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Full Length Research Paper

Distribution of TEM, SHV and CTX-M Genes among ESBL-producing Enterobacteriaceae isolates in Iran

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Antibiotic resistant especially to Extended Spectrum Beta-Lactamases (ESBLs) producing bacteria has been known as a healthy problem in treatment of patients with infection. The aim of this study is to determine the antimicrobial resistance patterns of *Enterobacteriaceae* isolated from clinical samples and also detect the phenotypic and genotypic ESBLs producing organisms. In this study, 420 Enterobacteriaceae strains isolated from 8000 clinical specimens were identified by biochemical standard tests. Their primary antimicrobial susceptibility was determined to seven antibiotic and ESBLs producing strains were also detected by a combined disk method (CDM). Finally, presence of bla_{TEM}, bla_{SHV} and bla_{CTX_M} genes was evaluated by Multiplex PCR Method. *Escherichia coli* and *Klebsiella pneumoniae* were the most common among of *Enterobacteriaceae* isolates. Drug resistance frequency of these strains to cefotaxime, ceftriaxone, ceftazidim, cefepim, cefepirom was 44, 44, 42, 39.5 and 39%, respectively. 128 of 420 strains (30.5 %) produced ESBLs by CDM. 73 of 128 (57%) ESBL-producing strains were positive for TEM and/or SHV by Multiplex PCR. The prevalence of bla_{TEM} and bla_{SHV} were 65.5 and 15%, respectively. 14 isolates (19%) had both bla_{TEM} and bla_{SHV} genes, but bla_{CTX-M} was not detected in ESBL-producing strains. The results of this study showed noticeable prevalence of ESBLs and multiple antibiotic resistant among of *Enterobacteriaceae* isolates, especially *E. coli* and *K. pneumoniae*. So, we suggest that beta-lactam antibiotics and beta-lactamase inhibitors or carbapenems to be limited only to patients with serious infections.

Key words: ESBLs, TEM, SHV, *Enterobacteriaceae* and multiplex PCR.

INTRODUCTION

Antibiotic resistant due to beta-lactamase is one of the most forms which are seen in many bacteria. There is a problem that occurred among Extended-Spectrum β-lactamase (ESBL)–producing Enterobacteriaceae which have widely caused the spread of infections worldwide (Quinteros et al., 2003; Al-Agamy et al., 2009; Apisarnthanarak et al., 2008). Although, today hundreds variant of ESBLs have been described (Jacoby and Munoz-Price, 2005; Rawat and Nair, 2010), but the most common of them are derivatives of TEM or SHV enzymes. Also, in recent years non-TEM and non-SHV ESBLs have been reported, mainly the CTX-M enzymes (Al-Agamy et al., 2009; Mohamudha et al., 2010; Munier et al., 2010).

Resistance to extended-spectrum cephalosporin is often related to plasmid which encoded ESLBs. However, inducible chromosomally encoded beta-lactamases in some species of Enterobacteriaceae may also contribute to resistance those species (Bradford, 2005). It has been known that gram-negative bacteria that produce ESBLs are increasingly implicated as cause of community-acquired infections (Ben-Ami et al., 2009; Pitout, 2005). The Clinical and Laboratory Standards Institute (CLSI) has proposed methods for detection of ESBLs in *Escherichia coli* and *Klebsiella* spp based on screening

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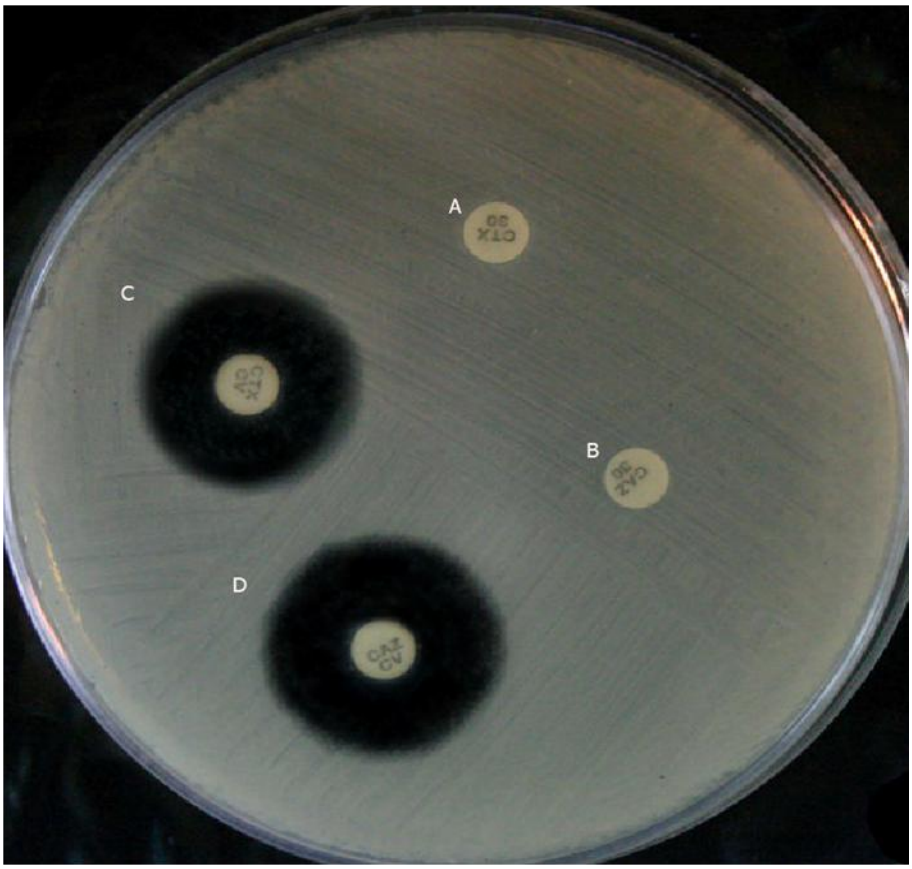

Figure 1. ESBLs phenotypic confirmation by Combination Disk Method. Disks of containing: (A) Cefotaxime (CTX=30µg) and (B) Ceftazidime (CAZ = 30µg) without inhibition zone and their combination (C and D) with Clavulanic acid (CA = 10 µg) with inhibition zone around of them, have been compared.

tests with disk containing cefpodoxime, ceftazidime or cefotaxime and confirmatory tests exploiting the inhibitory effect of clavulanic acid on hydrolyzing action of ESBLs (Hoffmann, 2006; CLSI, 2007). In this study, we used a combination of phenotypic tests and multiplex PCR to assess the frequency of ESBL genes among clinical isolates of Enterobacteriaceae.

MATERIALS AND METHODS

Bacterial isolates

Enterobacteriaceae strains were isolated from clinical specimens of patients who referred to Dezful Ganjavian hospital, dependent to Ahvaz Jundishapur University of Medical Sciences, Iran. In this study 8000 specimens including of urine, blood, CSF and wound discharge (except stool) were collected and examined from April 2007 to May 2008. Isolated bacteria were identified by Gram's stain and standard biochemical tests (Forbes et al., 2007).

Antimicrobial susceptibility test

Primary antimicrobial susceptibility test of the isolates Was performed by Kirby-Bauer disk diffusion method (Forbes et al., 2007) to seven antibiotics: cefotaxime(30 µg), ceftriaxone(30 µg), ceftazidime (30 µg), cefepime(30 µg), cefpirome(30 µg), imipenem(10 µg) and meropenem(10 µg).

Phenotypic detection of ESBLs

ESBLs producing strains detected using single or combined ceftazidime / cefotaxime-clavulanic acid disks (MAST Co. UK). This was a combination test for phenotypic confirmatory of ESBLs (CLSI, 2007, Thomson and Sanders, 1992). Phenotypic detection of ESBLs was defined by an increase ≥ 5 mm in the inhibition zone around clavulanic acid disk comparison with zone around the disks of without clavulanic acid (Figure 1). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 35218 were used as positive and negative(non-ESBL producer) controls (Akram et al., 2007; Adegoke and Okoh, 2011), respectively.

Multiplex PCR for TEM, SHV and CTX-M

Deoxyribonucleic acid (DNA) was extracted from phenotypic ESBLs-positive strains based on DNA kit procedure (Sinagen Co., Iran). Finally, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and 16S rRNA genes were detected using specific pair of primers (Metabion, Germany).

Table 1. The oligonucleotide primers used for amplification of Beta - lactamase genes.

NO	Primers	Sequences
1	TEM	5' ATAAAATTCTTGAAGACGAAA 3' 5' GACAGTTACCAATGCTTAATCA 3'
2	SHV	5' TCGGGCCGCGTAGGCATGAT 3' 5' AGCAGGGCGACAATCCCGCG 3'
3	CTX-M	5' TTAATGATGACTCAGAGCATTC 3' 5' GATACCTCGCTCCATTTATTG 3'
4		5`GGAATTCAAATGAATTGACGGGGG C 3 ' 16S rRNA CGGGATCCCAGGCCCGGGAACGTATTCAC3 '

Table 2. Frequency of Enterobacteriaceae species isolated from examined clinical specimens.

Name	No. of isolates	Percent (%)
E. coli	271	64.5
K. pneumoniae	84	20
K. oxytoca	13	3
E. cloacae	8	1.9
E. aerogenes	11	2.7
P. mirabilis	27	6.4
C. freundii	4	1
C. diversus	2	0.5
Total	420	100

The used primers (Quinteros et al., 2003; Geha et al., 1994) are listed in Table 1.

RESULTS

In this study, 420 clinical isolates of Enterobacteriaceae were identified which *E. coli* with 271 isolates (64.5%) and *K. pneumoniae* with 84 isolates (20%) were the most common of them (Table 2).

Primary antimicrobial susceptibility test of 420 species of Enterobacteriaceae showed the most resistance of these isolates was to third–generation cephalosporins such as cefotaxime (44%), ceftriaxone (44%) and ceftazidime (42%) while all of these isolates were susceptible to imipenem and meropenem. The results of phenotypic confirmatory test (Figure 1) showed that out of 420 isolates of Enterobacteriaceae, 128 (30.5%) were ESBLs-positive isolates, which the most common of them belonged to *Klebsiella* (45.4%) and *E.* coli (28.8%) (Table 3).

The results of PCR-products electrophoresis showed genomic patterns related to bla_{TEM} (1097 bp), bla_{CTX_M} (870 bp), bla_{SHV} (660 bp) and 16S rRNA (479 bp) genes (Figure 2). These results also demonstrated that 57% of the phenotypic ESBLs-positive isolates were genotypically ESBLs-positive. *Klebsiella* and *E. coli* with frequency of 79.5 and 43.6%, respectively, had the most frequency of EBLS genes (Table 4).

Multiplex polymerase chain reaction (PCR) of genes among ESBLs-positive bacteria (73 isolates) showed the presence of genes related to TEM and SHV in 65.8 and 15% of isolates, respectively. Also both of ESBL genes of TEM and SHV were seen in 14 isolates (19.2%)-(Table 5).

DISCUSSION

In the recent decade, extended–spectrum beta-lactamases (ESBLs) producing bacteria, especially Enterobacteriaceae, have caused increase of resistance to beta-lactam antibiotics and consequently it has been known as a healthy problem in the treatment of patients with infection due to these bacteria (Quinteros et al., 2003; Al-Agamy et al., 2009; Tasli and Bahar, 2005; Canton and Coque, 2006). In our study, primary antimicrobial susceptibility test of *E. coli* and *K. pneumoniae* isolates showed the most resistance to third-generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. Some reports also show resistance to β-lactam antibiotics (especially third-generation cephalosporins) and non-β- lactams, among which clinical isolates of gram-negative bacteria are increasing worldwide (Goossens, 2000; Andrews, 2001). However, similar to a study in Turkey (Tasli and Bahar, 2005), all clinical isolated strains in our study were susceptible to imipenem.

Most ESBLs have evolved by mutation from native β-lactamases, particularly TEM-1, TEM-2 and SHV-1. These parent enzymes are commonly found in gram-negative bacteria, especially Enterobacteriaceae (Pfaller and Segreti, 2006; Bradford, 2005). Also, some species of Enterobacteriaceae which newely recognized as New Delhi metallo-β- lactamase producers have been isolated from clinical specimens (Sarma et al., 2011). Out of 420 Enterobacteriaceae isolates from clinical specimens in present study, 128 isolates (30.5%) produced ESBLs which confirmed using a phenotypic combined disk method. Among of these bacteria, the most prevalent of ESBLs belonged to *Klebsiella* (45.5%) and *E. coli* (28.8%). A report from 10 European countries showed

Table 3. Frequency of phenotypic ESBLs-positive Enterobacteriaceae isolated from clinical specimens.

Name	Total isolates		Positive	
	(No.)	(%)	(No.)	(%)
E. coli	271	64.5	78	28.8
Klebsiella	97	23.1	44	45.4
Enterobacter	19	4.5	3	15.8
Proteus	27	6.4	2	7.4
Citrobacter	6	1.5	1	16.6
Total	420	100	128	30.5

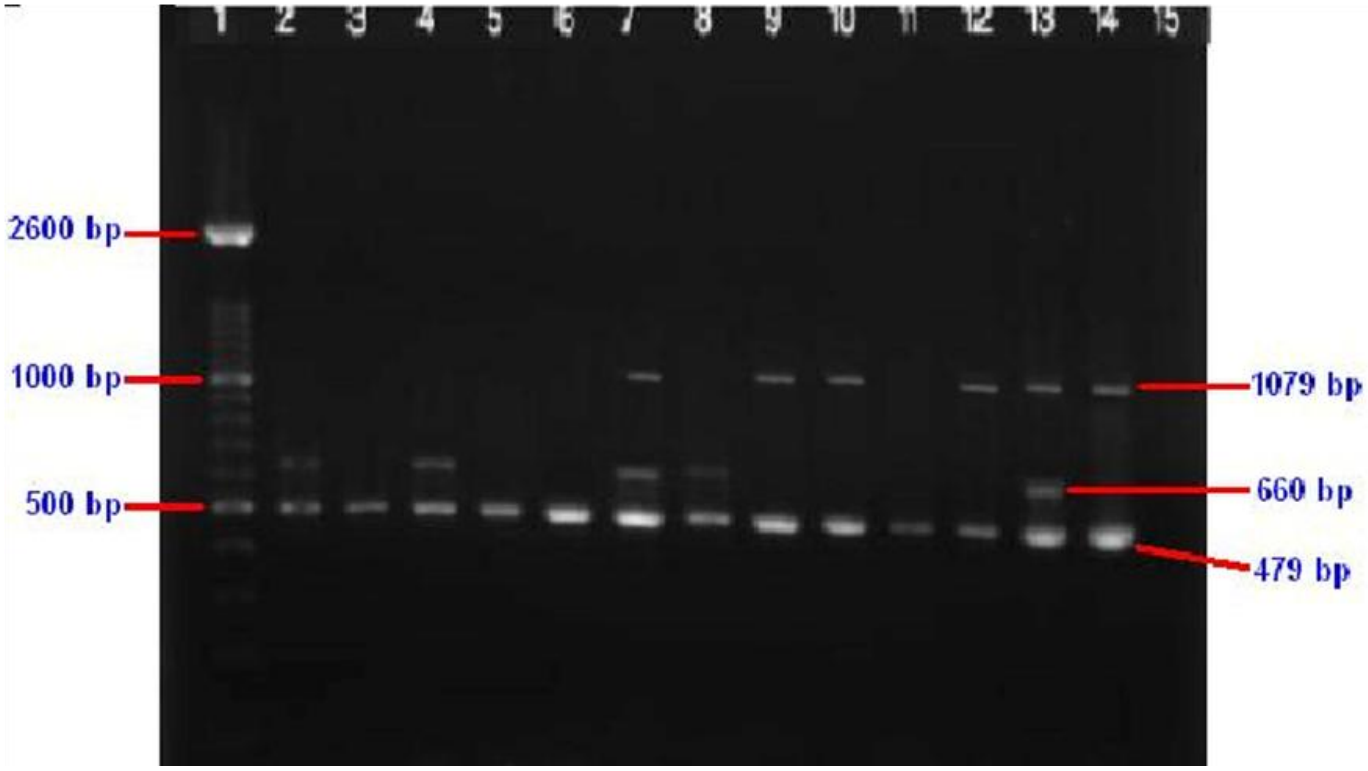

Figure 2. DNA genomic patterns of PCR products by Eletrophoresis. Bands 1079 and 660bp are related to TEM- and SHV- ESBL genes, respectively and band 479bp is related to the 16SrRNA gene which was used as an internal control. Lane 1: DNA size marker- Lanes 2 and 3: Positive (*K. pneumoniae* ATCC 700603) and Negative (*E. coli* ATCC 35218) controls - Lanes 9,10,12 and 14: TEM Gene- Lanes 4 and 8 : SHV Gene – Lanes 7 and 13: both TEM and SHV Genes- Lanes 5, 6, and 11 related to strains without TEM , SHV and CTX-M Genes- Lane 15: distilled water.

the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* ranged from at least 1.5% in Germany and 39 to 47% in Russia, Poland and Turkey (Goosens, 2001).

In our country, the prevalence of ESBL-producing Enterobacteriaceae has been reported in different rates by phenotypic confirmatory test. While Behroozi et al. (2010) showed 21 and 12% of *E. coli* and *K. pneumoniae* isolates, respectively were ESBL-producers in Tehran (Capital city of Iran), Feizabadi et al. (2010) reported that 72% *K. pneumoniae* strains isolated from Tehran hospitals were ESBLs-producing. However our study results showed that the most common of ESBL-producing Enterobacteriaceae isolated from another city (Dezful) in south of Iran, were *K. pneumoniae* (45.4%) *and E. coli* (28.8%).

The data analysis of epidemiologic studies related to ESBL-producing Enterobacteriaceae in Europe, Asia and North America demonstrated that 34.5% of isolates produced ESBLs. *E. coli* with 87.5% was the most common ESBL-producer organism (Ben-Ami et al., 2009). Tasli and Bahar (2005) and Al-Agamy et al. (2009) showed the rate of prevalence ESBL-producing *K. pneumoniae* as 57.1 and 55%, respectively, based on

Table 4. Genotypically frequency in phenotypic ESBLs-positive Enterobacteriaceae.

Phenotype-Pos. Bacteria	ESBL-genes				Total	
	Positive		Negative		(No.)	%
	(No.)	%	(No.)	%		
E. coli	34	43.6	44	56.4	78	100
Klebsiella	35	79.5	9	20.5	44	100
Enterobacter	3	100	0	0	3	100
Proteus	1	50	1	50	2	100
Citrobacter	0	0	0	100	1	100
Total	73	57	54	43	128	100

Table 5. Frequency of *TEM* , *SHV*, and *CTX-M* genes in ESBLs-positive Enterobacteriaceae isolates.

Genotype-Pos. bacteria	*TEM$^+$ SHV$^-$		TEM$^-$, SHV$^+$		CTX-M $^+$		TEM $^+$, SHV$^+$		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli	31	91	1	3	0	0	2	6	34	100
Klebsiella	17	48.5	8	23	0	0	10	28.5	35	100
Enterobacter	0	0	2	67	0	0	1	33	3	100
Proteus	0	0	0	0	0	0	1	100	1	100
Total	48	65.8	11	15	0	0	14	19.2	73	100

* (+) = Positive genotype (-) = Negative genotype.

phenotypic confirmatory test. This subject is important because of the isolates which are positive by phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycin, cefoxitin and cefotetan) and aztreonam, regardless minimum inhibitory concentration (MIC) of cephalosporin. This is CLSI criteria for ESBL-producing isolates (Rawat and Nair, 2010). Since ESBL-producing strains prevalence can vary greatly from one site to another and even over time for a given site, so regional and local estimates are probably more useful to clinical decision-making than are more global assessments (Pfaller and Segreti, 2006).

We used a 16S rRNA sequence which is common to all of bacteria as universal target for internal control in multiplex PCR. This is a control mechanism for identification of potential false-negative results (Geha et al., 1994). In our study, genotypic survey on 128 phenotypic ESBLs-positive strains by multiplex PCR revealed that 73 strains (57%) were genotypically ESBLs-positive, of which Klebsiella (79.5%) was the most positive of them (Table 4).

Among different ESBL-genes (Table 5), TEM + SHV- genotype in 48 strains (65.8%) and TEM- SHV + genotype in 11 strains (15%) were detected. While genotype of TEM + SHV + was detected in 14 strains (19.2%). The data analysis related to ESBL genes (Table 5) showed frequency of bla_{TEM} genes in *E. coli* and *Klebsiella* isolates was 97 and 77%, respectively. Also, frequency of bla_{SHV} genes in these bacteria was 9 and 1.5%, respectively.

The others studies in Iran, showed prevalence bla_{TEM} and bla_{SHV} genes in *K. pneumoniae* were 30.7 and 11.2% (Feizabadi et al., 2010) and in *E. coli* were 46.4 and 11.2% (Shahcheraghi et al., 2009), respectively.

Some reports show that most ESBLs are derivatives of TEM and SHV genes and there are now more than 90 TEM-type and more than 25 SHV-type β-lactamases (Tasli and Bahar, 2005; Bradford, 2005). The most ESBLs in our study were TEM-type and SHV-type enzymes. In comparison with some studies such as Tasli and Bahar (2005) and Hosseini-Mazinani et al. (2007) which showed 87.5 and 60% of ESBLs, respectively were TEM derived enzyme in *E. coli* isolates, prevalence of this gene (97%) in our study was higher.

The rate of TEM-type β-lactamase produced by *K. pneumoniae* in our study (77%) was more than that of Tasli and Bahar (2005) study (31.1%) and was less than that of Al-Agamy et al. (2009) study (84.1%). The results in our study showed prevalence of SHV-type ESBL among *E. coli* strains was 9% and among *Klebsiella* strains was 51.5%. These results were different from other studies (Tasli and Bahar, 2005; Ben-Ami et al., 2009) which detected SHV-type ESBL in 74.3 and 14% of these isolates, respectively.

The present study failed to detect CTX-M type ESBL in isolated strains. While CTX-M types as the most frequent ESBLs (65%) have been reported in some of studies (Ben-Ami et al., 2009), Champs et al. (2000) showed that CTX-M type was at least rate (1.2%) among of *Enterobacteriaceae* isolates.

The variation in our study results compared with others about prevalence rate of ESBLs may be arised from different reasons such as difference in type and volume of consumption of antibiotics and difference in time which the isolates were collected (Al-Agamy et al., 2009). In present study, 43% (55/128 strains) of phenotypic ESBL-positive strains lacked bla_{TEM}, bla_{SHV} and bla_{CTX_M}, genes based on multiplex PCR, which showed other factors such as presence of genes other than TEM, SHV and CTX-M were effective in producing resistance to beta-lactam antibiotics.

The results of this study showed that the prevalence rate of ESBLs, beta-lactamase genes and resistance to multiple antibiotics were noticeable among of *Enterobacteriaceae* isolates, especially *E. coli* and *K. pneumoniae*. We all should pay attention to the fact that using of many ineffective antibiotics and possibility in spreading of ESBL genes between the species of Enterobacteriaceae by transferable genes (Smet, 2010) will help to spread of ESBL-producing isolates. So, we suggest that combined therapeutic regimens such as beta-lactam antibiotics and beta-lactamase inhibitors or carbapenems be limited only to patients with serious infections and be designed based on the antibacterial susceptibility test.

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