

## Phylogenetic background of enterotoxigenic and enteroinvasive *Escherichia coli* from patients with diarrhea in Sirjan, Iran

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### ABSTRACT

**Background and Objectives:** Diarrheagenic *Escherichia coli* (DEC) strains are a major cause of intestinal syndromes in the developing countries. The aim of this study was to determine the prevalence of enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) in relation to phylogenetic background from patients with diarrhea.

**Materials and Methods:** A total of 110 *E. coli* isolates were obtained from diarrhea patients in Sirjan, southeast of Iran. The *E. coli* isolates were confirmed using biochemical and bacteriological tests. DNA of *E. coli* isolates was extracted by boiling method and checked for existence of ETEC (*LT* and *ST* genes) and EIEC (*ipaH* gene) pathotypes and also characterize the phylogenetic groups on the basis of presence or absence of the *chuA*, *yjaA* genes and an anonymous DNA fragment, TspE4.C2 by multiplex PCR.

**Results:** Out of 110 *E. coli* isolates, 32 (29.09%) were positive for ETEC (*LT* and *ST* genes) and 6 (5.45%) possessed EIEC (*ipaH* gene) pathotypes. Isolates fall into four phylogenetic groups: A (39.09%), B1 (20%), B2 (15.45%) and D (25.45%). Phylotyping of isolates of DEC indicated they were distributed in four phylogenetic groups including A (12 isolates), B1 (7), B2 (9) and D (10).

**Conclusion:** In this study, the DEC isolates were segregated into different phylogenetic groups. The majority of isolates belonged to phylo-groups A and D.

**Keywords:** Diarrheagenic *Escherichia coli*, Phylogenetic group, Diarrhea

### INTRODUCTION

Diarrhea is one of great concern throughout the world with high morbidity and mortality, especially in developing countries. The major causes of diar-

rhea differ considerably in developing and industrialized countries. Among the bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) is one of the important cause of endemic and epidemic diarrhea worldwide (1). In relation to diarrhea, *E. coli* strains have been classified into six groups, according to their serotypes and virulence factors and these include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAaggEC) and diffusely adherent *E. coli* (DAEC) (2). Among the six recognized diarrheagen-

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ic categories of *E. coli*, ETEC is the most common cause of *E. coli*-mediated diarrhoea, is contracted by consumption or use of contaminated food or water. Clinical human ETEC isolates produce one or both of two enterotoxins; the heat stable toxins (ST) and heat labile toxin (LT) (3). As expected, it has become clear that relative proportions of LT, ST, and LT/ST toxin-producing ETEC vary from one geographic area to another in patients with ETEC diarrhea (4-6). EIEC is mainly cause of bacillary dysentery that responsible for a substantial proportion of acute diarrhoeal diseases worldwide (7). The Invasion associated Pathogen Antigen or *ipaH* gene is a key virulence factor for EIEC, and thus dysentery is characterized by fever, painful abdominal cramps and diarrhea, sometimes vomiting and the stool contains blood and leukocyte (8).

Strains of *E. coli* are genetically diverse and have been categorized into four major phylogenetic groups, A, B1, B2, and D. Phylogenetic groups differ in their genotypic and phenotypic characteristics, comprising their antibiotic resistance and virulence genes profiles. The most diarrheagenic *E. coli* strains assign mostly to D phylo-group and the commensal strains to groups A and B1 (9, 10).

The main objectives of the present study were to determine the prevalence of ETEC and EIEC and distribution of phylogenetic groups in *E. coli* isolated from diarrhea patients in Sirjan, Iran.

## MATERIALS AND METHODS

**Bacterial strains.** One hundred and ten *E. coli* isolates were obtained from patients with diarrhea who referred to different hospitals and clinical laboratories in Sirjan city (southeastern of Iran) from May to December 2014. The samples were from 62 males and 48 females. Diarrheic samples were recognized as the incidence of unformed and watery stools with or without one of the following symptoms: fever, abdominal cramps, tenesmus and nausea. Stool specimens were directly streaked onto EMB and Mac-Conkey agar (Biolife Laboratories, Milan, Italy) for isolation of *E. coli*. After overnight incubation at 37 °C, lactose-fermenting colonies were selected and identified by the biochemical and bacteriological testes. From every sample, one confirmed colony of *E. coli* isolate was selected and was stored in Luria-Bertani broth (Invitrogen, Paisley, Scotland)

with 30% sterile glycerol at -70 °C.

**DNA extraction.** DNA of *E. coli* isolates and reference strains was extracted by boiling method. Approximately, 3 to 5 colonies from fresh pure cultures were suspended in 0.5 mL sterile distilled water. The suspension lysed by heating at 95 °C for 10 minutes. The supernatant (template DNA) was obtained by centrifugation at 8,000 rpm for 5 minutes.

**Diarrheagenic *E. coli* genes identification.** All isolates were examined by multiplex PCR assay for the presence of the ETEC (*ST* and *LT* genes) and EIEC (*ipaH* gene) pathotypes. In the present study two *E. coli* strains were used as positive controls for identification of diarrheagenic *E. coli* genes: *E. coli* 10407 for ETEC and *E. coli* 85b for EIEC. *E. coli* strain MG1655 was used as a negative control for virulence genes.

Three primers used in this study are listed in Table 1 as instructed by Aranda et al. (11).

**Phylogenetic background.** The phylogenetic analyses of the isolates were done by presence or absence of three genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2 by a triplex PCR method (9). ECOR62 was used as positive control for *chuA*+, *yjaA*+ and TspE4.C2+ and *E. coli* strain MG1655 as a negative control for phylogenetic grouping. The reference strains were from the bacterial collection of Microbiology Department of Ecole Nationale Vétérinaire Toulouse, France. The specific primers used in this study are presented in Table 1.

## RESULTS

Multiplex PCR study revealed that 38 *E. coli* isolates (34.54%) were DEC strains. In regard to individual DEC categories as expected, that ETEC was the most common (32 isolates) DEC isolated (15 with the *LT* and *ST* genes, 10 with only *ST* gene and 7 with *LT* gene) (29% of isolates). Out of *E. coli* isolates examined, 6 had *ipaH*, which characterized presumptively as EIEC pathotype (5.45% of isolates) because there are additional confirmatory phenotypic tests for EIEC (12) (Table 2) (Fig. 1).

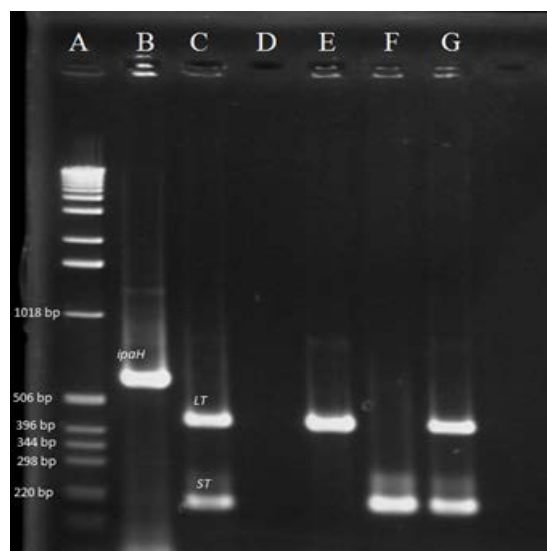
PCR phylotyping showed that 110 *E. coli* isolates segregated into phylogenetic groups A (39.09%), B1 (20%), B2 (15.45%) and D (25.45%) (Table 3) (Fig. 2).

**Table 1.** Oligonucleotide primers used in the present study

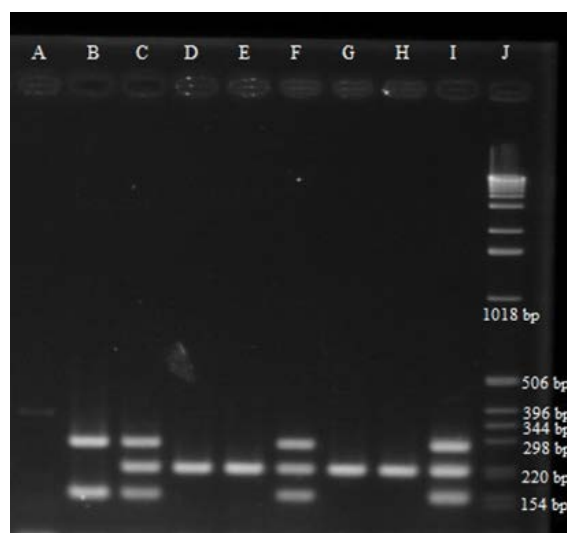
Gene	Primer Sequence (5'-3')	Product size (bp)
<i>LT</i>	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	450
<i>ST</i>	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	190
<i>ipaH</i>	G TTCCTTGACCGCCTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	600
<i>chuA</i>	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279
<i>yjaA</i>	TGAAGTGTGAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211
TspE4C2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152

**Table 2.** Distribution of ETEC and EIEC pathotypes in phylogenetic groups

DEC	Gene	TotalN (%)	Phylo-group			
			A	B1	B2	D
ETEC	<i>ST</i>	10 (10.00)	2 (20.00)	2 (20.00)	2 (20.00)	4 (40.00)
	<i>LT</i>	7 (6.36)	1 (14.28)	3 (42.85)	2 (28.57)	1 (14.28)
	<i>LT/ST</i>	15 (12.72)	8 (53.33)	2 (13.33)	3 (20.00)	2 (13.33)
EIEC	<i>ipaH</i>	6 (5.45)	1 (16.66)	2 (33.33)	-	3 (50.00)
Total		38 (34.54)	12 (31.57)	9 (23.68)	7 (18.42)	10 (26.31)



**Fig 1.** A: ladder 1Kb, B: positive control *E. coli* 85b, C: positive control *E. coli* 10407, D: positive control *E. coli* MG1655, E: the positive isolate for *LT* gene, F: the positive isolate for *ST* gene, G: the positive isolate for *LT* and *ST* genes.



**Fig 2.** A: negative control *E. coli* MG1655, B: D phylo-group, C, F: B2 phylo-group, D, E, G, H: A phylo-group, I: positive control *E. coli* ECOR62, J: ladder 1Kb

**Table 3.** The *E. coli* isolates distribution in phylo-groups

Phylo-group N (%)				Total
A	B1	B2	D	
43 (39.09)	22 (20.00)	17 (15.45)	28 (25.46)	110 (100.00)

Results of phylotyping of DEC isolates indicated that they were distributed in four phylogenetic groups including A (n=12), B1 (n=7), B2 (n=9) and D (n=10). Ten *ST* positive diarrheic isolates belonged to A (n=2), B1 (n=2), B2 (n=2) and D (n=4) groups, whereas 7 *E. coli* isolates possessed *LT* gene fell into A (n=1), B1 (n=3 isolates), B2 (n=2) and D (n=1) groups. Phylotyping of *ST/LT* positive isolates indicated that these belonged to A (n=8), B1 (n=2), B2 (n=3) and D (n=2) phylo-groups. Of the 6 *ipaH* positive isolates, 1, 2 and 3 belonged to A, B2 and D phylo-groups respectively (Table 2).

## DISCUSSION

Diarrheal disease caused by ETEC is the most common cause of travelers' diarrhea and diarrhea in all age groups in regions with poor sanitation in the developing countries including Iran (3, 12, 13). EIEC group represents an important enteric pathogen in different region of the world. A potential contributor to the lack of attention to the epidemiology of EIEC is that it is often observed to be an uncommon cause of diarrhea relative to other DEC (14, 15). The epidemiological features of DEC as a causative agent of diarrhea have a regional variation. A similar profile has also been observed between and within countries from the same geographical region (1, 2). In the present study, DEC was detected in 34.54% of all screened patients. Difference in the frequency of ETEC toxin types may occur from year to year and among different geographic regions. In this study, isolates carrying only the *ST* gene were more prevalent (10/32) than those carrying genes for *LT* (7/32) or both *ST* and *LT* genes (15/32). This result is similar to the report of previous studies in Iran (16), Bangladesh (5) and Tunisia (17) that the *ST*-expressing ETEC was the most common form. However, other studies conducted in Latin American countries, included Mexico, Peru, and Argentina reported that ETEC strains have shifted from *ST*-producers to significantly more *LT*-producing strains (18). On the other hand, in developing world the oc-

currence of ETEC related diarrhea decreases during the first five years of life whereas, children and adults from developed countries that travel to these countries are susceptible to this type of diarrhea, indicating that natural immunity develops (19). Infrequent studies have been done on EIEC in Iran. In this study, *ipaH* gene was used to identify EIEC from diarrheal samples. The low prevalence of EIEC isolates in the present study (5.45%) and the low rate of its recovery in previous studies suggest that this pathotype may play a less important role in diarrhea in developing areas (20). The prevalence of EIEC has been reported to be various in different geographical regions (21, 22). Vieira et al, reported high prevalence rates of EIEC (42/915) infection in diarrhea in 22 rural communities in northwestern Ecuador (13). In Colombia one (0.29%) EIEC isolate was identified from 466 stool samples in patients and 349 stool samples from controls (23).

*E. coli* strains fell into four main phylogenetic groups, each represents ecological specialization which prerequisite to understanding the epidemiology of infectious diseases (24). A strategy for recognizing the evolutionary origins of pathogenic *E. coli* is to determine the phylogenetic background of the virulence factors (25). In phylogenetic analyses performed to date, it has been reported that intestinal infection strains mostly belong to group D and groups A and B1 bacteria are considered to be commensal isolates (10). Phylogenetic group A was the most prevalent (39.09%) in *E. coli* isolates in this study. This result is in accordance with other studies in Iran and worldwide (26-28). Distribution of the other groups was as follows: D (25.45%), B1 (20%), and B2 (15.45%). According to the results, DEC (ETEC and EIEC) isolates were present among the isolates from A (n=12), B1 (n=7), B2 (n=9) and D (n=10) phylogenetic groups. Usein et al. in Rumania found that 51% of the examined DEC isolates from children belonged to group A, followed by group B2 (23%) (29). Another study analyzing commensal *E. coli* strains isolated from the stool of three geographically distinct human populations (France, Croatia and Mali) showed that strains from phylogenetic groups A and B1 were the most common, followed by phylogenetic group D strains (30). Several studies also revealed that EPEC and STEC strains segregate mainly into phylogenetic group B1 and confirms the rarity of the phylogenetic group B2. The remaining strains belonged to phylogenetic groups A and D (25, 31, 32). In a study in Costa

Rica, commensal *E. coli* isolates from children were associated to phylo-groups A and D (36%), while the studied DEC fell into B1 (35%), A (29%), B2 (23%), and D (14%) (33). These differences in distribution of the phylogenetic background among the strains of geographically distinct populations in different studies may be due to the use of antibiotics, differences arising from different sampling areas, dietary factors, health status of the host, geographic climatic conditions, or host genetic factors.

In conclusion, the DEC isolates were segregated into different phylogenetic groups and the majority of isolates fell into the phylogenetic groups A and D. ETEC was recovered at high rates from diarrhea in the study population.

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