Characterization of metallo-β-lactamase and extended-spectrum β-lactamases producing *Escherichia coli* isolates from urinary tract infections in southeast of Iran

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Abstract

**Background:** Urinary tract infection (UTI) is one of the most commonly encountered diseases in clinical settings and uropathogenic *Escherichia coli* (UPEC) is the major causative pathogen of UTI. The increase of antibiotic resistance among isolates of *E. coli* has become a main concern worldwide. The purposes of this study were to determine the phylogenetic background, prevalence and characterize of extended-spectrum β-lactamases and metallo-β-Lactamase produced by *E. coli* from UTIs. **Materials and Methods:** Two hundred and sixteen *E. coli* isolates were isolated from UTI. The isolates were screened to determine the phylogenetic background and prevalence of CTX-M-15, PER, VEB, IMP and VIM genes by PCR. The antimicrobial susceptibility of isolates was determined by disk diffusion and broth micro-dilution methods. The isolates were screened using a double-disc synergy test. **Results:** Phylotyping of isolates revealed that isolates segregated in phylo-groups A (40.74%), B1 (7.87%), B2 (18.05%) and D (33.34%). By disk diffusion test 61.57% of isolates were resistant to cefotaxime, 35.64% to ceftriaxone, 26.38% to aztreonam, 16.66% to cefepime and 6.48% to imipenem. Among the studied ESBL isolates, 72.41% isolates were positive for the CTX-M-15 gene. None of the isolates were positive for IMP, VIM, PER and VEB genes. **Conclusion:** The ESBL-producing strains were associated with shifts in phylogenetic distribution toward none-B2 phylo-groups and they mainly belonged to A and D groups. **Keywords:** *Escherichia coli*, Extended-spectrum β-lactamases, Metallo-β-lactamase, Urinary tract infection

Introduction

Uropathogenic *Escherichia coli* (UPEC) is involved in the spectrum of clinical syndromes as follows: asymptomatic bacteriuria, cystitis, pyelonephritis, urosepsis and infections in the central nervous, circulatory and respiratory systems (1). Urinary tract infections (UTIs) are extremely widespread outpatient problems among women that account for considerable morbidity, mortality and healthcare costs. The major aetiological agent of UTIs is well documented as *E. coli* (2). UTIs are also the most prevalent infection in long-term care facilities, where they account for 20–60% of all antibiotic prescription use (3). During the past decade, the increase of antibiotic resistance among isolates of *Enterobacteriaceae* both from community and health-care settings has become a main concern worldwide (4). *E. coli* is the most common producers of ESBLs and is responsible for many community and hospital-acquired infections (5). The presence of these enzymes compromises the efficacy of all β-lactams, except cephamycins and carbapenems, by hydrolysis of the β-lactam ring, and is inhibited by β-lactamase inhibitors (6, 7). Since the early 2000s, CTX-M enzymes have been increasingly detected, and these enzymes have now replaced other ESBLs such as TEM and SHV as the most common type of ESBL. CTX-M-producing *E. coli* is becoming increasingly involved in UTIs, especially among outpatients.
CTX-M-type ESBLs, particularly CTX-M-15 enzyme, have been involved in different epidemiological situations and have distributed all over continents (6). PER-1 (Pseudomonas extended resistance) is a clinically important enzyme with strong ESBL activity which can efficiently hydrolyze β-lactam ring. It has been found in several bacterial species from various geographic regions of Asia and Europe (8). Another enzyme that is somewhat related to PER-1 is the VEB-1 (Vietnamese extended-spectrum β-lactamase) β-lactamase. VEB-1 was first detected in E. coli isolate in a patient from Vietnam. The PER and VEB enzymes all confer resistance to oxymimino-cephalosporins, especially aztreonam and ceftazidime (9). Carbapenem resistance due to acquired carbapenemases has emerged in gram-negative bacilli and since the early 2000s spread in the worldwide (4). Metallo-β-lactamase (MBLs) enzymes are now widespread and found in Asia, Europe, Canada, Australia and South, and North America (10). Two major groups of MBLs have been described: IMP (Imipenemase) and VIM (Verona Imipenemase) enzymes. IMP-1 was the first identified acquired MBL. VIM variants are found throughout the world as well (11). Phylogenetic analyses have shown that E. coli strains belonged to four main phylogenetic groups (A, B1, B2, and D) and six phylo-subgroups (Aα, Aβ, B2α, B2β, Dγ, and Dδ). Strains that cause extra-intestinal infections, including pyelonephritis, cystitis, meningitis, and neonatal septicemia mostly belong to group B2 and, to a lesser extent, to group D, whereas most commensal and diarrheagenic strains belong to groups A and B1 (12, 13). Prevalence of antimicrobial resistance was shown to be greater in non-B2 phylogenetic group E. coli strains (14).

The goals of present study were to (i) analyze the distribution of phylogenetic group/subgroups, (ii) the occurrence of ESBLs (CTX-M-15, PER and VEB) and MBLs (IMP and VIM) genes and (iii) phenotypic characterization of antibiotic resistance E. coli isolates from UTIs cases in southeast of Iran.

Materials and Methods

Source of the E. coli isolates
From June to December 2013, two hundred and sixteen E. coli isolates were obtained from UTI samples of patients referring to the clinical laboratories of the Kerman province (southeastern), Iran. The samples were related to both female (n=184) and male (n=32). Their ages ranged from <5 years old (29), 5 to 15 years old (30), 15 to 40 years old (88) and 40 to 80 years old (69). Each sample was streaked on Mac Conkey agar and EMB plates (Biolife Laboratories, Milan, Italy) and incubated at 37 °C for 24 h. Bacterial colonies showing E. coli characteristics were submitted to Gram staining and were confirmed to be E. coli by using the biochemical and bacteriological tests. The confirmed E. coli isolates were stored in Luria–Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at -70 °C.

Phylogenetic group/subgroups
Several strains from the ECOR collection were used as positive controls for phylogenetic grouping: ECOR58 (B1 group), ECOR50 (D group), ECOR62 (B2 group) and E. coli strain MG1655 as a negative control for phylogenetic ECOR groups. The triplex PCR method developed by Clermont et al. was used to assign the E. coli isolates (12). Strains were categories to phylogenetic group/subgroups on the basis of presence or absence of the chuA, yjaA genes and an anonymous DNA fragment, TspE4.C2. Each phylo-group was subdivided as follows: chuA–, yjaA–, Tspe4.C2–, group A subgroup Aα; chuA–, yjaA+, Tspe4.C2–, group A subgroup Aβ; chuA–, yjaA–, Tspe4.C2+, group B1; chuA+, yjaA+, Tspe4.C2–, group B2 subgroup B2α; chuA+, yjaA+, Tspe4.C2+, group B2 subgroup B2β; chuA+, yjaA–, Tspe4.C2–, group D subgroup Dα; chuA+, yjaA–, Tspe4.C2+, group D subgroup Dβ. All the reference strains were from the bacterial culture collection of the Microbiology Department of Ecole Nationale Veterinaire Toulouse, France.

PCR assay
DNA from freshly grown overnight cultures of E. coli isolates was extracted by lysis method. ESBL-producing strains were screened by PCR for CTX-M-15 gene was performed with amplification conditions, as described Messai et al. (15), IMP and VIM genes were described by Garza-Ramos et al. (16). In addition, identification of VEB and PER genes was done as described by Udomsantisuk et al. (17), and Claeys et al. (18), respectively. The specific primers used for detecting sequences encoding MBLs, ESBLs and phylogenetic groups are presented in Table 1. PCR-amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide.

Disc diffusion method
The antimicrobial drug susceptibility of E. coli isolates was determined by a disc-diffusion method on Mueller–Hinton (MH) agar plates (BBL-Becton Dickinson), Clinical Laboratory Standards Institute (CLSI, 2013) guidelines (19). The following antimicrobial agents were used: cefotaxime (30 mg), ceftazidime (30 mg), cefepime (30 mg), imipenem (10 mg), aztreonam (30 mg). Quality controls were conducted using the reference strains E. coli ATCC 25922 and P. aeruginosa ATCC 27853.

Confirmatory for ESBL producing isolates
ESBL production was screened using a double-disc synergy test (DDST) as a standard disc-diffusion assay on MH agar. Discs containing cefotaxime (30 μg) and ceftazidime (30 μg) were placed at a distance of 30 mm (opposite sides) around discs containing cefotaxime / clavulanic acid (30/10 mg) and ceftazidime /clavulanic acid (30/10 mg) as recommended by the Clinical Laboratory Standards Institute (CLSI, 2013) guidelines. A
positive test result was defined as a ≥5 mm increase in zone diameter compared to a disk without clavulanic acid (19).

Results

Phylogenetic group/subgroups

PCR phylotyping indicated that the 216 E. coli isolates distributed into A 40.74% (88 isolates), B1 7.87% (17), B2 18.05% (39) and D 33.34% (72) phylogenetic groups. The results showed that the isolates belong to six phylo-subgroups, which mostly isolates fell into subgroup A0 with 27.31% (59 isolates) and D1 with 18.05% (39 isolates) (Table 2).

Phenotypic detection and antimicrobial resistance patterns

The most frequently observed resistance in E. coli isolates by the disc-diffusion method was to cefotaxime 61.57% (133 isolates), followed by resistance to ceftazidime 35.64% (77), aztreonam 26.38% (57), cefepime 16.66% (36) and imipenem 6.48% (4). Eight antibiotic resistance patterns were observed among the E. coli isolates, whereas 83 isolates were sensitive or intermediate for all antibiotics (Table 3). Phylogenetic background of antibiotic-resistant isolates demonstrated that these isolates mostly belonged to A (A0 phylogenetic subgroup) and D (D1 phylogenetic subgroup) groups (Table 3). Of 216 clinical samples, 58 (26.85%) isolates were producers of ESBL. The ESBL isolates mostly distributed into A (25 isolates) and D (20) phylogenetic groups, followed by B2 (11) and B1 (2) phylo-groups.

Detection of CTX-M-15, IMP, VIM, PER and VEB genes

Among the investigated ESBL isolates, 42 isolates (72.41%) possessed CTX-M-15 gene. The survey of ESBL isolates indicated that none of isolates were positive for IMP, VIM, PER and VEB genes. Forty-two CTX-M-15 positive isolates belonged to A (17 isolates), B1 (one), B2 (8) and D (16) phylogenetic groups.

Discussion

Resistance to β-Lactams (mainly extended-spectrum cephalosporins and carbapenems) has increasingly been reported worldwide with significant geographical differences in the epidemiology and prevalence of various types (11, 20). The European Antibiotic Resistance Surveillance System (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net) indicated a continuous increase since 2000 in pathogenic E. coli and K. pneumoniae isolates resistant to third-generation cephalosporins, with a prevalence of >10% for half of the 31 countries (6). E. coli strains segregate into four
main phylogenetic groups, termed A, B1, B2 and D. Previous studies in different parts of the world detect that group B2 and lesser extend D were the most frequent E. coli biotype in extra-intestinal diseases such as UTIs. Both groups have a higher prevalence of extra-intestinal virulence factors than the strains in the A and B1 groups (12-14, 21). The phylotyping results of the present study indicated that the highest prevalence of phylogenetic background was associated with A and D phylo-groups. Piatti et al. in Italy revealed that various geological areas affect the distribution phylogenetic background, antibiotic resistance and virulence factors of E. coli isolates (5). In a study in Iran phylogenetic groups A and D were predominant in E. coli isolated from UTI in Bam region (southeast of Iran) and also E. coli isolates resistant to antibiotics shifts to non-B2 phylogenetic groups (1). Another study, in Rigan area (southeast of Iran) phylogenetic analysis indicated that E. coli isolates mostly fell into phylogenetic groups B2 (42.22%) and D (33.33%), followed by B1 (15.56%) and A (8.89%) phylo-groups (22). Gordon et al. reported that the physiological, morphological and dietary differences that occur among human individuals of different sex or age may influence the distribution of E. coli phylogenetic groups (23). Grude et al. surveyed E. coli isolates from Norwegian and Russian of patients with significant bacteriuria; Russian isolates belonged to mostly to A phylo-group, whereas groups B2 and D were significant among the Norwegian isolates (24). In UTIs, resistant E. coli strains represented significantly

Table 2: Distribution of UTI E. coli isolates in detected phylo-group/subgroups

<table>
<thead>
<tr>
<th>Phylo-group</th>
<th>A no (%)</th>
<th>B1 no (%)</th>
<th>B2 no (%)</th>
<th>D no (%)</th>
<th>Total no (%)</th>
</tr>
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<tbody>
<tr>
<td>Phylo-subgroup</td>
<td></td>
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<tr>
<td>UTI isolates</td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>0</td>
<td>29 (13.42)</td>
<td>17 (7.87)</td>
<td>3 (1.39)</td>
<td>36 (16.66)</td>
</tr>
<tr>
<td>B1</td>
<td>59 (27.32)</td>
<td>17 (7.87)</td>
<td>3 (1.39)</td>
<td>72 (33.34)</td>
<td>216 (100.00)</td>
</tr>
<tr>
<td>B2</td>
<td>29 (13.42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D</td>
<td>216 (100.00)</td>
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</table>

Table 3: Distribution of antibiotic resistance patterns in detected phylo-group/subgroups

<table>
<thead>
<tr>
<th>Phylo-group</th>
<th>A no (%)</th>
<th>B1 no (%)</th>
<th>B2 no (%)</th>
<th>D no (%)</th>
<th>Total no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylo-subgroup</td>
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<tr>
<td>AZT, CAZ, CTX, FEP</td>
<td>6 (23.07)</td>
<td>2 (40.00)</td>
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<tr>
<td>AZT, CAZ, CTX, IMP</td>
<td>2 (50.00)</td>
<td>3 (37.50)</td>
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<td></td>
</tr>
<tr>
<td>AZT, CAZ, CTX, FEP</td>
<td>3 (37.50)</td>
<td>2 (25.00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZT, CTX, FEP</td>
<td>1 (100.00)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AZT, CAZ, CTX</td>
<td>4 (25.00)</td>
<td>2 (25.00)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AZT, CTX</td>
<td>2 (25.00)</td>
<td>1 (20.00)</td>
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<td></td>
</tr>
<tr>
<td>CAZ, CTX</td>
<td>34 (25.56)</td>
<td></td>
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<tr>
<td>CTX</td>
<td>37 (27.81)</td>
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AZT aztreonam, CAZ ceftazidime, CTX cefotaxime, FEP cefepime, IMP imipenem
isolates from Indian women -
- from polymicrobial -
- isolates -
- include VEB,
like from clinical specimens demonstrated that thirty-six in has increased in recent years, ranging from E. coli (Kerman province) found that from 94 ESBL positive other class A β-lactamases (29). Mobilized to plasmids almost ten times more frequently than previous study indicates that CTX-M genes are mobile among total ESBL enzymes are most probably associ-ated with high mobilization of the encoding genes. The activity of antibiotics against clinical isolates of ESBL producing E. coli by broth microdilution test in Spain showed that susceptibility of imipenem was 100%, ertapenem 95.7%, cefepime 80%, ceftazidime 67.8% and amoxicillin-clavulanate 81.7% (27). The results of double-disc synergy test indicated that 26.85% of isolates were producers of ESBL. Nasehi et al. in Iran found that resistance to ceftazidime and cefotaxime by disk diffusion test were 34.7% and 33.5% respectively (8). However, all K. pneumoniae strains were susceptible to imipenem. Eighty isolates showed MICs≥ 4 µg/ml for ceftazidime of which 96% were positive for ESBL production by a phenotypic confirmatory test. Another study revealed that 17% of the investigated E. coli isolates and 34.5% of K. pneumoniae isolates were ESBL production (17). The study of Lin et al. showed that most of the ESBL-producing E. coli isolates (98.6%) could be detected using cefotaxime discs with and without clavulanate (28). In Iran, the prevalence of ESBL producing strains of E. coli and K. pneumonia was 59.2% (8). In the present study, IMP, VIM, PER and VEB genes occurred at very lower frequencies and the predominance of CTX-M-15 indicates that this gene is common in patients with UTI in the Kerman province (southeastern), Iran. These higher rates of CTX-M among total ESBL enzymes are most probably associated with high mobilization of the encoding genes. The previous study indicates that CTX-M genes are mobilized to plasmids almost ten times more frequently than other class A β-lactamases (29). Kalantar et al. in Iran (Kerman province) found that from 94 ESBL positive E. coli isolates 23.4% isolates were positive for CTX-M gene (30). In Taiwan, the prevalence of ESBL producers has increased in recent years, ranging from 1.5 to 25.4% in E. coli (28). In Thailand, analyses of E. coli isolates from clinical specimens demonstrated that thirty-six E. coli isolates clinical producing carried TEM, CTX-M-like and VEB-like genes in 72.2%, 52.8%, and 16.7%, respectively (17). In addition, a number of β-lactamases include VEB, PER, GES-1, BEL, TLA, SFO and IBC, have already been reported in gram-negative bacte-ria with less prevalence (28). The possession of MBL genes is of particular concern for carbapenem resistance because they are able to hydrolyze most beta-lactams, such as imipenem and meropenem, drugs considered of reserve for the treatment of gram-negative pathogens. Therefore, the conclusive detection of the MBL-producing strains is necessary for the optimal treatment of infected patients and to control the nosocomial spread of resistance (31). The detection of resistance genes by PCR or similar techniques has limited utility, because only a few resistance genes are firmly relationship-with phenotypic resistance (eg, mecA, vanA, and vanB), whereas there are hundreds of β-lactamases, and numerous mutations, acquisitions, and expression mechanisms that result in resistance to other antibiotics (26). In conclusion, the results of the present study indicate that E. coli isolates, segregated into different phylogenetic group/subgroups, and the A and D phylogenetic groups represented the majority of strains involved in UTI. The resistant E. coli strains were associated with shifts in phylogenetic distribution toward none-B2 phylo-groups, in particular groups D and A. In addition, the results of this study revealed the lower prevalence of IMP, VIM, PER and VEB genes and higher frequency of CTX-M-15 gene in isolates. Although E. coli strains were distributed in some specific phylo-ge-netic background, the relationship was complex.

Acknowledgements

This study has an ethics approval from Hormozgan University of Medical Sciences, Bandar Abbas, Iran (Ethics code: HUMS.REC.1394.182).

Conflict of interest

The authors declare that there is no conflict of interest.

References


