTX-M-14 | B4    | + |

*BW, Birth weight of patient.
†GA, Gestational age of patient.
‡ATM, Aztreonam; C, chloramphenicol; CAZ, ceftazidime; CEC, cefaclor; CTX, cefotaxime; CZ, cefazolin; FEP, cefepime; 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.
The flanking structure of blaIMP-4 was therefore identical to the nucleotide sequence of a class I integron harbouring blaIMP-4 (GenBank accession no. FJ384365) described previously in Shanghai near to Shaoxing, China (Liu et al., 2009). The genetic relatedness of all K. pneumoniae isolates was also evaluated using PFGE. These results showed that two different band patterns, designated types A and B, were identified for these isolates. PFGE type A accounted for three clinical isolates (KpSX1, KpSX2 and KpSX3) and all three environmental isolates, whilst KpSX4 exhibited PFGE type B (Fig. 1). These data indicated that, with the exception of KpSX4, all of the isolates recovered in this study were closely related.

Our data complement those reported in recent outbreaks caused by K. pneumoniae producing carbapenemases and other β-lactamases as described elsewhere (Kassis-Chikhani et al., 2010; Souli et al., 2010; Steinmann et al., 2011). Ultimately, all four patients were isolated in single-bed rooms, where strict contact precautions were implemented. All NICU personnel were provided with additional training regarding standard precautions for prevention of infections in this setting, including appropriate hand hygiene and meticulous environmental cleaning. In addition, incubators, telephones, personal computers and door handles were cleaned with approved environmental disinfectants. Since the implementation of these procedures, multidrug-resistant K. pneumoniae isolates have not been isolated from patients or environmental sources in this NICU. We recommend early implementation of outbreak investigations with identification of two or more clinical cases of K. pneumoniae in the NICU setting in order to prevent additional cases and morbidity associated with these clinically challenging infections.

In conclusion, our data suggest that an outbreak due to multidrug-resistant K. pneumoniae occurred in this NICU.

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