

The Survey of Genes Encoding Beta-Lactamases, in *Escherichia Coli* Resistant to Beta-Lactam and Non-Beta-Lactam Antibiotics

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Abstract

Objective(s)

Resistance to the new generation of cephalosporins which is mediated by Extended-Spectrum beta-lactamases (ESBLs) has been found among *Escherichia coli* isolates throughout the world. These resistance genes and their producers, the micro-organisms carrying beta-lactamases, are responsible for serious clinical and therapeutic problems among inpatients and it is necessary to pay more attention to detection of ESBLs producing organisms.

Material and Methods

Collectively 260 isolates of *E. coli* were obtained from 6 hospitals in Tehran (Iran) during April-2006 to April-2007. The antibiotic susceptibility patterns of isolates were determined by disk diffusion method. phenotypic confirmatory test (PCT) was carried out for screening of ESBLs. Microbroth dilution assay was used to determine the minimum inhibitory concentration (MIC) of ceftazidime. Isolates showing MIC \geq 2 μ g/ml were subjected to polymerase chain reaction (PCR) targeting *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX} and *bla*_{PER} genes.

Results

The PCT showed that 48.08% of isolates are ESBL producers (125 of 260). The majority of cefotaxime resistant (90.8%) and ceftazidime resistant (92.5%) isolates were ESBL producers. The obtained results by PCR revealed that 5.77% (n=15 of 260) and 24.23 (n=63) of isolates can produce SHV and TEM type enzymes respectively. *bla*_{CTX} was detected in 20.38% of isolates (n=53) and none of them could produce *bla*_{PER} type beta-lactamases.

Conclusion

The results of our study showed that the ESBL genes have high prevalence among clinical isolates of *E. coli*. Such high dissemination of ESBLs is a serious problem for public health and therefore, it's necessary to seek a program for monitoring ESBLs in hospitals.

Keywords: Antibiotic resistance, *Escherichia coli*, Extended-spectrum beta-lactamases

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Introduction

Escherichia coli is a member of gastrointestinal tract micro flora in human and other animals. Certain serovars of this bacterium are potentially pathogenic and may cause various intestinal and extra intestinal diseases (1).

In the last century, bacteria have evolved mechanisms for antibiotics resistance. Resistance to beta-lactam antibiotics may occur as a result of permeability barriers, efflux pumps, altered penicillin binding proteins and production of beta-lactamases (2). Production of beta-lactamases, such as SHV, TEM, CTX, PER and others, is the most important mechanism of resistance to penicillins, cephalosporins, and monobactams and protect the enzymes producers against the lethal effects of these antibiotics (3, 4). Mutations of older legacy beta-lactamases, SHV-1 and TEM-1, altered their hydrolysis profiles leading to an entirely new class of β -lactamases now named ESBLs (Extended-Spectrum Beta- Lactamases) (2). Several ESBLs have emerged by such mutations which are able to hydrolyze stable antibiotics such as ceftazidime, cefotaxime, ceftriaxone and aztreonam but not carbapenems and cephamycins (1, 5, 6).

CTX-M family of beta-lactamases (including four major phylogenetic trees, CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9 groups) constitute a group of class A plasmid-encoded enzymes, and their carriers are highly resistant to cefotaxime (the cause of their designation). This family is well inhibited by clavulanate and tazobactam and has been described in various members of the Enterobacteriaceae mostly in *Salmonella enterica* serovar *typhimurium* and *E. coli* (7, 8). PER enzymes are another type of beta-lactamases with extended-spectrum in their hydrolytic activities.

This type of ESBLs was first identified in *Pseudomonas aeruginosa* (Paris, 1991), but it can be expressed in other organisms specially *Acinetobacter spp.* too. PER type beta-lactamases have high outbreak in Turkey and it's possible that the spreading of them in Western Europe is mostly related to the widespread immigration of Turkish nationals (9). There is limited data about the prevalence of ESBLs in Enterobacteriaceae in Iran (10).

The present study was conducted to determine the antibiotic susceptibility patterns and the prevalence rate of ESBL producing *E. coli* isolates cultured from patients in Tehran hospitals. Detection of *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX} and *bla*_{PER} -the important ESBL genes- was another aim of our research.

Materials and Methods

Bacterial isolates

The clinical specimens were collected from 6 hospitals in Tehran during April-2006 to April-2007. Bacterial isolates were identified to species level by conventional bacteriological tests (11) and 260 *E. coli* isolates were selected for study. The majority of *E. coli* isolates (n=232, 89%) were from urine followed by isolates from wound (n=14, 5.38%), faeces (n=10, 3.84%), sputum (n=3, 1.15%) and intra abdominal abscess (n=1, 0.51%).

Antibiotic susceptibility patterns

Susceptibility of isolates against various antibiotics including ceftazidime (CAZ: 30 μ g), cefotaxime (CTX: 30 μ g), ceftriaxone (CRO: 30 μ g), ceftizoxime (ZOX: 30 μ g), piperacilin (PC: 100 μ g), piperacillin/tazobactam (PT: 110 μ g), ciprofloxacin (CIP: 5 μ g), amikacin (Ak: 30 μ g), gentamicin (GM: 10 μ g), imipenem (IMP: 10 μ g) (BBL, Mast) was determined by Disk diffusion method [Clinical and Laboratory Standards Institute (CLSI)]. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35218 were used as controls (12). Ceftazidime MIC was determined by Microbroth dilution method (CLSI) (13).

Screening of ESBL producers

ESBL producers were detected by phenotypic confirmatory tests (PCT) (CLSI). In brief, CAZ/CA (10 μ g of clavulanic acid plus 30 μ g of CAZ) and CTX/CA (10 μ g of CA plus 30 μ g of CTX) disks were placed on the inoculated plates containing Muller Hinton agar. A positive test result was defined as a ≥ 5 mm increase in zone diameter comparison to a disk without clavulanic acid (13-15).

DNA extraction and PCR

Isolates were cultured in LB broth (Merk, Germany) at 37 °C overnight and total DNA was extracted according to the published method of Hammond *et al* (16). Primers SHV-F (Forward) and SHV-R(Reverse), TEM-F and TEM-R (17), CTX-F and CTX-R (18), PER-F and PER-R (19) were used to amplify a 200 bp fragment of *bla*_{SHV}, 800 bp fragment of *bla*_{TEM}, 550 bp fragment of *bla*_{CTX} and 925 bp fragment of *bla*_{PER} genes respectively (Table 1). PCR condition for detecting *bla*_{SHV} and *bla*_{TEM} was the same as described by Howard *et al* (20). This condition for detection of *bla*_{CTX} and *bla*_{PER} is shown in Table 1.

Klebsiella pneumoniae 7881 containing *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX} genes (Kindly provided by P. Nordmann) and *P. aeruginosa* KOAS containing *bla*_{PER} were used as controls. The amplicons were electrophoresed in 1% agarose gel and visualized after staining with ethidium bromide. A 100 bp ladder (Fermentase, Lithuania) was used as molecular weight marker.

RFLP

Following PCR the amplified DNA was digested with restriction enzymes *Pst*I (for *bla*_{TEM}) (21) and *Pvu*II (for *bla*_{CTX}) (22).

Results

Antibiotic susceptibility test

The resistance rate of isolates was as follows: piperacilin (81.15%), carbenicillin (87%), ciprofloxacin (43.1%), cefotaxime (33.5%), piperacilin/tazobactam (15.8%), ceftazidime (30.77%), ceftriaxon (31.5%), ceftizoxime (25.5%), gentamicin (27.6%) and amikacin

(12.7%). All of the isolates were susceptible to imipenem. Antibiotic resistance rate in ESBL producing isolates was as follows: piperacilin (93.5%), carbenicillin (94.5%), ciprofloxacin (62.4%), cefotaxime (65.6%), piperacillin/tazobactam (24.8%), ceftazidime (59.2%), ceftriaxon (60.8%), ceftizoxime (47.45%), gentamicin (40.74%) and amikacin (17.6%). More than 36% of isolates had MIC_{CAZ} ≥32 µg/ml and mostly had MIC=128 µg/ml.

Screening for ESBL genes

Using PCT method, ESBLs were detected in 48.08% of isolates (n=125). The majority of ESBL producers were from urine (n=111, 88.8%) followed by wound (n=8, 6.4%), faeces (n=5, 4%) and sputum (n=1, 0.8%). ESBL producing isolates were mainly resistant to carbenicillin and piperacillin but all of them were susceptible to imipenem. According to our results more than 70% of ESBL producers had MIC_{CAZ} ≥32 µg/ml. The majority of ESBL positives (29.8%) were placed in MIC_{CAZ}=128 µg/ml but none of them had 4<MIC<32 µg/ml (The distribution of these isolates based on their MIC_{CAZ} were described in Table 2).

Molecular detection

We didn't find any *bla*_{PER} gene among our isolates but other types of genes including *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX} existed in different frequencies based on their type (Figure 1). For example, SHV type beta-lactamases with 5.77% (n=15) had low but TEM and CTX type with 24.23% (n=63) and 20.38% (n=53) had relatively high frequency among *E. coli* isolates (Table 3).

Table 1. Primers used for detection of *bla* genes.

	Primer	5'-Sequence- 3'	Detected gene	Molecular weight (bp)	Annealing temperature (1 min/35 °C)
TEM	Forward	GAGTATTCAACATTTCCGTGTC	<i>bla</i> _{TEM}	800	45
	Reverse	TAATCAGTGAGGCACCTATCTC			
SHV	Forward	AAGATCCACTATCGCCAGCAG	<i>bla</i> _{SHV}	200	60
	Reverse	ATTCAGTTCCGTTTCCCAGCGG			
CTX	Forward	CGCTTTGCGATGTGCAG	<i>bla</i> _{CTX}	550	63
	Reverse	ACCGCGATATCGTTGGT			
PER	Forward	AATTTGGGCTTAGGGCAGAA	<i>bla</i> _{PER}	925	43
	Reverse	ATGAATGTCATTATAAAAAGC			

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 (%)	25.44	0.87	0	0	1.75	5.3	29.8	24.56	12.28

Table 3. The frequency of *bla* genes producers based on specimens source.

Gene source	Frequency (%)			
	SHV	TEM	CTX	PER
Urine	10 (66.7)	58 (92.1)	47 (88.7)	0 (0)
Stool	0 (0)	1 (1.6)	2 (3.8)	0 (0)
Wound	4 (26.7)	4 (6.3)	4 (7.5)	0 (0)
Sputum	1 (6.6)	0 (0)	0 (0)	0 (0)
Total	15 (100)	63 (100)	53 (100)	0 (0)

The majority of *bla*_{SHV} containing isolates (n=14 of 15) had MIC_{CAZ} >32 µg/ml. The *bla*_{TEM} containing isolates were also detected in lower ranges of MIC_{CAZ}. Some isolates had more than one *bla* gene (for example, *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX} genes together). The recent isolates mostly showed ceftazidime MIC=256 and 512 µg/ml. (Table 4).

Evaluation of ESBL producers showed that collectively 64.8% (n=81) could produce *bla*_{SHV}, *bla*_{TEM} or *bla*_{CTX} genes. The distribution of genes among ESBL positives is shown in Table 4 (The *bla* genes were detected among non-ESBL isolates too). The RFLP analysis showed that all of the amplified products had the same patterns (Figure 2).

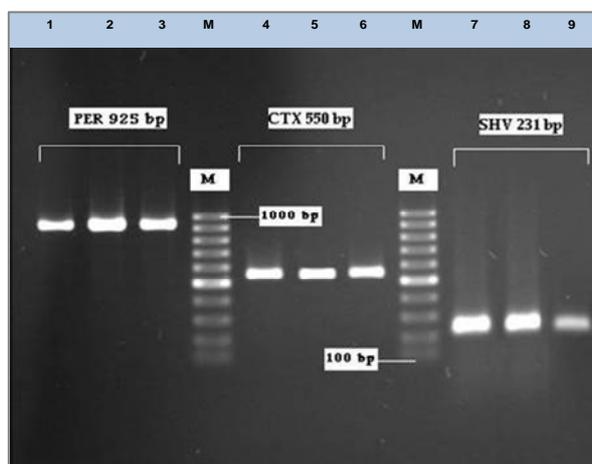


Figure 1. Agarose gel electrophoresis (*bla*_{TEM}, *bla*_{SHV}, *bla*_{PER} amplified fragments by PCR). M: marker (100 bp)/1, 2, 3: Positive control (*Pseudomonas aeruginosa* KOAS)/4, 7, 10: Positive control (*K. pneumoniae* 7881)/5 and 6: *bla*_{CTX} detected genes/8 and 9: *bla*_{SHV} detected genes/11 and 12 *bla*_{TEM} detected genes.

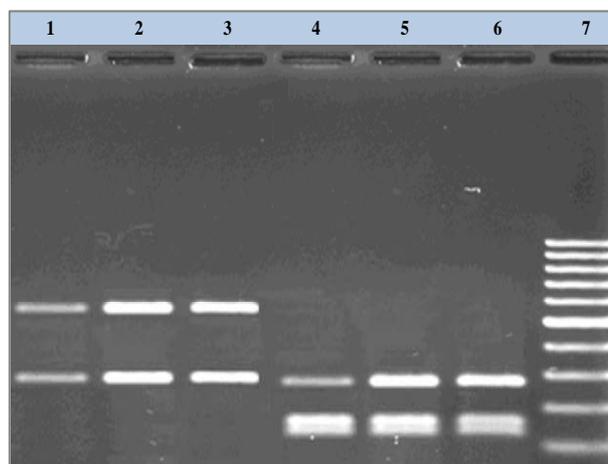


Figure 2. Agarose gel electrophoresis (*bla*_{CTX} and *bla*_{TEM} amplified fragments by RFLP-PCR). 1: Positive control (*Klebsiella pneumoniae* 7881), 2-3: *bla*_{TEM}, 4: Positive control (*K. pneumoniae*), 5-6: *bla*_{CTX}, 7: Marker (100 pb).

Table 4. Distribution of *bla* genes in various ranges of MIC_{CAZ}.

<i>bla</i> gene containing isolates	Frequency (%)		MIC (µg/ml)								
	Isolates number	ESBL producers number	≥2	4	8	16	32	64	128	256	512
SHV	3 (1.15)	3 (2.4)	---	---	---	---	---	1	1	1	---
TEM	33 (12.7)	30 (24)	12	2	---	---	1	---	8	6	4
CTX	20 (7.7)	17 (13.6)	3	---	---	---	1	---	8	5	3
Both SHV&TEM	2 (0.77)	1 (0.8)	---	---	1	---	---	---	1	---	---
Both SHV&CTX	5 (1.92)	5 (4)	---	---	---	---	---	---	2	2	1
Both TEM&CTX	23 (8.84)	20 (16)	---	---	---	---	---	---	10	11	2
SHV, TEM and CTX	5 (1.92)	5 (4)	---	---	---	---	---	1	---	2	2

Beta-Lactam and Non Beta-Lactam Resistance in *E. coli*

Table 5. Antibiotic resistance patterns of isolates which produce three *bla* genes together (*bla_{SHV}*, *bla_{TEM}* and *bla_{CTX}*).

No. of isolate	Source	Antibiotic resistance patterns											MIC (CAZ)	ESBLs
		CAZ	CTX	CIP	CRO	ZOX	CB	PC	PT	AK	GM	IMI		
48	urine	R	R	R	R	R	R	R	S	S	S	S	256	+
76	urine	R	R	R	R	R	R	R	S	S	R	S	256	+
130	urine	R	R	R	R	R	R	--	S	R	R	S	512	+
304	urine	R	R	R	R	R	R	R	S	S	R	S	64	+
505	urine	R	R	R	R	R	--	R	S	R	R	S	512	+

CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, ZOX: ceftizoxime, CB: carbenicillin, PC: piperacilin, PT: piperacillin/tazobactam, CIP: ciprofloxacin, Ak: amikacin, GM: gentamicin, IMP: imipenem.

Discussion

ESBLs have evolved remarkably during the last 20 years and the organisms producing these genes, are responsible for increasing in nosocomial infections, morbidity and mortality especially amongst patients on intensive care and high dependency units. The lower digestive tract of patients have been recognized as the major source of ESBL producing organisms (1, 14, 23, 24), hence this area is the important source for their spreading. According to a study the resistance rate to gentamicin and amikacin were reported 42% and 17% respectively (25) which were higher than our rates (27.6% and 12.7% respectively). In Saudi Arabia over 66% of isolates were found susceptible to piperacillin/tazobactam (26) but our study showed 84.2% susceptible isolates. Compared to reports of Jeong *et al* (3), the resistance to ceftazidime and cefotaxime in Iran were higher than South Korea (Korea: 11%, 14% and Iran: 30.77%, 33.5% for ceftazidime and cefotaxime respectively) but all of the isolates were jointly susceptible to imipenem in Iran and Korea. Considering other studies performed in the time period of 2004-2006 ESBL producing isolates of *E. coli* are prevalent in 5 our country and the percentage of this prevalence (48.08%) is more than other countries including Denmark: 0.8% (27), Korea: 9.2% to 10.2% (3, 28), Saudi Arabia: 10.3% (26), Turkish: 17% (29), United Kingdom: 21.6% (30), and India: 27% (31).

The studies regarding antibiotic susceptibility patterns of ESBL producing isolates show that the multi-resistance such as resistance to aminoglycosids, tetracyclines, chloramphenicol, trimethoprim (32), penicillins, first-, second-, and third generation cephalosporins, aztreonam (33), quinolone (34) and carbapenem (35-37) is common

among these organisms. We found fluoroquinolone, aminoglycoside and multi-drug resistance in 61.6%, 58.34% and 83.2% of ESBL producers respectively which were lower than results reported by Matar *et al* (40%, 33% and 18%, respectively) (38). The prevalence of fluoroquinolone resistance among ESBL producing strains, varies according to geographic regions, from 13.7% in Canada (lower than Iran) to 65.5% in the Western Pacific (which is nearly similar to our results) (33).

The ESBL genes which cause resistance to cefotaxime, ceftazidime and aztreonam (oxymino-cephalosporins), are seen now worldwide in all species of Enterobacteriaceae (39). In a study, all of the ESBL producing isolates were identified as CAZ resistant (40).

In comparison, our survey showed lower rate of ESBL producers as CAZ resistant (59.2%) but this amount is significantly high and alarming too. It's well established that the excessive consumption of third generation cephalosporins especially ceftazidime is not a suitable treatment plan because of its effects on acquisition of ESBL producing organisms (27). Therefore, this kind of antibiotic therapy should be discouraged in the medical society. Carbapenems are stable against ESBL producing organisms and their capability for treatment of infections caused by ESBL producing organisms is well established (28, 37). In a study, all of the isolates with ESBL phenotype and in another one, over 92% were identified as imipenem susceptible (26, 41). In this study, imipenem was more effective among 11 various tested antibiotics, and all of the isolates, including ESBL producers and non-ESBLs, were susceptible to this carbapenem. In another study we also

showed that all of the *K. pneumoniae* isolates were susceptible to imipenem (10), also in another research in Iran all of the clinical isolates of *K. pneumoniae* and *E. coli* were susceptible to imipenem (42).

It should be noted that the weakness of ESBL producing organisms in hydrolyzing carbapenems should not lead to routine prescription and excessive use of this antibiotic in the clinical setting. Although most ESBLs confer resistance to one or more of the oxymino-beta-lactams, the beta-lactamase does not always increase the MICs (2). In this study, 25.44% of ESBL producing isolates were found in CAZ MICs ≤ 2 $\mu\text{g/ml}$. This observation confirms that, it's necessary to test all isolates from inpatients using both ceftazidime and cefotaxime. It means that any organism showing reduced susceptibility to cefotaxime and ceftazidime, should be investigated for ESBLs production (16).

In this study, the frequency of *bla*_{TEM} (24.23%) and *bla*_{SHV} (5.77%) genes was lower than Lebanon, Turkey and Spain (*bla*_{TEM} 61%, 87.5%, 77.64% respectively and *bla*_{SHV} 21%, 33.3%, 37.64% respectively) (41, 43). Similar to these countries, we found *bla*_{TEM} genes more than *bla*_{SHV}. The *bla*_{CTX} was reported the most prevalent *bla* gene in Korea (44) and the *bla*_{SHV} in company with *bla*_{CTX} were most common in Denmark (45). However, in our country the *bla*_{TEM} and *bla*_{CTX} had high prevalence rate. Similar to Thailand (46) we couldn't find any *bla*_{PER} among the clinical isolates. Matar *et al* (Lebanon) (41) reported 21%, 61% and 18% of ESBL producers as *bla*_{SHV}, *bla*_{TEM} and both *bla*_{SHV}-*bla*_{TEM} containing isolates, respectively whereas our results showed lower rates (Table 6). They didn't find *bla*_{CTX} gene among *E. coli* isolates either, whereas we detected them with a similar frequency as *bla*_{TEM} genes. In other studies the prevalence rate of *bla*_{SHV}, *TEM*, *CTX* genes among the ESBL producers were reported 3.8 to 8% (*bla*_{SHV}), up to 77% (*bla*_{TEM}) and 78 to 99.6% (*bla*_{CTX}) (46, 47) which all are higher than our findings. Compared to our country, ESBL positives with both *bla*_{TEM} & *bla*_{CTX} genes have high prevalence rate in Thailand (67.56%, n=25 of

32) and 5.4% (n=2 of 37) of them were three genes (*bla*_{SHV} & *bla*_{TEM} & *bla*_{CTX}) producers (47) which is similar to Iran with 5.8%.

This is well-established that beta-lactamase inhibitors such as clavulanate and tazobactam are comparably good inhibitors of SHV and TEM enzymes and piperacillin/tazobactam seems to have a better prospect than other inhibitor combinations against isolates with TEM derivatives enzymes (28, 48). In our research the effectiveness of these inhibitors was confirmed, too.

Recently, the increasing incidence of infections with ESBL producing bacteria, and the high rates of antimicrobial resistances among them have been changed to microbiologist's challenges and it's necessary to control the transmission and outbreak of ESBL producing organisms such as *E. coli*. The emergence of ESBL producing organisms including *E. coli* seems to be the result of complex interactions between the type of ESBL, genetic background of the strain, and selective pressures existing in ecologic niches (39). The heavy antibiotic use is one of the selective pressures and a risk factor for acquisition of ESBL producing organisms (27). For example, the excessive use of expanded-spectrum cephalosporins in clinical practice is the main factor responsible for the appearance of ESBLs in the enteric bacteria and several studies have found a relationship between third generation cephalosporin use and acquisition of ESBL producing strains (47). Therefore, clinicians should be familiar with the clinical importance of these enzymes and potential strategies for dealing with them. Accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy (16).

Conclusion

It can be concluded that there is a respectively high prevalence of ESBL-producing *E. coli* isolates, as well as, high prevalence of CTX producing isolates in our country. This shows the need for improved and adequate infection control measures to be instituted and more rational use of third generation of cephalosporins in our country.

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