Prevalence study of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking inducible ampC from Saudi hospitals

Baha Abdalhamid,1,* Samar Albunayan,1 Alaa Shaikh,1 Nasreldin Elhadi2 and Reem Aljindan3

Abstract

**Purpose.** Enterobacteriaceae encoding plasmid-mediated AmpC (pAmpC) β-lactamases confer resistance to the third generation cephalosporins. pAmpC association with extended spectrum β-lactamases (ESBLs), plasmid-mediated quinolone resistance (PMQR) and aminoglycoside modifying enzymes (AMEs) is well documented. There are limited data regarding the epidemiology and clinical significance of pAmpC in Saudi Arabia. This study aimed to determine the prevalence of pAmpC and its coexistence with ESBLs, PMQR and AMEs in Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis isolates in Saudi hospitals from January to December 2015.

**Methodology.** The VITEK 2 system was used for organism identification and susceptibility testing. PCR and sequencing were used to detect pAmpC, ESBL, AME and PMQR genes.

**Results.** Out of 3625 isolates of E. coli, K. pneumoniae and P. mirabilis, 200 cefoxitin-resistant isolates were identified, making the prevalence of cefoxitin resistance 5.5% (200/3625). CMY-2 and DHA were detected in 24 and 12 isolates, respectively. The prevalence of pAmpC was 1% (36/3625). In several isolates, pAmpC β-lactamases were associated with PMQR genes including aac(6′)-Ib-cr and qnrB and/or with AMEs including aacA4, aacC2, aadA1, aphA6, armA and rmtB genes. No ESBLs were detected in pAmpC β-lactamase-harbouring isolates.

**Conclusions.** To our knowledge, this is the first study determining the prevalence of pAmpC β-lactamases and their association with PMQR and/or AME genes in Saudi Arabia and the Gulf States. CMY-2 is the most prevalent pAmpC β-lactamase in this study. These data emphasize the importance of surveillance studies and implementation of antimicrobial stewardship programmes to reduce infections caused by such resistant organisms.

INTRODUCTION

β-Lactams are the most common antibiotics prescribed to treat infections caused by Gram-negative rods [1–3]. The production of β-lactamases is the most common antimicrobial resistance mechanism against β-lactams in Gram-negative bacilli [1, 2, 4]. AmpC β-lactamases confer resistance to penicillins, cephalosporins except ceftepime, and monobactams, and when coupled with porin mutation they can mediate resistance to carbapenems [1, 3]. They are resistant to cephamycins and are not inhibited by clavulante and other β-lactamase inhibitors [1, 4]. AmpC β-lactamases are either chromosomal or plasmid encoded [1]. Plasmid-mediated AmpC (pAmpC) β-lactamases are derived from chromosomes and have moved onto plasmids [1, 3]. pAmpC variants are classified into five groups based on their origins: EBC variants (ACT-1 and MIR-1), CIT variants (CMY-2), (FOX and MOX) variants, DHA variants and ACC variants, originating from Enterobacter spp., Citrobacter freundii, Aeromonas spp., Morganella morgani and Hafnia alvei, respectively [1, 2]. In contrast to the other pAmpCs, ACC variants are unique in being susceptible to cephemycins [1]. pAmpC has been mainly detected in Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis and to a lesser extent in Salmonella spp. and Shigella spp. [1–3].

Organisms encoding pAmpC β-lactamases can cause a wide variety of both nosocomial and community acquired infections including urinary tract infections, sepsicaemia; wound infections; meningitis and pneumonia [1, 5]. In addition, several outbreaks have been reported worldwide caused by pAmpC β-lactamase-harbouring organisms [1, 3]. These
infections not only increase morbidity and mortality but also are an infection control concern since these genes are plasmid-mediated and so can transfer from one organism to another [5, 6, 7]. These genes are often associated with other resistance determinants such as extended spectrum β-lactamases (ESBLs), plasmid-mediated quinolone resistance (PMQR) and aminoglycoside modifying enzymes (AMEs) which cause resistance to β-lactams, fluoroquinolones and aminoglycosides, respectively [3, 6, 7].

There are minimal data about the presence of pAmpC β-lactamases in Saudi Arabia and the other Gulf States. DHA-1 and CMY-2 were reported in Saudi Arabia while CMY-4 and CMY-6 were reported in the United Arab Emirates and Kuwait, respectively [8–11]. However, the prevalence has not been reported before from countries of the Gulf Cooperation Council. In addition, there are very limited data determining the association of pAmpC with other antimicrobial resistance genes. Therefore, the aim of this study was to determine the prevalence of and characterize pAmpC β-lactamases in medical centres in Saudi Arabia. It also aimed to detect other resistance determinants associated with pAmpC such as ESBLs, PMQR and AMEs.

METHODS

Bacterial identification and antimicrobial susceptibility testing

The study was carried out in a 450-bed general hospital, King Fahad University Hospital, Alkhobar and a 650-bed tertiary hospital, King Fahad Specialist Hospital, Dammam in the Eastern Province of Saudi Arabia. From January to December 2015, non-duplicate wound, blood, respiratory and urinary tract specimens were collected from patients admitted to both hospitals. The VITEK 2 system (bioMérieux) was used to identify E. coli, K. pneumoniae and Proteus mirabilis isolates as instructed by the manufacturer. In order to maximize the study population, when several organisms of interest were isolated from patient specimens even from different body sources, only the first isolated organism was included in the study. Therefore, there is only one organism from each patient. The VITEK 2 system was also used to determine antimicrobial susceptibility, cefoxitin resistance and ESBL production using the VITEK 2 AST-N291 card. Minimum inhibitory concentration (MIC) and breakpoints were determined using the criteria of the Clinical and Laboratory Standards Institute (CLSI). The following antibiotics were tested: cefoxitin (FOX), imipenem (IMP), meropenem, ertapenem, cefotaxime, ceftazidime, cefepime, trimethoprim–sulfamethoxazole, gentamicin, amikacin and ciprofloxacin. Susceptibility testing for piperacillin–tazobactam (TZP) was performed using E test strips (epilometer assay; bioMérieux). The following quality control strains were used: E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853.

PCR analysis and sequencing

Cefoxitin-resistant isolates were screened for pAmpC β-lactamases using the Philisa ampC ID kit (Streck Company) as recommended by the manufacturer. The kit detects the following pAmpC genes: CMY, DHA, FOX, EBC, ACC and MOX. In addition, an internal control is included to detect any PCR inhibition. Briefly, one or two colonies from a freshly prepared blood agar plate were suspended in 100 μl molecular grade water, incubated at 95°C for 15 s, and centrifuged at 15,000 g for 5 min. The PCR was conducted in a 25 μl reaction containing 1 μl supernatant as DNA template, 1.25 U PhilisaFAST DNA polymerase, 1× primer mix, 0.2 mM dNTP, and 3 μl of 10× FB1 buffer. The PCR protocol was as follows: a hot start of 96°C for 30 s followed by 30 cycles of 96°C for 5 s, 58°C for 10 s, and 72°C for 7 s, followed by a final extension of 72°C for 10 s. The kit contains DNA templates as positive controls for the tested genes. ESBL- and carbapenem-producing isolates were subjected to PCR using primers specific for TEM, SHV, CTXm, IMP, VIM, KPC, NDM-1 and OXA-48 as previously described [12, 13]. The five CTXm subgroups were amplified using the PCR method described by Woodford et al. [12]. For fluoroquinolone resistance, PMQR was tested using PCR to examine qnrA, qnrB, qnrC, qnrD, qnrS, aac(6’)-Ib-cr and qepA [13]. For aminoglycoside resistance, AMEs were tested by PCR including aacA4, aac6I, aacC2, adaA1, adaB, aphA6, armA and rmtB genes as previously described [13]. Positive controls and molecular grade water reactions were included in each PCR. The amplified PCR products were sequenced using the ABI 3730xl DNA Analyzer (Applied Biosystems).

RESULTS

A total of 3625 non-duplicate Enterobacteriaceae isolates (2263 E. coli, 1083 K. pneumoniae and 279 Proteus mirabilis) were isolated from patients admitted to either King Fahad Specialist Hospital, Dammam or King Fahad University hospital from January to December 2015. Among these isolates, 200 isolates (108 E. coli, 80 K. pneumoniae, and 12 Proteus mirabilis) were cefoxitin-resistant. Therefore, the prevalence of cefoxitin resistance was 5.5% (200/3625). These 200 isolates were collected from intensive care units (123 isolates) and from the other wards (77 isolates) (Table 1). These cefoxitin-resistant isolates were collected from 112 females and 88 males with a mean age of 49.9 years and ranging from 1 to 86 years old. The most common specimen source for these isolates was urine (132 isolates) followed by respiratory samples (32), blood (28), and then wounds (8) (Table 1). Using VITEK 2 AST-N291 and CLSI breakpoints, the highest resistance rate was for trimethoprim–sulfamethoxazole at 88% (176/200) followed by ciprofloxacin at 80% (160/200) and both cefotaxime and ceftazidime at 78% (156/200). The resistance rates for aminoglycosides were 53.5% (107/200) and 42% (84/200) for gentamicin and amikacin, respectively. The lowest resistance rates were associated with meropenem at 19% (38/200) and both imipenem and ertapenem were 24% (48/200).

A total of 36 out of 200 isolates encoded pAmpC β-lactamases (Table 1). CMY-2 was detected in 24 isolates while DHA was detected in 12 isolates making the...
prevalence of pAmpC 18 % (36/200) of cefoxitin-resistant isolates and 1 % of the total number of isolates. ESBLs were detected in 128 cefoxitin-resistant isolates. CTX-M15 was the most common ESBL detected and accounted for 84.3 % (108/128) of the ESBLs. CTX-M14, SHV-12 and SHV-5 were detected in 12, 5 and 3 isolates, respectively. No ESBLs were detected in the pAmpC-encoding isolates. OXA-48 and NDM-1 were detected in 32 and 15 isolates lacking pAmpC β-lactamases, respectively. No IMP, VIM or KPC genes were detected in these isolates.

Fluoroquinolone resistance was determined in 80 % of isolates tested in this study. A total of 128 isolates harboured aac6-ib-cr while 52 isolates encoded qnrB. aac6-ib-cr and qnrB genes were detected in 32 and 8 pAmpC-encoding isolates, respectively. The genes qnrA, qnrC, qnrD, qnrS and qepA were not detected in any isolate.

For aminoglycoside modifying enzymes, aacA4 was the most common gene, being detected in 112 isolates, followed by aacC2 and aadA1 in 95 isolates. In addition, aphaA6 and armA were detected in 80 and 77 isolates, respectively, while rmtB was detected in only 12 isolates. In the pAmpC-harbouring isolates, aacA1, aacC2, aadA1, aphaA6, armA and rmtB genes were detected in 16, 13, 12, 8, 7 and 4 isolates, respectively. Neither aacC1 nor aadA1 were detected in this study.

**DISCUSSION**

There is a lack of data regarding the prevalence and clinical significance of pAmpC β-lactamases in Saudi Arabia and other Gulf States. This study is an attempt to determine the prevalence of and characterize pAmpC in two Saudi hospitals. The prevalence of cefoxitin resistance is 5.5 % in our study which was conducted on hospitalized patients. The prevalence of cefoxitin resistance in the community has not been determined yet in Saudi Arabia. This prevalence rate is comparable to that reported in Egypt (5.8 %) [14]. It is slightly higher than that in Algeria (2.18 %) and Greece (2.6 %) [6, 15], and lower than rates in Korea (7.2 %), China (17 %) and India (12.8 %) [16-18].

The prevalence of pAmpC is mainly unknown due to the unavailability of valid and rapid tests to accurately detect pAmpC. There are no algorithms to detect pAmpC like the ones used for ESBLs and carbapenemases. Therefore, organisms encoding pAmpC are basically missed in routine testing in clinical microbiology laboratories without having effective measures and protocols to limit their spread in hospital settings. The pAmpC prevalence is 1 % in this study. Cefoxitin resistance in *E. coli*, *K. pneumoniae* and *Proteus mirabilis* was the selection criterion in this study. Based on this, it is possible to miss the detection of ACC pAmpC-producing organisms due to their susceptibility to cefoxitin which may affect the overall prevalence of pAmpC in our study. However, this is very unlikely because ACC variants have never been reported in Saudi Arabia, the Gulf States and the Middle East region. In support of this conclusion, it is well known that ACC β-lactamases are fairly uncommon and other AmpC studies used only cefoxitin non-susceptibility as their screening method [1, 19]. To the best of our knowledge, this is the first report in Saudi Arabia and the Gulf Cooperation Council determining the prevalence of pAmpC β-lactamases. The prevalence of pAmpC has been reported to be 0.59 % in Tunisia, 0.4 % in Denmark, 0.09 % in Canada, 1.3 % in the Netherlands, 12 % in Pakistan and 13.6 % in India [5, 15, 20–23]. The pAmpC prevalence rates have increased worldwide during the last decade, including increases from 0.07 to 0.4 % in New Zealand, 0.06 to 1.3 % in Spain, 2.6 to 9.3 % in China, 0.32 to 13.2 % in the United States, and from 0.6 to 4.3 % in Korea [1, 5, 7, 16, 17].

In contrast to pAmpC, several studies have reported the prevalence of ESBLs in Saudi Arabia and the other Gulf States [24–28]. The prevalence of ESBLs varied in Saudi Arabia based on the geographical location. It was estimated to be 30.6 % in Dammam (Eastern Province) compared to 55 % in Riyadh (Central Province) and 27.5 % in Abha (Southern Province) [24, 25, 27]. The estimated prevalence of ESBLs was reported to be 41 % in the United Arab Emirates and 31.7 % in Kuwait [26, 28].

Increasing rates of alimentary tract colonization with ESBL and AmpC β-lactamase-producing Gram-negative rods have been reported [11, 23]. Therefore, it is not surprising that the majority of infections caused by pAmpC-encoding organisms were urinary tract infections since the gastrointestinal tract is the main source of organisms causing such infections. CMY-2 like β-lactamase was the most common pAmpC detected in this study, which correlates with other studies reporting CMY-2 as the most common pAmpC type detected worldwide [1, 7]. The majority of isolates harbouring pAmpC were also resistant to fluoroquinolones.

**Table 1.** Study population and sample source of cefoxitin-resistant organisms

<table>
<thead>
<tr>
<th>Criteria</th>
<th>ICU patients</th>
<th>Non-ICU patients</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>123</td>
<td>77</td>
<td>200</td>
</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Female</td>
<td>73</td>
<td>39</td>
<td>112</td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>38</td>
<td>88</td>
</tr>
<tr>
<td>Sample source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>90</td>
<td>42</td>
<td>132</td>
</tr>
<tr>
<td>Respiratory</td>
<td>14</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>Blood</td>
<td>15</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Wounds</td>
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<td>4</td>
<td>8</td>
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<tr>
<td>Organism identification</td>
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<td></td>
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<tr>
<td><em>E. coli</em></td>
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<td>47</td>
<td>108</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>57</td>
<td>23</td>
<td>80</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>5</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>pAmpC β-lactamase</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CMY-2</td>
<td>15</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>DHA</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

ICU, intensive care unit
and/or aminoglycosides. PMQR genes and AMEs such as methyltransferases, rmtB or armA genes were harboured in several organisms encoding pAmpC β-lactamases [1, 3, 15, 29]. To the best of our knowledge, this is the first report of the association of pAmpC β-lactamases with PMQR genes and/or AMEs in Saudi Arabia and the Gulf Cooperation Council. This type of co-resistance has been also reported internationally in several studies. This indicates that very few therapeutic options are available to treat infections caused by the multiple-drug-resistant organisms which in turn increase morbidity and mortality.

The resistance determinant genes of pAmpC, PMQR and AMEs tested in this project are known to be encoded on mobile DNA elements, mainly plasmids. Multiple-drug-resistant organisms harbouring such plasmids are of major concern since these plasmids can transfer among organisms and patients in hospital settings causing nosocomial outbreaks and creating significant infection control challenges [1, 3, 7]. These challenges become more difficult given the fact that there are no standardized protocols to detect pAmpC-harbouring organisms. In addition, these organisms have been also isolated from farm animals such as dogs, food products, drinking water supplies, and river beaches [30, 31]. These sources can be reservoirs for organisms encoding pAmpC, contributing to their spread and acquisition in both the community and hospitals [5, 30, 31]. The lack of surveillance studies regarding the epidemiology of pAmpC β-lactamases and the analysis of risk factors associated with their acquisition are also important risk factors for their spread.

In conclusion, this is to our knowledge the first report determining the prevalence of plasmid-mediated AmpC β-lactamases in Saudi Arabia and the Gulf Cooperation Council. It is also the first report, we believe, of organisms encoding pAmpC β-lactamases with PMQR genes and/or AMEs. CMY-2 β-lactamases are the most prevalent pAmpC β-lactamases in Saudi Arabia. This study emphasizes the urgent need for more surveillance and epidemiological studies to be conducted for better understanding of pAmpC β-lactamases and guiding effective prevention control measures.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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