Antibiotic susceptibility patterns in CTX-M-15-producing *Enterobacteriaceae* isolated from healthy Afghan refugees in Iran

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Extended-spectrum β-lactamases (ESBL), including the most wide spread CTX-M-15 enzyme, are major antibiotic resistant mechanisms of *Enterobacteriaceae*. Emergence of this plasmid-mediated enzyme poses a global threat particularly in Asian countries struggling with war. In this study, we investigated CTX-M-15 in clinical isolates of *Enterobacteriaceae* from healthy Afghan refugees in Iran and analyzed the association between CTX-M-15 and pattern of antibiotic resistance as well as the location of this gene among the isolates. No correlation was found between clonal groups and antimicrobial resistance patterns of the *Enterobacteriaceae* species. The frequency of cephalosporin resistance was significantly higher among CTX-M-15-producing isolates compared with other ESBL-producing isolates (P<0.05) with 70 and 30 resistant isolates, respectively; however the association between CTX-M-15 and quinolone resistance was not significant as 4 isolates were quinolone resistant in both CTX-M-15-positive and other ESBL-producing group of isolates. All 20 tetracycline resistant isolates were CTX-M-15-positive (P<0.05) and resistance to aminoglycosides among CTX-M-15-positive isolates were considerably higher (n=22) than other ESBL-producing isolates (n=7) (P<0.05). Resistance to meropenem, imipenem, aztreonam, piperacillin and ampicillin was not significantly associated with CTX-M-15-production. Plasmid analysis revealed that the CTX-M-15 gene is located on a large plasmid ranged between 90 and 100 kb. This is amongst the premier report describing the association between CTX-M-15-production and different antibiotic resistance patterns in *Enterobacteriaceae* isolates collected from healthy individuals. The significant association between cephalosporin, aminoglycoside and tetracycline resistance and CTX-M-15-production emphasizes a need for introducing new antibiotic choices for the treatment of infections caused by *Enterobacteriaceae*.

**Key words:** Extended-spectrum β-lactamases (ESBL), CTX-M-15, *Enterobacteriaceae*, antibiotic resistance, plasmid.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) represent a major threat among *Enterobacteriaceae* species (Jemima and Verghese, 2008; Leflon-Guibout et al., 2004). ESBLs are capable of hydrolyzing β-lactam antibiotics, and are...
plasmid-mediated β-lactamases that are easily transferable among different bacteria (Nemec et al., 2004). The production of ESBLs in Enterobacteriaceae confers resistance to the most of cephalosporins that have been commonly used to treat gram negative bacterial infections (Poirel et al., 2002). Combination therapy with β-lactams and aminoglycosides is one of the alternative choices for the treatment of systemic infections caused by Enterobacteriaceae (Livermore, 1995). However, co-resistance to non-β-lactam antibiotics such as aminoglycosides is also frequent, by the co-location and thus co-transfer of the resistance determinants in the same genetic elements. The β-lactamases can also be associated with quinolone resistance (Pitout and Laupland, 2008). The main mechanism of quinolone resistance was previously attributed to chromosomal mutations; however, since 1998, various plasmid-mediated horizontally transferable quinolone resistance genes have been reported worldwide in clinical isolates of Enterobacteriaceae (Jemima and Verghese, 2008; Pitout and Laupland, 2008; Mac et al., 2010; Sidjabat and Paterson, 2015). The emergence of concomitant quinolone resistance in ESBL-producing Enterobacteriaceae is a public health issue because the inappropriate use of quinolones will not only promote quinolone resistance, but will also raise transfer of ESBL genes on the same plasmid. Thus, the recent worldwide increase in the ESBL-producing Enterobacteriaceae has resulted in a therapeutic dilemma, as the antibiotic choices are limited because of ESBL production.

In recent decades, the ESBLs of the TEM, SHV and CTX-M type have emerged as significant mechanisms of resistance in Gram-negative bacilli including Enterobacteriaceae species (Apisarnthanarak et al., 2008); however, the most widespread plasmid-mediated ESBLs nowadays are the CTX-M enzymes (Hernandez et al., 2011). The first CTX-M-type β-lactamases were identified as plasmid-encoded enzymes in clinical isolates from the Enterobacteriaceae (Bogaerts et al., 2007). Five different groups of CTX-Ms containing a total of 100 different types, have been described so far, among which, CTX-M-15 type which belongs to group 1 CTX-M enzymes, is the most widespread in Enterobacteriaceae strains (Jemima and Verghese, 2008).

Since the mid-1990s, CTX-M-positive strains have been identified in most parts of the world including Asia, Europe, North America and South America (Bogaerts et al., 2007). However, little is known about the CTX-positive strains available in the Middle East, particularly among individuals from war-torn countries such as Afghanistan, which can result in the emergence of multi-drug resistance (MDR) species that can spread even worldwide over time due to lack of sufficient health care supports in these countries.

In this study, we investigated the existence prevalence of CTX-M-15 gene in Enterobacteriaceae isolates from healthy Afghan refugees in Iran and analyzed the antibiotic susceptibility patterns in CTX-M-15-positive strains.

MATERIALS AND METHODS

Clinical samples and bacterial isolates

We conducted a prospective review of the electronic and manual medical records of nearly 2500 newly arrived refugees at 100 specialized primary care clinics for Afghan refugees in Iran between August 2010 to February 2013. Newly arrived refugees were defined as persons, other than travelers or tourists, who had arrived in the last one month (less than 30 days) to Iran from their usual residence; Afghanistan, living in camps specialized for Afghan refugees with the same living conditions. Data were drawn from the earliest available standardized screening test results recorded in refugees’ charts as part of routine care for the newly arrived refugees. 1112 refugees who were without any urinary tract infection (UTI) and enteric infection sign and symptoms, as well as any underlying illness and no previous antibiotic treatment during the last 6 months, were subjected to our study in order to investigate Enterobacteriaceae healthy carriers. 678 fecal and 434 urine (total: 1112) specimens were collected in the camps, delivered in sterile plates and processed within 2 h after sampling. At least one sample (fecal and/or urine) was taken from a single refugee. MacConkey agar and Eosin Methylene Blue (EMB) agar designed to selectively isolate Gram-negative and enteric bacteria were inoculated, incubated aerobically at 37°C and examined after 24 and 48 h. Enterobacteriaceae species were identified by the standard microbiological methods and biochemical tests (Shahid et al., 2011).

Antimicrobial susceptibility testing and confirmation of ESBL production

Antimicrobial susceptibility testing was performed on Muller Hinton agar (Trek Diagnostic System Ltd., West Sussex, United Kingdom) by the standard disk diffusion method as per Clinical Laboratory Standard Institution (CLSI) guidelines (Boyd et al., 2004). K. pneumoniae ATCC700603 was used as control. Additionally, the MICs of the antibiotics were determined by broth microdilution method according to the Clinical Laboratory Standard Institution (CLSI) guidelines, as well. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organisms. The antibiotic disks (Mask, UK) used and their MICs are shown in Table 1. ESBL detection was done by a disk diffusion method. The test inoculum (0.5 McFarland turbidity) was streaked on Muller Hinton agar. A disk of ceftazidime-clavulanic acid (30/10 µg/ml) was placed at a distance of 30 mm, center to center, from ceftazidime (30 µg/ml). A parallel experiment was carried out using cefotaxime-clavulanic acid (30/10 µg/ml) and cefotaxime (30 µg/ml) to ensure ESBL production. A ≥ 5 mm increase of the zone diameter for the clavulanic-supplemented disks compared with the zone diameter...
Table 1. MICs of CTX-M-15-positive and other ESBL-producing *E. coli* and *K. pneumoniae* isolates to various antibiotics tested

<table>
<thead>
<tr>
<th>Antibiotics Tested</th>
<th>MIC(µg/ml)</th>
<th>K. pneumoniae</th>
<th>E. coli</th>
<th>Resistant</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTX</td>
<td>Other ESBL-</td>
<td>CTX</td>
<td>Other ESBL-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>producing</td>
<td>positive</td>
<td>producing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>isolates</td>
<td></td>
<td>isolates</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>1</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>≥64</td>
<td>16</td>
<td>≥64</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≥16</td>
<td>8-16</td>
<td>≥16</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥32</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32</td>
<td>8</td>
<td>8</td>
<td>≤8</td>
<td>11</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>&lt;0.25</td>
<td>0.25</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>≥128</td>
<td>64</td>
<td>≥128</td>
<td>≤4</td>
<td>18</td>
</tr>
<tr>
<td>Cefepime</td>
<td>≥64</td>
<td>32</td>
<td>16</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;64</td>
<td>64</td>
<td>&gt;64</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;512</td>
<td>16</td>
<td>&gt;512</td>
<td>4-16</td>
<td>10</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;128</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>≥128</td>
<td>64</td>
<td>64-128</td>
<td>32</td>
<td>26</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≥128</td>
<td>≤2</td>
<td>32</td>
<td>≤2</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 2. Sequences of primers used for CTX-M-15 gene detection.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaCTX-M-15</td>
<td>CTX-15/28F</td>
<td>ATAAAACCGGCAGCGGTG</td>
</tr>
<tr>
<td></td>
<td>CTX-15/28R</td>
<td>GAAATTTGACGATCGGGG</td>
</tr>
</tbody>
</table>

Random amplified polymorphic DNA (RAPD) typing

In order to distinguish whether ESBL-positive isolates were identical or different clones, a RAPD PCR typing assay was performed. Two different primers were used for RAPD: 5’- CGGCAGCCAA- 3’ for *Escherichia coli* and 5’- CGTGGGCCT- 3’ for *Klebsiella pneumoniae*. Total bacterial DNA was extracted using AccuPrepTM Genomic DNA Extraction Kit (Bioneer, Daejon, South Korea). RAPD polymerase chain reaction (PCR) was performed in a 20 µl AccuPowerTM PCR PreMix (BioNeer) with 100 pmol of each primer as follows: initial denaturation at 95°C for 2 min, followed by 24 cycles of 92°C for 30 s, 40°C for 1 min and 72°C for 1.5 min, and a final elongation at 72°C for 10 min.

The experiments were repeated twice to assess reproducibility. The amplified DNA fragments were separated on 2% (w/v) agarose gels, stained with ethidium bromide and photographed under ultraviolet light. DNA fingerprints were compared by visual inspection. Similar isolates with the same banding pattern were assigned to the same RAPD type.

Plasmid analysis

For the analysis of plasmids, plasmid DNA was obtained from the isolates using QIAGEN plasmid Midi Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions to screen for plasmid-encoded CTX-M-15 gene. Plasmids were digested with *EcoRI* or *BamHI*, and the resulting restriction fragments were separated by electrophoresis in a 1% agarose gel.

Detection of CTX-M-15 by PCR

The PCR was done on the bacterial whole genome to detect the CTX-M-15 gene. The primers used for the detection of CTX-M-15 gene are shown in Table 2. Briefly, bacterial DNA was prepared by suspending one loop of fresh colonies in 500 µl of sterile distilled water and heating the mixture at 95°C for 10 min. The reaction was carried out in a total volume of 50 µl. The cycling conditions were: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 1 min; and a final elongation at 72°C for 10 min.

Sequencing of representative isolates

PCR products were sequenced by BioNeer Co., South Korea and results were investigated using MEGA-4 and Chromas 1.45 softwares. The nucleotide sequences results thus obtained were compared with sequences from Genbank nucleotide database at www.ncbi.nlm.nih.gov/blast.

Statistical analysis

The significance of association between CTX-M-15 gene and
antibiotic resistance was analyzed by Fisher test and a P value of <0.05 was considered significant.

RESULTS

Clinical specimens and bacterial isolates

Of 1112 fecal and urine specimens, obtained from 637 male (mean age: 31±3) and 475 female (mean age: 29±2) refugees, 138 samples (96 fecal and 42 urine) yielded growth of Gram-negative bacteria from which, 130 were identified as Enterobacteriaceae species (82 E. coli and 48 K. pneumoniae) using microbiological methods and biochemical tests. ESBLs were phenotypically detected in 100 isolates (9% of the total 1112 samples) using ceftazidime as well as cefotaxime as an ESBL inhibitor including 62 E. coli and 38 K. pneumoniae.

RAPD analysis

RAPD assay for 62 ESBL-positive E. coli isolates revealed 3 clonal groups representing ≥ 2 isolates with > 80% similarity. These three clonal groups represented 58 of the 62 isolates; 4 isolates represented a single unique pattern.

RAPD assay for 38 ESBL-positive K. pneumoniae isolates also revealed 2 clonal groups representing ≥ 2 isolates with > 80% similarity. These 2 clonal groups represented 36 of the 38 isolates; 2 isolates represented a single unique pattern.

Antibiotic susceptibility testing results

The antimicrobial susceptibility testing results for the 100 ESBL-producing isolates and the association between CTX-M-15-production and antibiotic resistance pattern is shown in Table 3. E. coli and K. pneumoniae clones did not differ significantly with respect to the number of antimicrobial resistance patterns (P= not significant). Among the 100 ESBL-producing isolates, 99 were susceptible to meropenem and only 1 CTX-M-15-positive, was found to be resistant (P= not significant). The same pattern was seen for imipenem. 10 CTX-M-15-positive and 3 other ESBL-producing isolates were resistant to aztreonam (P= not significant) whereas all 10 resistant isolates to piperacillin were CTX-M-15-positive (P= not significant). The frequency of cephalosporin resistance, especially for cefpodoxim with 26 resistant isolates, was significantly higher among CTX-M-15-positive isolates compared with the other ESBL-producing isolates (P<0.05); however, among both CTX-M-15 (n=4) and other ESBL-producing isolates (n=4), 8 were resistant to quinolone (P=not significant). High resistance to ampicillin was detected among CTX-M-15 (n=13) and other ESBL-producing isolates (n=27) (P=not significant) while resistance to aminoglycosides among CTX-M-15-positive isolates were considerably higher (n=22) than other ESBL-producing isolates (n=5) (P<0.05). All 20 tetracycline resistant isolates were CTX-M-15-positive (P<0.05).

Plasmid analysis and CTX-M-15 gene location

32 isolates (including 24 strains of E. coli and 8 strains of
K. pneumoniae) of the 100 ESBL-producers confirmed phenotypically, had CTX-M-15 gene detected by PCR. PCR revealed that the CTX-M-15 gene was located on a large plasmid. This large plasmid carried CTX-M-15 gene confirmed by gene sequencing of the representative isolates (accession number: KF723592.1 for E. coli and KF513160 for K. pneumoniae). The size of this large plasmid harboring CTX-M-15 gene determined by the analysis of the EcoRI and BamHI restriction enzymes were estimated to range between 90 kb and 100 kb for all CTX-M-15-positive isolates, as previously investigated from Bosnia and Herzegovina and India (Poirel et al., 2002).

**DISCUSSION**

The CTX-M family of ESBLs has been increasingly detected worldwide (Ma et al., 2009). Dominant emergence of CTX-M types has been observed in Asia (Sidjabat and Paterson, 2015; Eckert et al., 2004; Robicsek et al., 2006; Stiles et al., 1981); recently high prevalence of CTX-M-15 was reported from South India as an Asian country neighboring Middle East countries (Ensor et al., 2006). However, data on CTX-M epidemiology in war-torn Asian countries such as Afghanistan are scarce. In the present investigation, our data suggest that CTX-M-15 could be one of the most prevalent genes among ESBL-producing Enterobacteriaceae isolated from Afghan refugees in Iran according to the fact that CTX-M-15 was present in about one third of the 100 ESBL-producing isolates confirmed by DNA sequencing.

In this study, only 32% of the isolates demonstrated the presence of CTX-M-15 gene as opposed to that of 100 phenotypically detected ESBL-producing isolates and we speculate that some other ESBL genes are also prevalent in the Middle East bacteria which we did not look for in this study. The antimicrobial resistance of gram-negative organisms has built up progressively during the last few decades, leading to increased incidence of outbreaks of infections due to existence of multi-resistant bacteria (Poirel et al., 2002). This issue is of great concern in war-torn countries since disrupted health care systems in these countries may result in rapid spreading of the resistant bacterial strains and thus incidence of outbreaks of infections. We also demonstrate that CTX-M-15 gene was found to be encoded on large transferable plasmid that might contribute to the endemic outbreak of infections that may be faced in countries struggling with war.

Clinical isolates expressing CTX-M β-lactamases often display high resistance to cephalosporins. In this collection of isolates, 34 and 36 of the CTX-M-15-producing K. pneumoniae and E. coli were resistant to cephalosporins, respectively. CTX-M-15 belongs to the first group of the CTX-M β-lactamases with measurable ceftazidime hydrolysis that results in higher ceftazidime resistance (Soge et al., 2006); however, in our study resistance to cefpodoxim was higher among CTX-M-15-positive isolates. Thus, universal susceptibility to cephalosporins in Enterobacteriaceae is no longer guaranteed as a result of high resistance rates to different members of this group of antibiotics since decades (Nemec et al., 2004).

Molecular characterization of plasmids encoding CTX-M-15 from gram-negative strains involved in outbreaks in different countries has demonstrated that they additionally carried other antibiotic resistance genes (Bonnet, 2004; Kanamori et al., 2011; Karisik et al., 2006). Our results show that 22 of the CTX-M-15-producing isolates were resistant to aminoglycosides which is similar to reports from other studies (Mac et al., 2010; Apisarnthanarak et al., 2008; Lavollay et al., 2006; Strahilevitz et al., 2009).

There is a possibility that Enterobacteriaceae harboring CTX-M-15 gene have reduced susceptibility to quinolones than other ESBL-producing strains (Pitout and Laupland, 2008; Shahid et al., 2011), whereas in this study significant quinolone resistance among CTX-M-15-positive isolates was not seen compared with other ESBL-positive isolates.

We revealed in the present study that the frequency of tetracycline resistance among CTX-M-15-producing isolates was considerably higher as all tetracycline resistant isolates had CTX-M-15 gene; this finding is consistent with the hypothesis that CTX-M genes are associated with large plasmids that also carry tetracycline resistance genes (Hernandez et al., 2011; Dedec-Ljubovic et al., 2010; Lee et al., 2006).

We found no correlation between clonal groups and antimicrobial resistance patterns since quinolone susceptibility and tetracycline resistance was seen in all E. coli and K. pneumoniae clonal groups independently. This indicates that antimicrobial resistance patterns cannot be predicted from the clonal status of the Enterobacteriaceae species.

In conclusion, we identified CTX-M-15 in ESBL-producing Enterobacteriaceae from the Afghan refugees, and also found a significant association between CTX-M-15 and cephalosporin, aminoglycoside and tetracycline resistance. Our data emphasize the importance of detecting antimicrobial resistance genes such as CTX-M group particularly in Asian countries with disrupted health care systems due to war in order to promote appropriate antimicrobial therapy and effective infection control. Future regional epidemiological data on antimicrobial resistance throughout Asia, including the war-torn countries such as Afghanistan, Iraq and Syria, will be required to implement strict national antibiotics policies to restrict the spread of these resistance bugs.

**Conflict of interests**

No competing financial or personal interests exist in any
part of the study.

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Ethical considerations

Study design and study materials were proposed to committee of ethics of the corresponding university. All individuals were described in the study design, study methods and were all aware of risks and benefits of study. All individuals signed informed consent form. Ethical committee of medical research at “Shahid Beheshti University of medical sciences” approved the protocol.

REFERENCES


