Hospital clonal dissemination of *Enterobacter aerogenes* producing carbapenemase KPC-2 in a Chinese teaching hospital

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Carbapenems are first-line agents for the treatment of serious nosocomial infections caused by multidrug-resistant *Enterobacteriaceae*. However, resistance to carbapenems has increased dramatically among *Enterobacteriaceae* in our hospital. In this study, we report clonal dissemination caused by carbapenem-resistant *Enterobacter aerogenes* (CREA). In 2011, CREA was identified from 12 patients admitted to the neurosurgical ward. All 12 clinical isolates were non-susceptible to ceftazidime, ceftazidime, cefoxitin, ertapenem, imipenem or meropenem. All isolates carried the gene encoding *Klebsiella pneumoniae* carbapenemase-2 (KPC-2), except for the isolate E₄. However, a remarkably lower expression level of the porin OmpF was detected in the non-KPC-2-producing isolate E₄ on SDS-PAGE compared with the carbapenem-susceptible isolate. Epidemiological and molecular investigations showed that a single *E. aerogenes* strain (PFGE type A), including seven KPC-2-producing clinical isolates, was primarily responsible for the first isolation and subsequent dissemination. In a case-control study, we identified risk factors for infection/colonization with CREA. Mechanical ventilation, the changing of sickbeds and previous use of broad-spectrum antibiotics were identified as potential risk factors. Our findings suggest that further studies should focus on judicious use of available antibiotics, implementation of active antibiotic resistance surveillance and strict implementation of infection-control measures to avoid the rapid spread or clonal dissemination caused by carbapenem-resistant *Enterobacteriaceae* in healthcare facilities.

INTRODUCTION

Carbapenems are the most potent and reliable β-lactam antibiotics for the treatment of serious infections caused by multidrug-resistant *Enterobacteriaceae* (Vardakas et al., 2012; Yang & Guglielmo, 2007). However, carbapenem resistance has been emerging and increasing in a wide variety of *Enterobacteriaceae* species worldwide (Gupta et al., 2011; Hu et al., 2012; Nordmann et al., 2012). Although the production of extended-spectrum β-lactamases and/or plasmid-borne AmpC β-lactamases, coupled with porin loss, can be responsible for carbapenem resistance, the main mechanism underlying resistance to carbapenems is the production of carbapenemases, especially *Klebsiella pneumoniae* carbapenemase (KPC) and metallo-β-lactamase (Hirsch & Tam, 2010). Carbapenemases have been detected in a wide range of species in the family *Enterobacteriaceae*, including *K. pneumoniae*, *Citrobacter freundii*, *Escherichia coli* and *Enterobacter aerogenes* (Peleg & Hooper, 2010).

Because carbapenemase-producing *Enterobacteriaceae* isolates are usually extensively drug resistant, infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) present a serious clinical challenge for physicians in healthcare settings (Lledo et al., 2009). Treatment options for these infections are limited and few clinical data are available on which to base antibiotic recommendations (Falagas et al., 2011). Moreover, the use of inappropriate empirical antibiotic therapy or delayed appropriate antibiotic therapy can lead to worse outcomes. Previous studies have found crude mortality rates ranging from 30 % to 44 % for diverse infections caused by CRE (Borer et al., 2009). The prevalence of CRE clinical isolates has increased significantly in our hospital since 2005 (Hu et al., 2012). In particular, the detection rate of carbapenem-resistant *E. aerogenes* (CREA) among CRE isolates rose...
from 0% (0/7 isolates) in 2005 to 21.1% (12/57 isolates) in 2011 (data not shown). Therefore, we keep a record of CREA strains isolated from the inpatient department in our hospital.

The purpose of this study was to describe the emergence and dissemination of CRE clinical isolates caused by CREA in a teaching hospital in Shanghai, China. Our findings provide valuable insight into possible strategies for treatment and controlling the spread of CREA isolates.

**METHODS**

**Bacterial strains.** All E. aerogenes isolates (n=57) obtained in 2011 at Huashan Hospital (Fudan University, Shanghai, China), a 1300-bed tertiary-care hospital, were screened to investigate the prevalence of carbapenem-resistant isolates. These isolates were non-duplicate and were collected on routine workdays without any specific exclusion criteria. Among the 57 isolates, a total of 12 carbapenem-resistant isolates were identified (the inhibitory diameter of imipenem or meropenem was ≤19 mm). Of the 12 isolates, 91.7% (11/12) were isolated from sputum and one was isolated from abdominal fluid. All of the isolates were identified from 12 hospitalized patients. A well-characterized strain of C. freundii 1641 (producing an IMP-9-type metallo-β-lactamase), K. pneumoniae 2528 (producing a KPC-2-type carbapenemase) (Chen et al., 2011b) and E. coli strain ATCC 25922 were used as positive and negative controls for screening carbapenemase genes and antimicrobial susceptibility testing, respectively. In addition, E. coli EC600 (resistant to rifampicin) was used as a recipient for conjugation. The carbapenem-susceptible E. aerogenes (CSEA) isolate E5 was randomly chosen as the control for analysis of outer-membrane protein profiles and real-time reverse transcriptase PCR.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed using the disc diffusion and the agar dilution methods recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). MICs of tigecycline, minocycline, doxycycline, amikacin, ceftoxitin, cefepime, ceftazidime, ciprofloxacin, polymyxin B and polymyxin E were determined following criteria of the CLSI, while the MIC of fosfomycin was determined using an Etest strip (bioMérieux) following the manufacturer’s instructions. Breakpoint MICs of tigecycline were determined following the guidelines of the US Food and Drug Administration (with MICs ≤2 µg ml⁻¹ denoting susceptibility and ≥8 µg ml⁻¹ denoting resistance). The MICs of imipenem, meropenem and etrapenem were analysed with or without the KPC inhibitor 3-phenylboronic acid (PBA; 400 µg ml⁻¹) or the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP; 25 µg ml⁻¹), respectively.

**Genotypic detection of β-lactamase genes.** The presence of genes encoding the β-lactamases TEM, SHV, PER, SFO, VEB, CTX-M and GES and genes encoding the plasmid-borne AmpC β-lactamases and carbapenemases KPC, NDM-1, VIM, IMP, SPM, SME, GIM, NMC, IMI, IND and OXA was investigated in all of the 12 clinical isolates by using previously described primers (Chen et al., 2011c; Murray et al., 2007; Pérez-Pérez et al., 2002). PCR amplicons were sequenced and the DNA sequences obtained were compared with those available in the NCBI GenBank database using BLAST searches.

**Transfer of carbapenem resistance.** Conjugation experiments were carried out in Luria–Bertani broth with rifampicin-resistant E. coli EC600 as the recipient, as described previously (Zhang et al., 2011). Transconjugants were selected on Trypticase soy agar plates containing rifampicin (600 µg ml⁻¹) and ampicillin (50 µg ml⁻¹) to select for plasmid-encoded resistance. The colonies grown on the selective medium were selected and identified using the Vitek 2 (bioMérieux) compact system and KPC-2 carbapenemase determination by PCR and DNA sequencing.

**PFGE analysis.** Clonal relationships were analysed using PFGE, which was performed according to a previously described protocol (Schlesinger et al., 2005). The DNA fingerprints generated were analysed according to the criteria proposed by Tenover et al. (1995).

**Analysis of outer-membrane protein profiles.** The outer-membrane proteins of all isolates were examined by SDS-PAGE, as described previously (Gayet et al., 2003).

**Real-time reverse transcriptase PCR.** Real-time reverse transcriptase PCR was performed to determine the expression of ompD and ompF porin genes, and of the acrB efflux pump gene relative to the rpoll housekeeping gene according to a previously described protocol (Szabó et al., 2006).

**Clinical epidemiology.** Epidemiological data were collected via chart review for each patient from the hospital’s uniform electronic database. The following parameters were assessed: (1) general demographics, such as age, sex and other background information; (2) the ward to which the patient was assigned after admission; (3) previous use of antibiotics, particularly carbapenems, extended-spectrum cephalosporin and quinolones; and (4) potential risk factors for the occurrence and spread of CREA. Infection or colonization with a CREA isolate was defined according to the Centers for Disease Control and Prevention’s definition of nosocomial infections (Garner et al., 1988). The medical histories of 12 randomly chosen patients from whom non-duplicated CSEA had been isolated were reviewed as controls.

**RESULTS**

**Antimicrobial susceptibility testing**

All 12 clinical isolates were non-susceptible to ceftoxime, ceftazidime, cefotixin, etrapenem, imipenem or meropenem. The proportions of the isolates that were susceptible to doxycycline, minocycline, ciprofloxacin and tigecycline were 50.0, 58.3, 66.7 and 83.3%, respectively. All remained susceptible to amikacin, fosfomycin, polymyxin B and polymyxin E (Table 1). The resistance patterns could be divided into two major groups according to the results obtained with carbapenem-susceptibility tests performed in the presence of the KPC inhibitor PBA or the efflux pump inhibitor CCCP (Table 2). For imipenem, PBA and CCCP exerted significant effects on the MICs, which were reduced four- or eightfold for all isolates except E4. For meropenem and etrapenem, PBA and CCCP also showed significant effects on the MICs of most isolates, where they were reduced by four- or eightfold. For isolates E3 and E7, the efflux pump inhibitor CCCP reduced the MIC of meropenem to a greater degree than the KPC inhibitor PBA.

**Characterization of β-lactamases**

All of the E. aerogenes strains carried the gene encoding KPC-2, except for E4. Isolates E3, E6, E8, E9, E11 and E12...
carried also \(\text{bla}_{\text{TEM}}\), while isolates \(E_6\) and \(E_{10}\) were \(\text{bla}_{\text{SHV}}\)-positive and isolate \(E_9\) carried the gene \(\text{bla}_{\text{CTX-M-15}}\). No PCR products were obtained for any of the other genes investigated.

### Transfer of carbapenem resistance

Two KPC-2-producing *E. aerogenes* were randomly selected for conjugation. Carbapenem resistance was successfully transferred from *E. aerogenes* to *E. coli* EC600. The two *E. coli* transconjugants exhibited significantly reduced carbapenem susceptibility, consistent with detection of \(\text{bla}_{\text{KPC-2}}\) in these transconjugants. The MICs of imipenem, meropenem and ertapenem ranged from 4 to 8 \(\mu\)g ml\(^{-1}\) and the antimicrobial susceptibility patterns of *E. coli* transconjugants were similar to those of the donor. They were resistant to cefoxitin, cefotaxime and ceftazidime, but were susceptible to doxycycline, minocycline and tigecycline.

### PFGE

Five distinct PFGE groups (PFGE types A–E) were observed among the 12 CREA isolates; one group (type A) included 58% (7/12) of the *E. aerogenes* isolates (\(E_1, E_2, E_3, E_6, E_8, E_{11}, E_{12}\)) and another group (type B) included 17% (2/12) isolates (\(E_7\) and \(E_9\)). The remaining three groups (C–E) included a single isolate each (Fig. 1).

### Analysis of outer-membrane proteins and gene expression of \(\text{ompF}, \text{ompD}\) and \(\text{acrB}\)

SDS-PAGE analysis of outer-membrane proteins revealed a remarkably lower level of expression of the porin OmpF in the clinical strain \(E_4\) compared with CSEA. The other 11 CREA isolates had comparable outer-membrane profiles to the carbapenem-resistant strain (Fig. 2). The transcription levels of both \(\text{acrB}\) and \(\text{ompD}\) were equivalent between carbapenem-resistant and -susceptible isolates. However,

<table>
<thead>
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<th>Drug</th>
<th>MIC ((\mu)g ml(^{-1}))</th>
<th>Range</th>
<th>MIC(_{50})</th>
<th>MIC(_{90})</th>
<th>Resistant (%)</th>
<th>Susceptible (%)</th>
</tr>
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<td>Imipenem</td>
<td></td>
<td>4–32</td>
<td>16</td>
<td>32</td>
<td>100</td>
<td>0</td>
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<tr>
<td>Meropenem</td>
<td></td>
<td>4–32</td>
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<td>32</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Ertapenem</td>
<td></td>
<td>32–128</td>
<td>64</td>
<td>128</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Tigecycline</td>
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<td>4</td>
<td>8.3</td>
<td>91.7</td>
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<td>Fosfomycin</td>
<td></td>
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<td>8</td>
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<td>100</td>
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<td>2–64</td>
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<td>64</td>
<td>25</td>
<td>75</td>
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<tr>
<td>Doxycycline</td>
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<td>4</td>
<td>64</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Ceefpimine</td>
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<td>2–1024</td>
<td>16</td>
<td>1024</td>
<td>41.7</td>
<td>58.3</td>
</tr>
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<td>16</td>
<td>64</td>
<td>83.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td></td>
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<td>64</td>
<td>1024</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cefoxitin</td>
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<td>32–1024</td>
<td>1024</td>
<td>1024</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
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<td>0.25</td>
<td>4</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>0.25–8</td>
<td>0.25</td>
<td>4</td>
<td>25</td>
<td>75</td>
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<tr>
<td>Polymyxin B</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Polymyxin E</td>
<td></td>
<td>0.25–0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table 1. *In vitro* activities of antimicrobial agents against CREA isolates

### Table 2. MICs of carbapenems, with or without PBA or CCCP, for 12 CREA isolates
the transcription levels of \textit{ompF} were five- to 100-fold lower in the E₄, E₁₀, E₁₁ and E₁₂ isolates compared with the susceptible control, while transcription was 16 times higher in isolate E₉.

**Epidemiology**

CREA isolates were identified from 12 patients (10 men and two women, ages 41–83 years) hospitalized on the neurosurgery ward of Huashan Hospital. The patients’ clinical characteristics are shown in Table 3. The first patient (patient 1), from whom the CREA isolate E₁ (PFGE type A) was identified (from sputum), had undergone surgery in May 2011 and experienced pneumonia post-operatively. Nearly 6 weeks after this first CREA isolate was identified, two patients (patients 2 and 3) on the same ward were found to have invasive infections with CREA isolates (E₂ and E₃, respectively; PFGE type A). In the next 10 days, a further two patients (patients 4 and 5) tested positive for CREA isolates (E₄ and E₅, respectively), which were considered colonizers. During the subsequent 5 weeks, seven additional patients tested positive for CREA isolates (PFGE type A for four isolates and PFGE type E for two isolates). Of these, six patients experienced an actual infection caused by CREA.

Overall, 75% of patients (9/12) in the CREA group and 58% (7/12) in the CSEA control group had undergone one or more surgical procedures ($P=0.387$). Half of the patients in the CREA group but only one patient in the CSEA group had received mechanical ventilation ($P=0.025$). Ten patients (83%) with CREA strains isolated had undergone one or more sickbed changes during hospitalization, compared with none in the control group ($P<0.00001$). In addition, eight patients with CREA (67%) harboured other multidrug- or pandrug-resistant pathogens (Falagas & Karageorgopoulos \textit{et al}., 2008), mostly \textit{K. pneumoniae}, \textit{Pseudomonas aeruginosa}, \textit{Acinetobacter baumannii}, \textit{E. coli} or meticillin-resistant \textit{Staphylococcus aureus}. However, 42% (5/12) of CSEA patients harboured susceptible pathogens. Patients in the CREA group were treated with various antimicrobial agents before isolation.
However, no antimicrobials were used before CSEA isolation in eight patients (67%, 8/12) in the control group ($P=0.0005$).

### DISCUSSION

Although carbapenemase-producing *Enterobacteriaceae* isolates are usually extensively drug resistant, some isolates can still remain susceptible to other antimicrobial agents (Petrosillo *et al.*, 2013). In the present study, 50% or more of the isolates were susceptible to minocycline and doxycycline, and 83.3 and 66.7% were susceptible to tigecycline and ciprofloxacin, respectively. None of the isolates was found to be resistant to amikacin, fosfomycin, polymyxin B or polymyxin E. Therefore, these agents, either alone or in combination, may be further explored as options for the treatment of infections caused by CREA. Overall, 92% (11/12) of CREA carried the gene encoding KPC-2, and the KPC inhibitor PBA reduced the MICs of the three tested carbapenems to these 11 isolates to various degrees. Other than $\beta$-lactamases, loss of outer-membrane porins or overexpression of genes that regulate or encode efflux pumps are among the mechanisms that contribute to CREA (Pfeifer *et al.*, 2010). Apart from in the E4 isolate, the efflux pump inhibitor CCCP reduced the MICs of meropenem and ertapenem by four- or eightfold (Table 2).

KPC-producing *Enterobacteriaceae* have become a public health concern worldwide, including in China (Cantón *et al.*, 2012; Hu *et al.*, 2012). In our hospital, CRE isolates have been detected in a wide range of species, especially *K. pneumoniae* (Chen *et al.*, 2011a; Hu *et al.*, 2012). In this study, epidemiological and molecular investigations showed that a single *E. aerogenes* strain (PFGE type A), including seven clinical isolates (E1, E2, E3, E6, E8, E11, E12), that produced KPC-2 was primarily responsible for the first isolation and subsequent dissemination (Fig. 1). Results of epidemiological and molecular investigations suggest that the increased prevalence of carbapenem-resistant isolates in our hospital may have been caused by a failure to control the spread of these resistant strains.

Studies have found reduced expression of *omp* genes in CREA (Fernández-Cuenca *et al.*, 2006; Thiolas *et al.*, 2004). The CRE isolate E4 was resistant to carbapenems but did not possess any $\beta$-lactamase genes implicated in carbapenem resistance, and its resistance was not compromised by CCCP. For this isolate, reduced expression of *ompF* was identified with SDS-PAGE, and a decreased gene transcription level of *ompF* was detected with real-time PCR. Therefore, we propose that the reduced expression of outer-membrane protein served as the major resistance mechanism for isolate E4. Several KPC-2-producing isolates exhibited identical MIC values despite different expression levels of *ompF* (e.g. E9 compared with E10–12). We suggest that compared with

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolate no.</th>
<th>Sex (age, years)</th>
<th>Underlying condition</th>
<th>Days of hospital stay before infection/total days</th>
<th>Specimen</th>
<th>Infection/colonization</th>
<th>Antimicrobial therapy</th>
<th>Status on hospital discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E1</td>
<td>F (41)</td>
<td>Epidural haematoma</td>
<td>5/14</td>
<td>Sputum</td>
<td>Infection</td>
<td>CSL, VAN, CIP</td>
<td>Recovered</td>
</tr>
<tr>
<td>2</td>
<td>E2</td>
<td>M (58)</td>
<td>Epidural haematoma</td>
<td>2/27</td>
<td>Abdominal fluid</td>
<td>Infection</td>
<td>MNO, MEM, PAN, VAN, BIA, CSL, CAS</td>
<td>Improved</td>
</tr>
<tr>
<td>3</td>
<td>E3</td>
<td>M (47)</td>
<td>Ventricular haemorrhage</td>
<td>12/74</td>
<td>Sputum</td>
<td>Infection</td>
<td>BIA, VAN, DOX, MEM, CAS</td>
<td>Improved</td>
</tr>
<tr>
<td>4</td>
<td>E4</td>
<td>M (83)</td>
<td>Adenoid tumour</td>
<td>15/87</td>
<td>Sputum</td>
<td>Infection</td>
<td>GEN, CXM, MEM, CAZ, FLU</td>
<td>Improved</td>
</tr>
<tr>
<td>5</td>
<td>E5</td>
<td>M (57)</td>
<td>Cerebral haematoma</td>
<td>5/15</td>
<td>Sputum</td>
<td>Infection</td>
<td>TZP, CIP</td>
<td>Recovered</td>
</tr>
<tr>
<td>6</td>
<td>E6</td>
<td>M (68)</td>
<td>Intracranial haematoma</td>
<td>1/12</td>
<td>Sputum</td>
<td>Colonization</td>
<td>CRO, MEM, CIP, ETP, TIP, CSL, CAS, TEC, TIP</td>
<td>Recovered</td>
</tr>
<tr>
<td>7</td>
<td>E7</td>
<td>M (55)</td>
<td>Cerebral trauma</td>
<td>13/27</td>
<td>Sputum</td>
<td>Infection</td>
<td>MEM, CSL, TZP</td>
<td>Improved</td>
</tr>
<tr>
<td>8</td>
<td>E8</td>
<td>M (42)</td>
<td>Cerebral trauma</td>
<td>20/41</td>
<td>Sputum</td>
<td>Infection</td>
<td>MEM, CSL</td>
<td>Improved</td>
</tr>
<tr>
<td>9</td>
<td>E9</td>
<td>M (76)</td>
<td>Craniocerebral trauma</td>
<td>17/30</td>
<td>Sputum</td>
<td>Infection</td>
<td>M, CAS</td>
<td>Recovered</td>
</tr>
<tr>
<td>10</td>
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<td>M (56)</td>
<td>Craniocerebral trauma</td>
<td>6/31</td>
<td>Sputum</td>
<td>Infection</td>
<td>M</td>
<td>Recovered</td>
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</table>
outer-membrane protein, KPC plays a conclusive role in carbapenem resistance mechanisms.

In a previous study on carbapenem-resistant \textit{C. freundii} among hospitalized patients, the risk factors for acquiring this organism included invasive procedures (especially surgical operations), indwelling urine catheters, the changing of sickbeds and previous in-hospital cephalosporin use (Chen \textit{et al.}, 2011b). We found very similar risk factors for the acquisition of CREA among hospitalized patients, including mechanical ventilation, the changing of sickbeds and previous use of broad-spectrum antibiotics. Determination of risk factors is important, because it will help to identify patients who require close monitoring and also optimize empirical antimicrobial drug therapy.

Because carbapenemase genes located on plasmids are readily transferable, infections caused by carbapenemase-producing \textit{Enterobacteriaceae} isolates may prove difficult to control once they have emerged (Lledo \textit{et al.}, 2009). Rapid dissemination of extensively drug-resistant isolates is a serious concern in clinical patient care. Therefore, prompt detection is critical for containing carbapenemase-producing strains and preventing nosocomial transmission. As only few novel antimicrobials are in development for the treatment of these extensively drug-resistant infections (Petrosillo \textit{et al.}, 2013), further studies should focus on the judicious use of available antibiotics and implementation of strict infection-control measures to avoid the rapid spread or clonal dissemination of carbapenemase-producing \textit{Enterobacteriaceae} in healthcare facilities.

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