Emergence of carbapenem-resistant clinical Enterobacteriaceae isolates from a teaching hospital in Shanghai, China

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Carbapenems such as imipenem and meropenem are first-line agents for the treatment of serious nosocomial infections caused by multidrug-resistant clinical isolates of bacteria belonging to the family Enterobacteriaceae. However, resistance to carbapenems has increased dramatically among members of the family Enterobacteriaceae isolated from a teaching hospital in Shanghai, China. In the present study, we investigated the prevalence and molecular characteristics of carbapenem-resistant clinical isolates of Enterobacteriaceae. None of the 77 clinical isolates collected from 2002 to 2009 were susceptible to ertapenem and only 6.5% and 1.3% of isolates were susceptible to imipenem and meropenem, respectively. Colistin and tigecycline were found to be the most active agents against carbapenem-resistant Enterobacteriaceae isolates, inhibiting 90% of isolates at a concentration of 1 μg ml⁻¹ and 4 μg ml⁻¹, respectively. The results of PFGE analysis suggested that many of the KPC-2-producing isolates of Citrobacter freundii and Klebsiella pneumoniae were clonally related. Most of these isolates were isolated from the same ward, namely the neurosurgical ward, suggesting horizontal transfer of the KPC-2-encoding gene in these isolates. Of the 77 isolates, 84.4% were found, by PCR, to be capable of carbapenemase production. SDS-PAGE analysis revealed that 75.3% (58/77) of the isolates had lost at least one porin protein. Our results suggested that the prompt detection of carbapenemase-producing strains is critical for the containment of nosocomial transmission. As no novel antimicrobials have been identified for use in the treatment of these pan-drug-resistant isolates, further studies should focus on the rational use of available antibiotics, the implementation of active antibiotic resistance surveillance and the strict implementation of infection control measures to avoid the rapid spread or outbreak of carbapenemase-producing Enterobacteriaceae in health-care facilities.

INTRODUCTION

Carbapenems such as imipenem and meropenem are first-line agents for the treatment of serious healthcare-associated infections caused by clinical multidrug-resistant Enterobacteriaceae (Brink et al., 2004). However, the phenomenon of carbapenem resistance is emerging in a wide variety of these species (Yigit et al., 2001). Carbapenemases have been widely detected in recent years and predominantly contribute to carbapenem resistance among members of the family Enterobacteriaceae including Klebsiella pneumoniae and Citrobacter freundii (Peleg & Hooper, 2010). Carbapenemases including KPC, VIM, IPM, SME and OXA belong to various families of β-lactamases (Queenan & Bush, 2007). Infections caused by carbapenem-resistant Enterobacteriaceae (CRE) have presented a great challenge to physicians in recent years (CDC, 2009; Munoz-Price & Quinn, 2009). In Shanghai, China, K. pneumoniae is the second most frequently isolated pathogen among Gram-negative bacilli according to the Shanghai Bacterial Resistance Surveillance Program (Zhu et al., 2006, 2007). Although the prevalence of carbapenem-resistant isolates among the Enterobacteriaceae is low, it has increased dramatically in recent years. In this study, CRE clinical isolates were collected and analysed for their molecular characteristics and similarities.

METHODS

Bacterial strains. All Enterobacteriaceae isolates obtained between January 2002 and April 2009 (n=7775) from Huashan Hospital, Fudan University, Shanghai, China, a 1300-bed tertiary care hospital,
were screened to investigate trends in the prevalence of the CRE isolates. These isolates were non-duplicate and were collected on routine workdays without any specific exclusion criteria. From the 7775 isolates, a total of 77 were selected for testing, including 43 isolates of *K. pneumoniae*, one isolate of *Klebsiella oxytoca*, four isolates of *Enterobacter cloacae*, one isolate of *Escherichia coli*, one isolate of *Serratia marcescens*, one isolate of *Providencia rettgeri*, one isolate of *Citrobacter freundii* and 25 isolates of *C. freundii*, most of which were resistant to all of three carbapenems tested, namely imipenem, meropenem and ertapenem, and had been isolated from different patients. Of the 77 isolates, 57.1 % (44/77) were isolated from urine, 39.0 % (30/77) from sputum, 1.3 % (1/77) from negative-pressure ball drainage, 1.3 % (1/77) from blood and 1.3 % (1/77) from cerebrospinal fluid. All of the strains were isolated from hospitalized patients. A well-characterized strain of *C. freundii*, producing an IMP-9-type metallo-β-lactamase (MBL), and *E. coli* strain ATCC 25922 were used as positive and negative controls, respectively, for antimicrobial susceptibility testing.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed using the disc diffusion method and the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). MICs of amikacin, cefotaxime, ceftazidime, cefepime, aztreonam, meropenem, doxycycline, minocycline and ciprofloxacin were determined following the guidelines of the US Food and Drug Administration (MICs were determined following the criteria of the European Committee on Antimicrobial Drug Administration (EUCAST, 2011) (MICs following the criteria of the CLSI. Breakpoint MICs of tigecycline or piperacillin–tazobactam. The proportions of the isolates found to be susceptible to ertapenem, cefotaxime, aztreonam and ciprofloxacin were determined following the criteria of the CLSI. Breakpoint MICs of tigecycline were determined following the guidelines of the US Food and Drug Administration (MICs ≤ 2 mg l⁻¹ denoting susceptibility and ≥ 8 mg l⁻¹ denoting resistance). MICs of colistin were interpreted following the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011) (MICs ≤ 2 mg l⁻¹ denoting susceptibility and ≥ 4 mg l⁻¹ denoting resistance).

**Genotypic detection of β-lactamases genes.** The presence of genes encoding the β-lactamases TEM, SHV, PER, SFO, VEB, CTX-M and GES, and genes encoding the plasmid-borne AmpCs and carbapenemases NDM-1, VIM, NDM-1, IPM, SPM, SME, GIM, NMC, IMI, IND and OXA was investigated in all of the 77 clinical strains by using previously described primers (Chen et al., 2011; Murray et al., 2007). Two conserved primers, forward primer KPC-F (5'-AGGACTTTG-GCGGCTCCAT-3') and reverse primer KPC-R (5'-TCCTCGAGCGCGAGTCTA-3'), were designed against the *bla*KPC gene to detect the sequences encoding KPC-1 to KPC-11, according to the published nucleotide sequences in GenBank. PCR amplicons were sequenced and the DNA sequences obtained were compared to those available in the NCBI GenBank database using BLAST searches.

**Epidemiological studies.** Isolates of *K. pneumoniae* and *C. freundii* were typed by using PFGE, which was performed according to a previously described protocol (Hunter et al., 2005). The DNA fingerprints generated were analysed according to the criteria proposed by Tenover et al. (1995).

**Outer-membrane proteins.** SDS-PAGE analysis of outer-membrane proteins, including OmpK35, OmpK36 and OmpK37, of all CRE isolates was performed as described previously (Woodford et al., 2004; Hosaka et al., 1995).

**RESULTS**

**Antimicrobial susceptibility testing and prevalence of CRE isolates**

The prevalence of CRE clinical isolates increased significantly in 2009, especially for *K. pneumoniae* (Fig. 1). The detection rate of carbapenem-resistant isolates of *K. pneumoniae* rose from 0.91 % (5/549) in 2005 to 12.87 % (82/637) in 2009. The proportion of imipenem-resistant isolates of *C. freundii* rose rapidly during 2005–2006, from 11.11 % (3/27) to 40.91 % (9/22), and remained high at 33.3 % (6/18) in 2009. Of the 77 clinical isolates selected for this study, none was found to be susceptible to ertapenem, cefotaxime, aztreonam or piperacillin–tazobactam. The proportions of the isolates that were susceptible to imipenem, meropenem, colistin and tigecycline were 6.5 %, 1.3 %, 96.1 % and 88.3 %, respectively, and >70 % of the isolates were susceptible to minocycline and doxycycline (Table 1).

**Characterization of β-lactamases in CRE isolates**

PCR was used to detect genes encoding NDM-1, VIM-2, SPM, GIM, NMC, IMI, IND, SME, OXA-48, OXA-50, OXA-55, OXA-60, OXA-69 and OXA-24 carbapenemases but did not result in amplicons with any of the clinical isolates. Of the 77 isolates, 65 (84.4 %) were found to be carbapenemase-producing. The *bla*KPC gene was detected in 58/77 (75.3 %) isolates and in all cases the gene encoded KPC-2-type carbapenemase. Among these 58 strains, this
gene was coupled with the *bla*<sub>VIM-1</sub> gene in two isolates, the *bla*<sub>IMP-2</sub> gene in two isolates and the *bla*<sub>IMP-1</sub> gene in one isolate. The genes encoding VIM-1, IMP-1 and IMP-2 carbapenemases were also detected in five (6.5 %), four (5.2 %) and three (3.9 %) isolates, respectively. The gene encoding CTX-M<sub>b</sub>-lactamase was detected in 65/77 (84.4 %) isolates, of which one possessed the *bla*<sub>CTX-M-2</sub> gene, 55 possessed the *bla*<sub>CTX-M-14</sub> gene and 20 possessed the *bla*<sub>CTX-M-15</sub> gene. Plasmid-borne AmpCs DHA-1 or CMY-2 were detected in 31/77 (40.3 %) isolates.

**PFGE and analysis of outer-membrane proteins**

The results of PFGE analysis, interpreted according to Tenover et al. (1995), revealed four and ten unrelated genotypes in isolates of *C. freundii* and *K. pneumoniae*, respectively (Table 2). SDS-PAGE profiles of outer-membrane proteins, including OmpK35, OmpK36 and OmpK37, indicated that 75.3 % (58/77) of the isolates possessed, among others, a single band probably corresponding to OmpK35 or OmpK36, suggesting that they have lost at least one porin protein (see Fig. 2).

**DISCUSSION**

The first CRE isolate (*E. coli*) collected from Huashan training hospital was recovered from the urine of patients in a medical ward in March 2002, later followed by the isolation of carbapenem-resistant strains of *C. freundii* and *K. pneumoniae*. In 2009, the incidence of imipenem-resistant *Enterobacteriaceae* rose rapidly. These results suggested that the increased prevalence of carbapenem-resistant isolates in the hospital may have been caused by a failure to control the spread of these strains. As CRE are some of the most important Gram-negative bacilli residing in hospitals and causing hospital-acquired infections, the emergence of CRE isolates has attracted much attention (Schwaber & Carmeli, 2008).

Although carbapenemase-producing *Enterobacteriaceae* isolates are usually pan-drug resistant, several isolates may still remain susceptible to amikacin and ciprofloxacin. In the present study, 10.4 % and 13.0 % of isolates were found to be susceptible to amikacin and ciprofloxacin, respectively.
Many studies have demonstrated the activity of colistin and tigecycline against carbapenemase-producing Enterobacteriaceae isolates (Platsika et al., 2007; Bratu et al., 2005). Colistin was found to be most active in our study, inhibiting 90% of isolates at a concentration of 1 µg ml⁻¹ (MIC₉₀). Of the nine tigecycline-resistant isolates, eight were found to be susceptible to colistin. However, the potential toxicity of colistin and the need for it to be used with other antimicrobial agents narrows its potential for clinical use (Walsh et al., 2005). In the present study, ~70% of carbapenem-resistant isolates were susceptible to both minocycline and doxycycline and ~89% were susceptible to tigecycline. Therefore, we presume that these drugs would be a rational option for the treatment of severe infections caused by CRE isolates.

Although the production of extended-spectrum β-lactamases (ESBLs) and/or plasmid-borne AmpC β-lactamases, coupled with porin protein loss, can be responsible for carbapenem resistance (Jacoby et al., 2004; Girlich et al., 2009; Carvalhaes et al., 2010), the main agents of resistance to carbapenemases are carbapenemases, especially KPC and MBLs, such as VIM and IPM (Hirsch & Tam, 2010). The genes encoding carbapenemases are usually carried by plasmids that often carry other resistance genes simultaneously, resulting in extensively drug-resistant bacteria (Gootz et al., 2009). In this study, out of the 25 C. freundii isolates, 18 carried KPC-2 carbapenemase genes and five produced both KPC-2 carbapenemase and MBL as well as CTX-M-14-type ESBL and CMY-2-type plasmid-borne AmpC. Out of the 43 K. pneumoniae isolates, nine carried carbapenemase genes encoding CTX-M-14-type ESBL and DHA-1-type plasmid-borne AmpC. In these two species, 23.3% (10/43) of K. pneumoniae isolates and 24% (6/25) of C. freundii isolates carried more than two kinds of carbapenemase genes. Although widespread dissemination of NDM-producing Enterobacteriaceae has been reported in India, Pakistan, the UK and other countries (Kumarasamy et al., 2010; Rolain et al., 2010), China still has no reports of NDM-producing Enterobacteriaceae, although there have been reports of NDM-producing strains of Acinetobacter baumannii (Chen et al., 2011). In this study, we screened 77 isolates of Enterobacteriaceae for the blaNDM-1 gene; however, none was found to be NDM-1-producing.

Since plasmids are readily transferable, carbapenem-resistance genes can easily spread within species and even from species to species, such as C. freundii and K. oxytoca (Schwaber & Carmeli, 2008; Sidjabat et al., 2009). When such plasmids enter a rapidly disseminating bacterial strain, the result may be a widespread outbreak of a multi-drug-resistant pathogen (Schwaber & Carmeli, 2008). The PFGE profiles suggested that many of the KPC-2-producing isolates of C. freundii and K. pneumoniae were closely related and most of these isolates were isolated from the same ward, namely the neurosurgical ward. Of the 25 C. freundii isolates, 15 possessed the same genotype and among the 43 K. pneumoniae isolates, 12 displayed the same PFGE pattern, suggesting that horizontal spread of the blaKPC-2 Gene occurs in C. freundii and K. pneumoniae. In contrast, the four isolates of E. cloacae showed four different banding patterns.

The emergence of conferred carbapenemase resistance in strains in a hospital in China is alarming because it adds to the already difficult task of treating antimicrobial-resistant infections. Because carbapenemase-resistance genes can potentially spread rapidly via transferable plasmids, infections caused by carbapenemase-producing Enterobacteriaceae may prove difficult to control once they emerge (Pourmaras et al., 2009), especially for both KPC- and MBL-producing isolates. Therefore, prompt detection is critical for the containment of carbapenemase-producing strains and for the prevention of nosocomial transmission (Hirsch & Tam, 2010; Lee et al., 2003; Pourmaras et al., 2010). As very few novel antimicrobials have been discovered for the treatment of these pan-drug-resistant isolates, further studies should focus on the rational use of available antibiotics, implementation of active antibiotic resistance surveillance and implementation of strict infection control measures to avoid the rapid spread or outbreak of carbapenemase-producing Enterobacteriaceae in health-care facilities.

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