High prevalence of VIM-4 and NDM-1 metallo-β-lactamase among carbapenem-resistant Enterobacteriaceae

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The purpose of this study was to identify the mechanisms leading to carbapenem resistance among multidrug-resistant Enterobacteriaceae isolates recovered from hospitalized patients with nosocomial infections in Mubarak Al Kabeer Hospital, Kuwait. Fourteen carbapenem-resistant Enterobacteriaceae isolates were obtained from inpatients in different wards and intensive care units between April 2009 and February 2011. Antibiotic susceptibilities were determined using the E-test method. Genes encoding β-lactamases were characterized by specific PCR amplification, sequencing and conjugation assays. All isolates were identified as metallo-β-lactamase (MBL) producers using phenotypic and molecular methods. Eleven of the 14 isolates produced VIM-4 (six Klebsiella pneumoniae, three Escherichia coli, one Enterobacter cloacae and one Klebsiella oxytoca). Three K. pneumoniae isolates produced the MBL NDM-1 and co-produced the plasmid-encoded AmpC CMY-4. The VIM-4-producing isolates co-produced extended-spectrum β-lactamases including CTX-M-15 and some SHV derivatives. The VIM-4 gene was not transferable by conjugation studies of six selected strains. We demonstrated here the emergence of VIM-4- and NDM-1-producing isolates in the largest teaching hospital in Kuwait.

INTRODUCTION

Carbapenems are one of the few treatment options available to treat serious infections caused by multidrug-resistant Gram-negative bacteria as these drugs are stable to organisms expressing extended-spectrum β-lactamases (ESBLs) or AmpC β-lactamases. However, extensive use of carbapenems has led to the selection of carbapenem resistance (Bush, 1998), creating antibiotic pressure on carbapenems and triggering resistance to this group of antimicrobial agents. Many reports worldwide now clearly show that carbapenem resistance among Enterobacteriaceae is emerging due to acquisition of carbapenem-hydrolysing β-lactamases (Nordmann et al., 2011), thus limiting the availability of antimicrobial agents for treating corresponding infections. The most prevalent carbapenemes are the molecular class B metallo-β-lactamases (MBLs), mainly VIM and NDM types, and the class A KPC-type β-lactamases (Queenan & Bush, 2007). The VIM-type enzymes have been identified previously in several countries from the Middle East (Nordmann et al., 2011). VIM-4 is a point mutation of VIM-1 and was initially described in Pseudomonas aeruginosa from Greece (Pournaras et al., 2002) and then identified in Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, Acinetobacter spp. and Aeromonas hydrophila (Figueiredo et al., 2008; Libisch et al., 2008; Luzzaro et al., 2004; Shevchenko et al., 2012). The blaVIM genes may be transferred horizontally as they are plasmid- and integron-borne genes (Poirel et al., 2000). Before 2009, carbapenem resistance in Enterobacteriaceae was extremely rare in Kuwait, but in the last 2 years we have encountered isolates showing resistance to carbapenems. The aim of this study was to investigate the occurrence of carbapenem-resistant determinants in Enterobacteriaceae isolated from hospitalized patients in Mubarak Al Kabeer Hospital, Kuwait.

MATERIAL AND METHODS

Bacterial isolates. Between April 2009 and February 2011, 14 clinical enterobacterial isolates were collected (one per patient) from patients who had been admitted to the Mubarak Al Kabeer Hospital. These were non-susceptible to at least one of the carbapenems with an

Abbreviations: CVP, central venous pressure; ESBL, extended-spectrum β-lactamase; ICU, intensive care unit; MBL, metallo-β-lactamase; PFGE, pulsed-field gel electrophoresis.
MIC of >1 μg ml⁻¹ for imipenem and meropenem, or >0.5 μg ml⁻¹ for ertapenem (CLSI, 2012). They were stored at −70 °C in 1% protease/peptone broth containing 7% glycerol for further evaluation. Bacterial identification to species level was carried out using VITEK II-ID cards (bioMérieux).

Susceptibility testing. Initial antibiotic susceptibility testing was carried out using a VITEK 2 card system with AST GN09 cards (bioMérieux). The susceptibility was then confirmed for 18 antibiotics by E-test (bioMérieux) according to the manufacturer’s instructions. The antibiotics tested were: amikacin and gentamicin (aminoglycosides); ampicillin, amoxicillin-clavulanic acid and piperacillin (β-lactams); aztreonam (monobactam); cefalothin, cefepime, cefotaxin, cefoxitin, and ceftriaxone (cephalosporins); ciprofloxacin (quinolone); colistin (polymyxin); ertapenem, imipenem and meropenem (carbapenems); and tigecycline (glycylcycline). Antibiotic susceptibility results were interpreted according to the Clinical Laboratory Standard Institute recommendations (CLSI, 2012), except for tigecycline and colistin. For tigecycline, we used breakpoints recommended by the US Food and Drug Administration, i.e. susceptible and resistant MIC: ≤2 μg ml⁻¹ and ≥8 μg ml⁻¹, respectively. For colistin, the breakpoint recommended by CLSI (2012) for Acinetobacter spp. was used (i.e. MIC breakpoints of ≤2 μg ml⁻¹ and ≥4 μg ml⁻¹ for susceptibility and resistance, respectively). Escherichia coli ATCC 25922 and ATCC 35218 strains were used as controls. All the carbapenem-resistant isolates were phenotypically screened for MBL carbapenemase production using an imipenem/EDTA double disc synergy test (Lee et al., 2001), modified Hodge test (Lee et al., 2010) and E-test according to the manufacturer’s instructions (bioMérieux). K. pneumoniae ATCC BAA-1705 and ATCC BAA-1706 were used as positive and negative controls, respectively.

PCR and sequencing. PCR was used to amplify genes encoding the ESBLs blaCTX-M, blaTEM, and blaSHV as described previously (Rotimi et al., 2008), as well as the genes encoding the carbapenemases blaVIM, blaOXA-48, bladCM, blamIM, bladIM and blaca1C as described elsewhere (Poirel et al., 2011c). The blaqury gene was detected using PCR amplification as described previously (Poirel et al., 2011a). Sequencing was performed using a GeneAmp PCR system 9700 by cycle sequencing with BigDye termination (AB Applied Biosystem).

Plasmid analysis and conjugation. All six VIM-4-positive K. pneumoniae strains were analysed for plasmid content (Table 1). Plasmid DNA was extracted using a Plasmid Mini kit (Qiagen) and separated by electrophoresis in 0.6% agarose in TAE buffer (0.04M Tris/HCl, 0.04M acetic acid, 0.001M EDTA, pH 8.3) at 85 V for 90 min. Plasmids were visualized after staining with ethidium bromide under UV light. Conjugation for resistance transfer was performed on these strains with the sodium azide-resistant Escherichia coli J53 as the recipient. Selection of transconjugants was carried out on MacConkey agar containing meropenem (2 mg l⁻¹) and sodium azide (100 mg ml⁻¹) (Jamal et al., 2012).

Pulsed-field gel electrophoresis (PFGE). The relationship of the strains was studied by macrorestriction analysis of genomic DNA with XbaI (New England BioLabs). DNA fragments were separated by PFGE in a CHEF-DR III system (Bio-Rad). Electrophoresis conditions were pulse times ranging from 5 to 45 s for 20 h at 6 V cm⁻¹ at 14 °C. Restriction patterns were analysed following previously established criteria (Tenover et al., 1995).

RESULTS

Bacteria

The origins of carbapenem-resistant isolates are shown in Table 1. Fourteen carbapenem non-susceptible enterobacterial isolates were recovered during the study period, comprising nine K. pneumoniae, three Enterobacter cloacae and one K. oxytoca. They were isolated from medical wards (n=9), adult intensive care units (ICUs) (n=3), paediatric ICUs (n=1) and a surgical ward (n=1). The specimens were from the following sites: blood culture (n=4), urine (n=5), wound (n=4) and central venous pressure (CVP) tip (n=1). Most patients were male (n=9) and all were Kuwaiti nationals except for a single patient (serial no. 4) who was an Indian but was resident in Kuwait.

Antibiotic susceptibility

The susceptibility of the isolates is shown in Table 2. All isolates showed a high-level resistance (MIC >256 μg ml⁻¹) to ampicillin, amoxicillin-clavulanic acid, cefoxitin, cefuroxime, cefalothin, cefotaxime, cefepime and piperacillin. In addition, all isolates were highly resistant to imipenem, meropenem and ertapenem. These isolates were also resistant to gentamicin; however, 33% remained susceptible to amikacin. Nine isolates were resistant to ciprofloxacin, two to tigecycline and one to colistin (Table 2).

Prevalence of genes encoding carbapenem resistance

All isolates were phenotypically positive for MBL production by the three screening methods used. As shown in Table 1, 11 of the 14 isolates produced VIM-4 (six K. pneumoniae; three Enterobacter cloacae and one K. oxytoca). Four of these strains were isolated from blood cultures of patients with septicemia, five from urine cultures of patients with lower urinary tract infection, four from wound infection and one from a CVP infected tip. In addition, three K. pneumoniae isolates produced NDM-1. These three isolates were recovered from three patients, one being an Indian resident in Kuwait and the two others being Kuwaitis with no history of travel abroad. The three NDM-1-positive K. pneumoniae isolates co-produced β-lactamase CMY-4. Additional β-lactamases were identified among all the isolates studied, corresponding to CTX-M-15 (n=7 isolates), TEM-1 (n=7), SHV-31 (n=2), SHV-11 (n=6), SHV-12 (n=1) and SHV-26 (n=1), and two K. pneumoniae isolates produced β-lactamases of the OKP-type, which are variants of the naturally occurring SHV-type enzymes (Fevre et al., 2005).

Plasmid and VIM-4 gene transfer

Six carbapenem-resistant and VIM-4-positive (K. pneumoniae strains 1, 2, 4, 6, 8 and 11) were analysed for plasmids and transferability of the VIM-4 gene by conjugation. All strains carried plasmids whose numbers varied from three to five (Fig. 1), but no growth of transconjugants on selective medium was seen after conjugation with these six donor strains.
Table 1. Patient information, bacterial isolates and β-lactamase genes

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Hospital location</th>
<th>Bacterium*</th>
<th>Site of isolation</th>
<th>Date of isolation (month/y)</th>
<th>Patient age/gender</th>
<th>Patient outcome</th>
<th>Carbapenemase isolated</th>
<th>Associated β-lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medical ward</td>
<td>KKP1</td>
<td>Blood</td>
<td>5/2009</td>
<td>42 years/F</td>
<td>Died</td>
<td>VIM-4</td>
<td>CTX-M-15, TEM-1, SHV-31</td>
</tr>
<tr>
<td>2</td>
<td>ICU</td>
<td>KKP2</td>
<td>Wound</td>
<td>4/2009</td>
<td>51 years/M</td>
<td>Discharged</td>
<td>VIM-4</td>
<td>SHV-11, TEM-1, CTX-M-15</td>
</tr>
<tr>
<td>3</td>
<td>Surgical ward</td>
<td>KKP4</td>
<td>Blood</td>
<td>7/2009</td>
<td>61 years/F</td>
<td>Discharged</td>
<td>VIM-4</td>
<td>SHV-11, CMY-4</td>
</tr>
<tr>
<td>4</td>
<td>ICU</td>
<td>KKP5</td>
<td>Wound</td>
<td>8/2010</td>
<td>52 years/M</td>
<td>Died</td>
<td>NDM-1</td>
<td>CTX-M-15, TEM-1, OKP-B</td>
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<tr>
<td>5</td>
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<td>KKP6</td>
<td>Urine</td>
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<td>Died</td>
<td>VIM-4</td>
<td>OKP-A</td>
</tr>
<tr>
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<td>KKP8</td>
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<td>SHV-26</td>
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<td>KKP9</td>
<td>Wound</td>
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<td>93 years/M</td>
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<td>NDM-1</td>
<td>CMY-4, SHV-11</td>
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<td>KKP10</td>
<td>Wound</td>
<td>1/2011</td>
<td>67 years/M</td>
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<td>NDM-1</td>
<td>TEM-1, SHV-11, CMY-4</td>
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<tr>
<td>9</td>
<td>Paediatric ICU</td>
<td>KKP11</td>
<td>CVP tip</td>
<td>1/2011</td>
<td>4 months/F</td>
<td>Discharged</td>
<td>VIM-4</td>
<td>SHV-11, CTX-M-15, TEM-1</td>
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<tr>
<td>11</td>
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<td>KEC1</td>
<td>Blood</td>
<td>5/2009</td>
<td>42 years/F</td>
<td>Died</td>
<td>VIM-4</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Medical ward</td>
<td>KEC6A</td>
<td>Urine</td>
<td>2/2011</td>
<td>71 years/M</td>
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<td>VIM-4</td>
<td>CTX-M-15, SHV-12</td>
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<td>13</td>
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<td>90 years/F</td>
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<td>14</td>
<td>Medical ward</td>
<td>KKOX6A</td>
<td>Urine</td>
<td>2/2011</td>
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<td>Died</td>
<td>VIM-4</td>
<td>SHV-11, CTX-M-15, TEM-1</td>
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</table>

*KKP1, K. pneumoniae strain 1; KKP2, K. pneumoniae strain 2; KKP4, K. pneumoniae strain 4; KKP6, K. pneumoniae strain 6; KKP8, K. pneumoniae strain 8; KKP11, K. pneumoniae strain 11; KECL3, Enterobacter cloacae strain 3; KKOX6A, K. oxytoca strain 6A; KEC1, Escherichia coli strain 1; KEC6A, Escherichia coli strain 6A; KEC7, Escherichia coli strain 7.

Table 2. Antibiotic susceptibility of the 14 Enterobacteriaceae isolates to eight selected antibiotics

All isolates were resistant (MIC >256 μg ml⁻¹) to ampicillin, amoxicillin-clavulanic acid, cephalothin, cefoxitin, cefturoxime, cefotaxime, cefazidime, cefepime and piperacillin. ATM, aztreonam; CIP, ciprofloxacin; COL, colistin; ERT, ertapenem; IMP, imipenem; MER, meropenem; TIG, tigecycline; AMK, amikacin.

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>ATM</th>
<th>CIP</th>
<th>COL</th>
<th>ERT</th>
<th>IMP</th>
<th>MER</th>
<th>TIG</th>
<th>AMK</th>
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<tr>
<td>K. pneumoniae strain 1</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>0.25</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1.5</td>
<td>16</td>
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<tr>
<td>K. pneumoniae strain 2</td>
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<td>&gt;32</td>
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<td>&gt;32</td>
<td>&gt;32</td>
<td>1.5</td>
<td>16</td>
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<td>&gt;32</td>
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<td>8</td>
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<td>0.38</td>
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<td>&gt;32</td>
<td>16</td>
<td>&gt;256</td>
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<tr>
<td>K. pneumoniae strain 6</td>
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<td>&gt;32</td>
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<td>16</td>
<td>&gt;256</td>
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<td>0.75</td>
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<td>&gt;32</td>
<td>16</td>
<td>0.25</td>
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<td>&gt;32</td>
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<td>&gt;256</td>
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<td>&gt;32</td>
<td>16</td>
<td>&gt;256</td>
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<td>Escherichia coli strain 7</td>
<td>32</td>
<td>&gt;32</td>
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<td>32</td>
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<td>0.25</td>
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<td>K. oxytoca strain 6</td>
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<td>16</td>
<td>32</td>
<td>&gt;32</td>
<td>3</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>
**PFGE analysis**

PFGE of the isolates showed that only two isolates, KKP1 and KKP2, were closely related, whilst the rest were not (Fig. 2).

**DISCUSSION**

The emergence of carbapenem resistance due to carbapenemases in *Enterobacteriaceae* is considered a serious threat in the treatment of Gram-negative infections. Recent reports suggest the potential for travel-related infections due to organisms with carbapenemases, especially NDM-1 (Kumarasamy et al., 2011). Although a recent study in Kuwait reported that 0.8% of *Escherichia coli* isolates were resistant to meropenem (Al Sweih et al., 2010), carbapenems are still among the most active agents used in this country.

Reports of emerging carbapenemases among *Enterobacteriaceae* have been published worldwide including Middle East countries such as Kuwait (Jamal et al., 2012), Oman (Dortet et al., 2012), United Arab Emirates (Sonnevend et al., 2012), Iraq (Poirel et al., 2011b), Egypt (Poirel et al., 2013), Lebanon (El-Herte et al., 2012), Tunisia (Ktari et al., 2006), Turkey (Poirel et al., 2012) and Israel (Marchaim et al., 2008). In particular, two NDM-1-producing K. *pneumoniae* were identified in Kuwait between 2010 and 2011 (Jamal et al., 2012). The carbapenemases identified in the present study corresponded to VIM-4 and NDM-1, with VIM-4 being predominant. It is noteworthy that none of the patients who harboured carbapenemase-producing isolates had a history of recent travel outside Kuwait. Interestingly, a recent report identified a single VIM-4-producing *Enterobacter cloacae* isolate recovered in a patient in Abu Dhabi, United Arab Emirates (Sonnevend et al., 2012). This may suggest that the VIM-4 determinant could be widely disseminated throughout countries of the Arabian Gulf region. A study of six selected strains showed that the VIM-4 gene is not transferable by conjugation. This is in agreement with previous studies (Galani et al., 2005; Kassis-Chikhani et al., 2006). In the study of Galani et al. (2005) from Greece, they could not transfer imipenem and ceftazidime resistance from an *Enterobacter cloacae* isolate on repeated attempts by conjugation. Similarly, Kassis-Chikhani et al. (2006) in France could not transfer the VIM-1 gene encoding resistance to imipenem in seven *K. pneumoniae* isolates by conjugation. However, it is possible that these strains could potentially transfer resistance by transposons and integrons under the right circumstances where they are infected by these elements. In contrast, Ktari et al. (2006) reported integron-borne transferable VIM-4 genes in *K. pneumoniae* in Tunisia and Luzzaro et al. (2004) also reported plasmid-borne transferable VIM-4 genes in *K.

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**Fig. 1.** Plasmid contents of VIM-4-positive *K. pneumoniae* isolates. Plasmid preparations were analysed by agarose gel electrophoresis and visualized after staining with ethidium bromide. Lane M contained plasmids from *Escherichia coli* V517 as molecular-mass markers. Plasmid preparations from each strain were analysed in duplicate lanes. Lanes 1–6 contained samples from *K. pneumoniae* strains 1, 2, 4, 6, 8 and 11, respectively. The molecular masses of the reference plasmids are indicated on the left of the gel.

**Fig. 2.** PFGE patterns of VIM-4-positive *K. pneumoniae* isolates after digestion with XbaI. Lanes: M, λ ladder (Bio-Rad); 1, *K. pneumoniae* strain 1; 2, *K. pneumoniae* strain 2; 3, *K. pneumoniae* strain 4; 4, *K. pneumoniae* strain 6; 5, *K. pneumoniae* strain 8; 6, *K. pneumoniae* strain 11.
*pneumoniae* and *Enterobacter cloacae* isolates in a patient in Italy.

Our study showed that VIM-4- or NDM-1-producing isolates co-expressed ESBLs. This observation is in accordance with previous studies often reporting co-expression of MBL and ESBL genes among multidrug-resistant enterobacterial isolates (Ktari et al., 2006; Poirel et al., 2011a, 2012; Mataseje et al., 2012). Treatment of patients infected with these organisms is therefore challenging because of limited therapeutic options. Thirteen of our 14 isolates were resistant to β-lactam antibiotics including aztreonam. This resistance rate may be explained by the production of other ESBLs such as CTX-M-15 and SHV by these isolates. Additional production of CMY-4, an AmpC β-lactamase, may also have contributed to the high MIC of >256 µg ml⁻¹ for the β-lactam antibiotics. Similarly, a very high proportion of our isolates were resistant to ciprofloxacin and amikacin, a finding that may be explained by the fact that the resistant genes are often carried on the same transposon (Bratu et al., 2005). In our study, ticagrycine and colistin were active against most isolates (with the exception of two isolates resistant to ticagrycine and one isolate resistant to colistin). However, their therapeutic use is restricted to treating only infections caused by multidrug-resistant isolates, as colistin is nephrotoxic, and ticagrycine, being a bacteriostatic drug, is not suitable to treat bloodstream infections. In addition, ticagrycine is not excreted in the urine and therefore is not suitable for treating urinary tract infections.

Our PFGE study on the genetic relatedness of six *K. pneumoniae* isolates carrying the VIM-4 gene showed that, except for two isolates that showed a similarity in banding patterns, the banding patterns of the isolates were different. This showed that the isolates were largely not clonal.

In conclusion, Kuwait, like many other countries, is now facing the threat of the emergence of carbapenem-resistant *Enterobacteriaceae*. To our knowledge, this is the first report of VIM-4-producing species of the family *Enterobacteriaceae* in Kuwait.

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