Original Article

Detection of ESBL- and AmpC-producing *E. coli* isolates from urinary tract infections

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Abstract

Background: Extended-spectrum β-lactamases (ESBLs) and AmpC enzymes have been observed in virtually all species of the family Enterobacteriaceae. The β-lactamase producing bacteria cause many serious infections, including urinary tract infections. These enzymes are predominantly plasmid mediated. There are no recommended guidelines for detection of this resistance mechanism and there is a need to address this issue as much as the detection of ESBLs. This study was undertaken to characterize ESBL and AmpC producers among *Escherichia coli* by polymerase chain reaction (PCR), which were initially screened by phenotypic method.

Materials and Methods: A total of 90 isolates of *E. coli* were recovered from the urinary tract during a 7-month period, and were screened for ESBLs and AmpC production by disk diffusion test using cefoxitin (30 μg) disks and confirmed by combined disk diffusion test using phenyl boronic acid. The presence of genes encoding CIT, FOX, and TEM was detected by PCR.

Results: On disk diffusion test, 59 of 90 isolates were resistant to third generation of cephalosporins; of these 37 (62.7%) and 3 (5%) were ESBL and AmpC producers, respectively. PCR showed that 29 (49.1%) and 3 (5%) were positive for $blaT_{EM}$ and bla_{CMY-2}, respectively.

Conclusion: ESBL- and AmpC-producing *E. coli* isolates cause significant resistance to cephalosporin. There is a need for a correct and reliable phenotypic test to identify AmpC β-lactamases and to discriminate between AmpC and ESBL producers. This work showed that boronic acid can differentiate ESBL enzymes from AmpC enzymes.

Key Words: AmpC, antibiotics, *Escherichia coli*, extended spectrum β-lactamase

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INTRODUCTION

Nosocomial infections caused by drug-resistant Gram-negative bacteria expressing extended-spectrum β-lactamases (ESBLs) pose a serious therapeutic challenge to clinicians due to limited therapeutic options.[1] Urinary tract infection (UTI) is the second most common type of infection in the body.[2] The most common cause of UTI is Gram-negative bacteria that belong to the family Enterobacteriaceae. Members of this family include *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Proteus*.[3] During recent years, infections caused by ESBL-producing organisms have been increasingly diagnosed in outpatients.[4] ESBLs were first identified in 1983 and often located on plasmids that are transferable from strain to strain and between bacterial species; most of the enzymes are members of TEM families,

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which have been described in many countries.[5-7] It is worth mentioning that ESBLs are enzymes capable of hydrolyzing and inactivating a wide variety of β-lactams, including third-generation cephalosporin, penicillin, and aztreonam, but are susceptible to β-lacatamase inhibitors such as clavulanate, sulbactam, and tazobactam.[8] The TEM was first reported in *E. coli* isolated from a patient named Temoniera in Greece.[9] Since that time, these have been identified worldwide and have been found in a number of different organisms, including *Klebsiella pneumoniae, Klebsiella oxytoca*, *E. coli*, *Proteus mirabilis, Enterobacter cloacae, Morganella morganii*, *Serratia marcescens*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Capnocytophaga ochracea*, *Citrobacter*, and *Salmonella* species.[10,11] Resistance to extended-spectrum cephalosporins can also be associated in *E. coli* with the production of plasmid class C β-lactamases, such as CMY-2 enzymes.[12] Plasmid-mediated AmpC β-lactamases represent a new threat since they confer resistance to cephamycins and are not affected by β-lactamase inhibitors. This resistance mechanism has been found around the world, can cause nosocomial outbreaks, and appears to be increasing in prevalence.[13] This study was undertaken to characterize ESBL and AmpC producers among *E. coli* by PCR, which were initially screened by phenotypic method.

MATERIALS AND METHODS

All 90 strains of *E. coli* were isolated from the urine culture of hospitalized patients (in three major hospitals in Zahedan, south-eastern Iran) who suffered from UTIs during the period 2011-2012. Each sample was streaked on the blood and MacConkey agar (Merck, Darmstadt, Germany) media and incubated at 37°C for 24 h. After incubation, *E. coli* isolates were detected by standard biochemical tests such as indole, methyl red, Voges-Proskauer, and citrate.

Antibiogram

Antibiotic susceptibility testing was performed by the Kirby Bauer method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) protocol.[14] In this method the bacteria were cultured on Muller-Hinton Agar plate then amoxicillin (25), tetracycline (30), trimethoprim-sulfamethoxazole (1.25 + 23.15), ceftazidime (30), ceftriaxone (10), gentamicin (10), nalidixin acid (30), difloxacin (25), and cefotaxime (30) disks (Himedia, Mumbai, India) were placed on the media in 20-30 mm with other disks. The plates were incubated for 18-24 h at 37°C.

ESBL screening

A 0.5 McFarland of test isolates was swabbed on Mueller-Hinton agar plates and ceftazidime (30 μg) and ceftazidime-clavulanic acid (30/10 μg) disks (Heimedia, India) were placed on the medium at a distance of 30 mm. Inoculated plates were incubated overnight at 35°C. An organism exhibiting zone size increase of 5 mm or greater around the ceftazidime-clavulanic acid disk compared to the ceftazidime disk was considered indicative of ESBL production.[15] *E. coli* ATTCC 25922 and *K. pneumoniae* ATTCC 700603 were used as control strains. In accordance with the CLSI criteria, isolates with resistance to cefoxitin were selected for further study.

AmpC screening

The boronic acid disk (Heimedia, Mumbai, India) test was used for AmpC screening by inoculating Mueller-Hinton agar by the standard disk diffusion method and placing a disk containing 30 μg of cefoxitin and another containing 30 μg of cefoxitin and 400 μg of boronic acid onto the agar surface. Inoculated plates were incubated overnight at 35°C. The organism that demonstrated 5 mm or greater zone around the disk containing cefoxitin and boronic acid compared to the disk containing cefoxitin was considered as AmpC producer.[16]

DNA extraction and PCR

DNA was extracted from colonies grown on agar medium using the (MBST, Tehran, Iran) extraction kit, following the manufacturer's instructions.

The specific primers and annealing temperatures used for amplifying the bla_{TEM}, bla_{CITM}, and bla_{FOX} genes by PCR are shown in Table 1. In this study, *K. pneumoniae* ATCC 7881 was taken as the positive control for bla_{TEM} expression, The PCR products were analyzed by agarose gels electrophoresis [Figure 1] and then the PCR product which was equivalent to expected amplification size in each cluster sent to Bioneer, Seoul, Korea for analyzing via sequencing.

RESULTS

Among the 90 isolates tested, 31 were susceptible to all antibiotics tested, including third-generation

Table 1: The primer sequences of the ESBL and AmpC genes amplified by PCR

Primer name	Sequence (5' to 3')	Expected amplicon size (bp)
TEM-F	GAGTATTCAACATTTCCGTGTC	848
TEM-R	TAATCAGTGAGGCACCTATCTC	
FOX-F	AACATGGGGTATCAGGGAGATG	190
FOX-R	CAAAGCGCGTAACCGGAT TGG	
CITM-F	TGGCCAGAACTGACAGGC AAA	462
CITM-R	TTT CTC CTG AAC GTG GCT GGC	

ESBL: Extended-spectrum β-lactamase, PCR: Polymerase chain reaction, Ampc: AmpC-type β-lactamases (Class C), TEM: Temorina, FOX: Cefoxitin, CITM: Cefotaxime hydrolyzing capabilities

cephalosporins. Of the remaining 59 isolates, 22 were resistant to cefoxitin and the remaining 37 were susceptible to it. ESBL phenotype was confirmed among all these 37 (62.7%) isolates by the combined disk diffusion (ceftazidime/ceftazidime-clavulanic acid). AmpC β-lactamase production was confirmed in 3 (13.6%) of 22 cefoxitin-resistant isolates and in the remaining (n = 19), it was not detectable. Antibiotic susceptibility of 90 *E. coli* isolates was evaluated for 10 antimicrobials. The majority of *E. coli* isolates were resistant to all 10 agents, including amoxicillin (93.3%), tetracycline (90%), cefixime (87.5%), nalidixic acid (85%), gentamicin (76.6%), trimethoprim (73.3%), difloxacin (72.2%), cefotaxime (62.2%), ceftriaxone (60%), and ceftazidime (54.4%) [Table 2].

PCR was performed on all 59 resistant isolates; the amplification of TEM, CITM, and FOX revealed 29 (49.1%), 3 (5%), and 0 (0%) isolates harbored the gene, respectively, and the rest of them were negative.

DISCUSSION

Resistance to β-lactam antibiotics of Gram-negative bacteria isolated from clinical samples has been increased worldwide.[17] For example, among 7054 *E. coli* samples collected between 1994 and 1996 in Barcelona (Hospital de la Santa Creu i Sant Pau), the prevalence of ESBL-producing strains was 0.14% (16). In 2001, this prevalence increased to 2.1%.[18] The prevalence of AmpC-producing *E. coli* isolates in Iran is not known due to limited number of studies and the difficulty that laboratories have in detecting the resistance mechanisms. The results of this study showed the prevalence of ESBL- and AmpC-producing *E. coli* to be 62.7% and 13.6%, respectively, by disk diffusion test, and 49.1% and 5% of the isolates harbored the genes encoding TEM and CITM with PCR method. The gene encoding FOX was not detected in any sample. In Iran, AmpC prevalence has been reported in *Klebsiella* spp. (5.95%) and *E. coli* (5.7%).[19,20] In another Iranian study, 3.3% of *E. coli* isolates produced AmpC β-lactamases.[13] A study from Canada showed that the annual incidence rates of AmpC were 1.7, 4.3, 11.2, and 15 per 100,000 residents for each year, respectively.[21] Jabeen *et al*. reported that the prevalence of the ESBL-producing *E. coli* and *K. pneumoniae* was 41% in *E. coli* and 36% in *K. pneumoniae* isolates.[22] The prevalence of the ESBL-producing organisms in Taiwan was in the range of 8.5-29.8% in *K. pneumoniae* and 1.5-16.7% in *E. coli*.[23] Behroozi *et al*. showed that 21% and 12% of *E. coli* and *K. pneumoniae* isolates, respectively, were ESBL producers in Tehran.[24] Feizabadi *et al*. reported that 72% *K. pneumoniae* strains isolated from Tehran hospitals were ESBL producing.[25] Tasli and Bahar and Al-Agamy *et al*. showed the rates of prevalence of ESBL-producing *K. pneumoniae* to be 57.1% and 55%, respectively.[26,27] Based on the criteria of CLSI, cefoxitin resistance is used a marker for detection of AmpC-producing isolates; but in our study, significant numbers of cefoxitin-resistant isolates were not positive for AmpC production, hence other mechanisms of resistance should be considered.[12] CMY-2 is the most prevalent of the plasmid-mediated AmpC enzymes in our hospitals. The rate of CMY-2 (5%) in this study was very similar to that reported in Spain and lower than that reported in Belgian hospitals.[28,29] ESBLs and AmpC β-lactamases were first described in 1983 (Germany) and 1988 (India), respectively.[30,31] Some ESBLs may fail to reach a level to be detectable by disk diffusion tests, but result in treatment failure in the infected patient. There is a need for a correct and reliable phenotypic test to identify AmpC ββ-lactamases and to discriminate between AmpC and ESBL producers. It seems necessary for clinicians and healthcare systems to be fully aware of ESBLs and AmpC-producing microorganisms. Also, the ESBLs and AmpC production monitoring is recommended to avoid treatment failure and for suitable infection control in Iran.

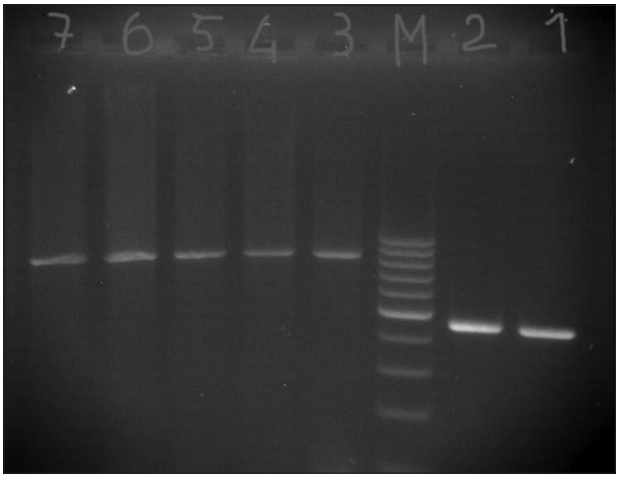

Figure 1: PCR amplification of *bla*TEM and *bla*CITM Lane M = 100 bp DNA marker, Lanes 1 and 2 = Clinical isolates expressing blaTEM, Lanes 3–7 = Clinical isolates expressing blaCITM

Table 2: Antimicrobial susceptibility of 90 strains of *E. coli*

Antimicrobial agent tested	No. (%)		
	Resistance	Intermediate	Sensitive
Ceftazidime	49 (54.4)	10 (11.1)	31 (34.4)
Ceftriaxone	54 (60)	17 (18.8)	19 (21.1)
Nalidixic acid	61 (67.7)	18 (20)	11 (12.2)
Amoxicillin	84 (93.3)	2 (2)	4 (4.4)
Cefotaxime	56 (62.2)	14 (15.5)	20 (22.2)
Cefixime	62 (68.8)	7 (7.7)	21 (23.3)
Gentamicin	69 (76.6)	15 (16.6)	6 (6.6)
Difloxacin	65 (72.2)	6 (6.6)	19 (21.1)
Tetracycline	81 (90)	3 (3.3)	6 (6.6)
Trimethoprim sulfamethoxazole	66 (73.3)	6 (6.6)	18 (20)

The increasing drug resistance of bacteria is the major cause of treatment failure of UTI. This study shows the necessity for a rapid and simple test based on CLSI recommendations and rational antimicrobial therapy.

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