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ORIGINAL ARTICLE

Detection of extended-spectrum β-lactamases (ESBLs) in Escherichia coli

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ABSTRACT
Background: Escherichia coli (E. coli) species are able to produce extended-spectrum β-lactamases (ESBLs) that cause high resistance to the beta-lactam antibiotics. Therefore, determining the antibiotic susceptibility patterns in resistant organisms is necessary for suitable therapeutic approaches.
Patients and methods: Totally, 260 clinical isolates of E. coli were collected from hospitals in Tehran during April-2006 to April 2007. All suspected isolates were screened by disk diffusion method and the production of ESBL genes was investigated by phenotypic confirmatory tests. Microbroth dilution method was applied to determine the MIC of ceftazidime. Subsequently, isolates showing $MIC_{CAZ} \geq 2\mu g/ml$ were subjected to PCR targeting bla_{TEM} and bla_{SHV} genes.
Results: Forty-nine percent of isolates contained ESBLs, among which 73.6% and 85.6% were ceftazidime- and cefotaxime-resistant, respectively. Molecular analysis showed 11.2% and 46.4% of ESBL producing isolates contain bla_{SHV} and bla_{TEM} genes, respectively.
Conclusion: Results revealed high percentage of ESBL genes among the clinical isolates of *E. coli*. Since the ESBL genes were detected in resistant isolates, it's necessary to test all isolates showing reduced susceptibility to third-generation cephalosporins. The isolation of patients infected with ESBL producing isolates can be useful in controlling associated outbreaks.

Keywords: *Escherichia coli, extended-spectrum β-lactamases, resistance.*
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INTRODUCTION

The β-lactams, such as penicillins, cephalosporins and carbapenems are the most commonly used antibiotics for treatment of nosocomial infections (1). In response to widespread use of β-lactams, bacteria have acquired the ability to produce β-lactamases that are capable to hydrolyze and inactivate β-lactam containing antibiotics (2). During the last century, bacteria have evolved the mechanisms to resist against antibiotics. Resistance to β-lactam antibiotics may be occurred as a result of permeability barriers, efflux pumps, altered penicillin binding proteins, AmpC-type β-lactamases and other products of β-lactamases (3). The production of beta-lactamase is the single most prevalent mechanism responsible for resistance to beta-lactams among clinical isolates of the family Entrobacteriaceae. Extended-spectrum β-lactamases (ESBLs) are clavulanate susceptible enzymes conferring broad resistance to penicillins, aztreonam and cephalosporins (with exception of

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cephamycins) and are detected most commonly in klebsiella pneumoniae and Escherichia coli (3). In recent years, there has been an increased incidence and prevalence of ESBLs enzymes with altered hydrolysis profiles, most of which are derived from the broad-spectrum beta-lactamases TEM-1 and SHV-1 (3). ESBLs are mostly plasmid mediated, and are mutants of the classic TEM and SHV enzymes (class A), with one or more amino acid substitutions around the active site (4).

In the present study, we detect ESBLs among the E. coli isolates cultured from in-patients. Meanwhile, determination of susceptibility patterns against third-generation cephalosporins and detection of bla_{SHV} and bla_{TEM}, two beta-lactamase genes, in ESBL producing isolates were also targeted.

PATIENTS and METHODS

Totally, 260 clinical isolates were cultured from inpatients and were assigned to species level by conventional bacteriological tests (5). Each isolate was obtained from a different patient, among whom 56% were females and 44% were males. All isolates were gathered during April 2006 and April 2007 from urine, wounds, stools, abscesses and sputum.

Susceptibility of isolates against various antibiotics including ceftazidime (CAZ: 30µg), cefotaxime (CTX: 30µg), carbenicillin (CB: 100µg), ceftriaxone (CRO: 30µg), ceftizoxime (ZOX: 30µg), piperacillin (PC: 100µg), piperacillin/tazobactam (PT: 110µg), ciprofloxacin (CIP: 5µg), amikacin (Ak: 30µg), gentamicin (GM: 10µg), and imipenem (IMP: 10µg) (MAST) was determined by disk diffusion method (CLSI). Those suspected for ESBL production, were screened for testing by phenotypic confirmatory test (CLSI). In brief, the antibiotic disks containing ceftazidime (30µg), cefotaxime (30µg), ceftazidime/clavulanic acid (30+10µg) and cefotaxime/clavulanic acid (30+10µg) were placed on the inoculated plates containing Muller Hinton agar. A positive test result defined as a ≥5 mm increase in zone diameter compared to a disk without clavulanic acid (6). Then the susceptibility patterns of ESBL producers to ceftazidime (CAZ: 30µg), cefotaxime (CTX: 30µg), ceftriaxone (CRO: 30µg), ceftizoxime (ZOX: 30µg) and imipenem (IMP: 10µg) were investigated by disk diffusion method (CLSI). Ceftazidime MIC was determined by Microbroth dilution method (7). Pseudomonas aeroginosa (ATCC 27853) and Escherichia coli (ATCC 35218) were used as controls (8). Finally, the isolates with ESBL positive phenotypes and MIC_{CAZ} ≥2µg/ml were selected for molecular detections.

DNA extraction and PCR: Isolates were cultured in LB broth at 37˚C overnight and total DNA was extracted according to the published method of Hammond et al (9). PCR condition was the same as described by Howard et al. (10). Table 1 represents PCR condition and primers used for detection of genes blaSHV and blaTEM.

Table 1. PCR condition and primers used for detection of genes blaSHV and blaTEM

Primers	Sequence (5' to 3')	Gene	Molecular weight	Annealing temperature (1min/35 c)
TEM-A	GAGTATTCAAC ATTTCCGTGTC	bla_{TEM}	800bp	45
TEM-B	TAATCAGTGAG GCACCTATCTC			
SHV-A	AAGATCCACT ATCGCCAGCAG	bla_{SHV}	200bp	60
SHV-B	ATTCAGTTCC GTTTCCCAGCGG			

A typical 25 µm PCR reaction mixture for every primer set consisted of 1x PCR raction buffer (Fermentase, Lithuania), 0.8µm Mgcl2 (25mM), 0.5µm of each deoxynucleotid (dNTP) (10mM), 0.5µm of each primer, 1U of Taq DNA polymerase 5U/µl (Fermentase, Lithuania) and 10µm of DNA template. The cycling conditions were an initial denaturation at 94°C for 3 minutes, template

denaturation at 94°C for 30 seconds, annealing at 43°C (for PER), 63°C (for CTX), 60°C (for SHV), 45°C (for TEM) for 1 minute and extension 72°C for 1 minute for a total of 30 cycles, with a final extension at 72°C for 10 minutes.

Klebsiella pneumoniae 7881 containing blaSHV and blaTEM genes (Kindly provided by P. Nordmann) were used as positive control for PCR assays. A negative control using sterile distilled water as template was included in every PCR assay. The amplicons were electrophoresed in 1% agarose gel and visualized after staining with ethidium bromide. A 100bp ladder (Fermentase, Lithuania) was used as molecular weight marker.

RESULTS

Of 260 clinical isolates of *E. coli,* 125 strains were confirmed by PCT as ESBL producers (49% of total specimens). (Figure 1)

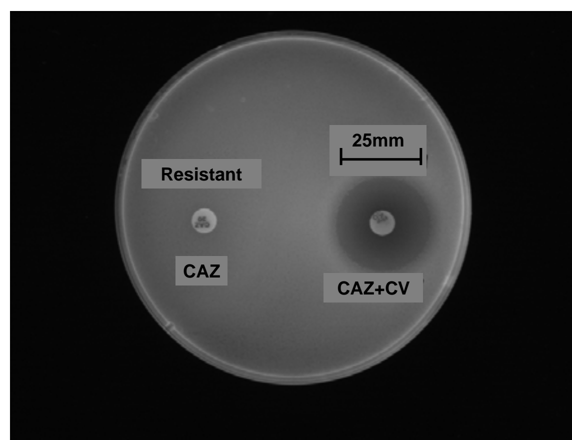

Figure 1. Phenotypic confirmatory tests for investigation of ESBL producing isolates.

The ESBL producers were mainly cultured from urine (n=111) followed by wound (n=8), fecal specimens (n=5) and sputum (n=1). A total of 73.6% of ESBL producers had MIC_{CAZ} ≥32µg/ml. The majority of these isolates were placed on MIC_{CAZ}=128µg/ml (29.8%), however, none were found in 8≤MIC≤16µg/ml (table 2).

Table 2. Distribution of ESBL producing isolates based on their MIC/CAZ

	MIC ranges (µg/ml)								
	≤2	4	8	16	32	64	128	256	512
Frequency (%)	25.4	0.9	0	0	1.8	5.3	29.8	24.6	12.2

According to our results, 59.2% of ESBL producing isolates were resistant to ceftazidime, 65.6% to cefotaxime, however, none were resistant to imipenem. Resistance rates to other antibiotics were as follow: Carbenicillin (94.5%), piperacillin (93.5%), ciprofloxacin (62.4%), ceftriaxone (60.8%), ceftizoxime (47.5%), gentamicin (40.7%), piperacillin/tazobactam (24.8%), and amikacin (17.6%).

The molecular analysis showed that 52.8% of ESBL producers (66 of 125) contain the *bla* genes (bla_{SHV} and bla_{TEM}) (figure 2).

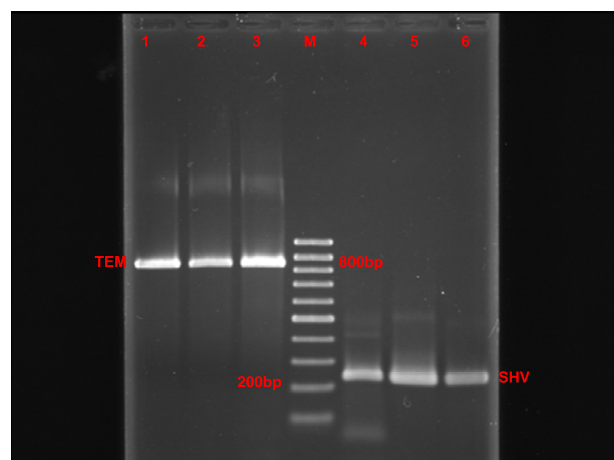

Figure 2. Agarose gel electrophoresis (blaTEM and blaSHV amplified fragments by PCR). 1: Positive control (Klebsiella pneumoniae 7881), 2 and 3: blaTEM, M: marker (100bp), 4: Positive control, 5 -6: blaSHV.

Evaluation of *bla* genes based on MIC_{CAZ} showed that the bla_{SHV} genes were placed on resistant ranges of MIC_{CAZ} (MIC_{CAZ}>32µg/ml) but the bla_{TEM} genes were found in lower ranges (table 3). Furthermore, the isolates had both bla_{SHV} and bla_{TEM} genes showed MIC_{CAZ} ≥64µg/ml.

Table 3. Distribution of bla genes in various ranges of MIC/CAZ

	Frequency (n)	MIC (µg/ml)								
		≥2	4	8	16	32	64	128	256	512
Only bla_{SHV} containing isolates	8	---	---	---	---	---	1	3	3	1
Only bla_{TEM} containing isolates	52	11	2	---	---	1	---	17	16	5
Both bla_{SHV} & bla_{TEM} containing isolates	6	---	---	---	---	---	1	1	2	2

DISCUSSION

ESBL-producing Enterobacteriaceae are among the most problematic multiresistant bacteria worldwide and are being isolated with increased frequency (11). In the past 20 years, ESBLs have evolved remarkably (12) and triggered the severity of nosocomial infections, morbidity and mortalities in ICUs and high dependency units (13). Extensive use of antibiotics such as expanded-spectrum cephalosporins in clinical practice is the main factor responsible for the appearance of ESBLs in enteric bacteria. The genes encoding the ESBLs cause resistance to oxyimino-cephalosporins have been found among all species of Enterobacteriaceae (12, 13).

ESBLs are acquired β-lactamases that are encoded mainly by genes located on plasmids. As such, they are a recent evolutionary development (14) and these genes may have been able to transfer within bacterial population of separated hospitals by means of patient transfer, or through acquisition from the community (15).

Based on the results of the current study, the prevalence of ESBL producing isolates of E. coli is high in our country in comparison with India (27%), Lebanon (13.3%), Korea (9.2%), Saudi Arabia (10.3%) and Turkey (17%) (16). Although most ESBLs confer resistance to one or more of the oxymino-β-lactams, the β-lactamase does not always increase the MIC_{CAZ} (17), for instance, we found 25.44% of ESBL producing isolates with MIC_{CAZ} <2µg/ml. In our study, all of the isolates including ESBL producers and non-ESBLs were susceptible to imipenem.

The effectiveness of carbapenems against ESBL producers and their ability for treatment of infections caused by ESBL producing organisms were well established (18), but the sensitivity of ESBL producers against carbapenems should not lead to excessive use of such drugs in clinical practice.

A total of 67.2% of isolates in a study conducted in Tehran were confirmed as ESBL-positive (19), however, in another study this rate was reported 89.3% (20). Mirsalehian et al found that 60.60% isolates of E. coli were producers of ESBLs (21), however, our results showed that 49% of E. coli was producers of ESBLs.

There is a considerable geographical difference in the occurrence of ESBLs in European countries. Within countries, hospital-to-hospital variability in occurrence may also be marked (22). This is in agreement with our findings.

Organisms that produce ESBLs are resistant to other antimicrobial agents, such as aminoglycosides, tetracycline, and trimethoprim-sulfamethoxazole, as many of these additional resistance genes are encoded on the ESBL-associated plasmid (14). The prevalence of Fluoroquinolone (ciprofloxacin) resistance among ESBL-producing strains varies according to geographic regions (23), from 13.7% in Canada, 34.8% in France and 34.2% in Europe to 65.5% in the western Pacific (14). In our study, 62.4% of strains were resistant to ciprofloxacin, which is close to the prevalence (65.5%) reported in western Pacific. The molecular analysis demonstrated that the blaTEM genes have high frequency compared

to blaSHV type. Similar results were reported by others. Furthermore, in our study the prevalence of blaTEM and blaSHV genes is lower than Lebanon, Turkey and Spain (blaTEM: 61%, 87.5% and 77.64% and blaSHV: 21%, 33.3%, 37.64%, respectively) (24).

Existing of extended-spectrum β-lactamases in bacteria and their potential for multiresistance will create serious problems in the future. Accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy. Ceftazidime is the best indicator for SHV and TEM derived ESBLs and cefotaxime is the best for CTX type ESBLs. Therefore any organism showing reduced susceptibility to ceftazidime and cefotaxime should be investigated for ESBL production.

In conclusion, there is a relatively high prevalence of ESBL-producing E. coli isolates, as well as, high prevalence of SHV and TEM producing isolates in our country. This leads to a need for improved, adequate infection control measures to be instituted and more rational use of third generation of cephalosporins in our country.

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