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Original Article

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 ceftazidime, 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.

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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 founded 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 oxyimino-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 (A_0, A_1, $B2_2$, $B2_3$, D_1, and D_2). 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 A_0; *chuA*–, *yjaA*+, Tspe4.C2–, group A subgroup A_1; *chuA*–, *yjaA*–, Tspe4.C2+, group B1; *chuA*+, *yjaA*+, Tspe4.C2–, group B2 subgroup $B2_2$; *chuA*+, *yjaA*+, Tspe4.C2+, group B2 subgroup $B2_3$; *chuA*+, *yjaA*–, Tspe4.C2–, group D subgroup D_1; *chuA*+, *yjaA*–, Tspe4.C2+, group D subgroup D_2 (12, 13). 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 as 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. aerug*inosa 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

Table 1: Specific primers used in this study

Primer	Primer sequence (5′-3′)	Annealing temp (°C)	Product size (bp)
IMP	GGAATAGAGTGGCTTAATTC GCCAAGCTTCTATATTTGCG	58	275
VIM	GTGTTTGGTCGCATATCGC CGCAGCACCAGGATAGAAG	58	380
PER	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC	50	925
VEB	CCTTTTGCCTAAAACGTGGA TGCATTTGTTCTTCGTTTGC	56	216
yjaA	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	55	211
chuA	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	55	279
TspE4.C2	CTGGCGAAAGACTGTATCAT CGCGCCAACAAAGTATTACG	55	152

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 A_0 with 27.31% (59 isolates) and D_1 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 (A_0 phylogenetic subgroup) and D (D_2 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

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

Phylo-group	A no (%)		B1 no (%)	B2 no (%)		D no (%)		Total no (%)
Phylo-subgroup	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	
UTI isolates	59 (27.32)	29 (13.42)	17 (7.87)	3 (1.39)	36 (16.66)	39 (18.06)	33 (15.28)	216 (100.00)
Total Phylo-group	88 (40.74)		17 (7.87)	39 (18.05)		72 (33.34)		216 (100.00)

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

Phylo-group	A no (%)		B1 no (%)	B2 no (%)		D no (%)		Total no (%)
Phylo-subgroup	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	
AZT, CAZ, CTX, FEP	6 (23.07)	4 (15.38)	3 (11.53)	-	5 (19.23)	3 (11.53)	5 (19.23)	26 (19.55)
AZT, CAZ, CTX, IMP	2 (50.00)	-	-	-	-	1 (25.00)	1 (25.00)	4 (3.00)
AZT, CAZ, CTX, FEP	3 (37.50)	2 (25.00)	-	-	-	1 (12.50)	2 (25.00)	8 (6.01)
AZT, CTX, FEP	1 (100.00)	-	-	-	-	-	-	1 (0.76)
AZT, CAZ, CTX	-	2 (40.00)	-	-	1 (20.00)	2 (40.00)	-	5 (3.75)
AZT, CTX	3 (25.00)	2 (16.66)	2 (16.66)	-	1 (8.33)	2 (16.66)	2 (16.66)	12 (9.02)
CAZ, CTX	7 (24.13)	12 (41.37)	1 (3.44)	-	2 (6.89)	3 (10.34)	4 (13.79)	29 (21.81)
CTX	12 (25.00)	15 (31.25)	2 (4.16)	-	3 (6.25)	7 (14.58)	9 (18.75)	48 (36.10)
Total no (%)	34 (25.56)	37 (27.81)	8 (6.01)	-	12 (9.02)	19 (14.28)	23 (17.29)	133 (100.00)

AZT aztreonam, CAZ ceftazidime, CTX cefotaxime, FEP cefepime, IMP imipenem

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

low-virulence phylogenetic group none-B2 (14). Branger *et al*. demonstrates that production of ESBL among *E. coli* clinical strains was associated with shifts in phylogenetic distribution toward none-B2 phylo-groups, in particular groups D and A, which is similar to the results of the current study (14). Most of the ESBL-producing *E. coli* isolates fell into A and B1 phylo-groups, perhaps due to greater antibiotic exposure of group A/B1 strains in the fecal flora (25). The results of the disc-diffusion method revealed the increase resistance to β-lactams, which were considered as cefotaxime and ceftazidime, followed by resistance to aztreonam, cefepime, and imipenem. The disc-diffusion and broth micro-dilution methods provide accurate detection of common antimicrobial resistance mechanisms. However, newer or emerging mechanisms (eg, carbapenemases) of resistance require constant vigilance regarding the ability of each test method to accurately detect resistance (26). 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 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 ESBL-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 bacteria 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 antibiotic groups (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 phylogenetic background, the relationship was complex.

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Conflict of interest

The authors declare that there is no conflict of interest.

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