Gastroenterology and Hepatology From Bed to Bench

ORIGINAL ARTICLE

Ribotyping of EPEC isolates from diarrheal and asymptomatic patients in Iran

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ABSTRACT

Aim: We intended to find out the diversity of EPEC isolates among asymptomatic or diarrheal children in Iran using ribotyping.

Background: Enteropathogenic *Escherichia coli* (EPEC) is responsible for gastroenteritis especially in young children.

Patients and methods: A total of 39 EPEC collected strains were serotyped and the presence of virulence genes as well as EAF plasmid among the strains was studied**.** Adherence assay was also performed. Clonal diversity of the isolates was investigated using ribotyping.

Results: Of 39 studied strains of *E. coli*, 6 serogroups of EPEC were represented. The presence of the *stx* gene was ascertained in 7 isolates and the *eaeA*, *eaeB* and *bfpA* genes were harbored by 5, 3 and 1 strains, respectively. Ribotyping yielded 9 different clusters.

Conclusion: According to our results there was not a significant correlation between the results of serotyping and those of ribotyping. However, different serotypes of *E. coli* may belong to the same ribotype clusters and vice versa.

Keywords: EPEC, Diarrhea, Serotyping, Ribotyping.

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Introduction

Enteropathogenic *Escherichia coli* (EPEC) is the oldest of the six categories of the organisms responsible for gastroenteritis in humans. It is one of the major causes of diarrhea in developing countries especially among infants (1, 2). EPEC strains are associated with the distinct set of serovars that can be defined serologically (3, 4). However, in recent literatures this pathotype of *E. coli* has been defined by detection of intimin gene (*eae*), an adhesion to bind intestinal cells, and lack of shiga toxin gene (*stx*) in the isolates. The *eae* may be considered as a factor with capability of dissemination of EPEC to extra-intestinal sites through producing attaching and effacing (A/E) lesions. Bundle-forming pili (BFP) located on EPEC-attaching factor plasmid (EAF) facilitate the adherence of bacteria to epithelial cells and the subsequent formation of A/E lesions. This mechanism has been showed to play a role in localization and interaction with the host cells (5-8).

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The presence of EAF plasmid has been observed in typical EPEC strains while an atypical strain lacks it (9, 10). Taking all these facts into consideration, that is, colonization of the intestinal mucosa and subsequent production of A/E lesions, atypical strains of EPEC are probably pathogenic and have been implicated as the cause of outbreak and endemic diarrhea (10-13). The association between atypical EPEC as emerging pathogens in human and animal hosts has been shown by epidemiological studies (14).

Atypical as well as typical strains of EPEC have been reported prevalent among Iranian children with diarrhea (15). The patients infected with EPEC may develop persistent diarrhea and probability of subsequent hospitalization (16).

However, literatures concerning the epidemiologic evidence on dissemination of such diarrheagenic pathogens in Iranian patients are rare. In the current study we intended to find out the diversity of EPEC isolates among asymptomatic or diarrheal children less than 5 years old at different provinces in Iran using a well-established molecular typing method, like ribotyping. This typing system has been found to be successful for epidemiologic and clonal investigation of clinically important bacteria including EPEC strains (17, 18).

Patients and Methods

Bacterial strains

A total of 39 EPEC strains originated from children with and without diarrhea in three provinces of Iran including Tehran (4 strains), Ilam (24 strains) and Mazandaran (11 strains) were collected from stool specimens in this study.

Serotyping of EPEC strains

EPEC strains were serotyped using standard procedures with O and H antisera following the instruction of the manufacturer's (Statens Serum institut, Copenhagen, Denmark).

Determination of virulence genes

PCR assay was performed to confirm the presence of *bfpA*, *stx*, *eaeA* and *eaeB* virulence genes as well as EAF plasmid among the studied strains using specific primers (19-21). Strain 2348/69 of EPEC prototype (serotype O127: H6) expressing intimin, BFP and EAF and *E. coli* EDL933 (stx^+) were used as positive controls.

Adherence assays

Adherence assay to HeLa cells (provided by National cell Bank, Institute Pasteur of Iran) was performed as previously described (22). The incubation of strains was prolonged up to 6 hours for localized adherence (LA) negative strains to find localized like adherence (LLA) pattern. D-mannose (1% wt/vol) was present throughout the tests. EPEC strain 2348/69 (serotype O127: H6), *E. coli* strain E17-2 (serotype O3: H2) and *E. coli* strain C1845 (serotype O75: NM) showing LA, AA (aggregative adherence) and DA (diffuse adherence), respectively, were used as positive controls.

Ribotyping

Genomic DNA of EPEC strains were extracted using phenol-chloroform method (23). Extracted DNAs were then cleaved by *Mlu*I restriction endonuclease (Roche Applied Science, Mannheim, Germany). Digested fragments were resolved by 0.7% LE (Low Electerophoresis) agarose gel electrophoresis (Roche Applied Science, Mannheim, Germany) and transferred onto positively charged nylon membrane using a vacuum blotter under alkali blotting condition. The membranes were hybridized at 55°C overnight in the hybridization solution containing freshly denatured oligomixed digoxigenin-labeled probes. The hybridized positive fragments were detected with alkaline phosphatase-labeled anti-digoxigenin antibodies followed by a color reaction using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) (Roche Applied Science, Mannheim, Germany.

Results

All of 39 strains studied in this research considered as EPEC based on serology method. Out of 39 studied strains of *E. coli* representing 6 serogroups of EPEC (O26, O111, O126, O127, O128 and O142), 18 strains were isolated from asymptotic individuals and the remaining 21 isolates were isolated from patients with diarrhea (Table 1). The details of serotyping and adherence assay findings of the isolates are shown in Table 1. The presence of the *stx* gene was ascertained by PCR in 7 out of 39 isolates. Apart from one strain with diffused adherence, all *stx* positive strains displayed non adherence (NA) or non-specific

Table 1. Characterization of studied isolates in detail

Ribotype pattern	stx	EAF	bfpA	eaeB	eaeA	adherence	serotype	Clinical	Source	Number of isolate	Row
I	-	-	-	+	+	LLA	O127:H21	D	T	E79	1
I	-	-	-	-	-	NSA	O127:H9	D	I	E76	2
I	-	-	-	-	-	NA	O127:H9	A	I	E62	3
I	-	-	-	-	-	NA	O111:H2	A	M	E53	4
I	-	-	-	-	-	NSA	O111:H48	A	I	E37	5
I	-	-	-	-	-	NA	O142:H48	A	I	E142	6
I	-	-	-	-	-	NSA	O142:H6	A	I	E140	7
I	+	-	-	-	-	NSA	O126:H2	A	I	E120	8
I	-	-	-	+	+	LLA	O127:H21	D	I	E72	9
I	-	-	-	-	-	DA	O111:H2	A	I	E33	10
I	-	-	-	-	-	NA	O111:H9	A	M	E45	11
I	-	-	-	-	-	DA	O111:H26	D	M	E44	12
I	-	-	-	-	-	NA	O111:H14	A	M	E43	13
I	-	-	-	-	-	NA	O111:H3	D	M	E54	14
II	-	-	-	-	-	NA	O127:H37	D	I	E68	15
II	-	-	-	-	-	NSA	O111:H2	D	T	E52	16
II	+	-	-	-	-	NA	O111:H34	D	T	E51	17
II	-	-	-	-	-	NA	O111:H4	A	I	E40	18
II	+	-	-	-	-	NSA	O126:H38	A	I	E117	19
II	-	-	-	-	-	AA	O111:H4	D	M	E49	20
II	+	-	-	-	-	NSA	O126:H20	D	M	E115	21
II	-	-	-	-	-	NSA	O26:H28	D	T	E99	22
III	-	-	-	-	-	NA	O127:H47	A	I	E74	23
III	-	-	-	+	+	LLA	O127:H28	D	I	E71	24
III	-	-	-	-	-	DA	O127:H47	D	I	E55	25
III	+	-	-	-	-	NSA	O128:H12/45	D	I	E125	26
III	+	-	-	-	-	DA	O126:H19	D	I	E118	27
III	-	-	-	-	-	NA	O127:H20	A	M	E67	28
III	-	-	-	-	-	NA	O127:H17	A	M	E66	29
IV	-	-	-	-	-	NA	O127:H42	A	I	E65	30
IV	-	-	-	-	-	NA	O127:H11	A	I	E64	31
IV	-	-	-	-	+	LLA	O127:H10	D	I	E61	32
IV	+	-	-	-	-	NSA	O127:H21	D	I	E69	33
V	-	-	-	-	-	NA	O111:H21	A	M	E50	34
V	-	-	-	-	-	AA	O127:H32	D	M	E77	35
VI	-	-	-	-	-	NA	O127:H25	D	I	E56	36
VII	-	+	+	-	+	LA	O111:H34	A	I	E24	37
VIII	-	-	-	-	-	DA	O127:H6	A	I	E58	38
IX	-	-	-	-	-	NSA	O127:H6	D	I	E70	39

A= Asymptomatic, D= Diarrheal, T= Tehran, I= Ilam, M= Mazandaran, LA= localized adherence, LLA= localized like adherence, DA= diffused adherence, NA= non adherence, NSA= non specific adherence, AA= aggregative adherence

adherence (NSA) pattern. The *eaeA*, *eaeB* and *bfpA* genes were harbored by 5, 3 and 1 strains, respectively. All *eaeB* positive EPEC strains were also positive for *eaeA* virulence gene; however the *eaeB* gene was not detected in two *eaeA* positive strains. All the strains with $eaeA^+$ whether they were $eaeB^+$ or $eaeB^-$ showed LLA pattern. All these strains were isolated from patients with diarrhea and identified as O127 serotypes. Plasmid EAF was found in one strain (Table 1) isolated from a healthy person. This strain which belonged to serogroup O111:H43 with $eaeA^+$, $eaeB^-$, $bfpA^+$, EAF^+, stx^- pattern could adhere to HeLa cells with LA pattern.

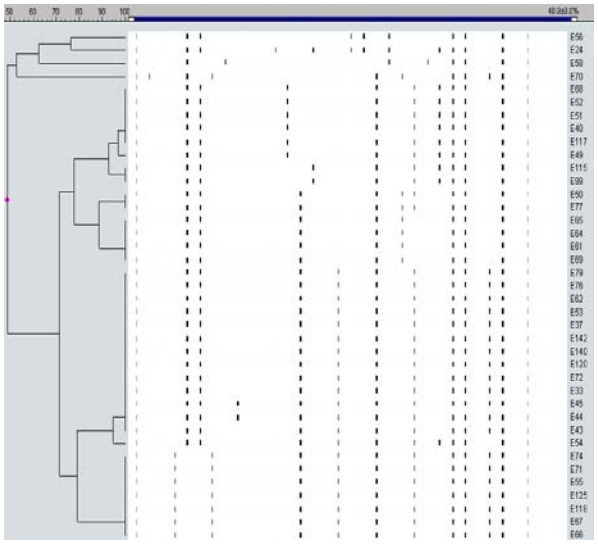

Figure 1. Dendrogram of 39 *E. coli* strains isolated from diarreal and asymptotic patients in this study.

The ribotyping of the isolates yielded 9 different clusters with a ribotype pattern consisting of an average 10 fragments per lane (Figure 1). They included 4 ribotypes that contained a single strain. Ribotype I was the dominant comprising 14 strains which belonged to 5 serogroups. Seven *stx* positive non EPEC strains of *E. coli* did not produce the same ribotype pattern. In our study it was found that different EPEC serotypes disseminated randomly in different ribotype clusters. There was no association between ribotype patterns and clinical outcome of persons (Table 1).

Discussion

Increasing importance of EPEC strains in gastroenteritis diseases is undeniable (2, 24). Epidemiologic study of EPEC strains using new typing methods have been carried out in recent years (25)**.** The role of typical and atypical strains of EPEC in children with diarrheal diseases in developing countries has been increased (9). These strains are responsible for thousands of deaths worldwide, mostly in infants and young children (1, 25).

Molecular epidemiologic study of these strains is essential in understanding the mode of spread between patients and in designing a better strategy for control of outbreaks. Discriminatory power of ribiotyping technique, first developed in 1986 by Grimont *et al*, (17) has been well evaluated by several investigations (26), the fact that has been reconfirmed by our study. In addition, integration of molecular with phenotypic data permitted us to define the relatedness of Iranian strains isolated from different geographic locations.

According to our study, there were not a significant correlation between the relatedness of the isolates, the pattern of virulence genes and their adherence pattern. Although we didn't found full systematic clustering based on geographical distribution of the strains by ribotyping, as it has shown in Table I this method of typing could cluster some of the isolates with the same geographic situation but different serogroups in the same pattern.

Similarly, we were not able to find a significant correlation between the results of serotyping and those of ribotyping. Random dissemination of serovars in different ribotype clusters in our study showed that we cannot correlate ribotypes patterns and serotypes of certain strains. Serotyping alone

was not useful since the majority of isolates could not be subtyped by this method. However, Machado et al showed that application of ribotyping using more than one endonuclease (*Mlu*I, *Cla*I and *Hind*III) might give a reseanable correlation between serotypes and ribotypes of EPEC (27).

In conclusion our results showed that different serotypes of *E. coli* may belong to the same ribotype clusters and vice versa and there has not been a correlation between strains isolated from similar geographic areas (Table 1).

This study is the first report of ribotyping for EPEC in Iran. The system of ribotyping can be approved when more serogroups are studied.

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