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The Distribution of Beta Lactamase Genes in *Escherichia Coli* Phylotypes Isolated from Diarrhea and UTI Cases in Northwest Iran

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Pathogenic *Escherichia coli* strains are a common cause of intestinal and extra-intestinal infections, especially in developing countries. Extended spectrum beta-lactamases ($ESBL_S$), a heterogeneous group of plasmid-encoded beta-lactamases, are common throughout the world.
Objectives. The aim of the present study was to determine the phenotypic and genotypic characteristics of $ESBL_S$ produced by *E. coli* isolates taken from patients with diarrhea and urinary tract infections (UTI) in northwest Iran.
Material and Methods. A total of 132 *E. coli* isolates (92 isolates from UTI and 40 isolates from diarrheic cases) were recovered and confirmed by biochemical tests. The isolates were examined for bla_{TEM} and bla_{SHV} genes and phylogenetic background by two multiplex PCR assays. The isolates were tested for antibiotic susceptibility against nine antibiotic agents by the disk diffusion method.
Results. The phylogenetic analysis showed that the UTI isolates mostly fell into phylo-group B2, followed by D, while the diarrheic isolates belonged to phylo-groups D and A. Out of 92 UTI isolates, 29.3% and 17.4% possessed bla_{TEM} and bla_{SHV} genes, respectively. Ten diarrheic isolates were positive for $bla_{TEM,}$ two isolates possessed the bla_{SHV} gene, and one isolate was positive for both genes. The UTI isolates that were positive for bla_{TEM} and bla_{SHV} genes mostly belonged to phylo-groups D and B2, whereas the diarrhea isolates were in phylo-groups D and A. Phylogenetic group D isolates have an accumulation of $ESBL_S$ genes in the diarrheic and UTI isolates. In both the UTI and diarrhea isolates, the maximum rate of resistance was against cefazolin, and the minimum rate of resistance was against nitrofurantoin. Twenty-four antibiotic resistance patterns were observed among the isolates. The amikacin, ciprofloxacin, cefotaxime, cefuroxime, cefazolin, gentamicin, nalidixic acid and trimethoprim/sulfamethoxazole resistance pattern was the most prevalent in the isolates that belonged to phylo-group D.
Conclusions. The correct choice of effective antibiotic policy is needed to limit the spread of antibiotic resistance in bacteria (**Adv Clin Exp Med 2014, 23, 4, 523–529**).

Key words: extended spectrum beta-lactamases (ESBLs), *Escherichia coli*, diarrhea, UTI, phylogeny.

Escherichia coli (*E. coli)* strains are versatile bacteria, comprising harmless commensal bacteria as well as different pathogenic variants with the ability to cause both intestinal and extra-intestinal diseases in humans and other hosts [1]. Some strains have specific virulence attributes, which confer the ability to adapt to new niches and cause a broad spectrum of diseases [2]. Several variants have been described, and the strains capable of causing infection of the gastrointestinal system are called intestinal pathogenic *E. coli,* while those that cause infections outside the gastrointestinal system are called extra-intestinal pathogenic *E. coli* (ExPEC) [3]. The most frequently reported extra-intestinal diseases caused by ExPEC are urinary tract infections (UTI); other neonatal diseases include meningitis, septicemia, nosocomial pneumonia, cholecystitis, peritonitis, osteomyelitis

and infectious arthritis [4, 5]. Diarrheagenic *E. coli* strains (DEC) are among the most important bacterial enteric pathogens, and are classified into six categories, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) [6]. Pathogenic *E. coli* isolates have a relatively high potential for developing resistance, so the treatment of *E. coli* infections is becoming increasingly difficult [7]. There are several mechanisms of drug resistance; one of the most important resistant mechanisms is the production of beta-lactamases [8]. Beta-lactamase enzymes hydrolyze the four-membered beta-lactam ring common to a wide range of beta-lactam type antibiotics [9].

Among the clinically bacterial isolates, bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ are the most frequently reported extended-spectrum beta-lactamase (ESBL) genes [10]. *E. coli* and *Klebsiella pneumoniae* (*K. pneumoniae*) are common species of *Enterobacteriacae* that both have pathogenic potential and frequently incorporate ESBL-encoding genes [11].

There are four well-recognized phylogroups of *E. coli* strains and these have been designated A, B1, B2 and D. These groups can be divided into six phylogenetic subgroups including (A_0, A_1, $B2_2$, $B2_3$, D_1, and D_2), according to the combination of the three genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2 [12]. Phylogenetic group distribution may be related to antimicrobial resistance prevalence in isolates of *E. coli* [13].

The aim of this study was to determine the bla_{TEM} and bla_{SHV} genes, phylogenetic background and antibiotic resistance profile of *E. coli* isolates from diarrhea and UTI samples in the Hamadan province in northwest Iran.

Material and Methods

Sampling and Bacteriological Examinations

The study was carried out on 92 UTI samples and 40 diarrhea specimens from patients referred to laboratories in Hamadan, in northwest Iran. The samples were obtained from patients aged between one and 65 years old. They were collected between April and November 2012. The specimens were cultivated on 5% sheep blood agar and MacConkey agar plates (Biolife Laboratories, Milano, Italy). The isolates were confirmed to be *E. coli* based on colony morphology, gram stain and standard biochemical methods. A single colony was obtained from each sample. Cultures of each isolate confirmed as *E. coli* were maintained in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at –80°C.

Polymerase Chain Reaction (PCR) and Reference Strains

DNA was extracted from the *E. coli* isolates and reference strains by the lysis method. The *E. coli* reference strain ECOR62 (*chuA*+, *yjaA*+ and Tspe4. C2+), ATCC *E. coli* 35218 strain (bla_{TEM}+) and *K. pneumoniae* 700603 strain (bla_{SHV}+) were used as positive controls. *E. coli* strain ATCC 25922 was used as a negative control. The PCR primers specific for bla_{TEM} and bla_{SHV} were described by Sharma et al. [14]; phylogenetic groups (A, B1, B2 and D) were described by Clermont et al. [15]. Classification of the isolates in to phylogenetic subgroups (A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2) was undertaken as described by Escobar-Paramo et al. [12]. The sequences and sizes of the PCR products are shown in Table 1.

Table 1. Oligonucleotide primers used in this study

Reference	Product size (bp)	Primer sequence (5'–3')	Gene
Sharma et al. (2010)	1080 bp	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	bla_{TEM}
	928 bp	GGGTAATT CTTATTTGTCGC TTAGCGTT GCCAGTGCTC	bla_{SHV}
Clermont et al. (2000)	211 bp	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	*yjaA*
	152 bp	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	*TspE4C2*
	279 bp	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	*chuA*

Antimicrobial Susceptibility Test

The antibiotic resistance of all the isolates to nine antibacterial agents was assessed by the disk diffusion method according to the Clinical and Laboratory Standards Institute's guidelines [16]. The antibiotic disks (Mast, England) used in this study were amikacin (30 μg), cefazolin (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). *E. coli* ATCC 25922 was used as the positive control for the disk diffusion test.

Statistical Analysis

The statistical analysis for the descriptive data was carried out using SPSS software, version 17.

Results

Following the bacteriological tests, 132 confirmed *E. coli* isolates (92 urine samples and 40 diarrhea samples) were chosen for antibiogram and molecular examinations. The isolates were recovered from both female (88) and male (44) patient samples.

The phylogenetic analysis showed that the 132 *E. coli* isolates segregated into phylogenetic groups A (33.3%), B1 (6.8%), B2 (25%) and D (34.9%). The PCR assays for phylotyping of the 92 UTI *E. coli* isolates indicated that those isolates fell into 4 phylogenetic groups: 23 of them belonged to A (25%), 8 belonged to B1 (8.7%), 29 were B2 (31.5%) and 32 isolates belonged to group D (34.8%). The PCR assays of the 40 diarrheic *E. coli* isolates showed that 21 isolates (52.5%) belonged to phylogenetic group A, one isolate (2.5%) to B1, 4 isolates (10%) to B2 and 14 isolates (35%) to group D.

Further analysis of the PCR phylotyping of UTI *E. coli* isolates showed that the isolates fell into five phylogenetic subgroups: A_0, A_1, $B2_3$, D_1 and D_2; most of them were in subgroups $B2_3$ (31.5%) and D1 (26.1%). The 40 diarrheic *E. coli* isolates also fell into five phylogenetic subgroups: A_0, A_1, $B2_3$, D_1 and D_2; the A_0 35% (14 isolates) and D_1 25% (10 isolates) were the most prevalent subgroups (Table 2).

Table 2. Distribution of diarrhea and UTI isolates in phylogenetic groups/subgroups

Phylo		Diarrhea isolates (no.: 40)		UTI isolates (no.: 92)	
group	subgroup	no.	(%)	no.	(%)
A		21	(52.50)	23	(25.00)
	A_0	14	(35.00)	13	(14.10)
	A_1	7	(17.50)	10	(10.90)
B1		1	(2.50)	8	(8.70)
B2		4	(10.00)	29	(31.50)
	$B2_2$	0	(0.00)	0	(0.00)
	$B2_3$	4	(10.00)	29	(31.50)
D		14	(35.00)	32	(34.80)
	D_1	10	(25.00)	24	(26.10)
	D_2	4	(10.00)	8	(8.70)

The genotyping of the *E. coli* isolates revealed that out of 92 UTI isolates and 40 diarrheic isolates, 56 (42.4%) isolates were positive for the bla_{TEM} and bla_{SHV} genes. Specifically, 37 isolates (28.03%) were positive for $bla_{TEM;}$ 18 (13.6%) were positive for bla_{SHV} genes; and 1 isolate (0.76%) was positive for both genes. Thirteen diarrheic isolates (32.5%) and 43 UTI isolates (47%) contained bla_{TEM} and/or bla_{SHV} genes (Table 3). Out of 13 (32.5%) ESBL positive isolates from diarrheic cases, 10 (25%) isolates carried bla_{TEM} genes and 2 (5%) had bla_{SHV} genes, while 1 (2.5%) isolate included both genes. Out of the 43 (47%) ESBL positive isolates from UTI cases, 27 isolates (29.3%) possessed bla_{TEM} genes and 16 (17.4%) had bla_{SHV} genes (Table 3).

Table 3. Combination of beta-lactamase genes in 40 diarrheic and 92 UTI isolates in relation to phylogenetic groups/subgroups

Gene	Diarrhea isolates								UTI isolates							
	A		B1	B2		D		Total	A		B1			D		Total
	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2		A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	
bla_{TEM}	1	2	1	–	2	3	1	10	3	2	1	–	11	7	3	27
bla_{SHV}	–	–	–	–	–	1	1	2	3	2	2	–	3	6	–	16
bla_{TEM},bla_{SHV}	–	–	–	–	–	1	–	1	–	–	–	–	–	–	–	–
Negative	–	–	–	–	–	–	–	27	–	–	–	–	–	–	–	49
Total	1	2	1	–	2	5	2	40	6	4	3	–	14	13	3	92

The phylotyping of 56 diarrhea and UTI *E. coli* isolates possessing beta-lactamase genes indicated that these isolates belonged mainly to phylogenetic groups D and B2. The 10 bla_{TEM} positive isolates from diarrheic cases fell into phylogenetic groups A (3 isolates), B1 (1), B2 (2) and D (4); the 27 UTI isolates belonged to phylogenetic groups A (5 isolates), B1 (1), B2 (11) and D (10). Both of the 2 bla_{SHV} positive isolates from diarrheic cases fell into phylogenetic group D; the 16 bla_{SHV} positive UTI isolates belonged to groups A (5 isolates), B1 (2), B2 (3) and D (6). The 1 isolate (0.76%) that was positive for both genes fell into phylogenetic group D (Table 3).

According to the results of the antibiotic susceptibility test, in the diarrheic isolates the maximum rate of resistance was against cefazolin 97.5% (39 isolates) and the minimum resistance rate was against nitrofurantoin 10% (4 isolates). The rates of resistance to other antibiotics were as follows: cefotaxim 34 (85%), nalidixic acid 25 (62.5%), cefuroxime 24 (60%), cotrimoxazole 20 (50%), amikacin 19 (47.5%), ciprofloxacin 13 (32.5%), and gentamicin 14 (35%). In the UTI isolates the maximum rate of resistance was against cefazolin 95.7% (88 isolates), followed by cefotaxim 87% (80), cotrimoxazole 66% (61), cefuroxime 65.2% (60), nalidixic acid 59% (54), gentamicin 53.3% (49), amikacin 39.1% (36), ciprofloxacin 39.1% (36), and nitrofurantoin 23.9% (22). The results showed that 24 antibiotic resistance patterns were found among the *E. coli* isolates. Fourteen antibiotic resistance patterns were detected in the diarrheic isolates. The cefotaxime, cefuroxime, cefazolin, nalidixic acid and trimethoprim/sulfamethoxazole resistance pattern was the most prevalent pattern in the diarrheic isolates that belonged to phylogenetic groups A and D. Among the UTI isolates, 24 antibiotic resistance patterns were detected. The most frequent antibiotic resistance pattern among these isolates was against amikacin, ciprofloxacin, cefotaxime, cefuroxime, cefazolin, gentamicin, nalidixic acid and trimethoprim/sulfamethoxazole, which was observed in phylogenetic group D. Twenty-four antibiotic resistance patterns were determined, distributed among the isolates in the 4 phylogenetic groups (Table 4).

Discussion

E. coli is one of the principal bacterial pathogen causes of UTIs, enteric infections and systemic infections in humans and other hosts [3]. Diarrheagenic *E. coli* strains (DECs) are an important cause of diarrhea, especially in developing countries and constitute an important public health problem [6, 17]. Extensive use of antibiotics to treat *E. coli* infections caused by resistant isolates will increase the problem of resistance [18, 19]. bla_{TEM} and bla_{SHV} genes, which are commonly found in *E. coli* and *K. pneumoniae,* confer resistance to a broad spectrum of beta lactam antibiotics and are a significant therapeutic concern for infections caused by many species of gram-negative bacteria [20–23]. At the same time, the epidemiology of ESBL-producing *E coli* is complicated and varies among hospitals and countries [24]. There are alarming trends of associated resistance to other classes of antimicrobial drugs among ESBL-producing organisms isolated from the community [25].

In the current study, antibiotic resistance patterns were studied for *E. coli* isolates. Resistance was observed to antibiotics in widespread use, such as cefazolin, ciprofloxacin, cotrimoxazole, cefotaxime, gentamicin, amikacin and nalidixic acid. Most of the isolates in the present study were resistant to multiple antibiotic groups. High resistance to common antibiotics has also been reported in previous studies [7, 11, 26]. The present study offers the first characterization of TEM-type and SHV-type ESBL variants produced by *E. coli* circulating in hospitals in Hamadan (northwest Iran). The progressive spread of ESBL-producing *E. coli* seems to be caused mainly by the extensive use of broad-spectrum beta-lactam antibiotics in empiric therapy and rapid plasmid mediated distribution of resistance genes between bacterial species [23]. In this study, the bla_{TEM} gene is the most prevalent ESBL, followed by the bla_{SHV} gene. Therefore, it seems that these two genes are appropriate candidates for molecular screening of ESBL positive samples [8].

Enterobacteriaceae harboring transferable bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{OXA} genes have been reported worldwide in several studies of clinical isolates. Isendahl et al. [11] reported that 32.6% fecal samples from children were carriers of ESBL-producing *E. coli* or *K. pneumonia*. Shahid et al. [22] reported findings similar to those of the present study: 38.4% of the isolates in their study were positive for *bla* genes, and the bla_{TEM} gene was more prevalent than the bla_{SHV} gene. In another study, the prevalence of bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes among *E. coli* isolates were 100%, 37.5% and 4.1% respectively [24]. The incidence of ESBL-producing isolates from 2002 to 2010 increased from 9.2% to 21.2%. The highest rates were observed in Asia (38.3%) and Latin America (22.9%), and the lowest rates in Africa (6.3%), North America (6%), and the South Pacific (5.8%) [27]. In an investigation on patients with UTI in the capital of Iran (Tehran) a combined disk test indicated that of 109 ESBL-positive isolates, 77 isolates (70.6%)

Table 4. Antibiotic resistance patterns in relation to phylogenetic groups

Phylogroups (Diarrhea)						Phylogroups (UTI)					Antibiotic resistance patterns
Total	Total	D	B2	B1	A	Total	D	B2	B1	A	
9	4	2	2	–	–	5	2	3	–	–	AK, CIP, CTX, CXM, CZ, GM, NA, NI, TS*
23	6	2	1	–	3	17	7	5	2	3	AK, CIP, CTX, CXM, CZ, GM, NA, TS
3	–	–	–	–	–	3	2	1	–	–	AK, CIP, CTX, CXM, CZ, GM, NA, NI
2	–	–	–	–	–	2	–	–	2	–	AK, CTX, CXM, CZ, GM, NA, NI, TS
6	1	–	–	–	1	5	2	2	1	–	CIP, CTX, CXM, CZ, GM, NA, NI, TS
6	2	–	1	1	–	4	–	4	–	–	AK, CIP, CTX, CXM, CZ, GM, TS
7	2	1	–	–	1	5	1	–	–	4	AK, CIP, CTX, CXM, CZ, NA, TS
2	1	–	–	–	1	1	1	–	–	–	AK, CTX, CXM, CZ, GM, NA, TS
4	1	–	–	–	1	3	1	–	–	2	AK, CTX, CXM, CZ, NA, NI, TS
1	–	–	–	–	–	1	1	–	–	–	CTX, CXM, CZ, GM, NA, NI, TS
1	–	–	–	–	–	1	–	1	–	–	AK, CIP, CTX, CXM, CZ, TS
2	–	–	–	–	–	2	–	1	–	1	CIP, CTX, CXM, CZ, GM, NA
2	–	–	–	–	–	2	–	–	1	1	CTX, CXM, CZ, GM, NA, TS
5	–	–	–	–	–	5	3	1	1	–	CTX, CXM, CZ, GM, NA, TS
4	–	–	–	–	–	4	4	–	–	–	CTX, CXM, CZ, NA, NI, TS
3	–	–	–	–	–	3	–	2	–	1	AK, CTX, CXM, CZ, GM
10	3	1	–	–	2	7	1	2	1	3	CTX, CXM, CZ, GM, NA
5	1	1	–	–	–	4	3	1	–	–	CTX, CXM, CZ, GM, TS
15	8	4	–	–	4	7	3	–	–	4	CTX, CXM, CZ, NA, TS
3	2	1	–	–	1	1	–	–	–	1	AK, CTX, CZ, NA
4	1	1	–	–	–	3	1	1	–	1	CTX, CZ, NA, NI
4	–	–	–	–	–	4	–	3	–	1	CTX, CZ, TS
4	3	1	–	–	2	1	–	1	–	–	CTX, NA
7	5	–	–	–	5	2	–	1	–	1	CXM, CZ
132	40	14	4	1	21	92	32	29	8	23	Total

* AK – amikacin, CIP – ciprofloxacin, CTX – cefotaxime, CXM – cefuroxime, CZ – cefazolin, GM – gentamicin, NA – nalidixic acid, NI – nitrofurantoin, TS – trimethoprim/sulfamethoxazole.

were carrying bla_{SHV}, 75 isolates (68.8%) were carrying $bla_{CTX\text{-}M}$, and 95 isolates (87.1%) were carrying bla_{TEM} genes, while 40 isolates (36.6%) isolates had all three of these genes [8].

Phylogenetic analyses have shown that extra-intestinal and intestinal *E. coli* are segregated in four main phylogenetic groups: A, B1, B2, and D [15]. In the present study, UTI isolates fell mostly into phylogenetic group B2, followed by D; and diarrheic isolates belong to groups D and A. Similarly, previous reports indicated that most ExPEC strains belonged to group B2, although some fell into group D [3, 28]. In this study, UTI isolates that were positive for bla_{TEM} and bla_{SHV} genes mostly belonged to phylogenetic groups D and B2, while diarrhea isolates that were positive for these genes were in groups D and A. A study of community-acquired fecal carriage of ESBL-producing *E. coli* isolates in children showed that phylo-group A predominated and that the most frequent ESBL was *CTX-M-1*. The *CTX-M* producing isolates mainly carried one other beta-lactamase-encoding gene (*TEM-1* and *SHV*). Most of the ESBL-producing *E. coli* isolates belonged to groups A and B1, possibly because of greater antibiotic exposure of group A/B1 strains in the fecal flora [28].

In conclusion, as Abdou et al. wrote: "Resistance in gram negative bacteria (such as *E. coli*) presents a major challenge for the antimicrobial therapy and significantly narrows the treatment options of human infections" [29]. Pathogenic isolates of *E. coli* have relatively high potentials for developing resistance and can make the treatment of ESBL-producing *E. coli* infections difficult [7]. Therefore, correctly detecting drug-resistant *E. coli* is important to prevent the further spread of beta-lactamase-positive resistance in humans and other hosts [20]. The correct choice of safe and effective antibiotic policy is needed to limit the emergence and spread of antibiotic resistance in bacteria [7]. The present study examined the genes codifying for beta-lactamases, but this does not necessarily reflect the expression of these genes during infection; and the results might not be applicable to areas with a different epidemiology of ESBL-producing strains of *E. coli.* Discrepancies in the distribution of genotypes of ESBLs may be due to differences in the patterns of antibiotic use in various geographical areas. In addition, frequent surveillance of resistance to antimicrobial agents is necessary.

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