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Molecular study on diarrheagenic *Escherichia coli* pathotypes isolated from under 5 years old children in southeast of Iran

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ABSTRACT

Objective: To determine the phylogenetic groups and prevalence of diarrheagenic *Escherichia coli* (*E. coli*) (DEC) genes from children less than five years of age with diarrhea in southeast of Iran.
Methods: A total of 142 *E. coli* isolates were isolated from diarrheic samples. The isolates were examined for detection of virulence determinants and their phylogenetic background by PCR technique.
Results: The *E. coli* isolates fall into four phylogenetic groups: A (40.14%), B1 (18.31%), B2 (16.90%) and D (24.65%). Eighty isolates were positive for at least one of the examined DEC genes. *E. coli* isolates were classified in enterotoxigenic *E. coli* (52 isolates), enteroaggregative *E. coli* (23), atypical enteropathogenic *E. coli* (9), enteroinvasive *E. coli* (2).
Conclusions: This study demonstrated the importance of enterotoxigenic *E. coli* and enteroaggregative *E. coli* pathotypes in the childhood diarrhea. An epidemiologic surveillance especially for DEC, would be useful in control and prevention of infectious diarrhea in children.

1. Introduction

Gastrointestinal infections due to pathogenic *Escherichia coli* (*E. coli*) are significant causes of morbidity and mortality in children, particularly in developing countries[1]. Clinical categories of *E. coli* comprise commensal, intestinal pathogenic and extra-intestinal pathogenic strains. Diarrheagenic *E. coli* (DEC) pathotypes include enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and diffusely adherent *E. coli*[2]. ETEC pathotype defined by the presence of plasmid-encoded enterotoxins, comprise thermostable toxin (*ST*) and the thermolabile toxin (*LT*). ETEC strains are the most common cause of childhood diarrhea among all *E. coli* pathotypes and the major cause of diarrhea in travelers to developing countries[3]. Several virulence factors of EAggEC associated with diarrhea in children. Most of the genes encoding these virulence factors are located in the pAA plasmid, such as probe CVD432 and transcriptional factor encoded by the *aggR* gene. The pAA plasmid also carries the *aap* gene, which secreted low-molecular weight protein that promotes dispersal of EAggEC on the intestinal mucosa and facilitates efficient colonization[4,5]. Outbreaks of EIEC diarrhea are usually food or water-borne. However, through person-to-person transmissions have also been reported[6]. EIEC strains are able to attack intestinal epithelial cells. The invasion plasmid antigen H

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(*ipaH*) gene sequence is used for the diagnosis of EIEC[7,8]. EPEC strains express *eaeA* gene, which produce intimin, and bundle forming pili (*bfpA*) responsible for the attaching and effacing lesions of intestinal microvilli[3,9]. Shiga-toxin-producing *E. coli* or EHEC are principal emerging pathogens that cause food and water-borne diarrheal diseases in humans. All Shiga-toxin-producing *E. coli* strains possess *stx1* and/or *stx2* genes that produce two powerful cytotoxins, called Shiga toxin[10]. The *eaeA* gene of EHEC shares considerable homology with the *eaeA* gene of EPEC. Attaching and effacing *E. coli* strains (*eaeA*+) that harbor the *bfpA* gene are classified as typical EPEC and strains that do not possess *bfpA* gene are classified as atypical EPEC[11,12]. There are important regional differences in the prevalence of different categories of DEC in South and Southeast Asia[13].

Strains of the phylogenetic groups differ in their genotypic and phenotypic characteristics, comprising their antibiotic-resistance profiles, their ability to exploit different sugars sources and their growth rate temperature relationships. Phylogenetically, *E. coli* strains are divided upon amplification of *chuA* and *yjaA* genes and DNA fragment TSPE4.C2. The patterns of amplicons assigned four groups A, B1, B2 and D. DEC strains are derived from groups A, B1 and D, non-pathogenic commensal strains from A and B1, and extra-intestinal pathogenic strains usually belong to groups B2 and D[2,14].

The purpose of this study was to analyze the distribution of phylogenetic groups and occurrence of diarrheagenic genes in *E. coli* isolated from children less than five years of age with diarrhea in southeast of Iran by PCR.

2. Materials and methods

2.1. Sampling and bacteriological identification

One hundred and forty two *E. coli* isolates were obtained from diarrheal samples of children under five years old. Isolates were collected between 2010 and 2012 from children referring to the laboratories of Kerman Province, southeastern Iran. Samples were cultured on Mac Conkey agar and eosin methylene blue (Biolife Laboratories, Milano, Italy). Standard bacteriological methods were used to confirm the *E. coli* isolates. Isolates were stored in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at -70 °C for further analysis.

2.2. Reference strains

Five *E. coli* strains were used as positive controls: *E. coli* H10407 for ETEC (*LT*+, *ST*+), *E. coli* 85b for EIEC (*ipaH*+), *E. coli* O42 for EAggEC (probe CVD432+, *aggR*+ and *aap*+), *E. coli* Sakaï for EHEC and atypical EPEC (*stx1*+, *stx2*+ and *eaeA*+) and *E. coli* ECOR62 for (*chuA*+, *yjaA*+ and Tspe4. C2+). *E. coli* strain MG1655 was used as a negative control for virulence genes. All the reference strains were from the bacterial collection of Microbiology Department of Ecole Nationale Vétérinaire Toulouse, France.

2.3. PCR protocol

DNA was extracted from *E. coli* isolates and reference strains by lysis method. All isolates were tested by multiplex PCR assay for the presence of the *LT*, *ST* and *ipaH* genes by Aranda *et al*.[4], for *stx1*, *stx2* and *eaeA* genes by China *et al.* and probe CVD432, *agg*, *aap* genes by Cerna *et al*[15,16]. The phylogenetic groups (A, B1, B2, and D) of each *E. coli* isolate were carried out by triplex PCR method as described previously[17]. The primers used for detecting sequences encoding virulence genes and phylogenetic groups are described in Table 1.

Table 1

Oligonucleotide primers used in this study.

	Gene or probe name	Primer sequence (5′-3′)	Product size (bp)	Reference
ETEC	*LT*	GGC GAC AGA TTA TAC CGT GC	450	[4]
		CGG TCT CTA TAT TCC CTG TT		
	ST	ATT TTT CTT TCT GTA TTG TCT T		
		CAC CCG GTA CAA GCA GGA TT	190	
EAggEC	Probe CVD432	CTG GCG AAA GAC TGT ATC AT	600	[16]
		CAA TGT ATA GAA ATC CGC TGT T		
	aggR	CTA ATT GTA CAA TCG ATG TA	457	
		AGA GTC CAT CTC TTT GAT AAG		
	aap	CTT GGG TAT CAG CCT GAA TG	310	
		AAC CCA TTC GGT TAG AGC AC		
EIEC	*ipaH*	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	600	[4]
		GCC GGT CAG CCA CCC TCT GAG AGT AC		
EPEC & EHEC	*eaeA*	AGG CTT CGT CAC AGT TG	570	[15]
		CCA TCG TCA CCA GAG GA		
	stx1	AGA GCG ATG TTA CGG TTT G	388	
		TTG CCC CCA GAG TGG ATG		
	stx2	TGG GTT TTT CTT CGG TAT C	807	
		GAC ATT CTG GTT GAC TCT CTT		
Phylo-group	*yjaA*	TGA AGT GTC AGG AGA CGC TG	211	[17]
		ATG GAG AAT GCG TTC CTC AAC		
	TspE4.C2	CTG GCG AAA GAC TGT ATC AT	152	
		CGC GCC AAC AAA GTA TTA CG		
	chuA	GAC GAA CCA ACG GTC AGG AT	279	
		TGC CGC CAG TAC CAA AGA CA		

3. Results

3.1. Phylogenetic grouping

The triplex PCR assays for phylotyping of isolates revealed that isolates fall into four phylogenetic groups, whereas 40.14% (57 isolates) belonged to A, 18.31% (26 isolates) to B1, 16.90% (24 isolates) to B2 and 24.65% (35 isolates) to D phylogenetic groups.

3.2. Detection of DEC isolates

Multiplex PCR were performed to detect the main five categories of *E. coli*. PCR assays revealed that 80 isolates were positive for at least one of the examined DEC genes. Fifty two (36.62%) isolates were positive for *LT* and/or *ST* genes. The ETEC pathotype coding genetic marker *ST* and *LT* were the most prevalent genes in the isolates, while were detected in 11.97% and 9.86% of isolates respectively. Among 52 isolates possess ETEC pathotype genes 21 isolates (14.79%) were positive for both *LT* and *ST* genes (Table 2). Overall 23 (16.20%) of the 142 *E. coli* isolates analyzed carried the EAggEC encoding genes, while probe CVD432 and *aap* genes were detected in 9.86% and 6.34% of isolates respectively. None of the isolates were positive for *aggR* gene (Table 2). Of the 142 isolates investigated, nine (6.34%) isolates were positive for atypical EPEC pathotype coding genetic marker *eae*. Out of *E. coli* isolates analyzed 2 (1.41%) isolates had the gene genetic marker for *ipaH*, which characterized as EIEC pathotype. Of the all isolates surveyed, none were positive for the EHEC encoding genes (*stx1* and *stx2*) (Table 2).

Table 2
Distribution of pathotypes in phylogenetic groups from children less than five years old.

DEC	Gene	Total No. (%)	Phylo-group A	B1	B2	D
ETEC	*LT*	14 (9.86)	–	7 (50.00)	5 (35.71)	2 (14.29)
	ST	17 (11.97)	5 (29.41)	7 (41.18)	–	5 (29.41)
	LT/ ST	21 (14.79)	17 (80.96)	2 (9.52)	2 (9.52)	–
EAggEC	Probe CVD432	14 (9.86)	–	–	5 (35.71)	9 (64.29)
	aap	9 (6.34)	2 (22.22)	–	2 (22.22)	5 (55.56)
	aggR	–	–	–	–	–
EIEC	*ipaH*	2 (4.92)	–	–	–	2 (100.00)
EPEC	*eaeA*	9 (6.34)	2 (22.22)	–	7 (77.78)	–
EHEC	*stx1*	–	–	–	–	–
	stx2					
Total		86 (60.56)	26 (18.30)	16 (11.26)	21 (14.79)	23 (16.20)

3.3. Distribution of DEC genes in phylo-groups

Among 142 *E. coli* isolates 56.34% (80 isolates) and 43.66% (62 isolates) were positive and negative for at least one of the examined DEC genes respectively which, distributed in four phylo-groups (Table 3). ETEC strains were present among the isolates from A (21 isolates), B1 (17 isolates), B2 (7 isolates) and D (7 isolates) phylogenetic groups. Fourteen *LT* positive isolates belonged to B1 (7 isolates), B2 (5 isolates) and D (2 isolates) phylogenetic groups, while 17 isolates possess *ST* gene segregated in phylogenetic group A (5 isolates), B1 (7 isolates) and D (5 isolates). Phylotyping of *LT/ST* positive isolates showed that the isolates belonged to A (17 isolates), B1 (2 isolates) and B2 (2 isolates) phylo-groups. EAggEC strains encoding probe CVD432 fell into B2 (5 isolates) and D (9 isolateds) phylogenetic groups. The *aap* positive isolates were distributed in A (2 isolates), B2 (2 isolates) and D (5 isolates) phylogenetic groups. The atypical EPEC isolates were segregated in A (2 isolates) and B2 (7 isolates) phylo-groups. The EIEC strains coding genetic marker *ipaH* belonged to D (2 isolates) phylogenetic group (Table 2).

Table 3
The positive and negative isolates for at least one of the examined DEC genes distribute in phylo-groups.

	Phylo-group No. (%) A	B1	B2	D	Total
Positive	24 (30.00)	16 (20.00)	16 (20.00)	24 (30.00)	80 (100.00)
Negative	33 (53.23)	10 (16.13)	7 (11.29)	12 (19.35)	62 (100.00)
Total	57 (40.14)	26 (18.31)	23 (16.20)	36 (25.35)	142 (100.00)

4. Discussion

DEC is recognized as an important cause of both outbreaks and sporadic cases throughout the world. There are at least six pathotypes of *E. coli* including ETEC, EAggEC, EIEC, EPEC, EHEC and diffusely adherent *E. coli*, which can cause intestinal infection in humans. Phylogenetic analysis of *E. coli* isolates showed that DEC strains were distributed among groups A, B1 and D and commensal strains in groups A and B1[14]. On the other hand, surveying the evolutionary origins of pathogenic *E. coli* is to determine the phylogeny distribution of the virulence genes[18]. DEC are second most common cause of diarrhea among children under five years old[3].

The results of the present study highlight the importance of ETEC as a cause of childhood diarrhea in the studied region of Kerman, Iran. ETEC is the major etiologic agents but under-recognized bacterial cause of either infantile diarrhea in all age groups in areas with poor sanitation. This pathotype is the most important cause of traveler's diarrhea; the organism is regularly imported to the developed world[19,20]. According to the results *ST*+ and *LT*+ isolates were detected in 11.97% and 9.86% of isolates respectively. In the other parts of world, there were reports differences from prevalence of ETEC pathotype. In studies on capital of Iran (Tehran) and Nicaragua 6.73% and 20.5% of diarrheic isolates obtained from children were positive for ETEC pathptype respectively[21,22]. Perez *et al.* indicated that 7.69% of *E. coli* isolates possessed ETEC encoding sequences[3]. In this study PCR results of phylogenetic determination, showed that ETEC pathotype mostly fell into group A, followed by B1, B2 and D. Escobar-Paramo *el al.* indicated that ETEC strains were found in A and B1 phylogenetic groups[23]. In a study, distribution of ETEC strains in phylo-groups were B1, A and D[3]. In the current study EAggEC pathotype encoding genes were examined. According to the results, probe CVD432+ and *aap*+ isolates were detected in 9.86% and 6.34%

of isolates respectively. The EAggEC pathotype has been implicated in endemic diarrhea among children in both industrialized and resource-poor countries[24]. In Tanzania a study on EAggEC isolates obtained from children less than five years old showed that prevalence of *aggR*+, *aap*+ and *astA*+ isolates were 61.6%, 26.7% and 15.1% respectively[5]. In Romanian, 11.6% of diarrheic isolates were positive for EAggEC pathotype that segregated to A (19 isolates), B1 (2 isolates), B2 (5 isolates) and D (3 isolates) phylogenetic groups[25], whereas in the current study 16.20% of isolates were positive for EAggEC pathotype and belonged to A, B2 and D phylo-groups. Boisen *et al.* surveyed potential virulence factors among 121 EAggEC strains isolated as part of a case-control study of moderate to severe acute diarrhea among children[24]. Among examined isolates prevalence of *aggR* and *aap* genes were 69.40% and 71.90% respectively and belonged to four phylogenetic groups A, B1, B2 and D. In the current study among 142 isolates nine *eaeA*+ isolates were detected which considered as atypical EPEC pathotype. Strains of EPEC are a well-known cause of diarrhoea particularly in infants and young children in less developed countries[26]. The results of an investigation on children with and without diarrhea in three Iranian Provinces, Tehran, Ilam and Mazandaran as a reservoir for intimin gene positive *E. coli* types showed that 40.5% and 20.0% of children with and without diarrhea harbored *eaeA* gene respectively[12]. On another study on 1610 *E. coli* isolates from patient age ranged from a few days to 98 years, 8.9% isolates were positive for EPEC and 4.8% positive for EAggEC pathotypes and 17 isolates were positive for both pathogens[27]. Phylogenetic analysis of DEC showed that EPEC strains were clustered mostly in groups B1, B2 and E[23]. The EIEC coding genetic marker (*ipaH*+) was a low frequency gene in the diarrheic isolates (4.92%). Similar reports showed that two isolates of the *E. coli* isolates from diarrheic children were positive for *ipaH* gene[22,28]. EIEC outbreaks are usually food or water borne; however, person-to-person transmission has also been reported[6]. This pathotype is extremely rare in southeast of Asia[8]. In a study, presence of the invasion-associated locus (*ial*) of the invasion plasmid was reported in 5% of children under two years old[29]. In Costa Rica, distribution of EIEC pathotype in each phylo-group indicated that isolates fell into A, B1 and D groups, whereas according to the results *ipaH* gene belonged to D phylogenetic group[3]. Phylogenetic analyses have shown that DEC strains fall into A, B1 and D phylo-groups[2]. None of the isolates possessed *stx1* and *stx2* genes and were not categorized as EHEC. This pathotype cannot be considered a main cause of childhood diarrhea in this region. These results are in accordance with the previous studies which were done on Thailand and Myanmar[30,31].

In conclusion ETEC and EAggEC were recovered at high rates from children with diarrhea, indicating a wide spread of these pathotypes in the study population. It is maybe that the proportion of *E. coli* pathotypes difference according to the geographic region. The PCR assay can facilitate epidemiologic surveillance of DEC contamination. It is may also be used in epidemiologic surveillance of water for human consumption and food samples for *E. coli* contamination. Moreover, these contaminations can be transmitted from adults to children. Detection of epidemiological information may contribute to the prevention, including vaccines and control of infectious diarrhea in children.

Conflict of interest statement

We declare that we have no conflict of interest.

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