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Research Article

Phylotyping of *bla*$_{CTX-M-15}$ gene in extended spectrum beta lactamase producing *Escherichia coli* isolates from clinical samples in Iran

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Abstract. Aim: *Escherichia coli* (*E. coli*) producing extended-spectrum β-lactamase (ESBL) has become prevalent in worldwide. The ESBL-producing *E. coli* is genetically various, and isolates have been divided into four phylog-groups (A, B1, B2 and D). Material and method: One hundred and fourteen (ESBL) producing *E. coli* isolates were isolated from urinary tract infections (58 isolates) and diarrhea (56 isolates) samples. Screening and confirmation tests of ESBLs were done by disk-diffusion and broth micro-dilution methods according to CLSI. The isolates were studied to determine the phylogenetic groups and presence of and beta-lactamase (*bla*$_{CTX-M-15}$) gene. Results: Phylogenetic analysis revealed that ESBL producing UTI isolates belonged to four phylogenetic groups A (43.10%), B1 (3.44%), B2 (18.96%) and D (34.48%). PCR assays of ESBL producing diarrheic *E. coli* isolates indicated that 67.85% fell into A, 7.14% to B1, 10.71% to B2 and 14.28% to D phylogenetic groups. Forty one isolates from urine samples and 52 isolates from diarrheic samples were positive for the *bla*$_{CTX-M-15}$ gene respectively. Phylotyping of isolates possessed β-lactamase gene indicate that the isolates distributed in four phylogenetic groups including A (52 isolates), B1 (5), B2 (12) and D (24) phylogenetic groups. Conclusion: Production of ESBL among *E. coli* strains isolated from UTI and diarrheic was relation to change in phylogenetic distribution toward non-B2 phylogenetic groups, in particular groups A and D. In addition, results of current study indicated the higher prevalence of *bla*$_{CTX-M-15}$ gene in investigated isolates.

Key Words: *Escherichia coli*, Phylogenetic group, ESBL, *bla*$_{CTX-M-15}$

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Introduction

Infections with antibiotic-resistant bacteria are relation to higher rates of illnesses, which have an effect on rising health care costs (Overdevest et al 2011). *Escherichia coli* (*E. coli*) producing extended-spectrum β-lactamase (ESBL) has become prevalent in hospitals throughout the world since the late 1980s (Paterson & Bonomo 2005). Pathogenic *E. coli* strains can cause a wide variety of extra-intestinal and intestinal diseases such as urinary tract infection (UTI), septicemia, neonatal meningitis and diarrhea (Clermont et al 2000). Uropathogenic *E. coli* (UPEC) are the most frequent agent causing in both community-based and hospitalized patients. On the other hand, diarrheagenic *E. coli* are an important cause of endemic and epidemic diarrhea worldwide (Alizade et al 2013; Jafari et al 2008).

ESBL genes are located on mobile genetic elements (plasmids or transposons) which can be easily mobilized between different bacterial species. Some ESBL encoding genes are mutations of primary plasmid-mediated β-lactamases (e.g., *bla*$_{TEM/SHV}$), and others ESBL genes are transferred from environmental bacteria (e.g., *bla*$_{CTX-M}$) (Overdevest et al 2011; Ruppé et al 2009). The recent global ESBL genes increase has been caused significantly by CTX-M type enzymes. The epidemiology of ESBL encoding genes is changing rapidly and show geographic regions differ in distribution of genotypes of *bla*$_{CTX-M}$ β-lactamases (Hawkey & Jones 2009). Since the turn of the century there have been dramatic shifts reported in both the prevalence and types of ESBLs reported in the world, reports of isolates producing CTX-M remain sporadic, while in Asia, much of Europe and South America, endemic prevalence has been reached (Canton & Coque 2006).

A triplex PCR method has been developed for categories *E. coli* strains to phylogenetic groups A, B1, B2 and D (Clermont et al 2000). Strains that cause extra-intestinal infections assign mostly to B2 phylo-group and lesser extent to D phylogenetic group, whereas commensal *E. coli* strains belong mostly to group A and group B1. Prevalence of antibacterial resistance was shown to be greater in non-B2 phylogenetic group *E. coli* strains (Ahmed et al 2013).

Table 1. Primers used in this study

Primer	Primer sequence (5′-3′)	Annealing temp (°C)	Product size (bp)
bla $_{CTX-M-15}$	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	60	550
yjaA	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	55	211
TspE4.C2	CTGGCGAAAGACTGTATCAT CGCGCCAACAAAGTATTACG	55	152
chuA	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	55	279

This study was set to determine of phylogenetic groups and prevalence of the *bla* $_{CTX-M-15}$ gene in extended spectrum beta lactamase producing *E. coli* isolates from diarrhea and UTI samples in Kerman province of Iran.

Materials and Methods

Bacterial isolates

Totally 114 extended spectrum beta lactamase producing *E. coli* were isolated from the clinical specimens including diarrhea and urine of patients admitted to medical laboratories in Kerman, southeast Iran. The *E. coli* isolates (n=58) were recovered from urine followed by isolates from diarrehea (n=56). In order to confirm *E. coli* isolates, standard biochemical and bacteriological tests were used. After identification, all isolates were stored at –70°C in Luria-Bertani broth (In vitrogen, Paisley, Scotland) with 30% glycerol till further tests.

ESBL confirmatory test by disk diffusion method

Disk confirmation test using cefotaxime (CTX) (30 ug) and ceftazidime (CAZ) (30 ug) disks in combination with and without 10 ug of clavulanate (CLA) were done and interpreted by Clinical Laboratory Standards Institute (CLSI, 2013) guidelines for ESBL screening and disk confirmation tests (CLSI, 2013). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls, respectively.

ESBL confirmatory test by broth micro-dilution method

Minimum inhibitory concentrations (MICs) for cefotaxime (0.25–64 µg/mL), ceftazidime (0.25–128 µg/mL), ceftazidime-clavulanic acid (0.25/4–128/4) µg/mL and cefotaxime-clavulanic acid (0.25/4–64/4 µg/mL) were determined by broth micro-dilution method. A ≥ 3 twofold concentration decrease in an MIC for both ceftazidime and cefotaxime tested in combination with clavulanic acid vs when these agents tested alone (CLSI, 2013). Quality controls were conducted using the reference strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

DNA extraction

The several colonies (3 to 5) from fresh pure cultures were suspended in 0.5 mL sterile distilled water. The suspension lysed by heating at 95oC for 10 minutes. The supernatant (template DNA) was obtained by centrifugation at 8,000 rpm for 5 minutes.

Phylogenetic groups typing

The triplex PCR method described by Clermont et al. (2000) was used to assign the *E. coli* isolates. The presence/absence of the three PCR products (*chuA*, *yjaA* and TspE4.C2) is used to assign an unknown isolate to one of the phylo-groups. *E. coli* strains belonged to one of the four phylo-groups typing: A, B1, B2 or D. The phylogenetic subgroups (A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2) of each isolate were determined as described previously (Escobar-Paramo et al 2004). In this study the *E. coli* strain ECOR62 fell into phylogenetic group B2, was used as a positive control. *E. coli* strain MG1655 as a positive control for phylogenetic group A. The primers and sizes of the expected amplification product are listed in Table 1.

β-Lactamase gene identification

All isolates were screened for the presence of *bla* $_{CTX-M-15}$ gene. Primer sequences of *bla* $_{CTX-M-15}$ gene in this study are shown Table 1. PCR conditions were as follow: Hot start: 94˚C 5 minutes; 30 cycle (94˚C in 30 seconds, 60˚C in 30 seconds, 72˚C in 30 seconds) final extension: 72˚C in 10 minute. The PCR products were observed by gel electrophoresis on 1.5% agarose gels (BIONEER) stained with ethid¬ium bromide.

Results

Disk diffusion indicated that 114 isolates were resistant to cefotaxime and ceftazidime and positive for ESBL production by disk confirmatory test using cefotaxime/clavulanate and ceftazidime/clavulanate. The MICs of β-lactams and β-lactam/inhibitor combinations were determined by broth microdilution method. PCR phylotyping revealed that the 114 ESBL producing *E. coli* isolates distributed in phylo-groups A (55.26%), B1 (5.26%), B2 (14.91%) and D (24.56%). The results showed that the isolates belong to six phylogenetic subgroups, including 44 isolates (38.59%) to A_0, 19 isolates (16.66%) to A_1, 5 isolates (4.38%) to $B2_2$, 12 isolates (10.52%) to $B2_3$, 19 isolates (16.66%) to D_1 and 9 isolates (7.89%) to subgroup D_2 (Table 2). Phylogenetic analysis revealed that 58 ESBL producing UTI isolates segregated in four phylogenetic groups including 25 isolates (43.10%) in A, 2 isolates (3.44%) in B1, 11 isolates (18.96%) in B2, and 20 isolates (34.48%) in D group. PCR assays of 56 ESBL producing diarrheic *E. coli* isolates showed that 38 isolates (67.85%) belonged to A, 4 isolates (7.14%) to B1, 6 isolates (10.71%) to B2 and 8 isolates (14.28%) to D phylogenetic groups. Phylotyping of urine and diarrheic isolates showed that the isolates fell into six subgroups A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2 (Table 2).

Among the investigated isolates, 93 isolates (81.57%) possessed *bla* $_{CTX-M-15}$ gene. Forty one isolates (70.68%) from 58 urine samples and 52 isolates (92.85%) from 56 diarrheic samples were positive for the *bla* $_{CTX-M-15}$ gene respectively.

Table 2. Distribution of UTI and diarrhea *E. coli* isolates in detected phylo-groups/subgroups

Phylo-group	A no (%)		B1 no (%)	B2 no (%)		D no (%)		Total no (%)
Phylo-subgroup	**A_0**	**A_1**	**B1**	**$B2_2$**	**$B2_3$**	**D_1**	**D_2**	
UTI isolates	18 (31.03)	7 (12.06)	2 (3.44)	1 (1.72)	10 (17.24)	11 (18.96)	9 (15.51)	58 (100.00)
Diarrhea isolates	26 (46.42)	12 (21.42)	4 (7.14)	4 (7.14)	2 (3.57)	8 (14.28)	-	56 (100.00)
Total Phylo-group	44 (38.59)	19 (16.66)	6 (5.26)	5 (4.38)	12 (10.52)	19 (16.66)	9 (7.89)	114 (100.00)

Table 3 Distribution of isolates possess $bla_{CTX\text{-}M\text{-}15}$ in phylo-group/subgroups

Phylo-groups	A no (%)		B1 no (%)	B2 no (%)		D no (%)		Total no (%)
	A_0	**A_1**	**B1**	**$B2_2$**	**$B2_3$**	**D_1**	**D_2**	
Positive UTI isolates	10 (24.39)	6 (14.64)	1 (2.44)	-	8 (19.52)	7 (17.07)	9 (21.94)	41 (100.00)
Negative UTI isolates	8 (47.06)	1 (5.88)	1 (5.88)	1 (5.88)	2 (11.76)	4 (23.52)	-	17 (100.00)
Positive diarrhea isolates	24 (46.15)	12 (23.07)	4 (7.69)	4 (7.69)	-	8 (15.38)	-	52 (100.00)
Negative diarrhea isolates	2 (50.00)	-	-	-	2 (50.00)	-	-	4 (100.00)
Total no (%)	44 (38.59)	19 (16.66)	6 (5.26)	5 (4.38)	12 (10.52)	19 (16.66)	9 (7.89)	114 (100.00)

Phylotyping of isolates possessed β-lactamase gene indicate that the isolates distributed in four phylogenetic groups including A (52 isolates), B1 (5), B2 (12) and D (24) phylogenetic groups. Fifty two $bla_{CTX\text{-}M\text{-}15}$ positive diarrheic isolates belonged to A (36 isolates), B1 (4), B2 (4) and D (8) groups, whereas the 41 $bla_{CTX\text{-}M\text{-}15}$ positive UTI isolates fell into A (16 isolates), B1 (one), B2 (8) and D (16) phylogenetic groups (Table 3).

Discussion

This study was designed to evolution the role of the genetic background of *E. coli* isolates in the present of $bla_{CTX\text{-}M\text{-}15}$ gene. The molecular biology details of *E. coli* are poorly understood. (Navidinia et al 2013). The pathogenic strains causing UTI mostly fell into B2 and D phylo-groups, which was similar with previous studies (Duriez et al 2001; Carlos et al 2010; Escobar-Paramo et al 2004). Phylogenetic analysis of *E. coli* isolates showed that diarrheagenic *E. coli* strains were distributed among groups A, B1 and D. However, in contrast to the studies were done in developed countries (France and USA); most commensal strains assigned to group A or B1, as many as the commensal strains fell into B2 or D phylogenetic groups. Reports of the phylogenetic distribution of *E. coli* pathogenic strains relation to acute diarrhea are distributed in non-B2 and D phylo-groups. As tropical populations seem too preferably carrier strains of A and to a lesser extent B1 phylogenetic groups, these strains might have the genetic background essential for the emergence of diarrheagenic *E. coli* strains (Escobar-Paramo et al 2004). Alizade et al (2013) reported that phylogenetic groups A and D were predominant in *E. coli* isolated from UTI in Bam area (southeast of Iran) and also *E. coli* isolates resistance to antibiotics shifts to non-B2 phylogenetic groups. In another study in Rigan area (southeast of Iran) phylogenetic analysis indicated that UTI *E. coli* isolates mostly fell into phylogenetic groups B2 and D and mostly diarrheic isolates belonged to A and D phylo-groups (Alizade et al 2014). This difference can maybe attribute to the bacterial characteristics in different geographic areas under the effect of host genetic elements or antibiotics usage (Duriez et al 2001; Lee et al 2010).

ESBL-producing bacteria, which are resistant to β-lactams, except carbapenems and cephamycins, are mostly responsible for infections in immunocompromised patients. ESBL-positive bacteria also frequently colonize the lower intestinal system, and therefore are a major source for ESBL distribution (Lucet & Regnier 1998). PCR assays revealed that 70.68% from urine samples and 92.85% from diarrheic samples were positive for the $bla_{CTX\text{-}M\text{-}15}$ gene. A report from 10 European countries indicated the prevalence of ESBL producing *E. coli* ranged from 39 to 47% in Turkey, Poland and Russia (Ahmed et al 2013). According to the widespread findings of CTX-M-type enzymes in China and India, it could be suggested that CTX-M-type ESBLs are now really the most frequent ESBL type throughout the world (Paterson & Bonomo 2005). CTX-M type's class A ESBLs are most active against cefotaxime. However, some CTX-M types can hydrolyze ceftazidime includes $blaC_{TX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}19}$. CTX-M ESBLs have been reported in Egypt, with $bla_{CTX\text{-}M\text{-}15}$ being the most common ESBL reported in the Middle East area and North Africa (Khalaf et al 2009). ESBL producing bacteria are resistant to other antibacterial agents, such as tetracycline, aminoglycosides and co-trimoxazole, whereas most these resistance genes are encoded on the plasmids associated to ESBL producing bacteria (Martinez-Martinez et al 1998).

It had been estimated which strains belonged to group B2 account for almost two thirds of all extra-intestinal infections are non-ESBL producing *E. coli* (Branger et al 2005). When all ESBL-positive *E. coli* isolates were considered, whatever their types were group B2 indicated only 18.96% of the strains responsible for UTI in current study. Thus, production of ESBL among *E. coli* strains isolated from UTI and diarrheic was relation to shifts in phylogenetic propagation toward non-B2 groups, in particular groups A and D. An association was seen between CTX-M type and subgroup A_0. $bla_{CTX\text{-}M\text{-}15}$ positive *E. coli* isolates from UTI samples mostly fell into A_0 and D_2 phylogenetic subgroups, whereas mostly isolates of diarrheic belonged to A_0, A_1 and D_1 phylogenetic subgroups. On the other hand, genotyping of *E. coli* from clinical samples in France showed that strains carrier ESBL of CTX-M type enzymes were relation to D_2 phylogenetic subgroup, had few virulence factors (VFs) (Branger et al 2005). In another study in Kerman province (southeast of Iran) the isolates that possessed the VFs mostly belonged to A and B2 phylo-groups, whereas isolates possessed antibiotic resistant fell into groups A and D (Adib et al 2014). Therefore,

E. coli strains acquired the resistance by mutation, which were distributed in some specific phylogenetic background, the relationship was complex.

Conclusion

In conclusion, the ESBL producing *E. coli* isolates of UTI and diarrhea samples were segregated into dif¬ferent phylog-groups, which A and D phylogenetic groups were observed the majority of isolates. In addition, results of current study revealed the higher prevalence of $bla_{CTX\text{-}M\text{-}15}$ gene in investigated isolates. *E. coli* strains acquired the resistance, which were distributed in some specific phylogenetic background, the relationship was complex.

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