Original article

Emergence of multiple β-lactamases produced by *Escherichia coli* clinical isolates from hospitalized patient in Kerman, Iran

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Abstract

Introduction and objective: *Escherichia coli* is a major cause of urinary tract and other opportunistic infections. Emergence of antibacterial resistance and production of extended spectrum β-lactamases (ESBLs) are responsible for the frequently observed empirical therapy failures. ESBLs producing bacteria and AmpC are serious threat in treating bacterial infections. Existence of various mechanisms which create resistance to antibiotics accounts for treatment failure in infections with these bacteria. The aim of this study was to determine the presence and the prevalence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{AmpC} β-lactamases genes in clinical isolates of *E. coli* in Kerman.

Materials and methods: Agar dilution method was used to determine the minimum inhibitory concentration of cefotaxime, ceftazidime and ceftizoxime in138 *E. coli* isolates. Resistance to imipenem, cefepime and cefoxitin was determined by disk diffusion method. Phenotypes of ESBLs and AmpC were also determined by combined disk method and non β-lactam inhibitor based method (boronic acid) respectively. PCR was used to determine $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{AmpC} genes in the ESBLs positive isolates.

Results: From 138 *E. coli* isolates 68.1% produced ESBLs by phenotypic method. Incidence rate of bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ among ESBL producing isolates were 63.8%, 51% and 23.4%, respectively. Presence of *bla*TEM, *bla*SHV and *bla*CTX-M genes at the sametime was detected in10.6% of isolates. Simultaneous production of AmpC and ESBLs was observed in 6.5 % of isolates. *bla*AmpC gene by PCR was seen in three isolates.

Conclusion: TEM and SHV β-lactamases are the dominant β-lactamases in this area. Simultaneous production of various β-lactamases in these isolates reflects the increased ability of these isolates against antibacterial agents and; therefore, this can cause serious problems in future in the treatment of infections especially nosocomial infections of such isolates.

Keywords: *Escherichia coli*, Antimicrobial resistance, Extended spectrum β-lactamase, AmpC β-lactamase, SHV, TEM

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Introduction

Nowadays, curing bacterial infections has faced several problems due to increased resistance to antibacterial agents. In this respect, isolates with multiple drug resistance phenotypes, ESBLs producers and AmpC β-lactamases, are of great importance [1]. Widespread use and abuse of antibacterial agents for curing infections among other factors may be responsible for the creation of multiple drug resistant bacteria [2]. *E. coli* is one of the most common bacteria which cause nosocomial infections especially urinary infections and meningitis in infants, and more than 85% of urinary infections are attributed to this organism [3].

One of the main mechanism of resistance to antibacterial agents is the appearance of β-lactamase enzymes. In most cases, β-lactamase causes bacteria to get resistant to a broad spectrum of antibiotics like fluoroquinolones, aminoglycosides and trimethoprime [4]. According to Ambler classification, these enzymes, most of which are called ESBLs, are divided into four main groups from A to D [5]. ESBL enzymes of CTX-M, TEM and SHV, from group A, have been widely reported to be produced by *E. coli*. These enzymes can hydrolyze ampicillin, carbenicillin, oxacillin and an extended spectrum of cephalosporins like ceftazidime and cefotaxime; and are usually encoded by plasmids [6].

AmpC β-lactamases are able to hydrolyse monobactams, cephamycins and cephalosporins and are not inhibited by ESBL inhibitors such a clavulanic acid [7]. Incidence of these resistant factors and multiple β-lactamase producers which are able to produce different types of ESBLs and AmpC can cause serious problems in future regarding the treatment of infections caused by this bacterium [8]. Most of encoding genes of resistant factors such as TEM, CTX-M, SHV and AmpC β-lactamases are on transferrable plasmids and can be transferred to other sensitive isolates. Therefore, determination of common β-lactamases genes which are among the main causes of resistant bacteria such as *E. coli*, is essential. It may reduce the emergence of more resistant strain, and the result can be a guide for the physician in the case of empirical therapy. The aim of this study was to determine the presence and prevalence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{AmpC} β-lactamases genes in clinical isolates of *E. coli* recovered from hospitalized patients in Kerman.

Materials and methods

Bacterial strains

Totally 138 consecutive nonduplicate *E. coli* were isolated from the clinical specimens including blood, urine and body fluids of patients admitted to three major hospitals (Afzali Poor, Kashani and Bahonar) located in three different regions in Kerman, southeast Iran, November 2007 to July 2008. The isolates were identified by their cultural characteristics and reactions to standard biochemical tests [9]. The majority of *E. coli* isolates (n=117, 84.8%) were recovered from urine followed by isolates from blood (n=13, 9.4%) and body fluids (n=8, 5.8%).

Antimicrobial susceptibility of E. coli isolates

Resistant to cefotaxime, ceftazidime and ceftizoxime antibiotics (Himedia, India) was tested using standard agar dilution method [10]. Minimum concentration of a drug which inhibited bacterial growth was considered as MIC. Resistant to imipenem (10μg), cefepime (30μg) and cefoxitin (30μg) (Himedia, India) antibiotics were determined using standard disk diffusion

method according to the recommendation of the Clinical and Laboratory Standard Institute (CLSI) [11].

Phenotype of ESBLs among E. coli isolates

To distinguish the isolates resistant to any of the third generation of cephalosporins, ESBL production was tested by the MAST combined disk method (MAST Chemical Co, England). The bacterial suspension was prepared by agar dilution method matching the 0.5 McFarland standards. Three sets of disks of the following antibiotics were used in this study: ceftazidime (30µg), ceftazidime (30µg) plus clavulanic acid (10µg), cefotaxime (30µg), cefotaxime (30 µg) plus clavulanic acid (10µg), and cefpodoxime (30µg), cefpodoxime (30µg) plus clavulanic acid (10µg) [12].

Muller Hinton agar (Himedia, India) was inoculated with the bacterial suspension, and ESBL detection disks were placed on the surface of agar. Diameter of inhibition zone was measured after 18-24 hours of incubation at 37°C. In accordance with the MAST instruction for ESBL detection, the following formula was used to determine the presence of ESBL in the test organisms:

$$\text{Diameter of Inhibition zone (mm)} = \frac{\text{ceftazidime}}{\text{Ceftazidime plus clavulanic acid}} =$$

≥ 1.5mm, Positive and ≤ 1.5mm, Negative

E. coli (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *K. pneumoniae* (ATCC 700603) were used as control in the susceptibility and ESBL production tests [13].

AmpC phenotype

AmpC phenotype is generally specified by means of combined disk method using cefoxitin disk (30µg) alone, and/or in combination with boronic acid (400µg). To prepare the combination disk, we added 120mg phenyl boronic acid (Sigma Co. USA) to 3ml Dimethyl sulfoxide (DMSO) (Merck, Germany). DMSO boronic acid solution was diluted with an equal volume of sterilized distilled water, and 20µl of this solution was added to cefoxitin disks (MAST Chemical Co., England).

The disks were then used after one hour of their preparation at room temperature. Cefoxitin and cefoxitin/boronic acid disks were placed on Mueller Hinton Agar plates (Himedia, India) inoculated with bacteria. According to CLSI standard, increased growth inhibition zone diameter around cefoxitin disk with boronic acid (≥5mm) is considered as AmpC β-lactamases in comparison with cefoxitin disk alone [14]. *E. coli* MK148 with bla_{AmpC} was used as a positive control [15,16]. Presence of bla_{AmpC} by PCR method was also tested to confirm the phenotypic tests.

Extraction and amplification DNA

Isolates producing ESBLs were subjected to polymerase chain reaction (PCR) targeting bla_{SHV}, bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{AmpC} genes. Genomic DNA was extracted by phenol/chloroform method [17]. PCR amplification was performed using the primers listed in Table 1, the primers were obtained from CinnaGen Inc., Iran.

PCR conditions were as follows: reactions were carried out in MWG thermo cycler in 25µl mixtures containing 12.5µl PCR Master Kit (CinnaGen Inc., Iran), 9.5µl sterile deionized water, 1µl template DNA and 1µl of each oligonucleotide primer. Initial denaturation at 95°C for 4min followed by 30cycles of denaturation at 95°C for 1min, annealing for 1min and at 48°C for TEM, and 60°C for SHV, CTX-M and AmpC, extension at 72°C for 1min. The final extension step was extended to 10min at 72°C for all genes [16,19,20].

Table 1: Primers used for amplification

Target	Primer sequence	Product size (bp)	Reference
SHV-F	5′-AAG ATC CAC TAT CGC CAG CAG-3′	200	[18]
SHV-R	5′-ATT CAG TTC CGT TTC CCA GCG G-3′		
TEM-F	5′-GAG TAT TCA ACA TTT CCG TGT C-3′	800	[18]
TEM-R	5′-TAA TCA GAG GCA CCT ATC TC-3′		
AmpC-F	5'- ATG CAA CAA CGA CAA TCC ATC-3′	1150	[16]
AmpC-R	5'- GTT GGG GTA GTT GCG ATT GG-3'		
CTX-M/F	5'- CGC TTT GCG ATG TGA AG-3′	550	[19]
CTXM/R	5'- ACC GCG ATA TCG TTG GT-3′		

The PCR products were separated on 1.2% agarose gels (Sigma, Co. USA) in TBE 1X (Tris/borate/EDTA) buffer. Bands were visualized under UV gel documentation after being stained with ethidium bromide (Merck, Germany) and photographed. *E. coli MK148* carrying the AmpC gene [15,16] and *K. pneumoniae* ATCC 700603 carrying the CTX-M, SHV and TEM genes [13], were used as positive controls for DNA amplification of CTX-M, TEM, SHV and AmpC specific primers.

Results

Antibacterial resistance pattern and minimum inhibitory concentration (MIC)

Using disk diffusion method, all the isolates were found to be susceptible to imipenem and susceptibility to cefepime and cefoxitin was found in 56(40.5%) and 87(63%) of the isolates, respectively. Ceftazidime has the highest level of resistance and high rate of MIC (Table 2).

ESBLs and AmpC β-lactamases production

Of 138 clinical samples, 94 (68%) isolates were producers of ESBL using combined disk method (Fig. 1). By phenotypic method 9(9.6%) of ESBL positive isolates produced AmpC (Fig. 2).

Detection of ESBLs genes by PCR

The rate of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ in the isolates were 60(43.5%), 48(34.8%) and 22(15.9%), respectively. In 32(23.1%) of the isolates, both bla_{TEM} and bla_{SHV} were detected. Simultaneous appearance of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ was observed in 10(7.2%) of isolates. The PCR product bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ are shown in figures 3-5. From nine isolates producing AmpC β-lactamases by phenotypic method in three isolates, AmpC production was positive by molecular method. Simultaneous appearance of bla_{TEM}, $bla_{SHV,}$ $bla_{CTX\text{-}M}$ and bla_{AmpC} was observed in two isolates, one from urinary tract infection and the other one from blood culture. PCR detection of these isolates is presented in figure 6.

Table 2: Antibacterial resistant pattern of 138 *E. coli* isolates to the tested agents

Antimicrobial agents	Resistance No (%)	MIC (µg/ml)		MIC range (µg/ml) Minimum/Maximum
		MIC_{50}	MIC_{90}	
Ceftizoxime	23 (16.6%)	16	64	32/128
Cefotaxime	51(37%)	64	128	32/1024
Ceftazidime	93(67.3%)	128	512	32/1024

Fig. 1: Broad spectrum β-lactamases producing *E. coli*: (A=Cefotaxime B=Cefotaxime + Clavulanic acid C=Ceftazidime D=Ceftazidime + Clavulanic acid) Clavulanic acid has inhibited the broad spectrum β-lactamases with the formation of growth inhibition zone

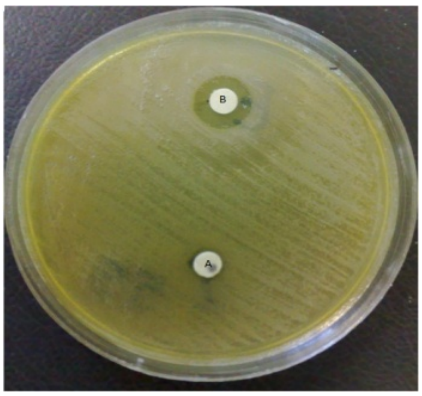

Fig. 2: AmpC β-lactamases producing *E. coli*: (A=Cefoxitin, B=Cefoxitin + Boronic acid) Boronic acid inhibits β-lactamases activity

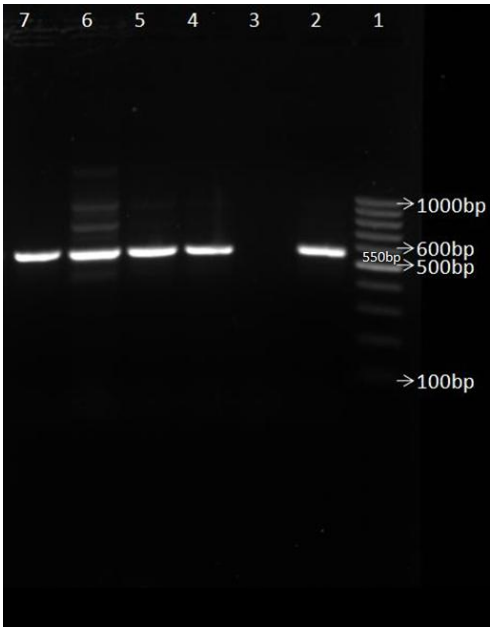

Fig. 3: Electrophoresis of PCR products for specifying CTX-M broad spectrum β-lactamases genes. No.1: DNA Marker (100bp), No 2: positive control for $bla_{\text{CTX-M}}$, No. 3: negative control No. 4, 5,6,7 isolates with CTX-M (550bp) gene

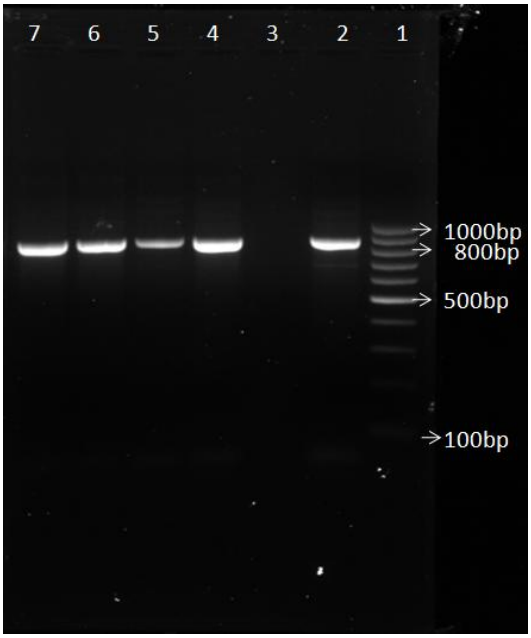

Fig. 4: Electrophoresis of PCR products for specifying TEM broad spectrum β-lactamases genes. No.1: DNA Marker (100bp), No 2: positive control for bla_{TEM}, No. 3: negative control, No. 4,5,6,7 isolates with TEM (800bp) gene

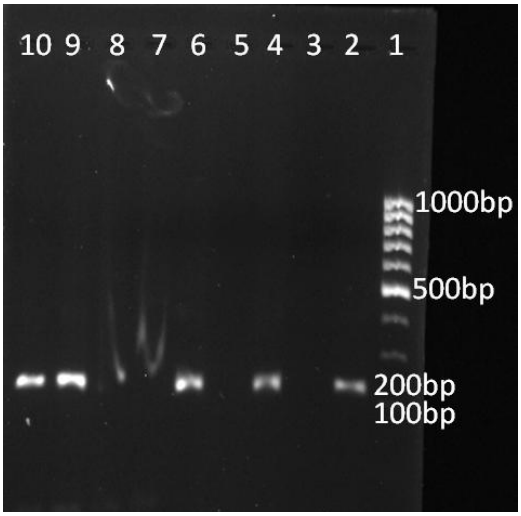

Fig. 5: Electrophoresis of PCR products showing SHV broad spectrum β-lactamases genes. No.1: DNA Marker (100bp), No 2: positive control for *bla*SHV, No. 3: negative control, No. 4,6,9,10 isolates with SHV (200bp) gene, No.5,7,8. Isolates negative for SHV beta-lactamase gene

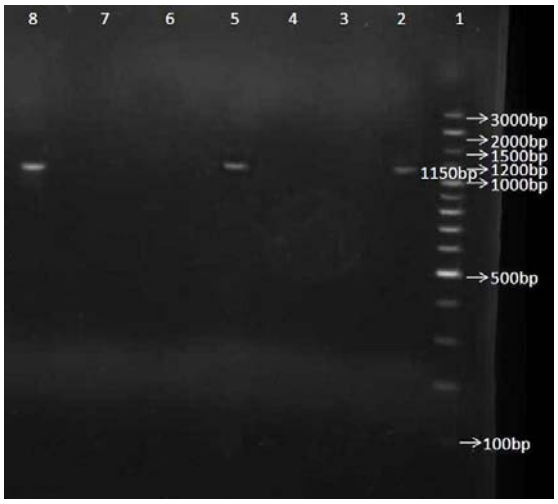

Fig. 6: Electrophoresis of PCR products for showing AmpC broad spectrum β-lactamases genes. No.1: DNA Marker (100bp), No 2: positive control for *blaAmpC*, No. 3: negative control, No. 5,8 isolates with AmpC (1150bp) gene, No.3,4,6,7 Isolates negative for AmpC beta-lactamase gene

Discussion

Escherichia coli is among the most important causes of nosocomial infections especially neonatal meningitis and urinary tract infections [21]. Unfortunately, extensive use of antibiotics is the cause of resistance phenomena, and treatment of these infections especially nosocomial infections faces a serious problem.

In our study, imipenem was found to be the most active agent, while resistance to ceftazidime was high. Although resistance to ceftazidime is reported to vary in different areas, and imipenem resistance is still very rare around the world including Iran [20,22]. In this study, prevalence of ESBLs in *E. coli* isolates was 68%, which is slightly higher than the ESBL production in clinical isolates of *E. coli* reported in Tehran (45.2%, 59.5%) [23,24].

Our results showed that the prevalence of ESBLs production is higher in Iran compared to developed countries, for example ESBL production in *E. coli* isolates from Australia, Italy and USA were reported to be 2.1%, 1.59% and 2.8%, respectively [25-27]. In countries like Turkey 17% and in Egypt 60.9% of *E. coli* were positive for ESBL production [28,29]. These could be due to the fact that in more developed countries effective strategies for the control of antimicrobials are present, which effectively prevents the emergence of ESBLs.

In this study, the most common β-lactamases gene among body fluids, urine and blood samples was TEM (43.5%) followed by SHV (34.8%). No meaningful relationship was observed between appearance of TEM or SHV β-lactamases and resistance to tested antibiotics. TEM β-lactamases in urinary isolates of *E. coli* in Tehran is reported to be 40% which is similar to our finding which is 41% but

SHV β-lactamases is in contrast to our study and is much higher in this area (8% and 33%, respectively) [16].

Eftekhar *et al.* [16] in Tehran reported the AmpC type β-lactamases to be 4% in urinary isolates of *E. coli*, which is not much higher than this study and showed that AmpC type β-lactamases were not high in the studied areas. In Algeria and England the prevalence of AmpC was reported to be 1% and 7.15 %, respectively [30,31]. Diagnosis of AmpC producing isolates is facing a serious problem because AmpC β-lactamases are not inhibited by ESBLs inhibitors like clavulanic acid and sulbactam [32].

AmpC type β-lactamases are cephalosporinase which are able to hydorolyse all β-lactamases to some extent. Both ESBL and AmpC β-lactamases may co-exist and therefore their detection is difficult because they mask each other. Boronic acid disk test is a practical and efficient method to detect AmpC β-lactamases [13]. In this study, we only used one type of primer for AmpC, and the phenotypic methods used may be more reliable than molecular method for AmpC detection.

Cefotaximases, CTX-M β-lactamases are reported to be quite common in the members of *Enterobacteriaceae* family in European countries around the world [31]. However, in the present study, they were not as common as TEM or SHV type β-lactamases. There was a significant correlation between presence of CTX-M gene and resistance to cefotaxime and ceftizoxime in our isolates ($p<0.05$). In a study by Mirzaee *et al.* [33] in Tehran at 2007 all the *E. coli* isolates were positive for CTX-M, which is not much higher than our study (23.4% compared to 15.9%).

In conclusion, our study like other similar studies in Iran showed a high prevalence of ESBLs in Iran. Fortunately, AmpC β-lactamases which are very important and usually are not detected in clinical laboratories are still low in this area. CTX-M type β-lactamases are emerging type of ESBLs in European countries but in this area they were not as common as TEM or SHV type β-lactamases. Detecting the prevalence of other kind of ESBLs in enteric bacteria and gram-negative non-enteric bacilli in the hospital isolates is necessary in the area.

Conclusion

We must be conscious about administration and consumption of antibiotics in the treatment of infections especially nosocomial infection, in order to prevent the emergence of multi β-lactamases producing isolates and to reduce the chance of mutations in β-lactamases genes by reducing the antibiotics consumption.

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