Original Article

PREVALENCE OF AMBLER CLASS A EXTENDED-SPECTRUM- β - LACTAMASES (ESBLS) AMONG UROPATHOGENIC *Escherichia coli* STRAINS ISOLATED FROM RASHT CITY, IRAN

PREVALÊNCIA DE AMBLER EXTENDED-SPECTRUM CLASSE A- β -LACTAMASES (ESBLs) ENTRE UROPATHOGENIC Escherichia coli *ISOLADOS DA CIDADE DE RASHT, IRÃ*

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ABSTRACT: Extended spectrum β-lactamases (ESBLs), a group of bacterial enzymes which are the major cause of resistance to penicillins, broad spectrum cephalosporins and monobactams, are found among the member of *Enterobacteriaceae*. The class A ESBLs are mainly encoded by the plasmid mediated bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes. In this study, prevalence of Ambler class A ESBL genes among uropathogenic *E. coli* (UPEC) isolates associated with community acquired infections and their antibiotic susceptibility pattern was investigated. Seventy UPEC strains were isolated from urine samples and subjected to antimicrobial susceptibility assay using disk diffusion method. Phenotypic screening of ESBL production was evaluated according to the CLSI combined disk method. Genotyping of Ambler class A ESBLs was investigated using PCR. According to the results, ESBLs was identified in 37 isolates while molecular assay showed 47 isolates harbored ESBL genes. The most prevalence was recorded for bla_{TEM} (74.2%) followed by bla_{CTX-M} (43.2%) and bla_{SHV} (12.2%). Imipenem was the most effective drug and ESBL producing isolates showed higher resistance to CAZ, CRO, CFZ, CTX and FOX compared to non ESBL isolates. In conclusion, high prevalence of class A ESBL genes was observed in our study which needs more consideration and rational antibiotic prescription.

KEYWORDS: Antibiogram. ESBL. Phenotypic combined disk. Uropathogenic *E. coli*.

INTRODUCTION

β-Lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis of β-lactam ring, which result in ineffective compounds. At least 400 different types of b-lactamases, originating from clinical isolates, have been described (PARVEEN et al. 2012). Extended-Spectrum-β-Lactamases (ESBLs) are regarded as the major cause of resistance to penicillins, broad spectrum cephalosporins and monobactams among *Enterobacteriaceae*. These enzymes are found predominantly in *Escherichia coli* (*E. coli*) and *Klebsiella* species and have been described in other *Enterobacteriaceae* as well (GARZA-GONZÁLEZ et al. 2011).

The Ambler class A ESBLs are mainly encoded by the bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes which are generally found in plasmids and readily spread among different members of the *Enterobacteriaceae*. Currently, hundreds variants of ESBLs have been reported worldwide which arise mainly due to point mutations in ESBLs encoding genes (GARZA-GONZÁLEZ et al. 2011; KAUR; AGGARWAL 2013). Although *SHV* and *TEM* variants are the most frequent ESBLs, the *CTX-M* family of ESBLs have became prevalent in many

regions. The *CTX-M* family of enzymes appears to have arisen by initial transfer of the chromosomal β-lactamase gene from *Kluyvera* species to conjugative plasmids that have readily spread among different members of the *Enterobacteriaceae* and certain other gram-negative bacteria (JORGENSEN et al. 2010).

Uropathogenic *Escherichia coli* (UPEC) strains are important causal agent of Urinary Tract Infections (UTIs). ESBL producing bacteria show resistance to penicillins, extended spectrum cephalosporins and monobactams, resulting in failure of antibiotic therapy. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (e.g. aminoglycosides, tetracycline, chloramphenicol). Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited (CANTON et al. 2008). Thus, detection of the common ESBL genes such as bla_{TEM}, bla_{SHV} and bla_{CTX-M} by molecular methods in the ESBL producing bacteria and their patterns of antimicrobial resistance can provide valuable information about their epidemiology and can aid a rational antimicrobial therapy.

The present study was conducted to evaluate the prevalence of ESBL producing

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uropathogenic *E. coli* and to detect the presence of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes among ESBL producing isolates. In addition, antibiotic resistance profile of the isolates was investigated.

MATERIAL AND METHODS

Clinical isolates

In this study 70 urinary *E. coli* isolates were collected from patients with community acquired infections referring to clinical laboratory of Rasht city (Iran) during the period of February 2015 to January 2016. Clinical isolates were obtained from patients within an age range of 17 and 66 years (mean age= 37.9 years). Fifty-three strains were isolated from females (with a mean age of 35.4 years), and the remaining were obtained from males (with a mean age of 46.4 years). Bacterial identification was performed by conventional biochemical methods and all isolates were stored at -70ºC in Tryptic Soy broth containing 20% glycerol for subsequent analysis.

Antimicrobial susceptibility testing

The isolates were tested for their antimicrobial susceptibilities by the Kirby-Bauer disc diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) guideline using the following antibiotics(CLSI 2012): Ceftazidime (30 µg), Cefazoline (30 µg), Imipenem (10 µg), Ceftriaxone (30µg), Cefotaxime (30µg), Piperacillin (100µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Cefoxitin (30µg). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as the reference strains to control the quality of the applied antimicrobial agents (CLSI 2012).

Phenotypic detection of ESBLs

The ESBL phenotypic detection test was performed using the combination disc method on all isolates. Briefly, a disc of ceftazidime (30 µg) alone and ceftazidime + clavulanic acid (30 µg/10 µg) were placed at a distance of 25 mm, center to center, on a Muller Hinton Agar (MHA) plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight at 37°C. An increase in the inhibition zone diameter of 5 mm for a combination disc versus ceftazidime disc alone confirmed ESBL production (CLSI 2012). According to the CLSI guideline, cefotaxim could also be used instead of ceftazidime in the phenotypic detection assay (CLSI 2012).

Molecular identification of ESBL genes

Polymerase Chain Reaction (PCR) was employed to detect *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes among all 70 UPEC isolates. Bacterial plasmid extraction was performed using Viogene® plasmid DNA extraction kit according to the manufacturer's protocol. Briefly, the isolates were grown in Luria broth (Merck, Germany) overnight at 37ºC, centrifuged at 8000rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 200 µL MX buffer. Then, 250 µL of MX$_2$ buffer was added and incubated for 1-2 minutes at room temperature in order to lyse bacterial cells. After that, MX$_3$ buffer (350 µL) was added to neutralize the lysates. The cell lysates were centrifuged at 10000 ×g for 5 min and plasmid DNA precipitated by WN buffer and purified using spin columns.

The master mix for the PCR was prepared as follows: 3µL of 10× PCR buffer, 3µL of 25mM MgCl$_2$, 3µLof 10mM dNTP mix, 0.5µL of Taq DNA Polymerase , 9.5µL of MilliQ water and 1µL of each of the forward and reverse primers. Finally, 4µL of each DNA template was added in the corresponding tubes to make up the final reaction volume of 25µL. The PCR primer pairs used in this study were presented in Table 1.

Table1. Primer pairs used for amplification of Ambler class A ESBL genes

Target gene	Primers (5'-3')	Size of PCR product (bp)	Reference
bla$_{TEM}$	F: TCC GCT CAT GAG ACA ATA ATA ACG R: TTG GTC TGA TGA CAG TTA CCA ATG	930	(Kiratisin et al. 2008)
bla$_{SHV}$	F: TGG TTA TGC GTT ATA TTC GCC R: GGT TAG CGT TGC CAC TGCT	868	(Park et al. 2006)
bla$_{CTX-M}$	F: TCTTCCAGAAGGAATCCC R: CCGTTTCCGCTATTACAAAC	909	(Kiratisin et al. 2007)

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The reaction mix was incubated at 94ºC for 5 min followed by 30 cycles of PCR, denaturation for 50 s at 94 ºC, primer annealing at 53 ºC for both bla_{SHV} and bla_{TEM} and 54ºC for bla_{CTX-M} for 30 s and polymerization at 72 ºC for 1 min, was conducted. The polymerization was concluded by an extension period of 10 min at 72 ºC. Then, PCR products were mixed with 3µL Power load DNA stain and were visible after electrophoresis in a 1% agarose gel in TBE buffer and under UV transillumination.

RESULTS

Clinical isolates
A total number of 70 UPEC strains were isolated from UTIs. The majority of the isolates

associated to women's UTI (63 isolates), whereas only 7 UPEC were isolated from men's urine samples.

Antibiotic susceptibility profile
Among 70 UPEC isolates, 25 isolates (35.7%) displayed resistance to at least three cephalosporines (3rd and 4th generations). None of isolates were piperacillin susceptible while imipenem was found to be the most efficient antibiotic with 88.6 % susceptibility of the isolates. In addition the isolated strains indicated 50% and 64.28% resistance to ciprofloxacin and nalidixic acid, respectively. The antibiotic susceptibility patterns of the isolates were presented in Table 2.

Table 2. antimicrobial drug resistance among the isolates.

ESBL genes	Prevalence (n)	Antibiotic Resistance (%)								
		CAZ*	CRO*	CFZ*	CTX*	FOX*	NX	PIP	CIPX	IPM
ESBL strains	47	42.5	59.5	55.3%	44.6	40.4	63.8	87.2	53.2	10.6
Non ESBL strains	23	21.7	34.7	26.0%	17.4	21.7	65.2	91.3	47.8	15.0

CAZ, ceftazidime; CRO, ceftriaxone, CFZ, cefazolin; CTX, cefotaxime; FOX, cefoxitin NX, Nalidixic acid; PIP, piperacillin; CIPX, ciprofloxacin; IPM, imipenem;*Significant with P value less than 0.01.

Prevalence of ESBLs
ESBLs production among UPEC isolates was evaluated using phenotypic confirmatory test (Fig 1). According to the results, ESBL was noted in 37 isolates (52.8%). Prevalence of Ambler class A ESBL genes was investigated among all 70 isolates and the results were compared to the results from phenotypic test. Molecular detection of ESBL genes among UPEC isolates showed that 67.1 % (n= 47) of the isolates harbor at least one ESBL genes. Our

results indicated that the bla_{TEM} was the most prevalent ESBL gene (50%) while the least frequency was observed for bla_{SHV} (8.5%). The molecular characterization revealed that 22.8 % of isolates possessed the bla_{CTX-M} gene alone, while 35.7% and 8.5% of the isolates had bla_{TEM} and bla_{SHV} genes alone, respectively. The bla_{CTX-M} and bla_{TEM} genes together were found in 10% of isolates and 4.2%
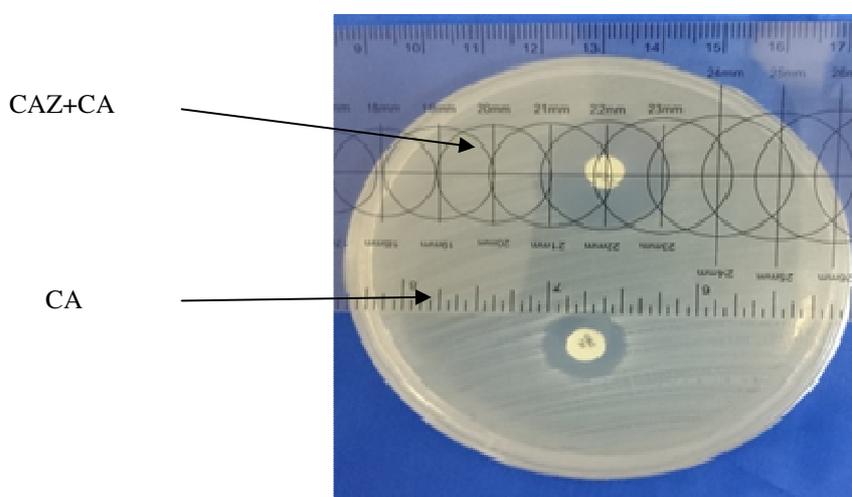

Figure 1. Screening of ESBLs producers by phenotypic combined disk assay. CAZ: ceftazidime; CA: clavulanic acid.
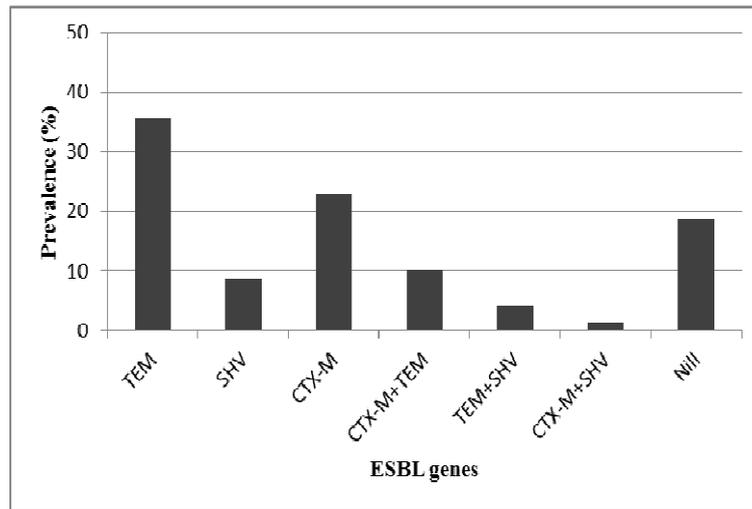
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Figure 2. Prevalence of different ESBL genes among the isolates had bla_{TEM} and bla_{SHV} together. In addition, only one isolate harbored both bla_{CTX-M} and bla_{SHV} and none of isolates harbored all the three ESBL genes (Figures 2 and 3).
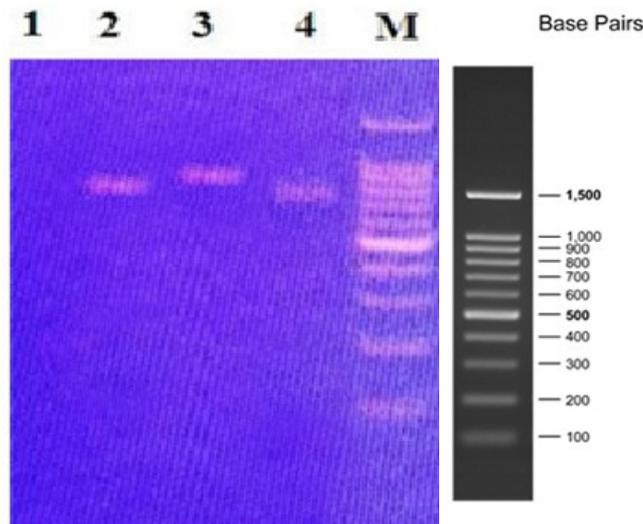

Figure 3. PCR amplification of Ambler class A ESBL genes. M: 100bp DNA ladder; 1: negative control; 2: bla_{CTX-M} gene (909 bp) ; 3: bla_{TEM} gene (930 bp); 4: bla_{SHV} gene (868 bp).

DISCUSSION

The emergence of ESBL genes mediated drug resistance has been increased worldwide. Overuse of broad spectrum antibiotics such as the second, third and fourth generation cephalosporins and other β-lactams could be associated with increase of ESBL producing strains. Infections caused by such pathogens often limit therapeutic options and cause treatment failures (ROSSOLINI et al. 2007; ZAHAR et al. 2009). The effective control of infections and reduction of the occurrence of drug-resistant bacterial strains, it is imperative to investigate the current status of antibiotic resistance as well as the mechanisms which are involved in drug resistance.

High prevalence of ESBLs among *enterobacteriaceae* causing human infections in Iran has been reported by several researchers (FEIZABADI et al. 2010; RIYAHI ZANIANI et al. 2012). In our study, prevalence of ESBLs among UPEC isolates was investigated by CLSI phenotypic confirmatory assay and the ESBL genes were also genotyped using molecular assay. According to the phenotypic confirmatory test, only 52.8% of the isolates were identified as ESBL producers while molecular analysis detected at least one ESBL genes in 67.1% of the isolates. There are multiple mechanisms of resistance to β-lactam antibiotics in a single isolate (BUSH et al. 2011; DRIEUX et al. 2008). Co-existence of ESBLs with chromosomal cephalosporinases, plasmid mediated AmpCs and

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carbapenemases may complicate the phenotypic detection of ESBLs (POULOU et al. 2014; TZELEPI et al. 2008). Thus, the lower sensitivity of phenotypic assay could be attributed to the expression of other β-lactamases and repression of ESBL genes. The expression of the non ESBL β-lactamases can mask the presence of ESBLs, so that, in terms of phenotypic screening, the prevalences of ESBLs may be underestimated (GARREC et al. 2011; JEONG et al. 2008). Therefore, molecular detection of ESBL genes is a more accurate method compared to the phenotypic screening assay.

This study documented that there was significant difference in antibiotic resistance pattern between ESBL and non ESBL strains. ESBL producing strains displayed significantly higher resistance to ceftazidim (CAZ), ceftriaxone (CRO), cefazolin (CFZ), cefotaxime (CTX) as well as cefoxitin (FOX), while, there was no significant difference in resistance to other antibiotics. In addition, imipenem (IMP) was found as the most effective antibiotic against UPEC isolates. In addition, higher resistance rate to ciprofloxacin among ESBL producing UPEC isolates was observed in our study. ESBL genes are often co-transferred with plasmid-mediated fluoroquinolone and aminoglycoside resistance genes, which results in multidrug resistance of the isolates (CARATTOLI, 2009).

The types and frequencies of ESBL production by members of the *Enterobacteriaceae* have significantly changed recently. bla_{SHV} and bla_{TEM} derived ESBLs have arisen from mutations in the very common genes that encode TEM-1 and SHV-1 enzymes (Jacoby and Munoz-Price 2005). In this study we reported the prevalence of bla_{TEM} and bla_{SHV} genes to be 74.2 and 12.2% respectively. This finding was higher than the values reported by Riyahi Zaniani et al. (RIYAHI ZANIANI et al., 2012) who reported a prevalence of bla_{TEM} and bla_{SHV} ESBL genes in *E. coli* isolates around 13.4 and 0% respectively. The new group of ESBLs, the bla_{CTX-M} family, has rapidly emerged and become the predominant ESBL type in many parts of the world (PATERSON et al. 2003; PITOUT; LAUPLAND 2008). In our study we found bla_{CTX-M} was the second dominant ESBL gene with the prevalence of 43.2%. This was not in accordance with the results reported by Yazdi et al. (2012) and Nakhaei Moghaddam et al. (2012) who reported a significantly higher prevalence of bla_{CTX-M} among pathogenic *E. coli* strains. Thus, our study indicated that the prevalence of different ESBL genes varies in different geographic areas.

The high prevalence of ESBL genes among UPEC strains is a major challenge which needs more consideration. In addition, although phenotypic assay is an easy, time-saving and cost effective method, the molecular assay is more accurate and reliable. In our study, we determined prevalence of different ESBL genes among UPEC isolates as well as their antibiotic susceptibility profile. However, lack of molecular sub-typing of each ESBL genes is the limitation of our work which needs more investigation in future.

RESUMO: As β-lactamases de espectro alargado (ESBLs), um grupo de enzimas bacterianas que são a principal causa de resistência às penicilinas, cefalosporinas de largo espectro e monobactamas, encontram-se entre os membros das Enterobacteriaceae. As ESBLs de classe A são principalmente codificadas pelos genes bla_{SHV}, bla_{TEM} e bla_{CTX-M} mediados por plasmídeo. Neste estudo, foi investigada a prevalência dos genes ESBL de classe A de Ambler entre isolados de *E. coli* uropatogênicos (UPEC) e seu padrão de suscetibilidade aos antibióticos. Setenta cepas UPEC foram isoladas a partir de amostras de urina e submetidas a ensaio de susceptibilidade antimicrobiana utilizando o método de difusão em disco. O rastreio fenotípico da produção de ESBL foi avaliado de acordo com o método de disco combinado CLSI. A genotipagem de ESBL de classe A de Ambler foi investigada usando PCR. De acordo com os resultados, as ESBLs foram identificadas em 37 isolados enquanto que o ensaio molecular mostrou 47 isolados portadores de genes ESBL. A maior prevalência foi registrada para bla_{TEM} (74,2%), seguida de bla_{CTX-M} (43,2%) e bla_{SHV} (12,2%). O imipenem foi o fármaco mais eficaz e os isolados produtores de ESBL apresentaram maior resistência a CAZ, CRO, CFZ, CTX e FOX em comparação com os isolados não ESBL. Em conclusão, a alta prevalência de genes ESBL de classe A foi observada em nosso estudo, que necessita de maior atenção e prescrição de antibióticos racionais.

PALAVRAS-CHAVE: Antibiograma. ESBL. Discos combinados fenotípicos. Uropathogenic E. coli.

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