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Saeid Bouzari, Mohammad M Aslani, Mana Oloomi, Anis Jafari, Amir Dashti. Comparison of multiplex PCR with serogrouping and PCR-RFLP of *fliC* gene for the detection of enteropathogenic *Escherichia coli* (EPEC).. *Brazilian Journal of Infectious Diseases*, Elsevier, 2011, 15 (4), pp.365-369. 10.1016/S1413-8670(11)70206-9 . pasteur-00747971

HAL Id: pasteur-00747971

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Submitted on 3 Nov 2012

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Comparison of multiplex PCR with serogrouping and PCR-RFLP of *fliC* gene for the detection of enteropathogenic *Escherichia coli* (EPEC)

ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) comprise one of the six categories of diarrhoeagenic *E. coli* (DEC). EPEC is subgrouped into typical (tEPEC) and atypical (aEPEC). The identification of DEC cannot be based only on cultural and biochemical criteria, since they are indistinguishable from the non-pathogenic *E. coli* commonly found in human feces. Several PCR methods, with both single and multiple target genes, have been reported for detecting the different DEC pathotypes. In the present study five hundred *E. coli* isolates from children with diarrhea were subjected into multiplex PCR. Furthermore the strains were typed serologically with O antisera and their *fliC* gene was characterized by PCR-RFLP. The results obtained revealed that overall 41 (8.2%) isolates could be detected as EPEC by this multiplex PCR assay. Of these isolates; 27 (66%) were typical (*escv+*, *bfp+*) and 14 (34%) atypical EPEC (*escv+*, *bfp-*). None of these 41 isolates contained the *Stx1* and *Stx2* genes. Among 37 (90%) typeable strains, nine different serogroups were present. The most common serogroups were O111, followed by O86, O55 and O119 and 10 different H types were found among these isolates. The multiplex PCR assay was found to be rapid and reliable in comparison to serological test; especially when screening the large number of isolates.

Keywords: diarrhea; enteropathogenic *Escherichia coli*; polymerase chain reaction.

INTRODUCTION

Diarrhea is an important public health problem with high infant mortality especially in developing countries. Systematic surveys have shown that diarrhoeagenic *Escherichia coli* (DEC) are a common cause of diarrhea in both developing and developed countries. To date six categories of DEC have been defined on the basis of specific virulence properties.^{1,2} Enterotoxigenic *E. coli* (ETEC) cause diarrhea via secretion of heat stable (ST) and/or heat labile (LT) enterotoxin. Enteroinvasive *E. coli* (EIEC) strains are closely related to *Shigella* spp. and the responsible genes are carried on the pINV plasmid. Shiga-toxin producing *E. coli* (STEC) or enterohaemorrhagic non-bloody diarrhea as well as a hemorrhagic colitis may trigger haemolytic uraemic syndrome (HUS).

Infant diarrhea in developing countries is linked to EPEC strains,¹ but they are also found with increasing frequency from diarrheal cases in industrialized countries.² The ability to

induce attaching-effacing (A/E) lesions in the intestine is main characteristic of EPEC.³⁻⁵ The genes required for the production of these lesions are located on a pathogenicity island known as the locus for enterocyte effacement (LEE). The *eae* and *escV* genes of EPEC encode factors responsible for the attaching and effacing lesions that intimate bacterial adherence to the intestinal epithelial cells. These lesions are comprised of bacteria intimately attached to the intestinal mucosa at sites of cytoskeletal rearrangements leading to characteristic morphological changes, known as cupping and pedestal formation, accompanied by the absence of microvilli.⁶

EPEC is divided into two groups: typical and atypical. The basic difference between the typical EPEC (tEPEC) and atypical EPEC (aEPEC) is a ca. 90-kb EPEC adherence factor plasmid (pEAF) that encodes type IV-like bundle-forming pili (BFP), whereas atypical EPEC lacks this adherence promoting factor.⁷ The tEPEC produce localized adherence to epithelial cells in

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Submitted on: 10/10/2010
Approved on: 11/17/2010

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We declare no conflict of interest.

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cultures forming microcolonies on the cell surface.^{8,9} Adult volunteers have demonstrated that these genes are essential virulence determinants of EPEC.¹⁰⁻¹² The involvement of some aEPEC strains with diarrheal outbreak support the concept that they are also pathogenic.²

Since DEC cannot be diagnosed adequately by culture and biochemical criteria alone, identification of these strains is difficult. Traditional methods to detect diarrheagenic *E. coli* have been very laborious and not suitable for daily clinical use, except for EHEC. Therefore, systematic epidemiological data of diarrheagenic *E. coli* prevalence in different patient groups have not been available either.¹³ To overcome this obstacle, molecular methods detecting all major diarrheagenic *E. coli* have recently been described. Many PCR techniques detecting various genes coding for virulence traits of the different categories of DEC have been reported.¹⁴⁻¹⁹ In this study we screened large number of clinical isolates of *E. coli* by multiplex PCR.²⁰ The results of PCR assay was compared with traditional typing, i.e., serology for O typing and PCR-RFLP technique for H typing of the isolates. Therefore the main features of these techniques are presented and discussed in this present communication.

MATERIAL AND METHODS

Bacterial strains

In this study 500 *E. coli* isolates from diarrheal patients were subjected to multiplex PCR and PCR- RFLP and serological typing. *E. coli* strains E2348/69 were used as positive control.

DNA extraction

The genomic DNA of the isolates was extracted with alkaline lysis method²¹ and was used for the PCR purpose. The method²² was followed for total genomic DNA extraction that is used for PCR-RFLP.

Multiplex PCR

Molecular typing was performed using multiplex PCR that can identify STEC and EPEC pathogroups based on sequences complementary to *escV*, *bfpB*, *Stx1*, and *Stx2*.

This multiplex PCR differentiates STEC and EPEC and also divides the later into two groups, i.e. typical and atypical EPEC. Strains with *escv+*, *bfp+* traits are considered typical and isolates with *escv+*, *bfp-* are atypical EPEC. The PCR was performed in a 25 µL reaction mixture consisting of 1 U of *Taq* DNA polymerase with the corresponding *Taq* polymerase buffer, a 0.3 mM concentration of each deoxynucleoside triphosphate, and a 0.4 mM concentration of each PCR primer. Thermocycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The sequence of primers and expected PCR product with the appropriate size are shown in Table 1. The PCR products hence obtained were run on 1% gel and the gels were visualized by gel Doc system (Figure 1).



Figure 1: Multiplex PCR for detection of EPECs. Lanes 1-7 showing strains identified as typical and atypical EPEC.

aEPEC, atypical enteropathogenic *Escherichia coli*; A/E, attaching-effacing; BFP, bundle-forming pili; DEC, diarrhoeagenic *E. coli*; EIEC, enteroinvasive *E. coli*; *escv*, gene encoding a predicted inner membrane protein; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; HUS, haemolytic uraemic syndrome; LEE, enterocyte effacement; LEE, locus of enterocyte effacement; LT, heat labile enterotoxin; PCR, polymerase chain reaction; pEAF, plasmid EAF; RFLP, restriction fragment length polymorphism; ST, heat stable enterotoxin; STEC, Shiga-toxin producing *E. coli*; *Stx*, Shiga-toxins; tEPEC, typical enteropathogenic *E. coli*.

Table 1. Primer pairs used for detection of the pathotypes

Target gene	Primer pairs genes	Primer sequence (5 to 3)	Product size (bp)
<i>escV</i>	<i>escV</i> -F <i>escV</i> -R	GGCTCTCTTCTTTATGGCTG CCTTTTACAAACTTCATCGCC	534
<i>bfpB</i>	<i>bfpB</i> -F <i>bfpB</i> -R	GATAAACTGATACTGGGCAGC AGTGACTGTTCCGGGAAGCAC	826
<i>Stx1</i>	<i>Stx1A</i> -F <i>Stx1A</i> -R	GCGTTCCTTATGTAATGACTGC ATCCCACGGACTCTTCCATC	250
<i>Stx2</i>	<i>Stx2A</i> -F <i>Stx2A</i> -R	CGTTTTGACCATCTTCGTCTG AGCGTAAGGCTTCTGCTGTG	325

O typing

The O antigens of the isolates were typed with commercially available "O" antisera with slide agglutination.

fliC gene amplification

The PCR-RFLP technique²³ was employed for determination of H type of the isolates. For this purpose the *fliC* gene of the isolates was amplified using specific primers; the sequence of primers and PCR condition is as follows:

Forward: FSA F\1Sc: 5'-CAAGTCATTAATACMAACAGC-3'
(M = A or C)

Reverse: FSA R\1Sc: 5'-GACATRTTRGAVACTTCSGT-3'
(R = A or G) (V = A or G or C) (S = G or C)

Hot start: 94°C 5 min

Denaturation: 94°C 1 min

Annealing: 55°C 1 min

Extension: 72°C 1 min

Final extension: 72°C 5 min

The PCR products obtained were eluted from the gel and digested with HhaI and the digested patterns of the isolates were compared and thereafter the H types of the isolates were determined (Figure 2).

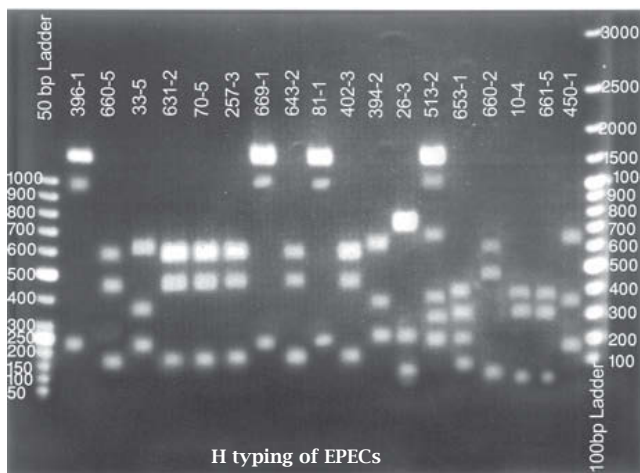


Figure 2: PCR-RFLP of the *fliC* gene for H typing. The patterns of different strains digested with HhaI.

aEPEC, atypical enteropathogenic *Escherichia coli*; A/E, attaching-effacing; BFP, bundle-forming pili; DEC, diarrhoeagenic *E. coli*; EIEC, enteroinvasive *E. coli*; *escv*, gene encoding a predicted inner membrane protein; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; HUS, haemolytic uraemic syndrome; LEE, enterocyte effacement; LEE, locus of enterocyte effacement; LT, heat labile enterotoxin; PCR, polymerase chain reaction; pEAF, plasmid EAF; RFLP, restriction fragment length polymorphism; ST, heat stable enterotoxin; STEC, Shiga-toxin producing *E. coli*; *Stx*, Shiga-toxins; tEPEC, typical Enteropathogenic *E. coli*.

Table 2. Characteristics of EPEC isolates

Iso-lates no.	<i>escv</i>	<i>bfpA</i>	<i>Stx1</i>	<i>Stx2</i>	Typical/atypical EPEC	O type	H type
1	+	-	-	-	A	O111	-ND
2	+	+	-	-	T	O55	H7
3	+	+	-	-	T	O111	-ND
4	+	-	-	-	A	O86	H21-3
5	+	-	-	-	A	O55	H7
6	+	-	-	-	A	O26	-ND
7	+	-	-	-	A	NT	H7
8	+	+	-	-	T	NT	H41
9	+	+	-	-	T	O111	H2
10	+	+	-	-	T	O111	H2
11	+	-	-	-	A	NT	H50
12	+	-	-	-	A	O111	H7
13	+	+	-	-	T	O127	-ND
14	+	+	-	-	T	O86	-ND
15	+	+	-	-	T	O142	H41
16	+	+	-	-	T	O119	H6
17	+	+	-	-	T	O127	H2
18	+	-	-	-	A	O55	H7
19	+	+	-	-	T	O86	-ND
20	+	+	-	-	T	O114	H2
21	+	-	-	-	A	O142	H41
22	+	+	-	-	T	O126	H41
23	+	+	-	-	T	O119	H41
24	+	+	-	-	T	O114	H17
25	+	-	-	-	A	O86	-ND
26	+	+	-	-	T	O111	H21
27	+	+	-	-	T	O119	-ND
28	+	+	-	-	T	O127	H41
29	+	+	-	-	T	NT	H41
30	+	-	-	-	A	O86	-ND
31	+	+	-	-	T	O126	H2-c
32	+	+	-	-	T	O119	-ND
33	+	-	-	-	A	O55	H6
34	+	+	-	-	T	O111	H41
35	+	+	-	-	T	O127	-ND
36	+	+	-	-	T	O126	H2
37	+	+	-	-	T	O126	H2
38	+	+	-	-	T	O114	H2
39	+	-	-	-	A	O86	H47
40	+	-	-	-	A	O119	H6
41	+	+	-	-	T	O55	H6

A, atypical EPEC; T, typical EPEC; ND, not determined; NT, non-typeable.

RESULTS

In the present study a multiplex PCR was employed for the detection of typical and atypical EPEC among 500 *E. coli* isolates. The results obtained revealed that overall 41(8.2%) isolates could be detected as EPEC by this multiplex PCR assay. Of these isolates; 27 (66%) were typical (*escv+*, *bfp+*) and 14 (34%) atypical EPEC (*escv+*, *bfp-*) (Figure 1). The isolates were then subjected to serogrouping and nine different serogroups were detected. On the other hand, strains typeable with O antisera were 37 (90.2%) and 4 (9.8%) were found to be non-typeable with O antisera. Among typeable strains, nine different serogroups were present as follows: O111, O86, O55, O119, O127, O126, O114, O142, and O26. The most common serogroup was O111 followed by O86, O55, each by 7, 6 and 5 strains respectively. All these serogroups were distributed among typical and atypical EPEC, and only among strains with serogroups O114, O126 and O127 all the isolates were found to be typical EPEC by PCR analysis. For the rest of the serogroups typical and atypical isolates were distributed randomly. Of the 37 typeable strains, 25 (67.6%) were typical and 12 (32.4%) atypical and for 4 non-typeable isolates 2 were typical and the rest 2 were atypical. H typing with PCR-RFLP revealed 10 different H types, being the most common H types H41, H2 and H7 (each with 8, 7 and 5 isolates, respectively – Figure 2). Overall in 37 typeable strains, 11 (29.7%) strains could not have the H determined, and for 4 non-typeable strains the H types, was determined. On the basis of O:H typing, 26 strains (5.2%) could be fully characterized (Table 2).

DISCUSSION

Infant diarrhea in developing countries is linked to EPEC strains,¹ but they are also found with increasing frequency from diarrheal cases in industrialized countries. In 1995, EPEC was divided into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC). The basic difference between the two groups is the presence of the EPEC adherence factor plasmid (pEAF) in tEPEC and its absence in aEPEC.²

The World Health Organization recognized that EPEC comprises strains of 12 O serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158,²⁴ also known as the classical EPEC serogroups. Identification of EPEC and STEC strains is currently usually based on serotyping with specific antisera in a time-consuming process demanding some technical expertise.²⁰ In the present investigation, the traditional method of serotyping targeting exclusively the 12 serogroups considered to represent the EPEC pathotype was performed. Only 37 (7.4%) strains were found to be typeable with O antisera. The PCR-RFLP was performed for H typing of the isolates in conjunction with O typing and with combination of O:H typing 26 strains (5.2%) could be fully characterized.

Since DEC cannot be diagnosed adequately by culture and biochemical criteria alone, identification of these strains is difficult. Traditional methods to detect diarrheagenic *E. coli* have been very laborious and not suitable for daily clinical use. To facilitate diagnostic and therapeutic measures, many PCR techniques detecting various genes coding for virulence traits of the different categories of DEC have been reported.¹⁴⁻²⁰ Several PCR assays have been developed for rapid identification and differentiation of diarrheagenic *E. coli* belonging to EPEC and STEC pathotypes. The study of Abe et al.²⁵ from Brazil indicated that aEPEC comprise a very heterogeneous group. Typing of O and H antigens showed a large variety of serotypes (52 O:H combinations), including the O non-typeable strains. Most of the strains belonged to serotypes not included in classical EPEC serogroups, and several strains were non-motile. EHEC strains cause acute inflammation in the intestine and bloody diarrhea, including abdominal pain with, rare but severe, secondary complications such as haemolytic uremic syndrome (HUS). The main virulence factors of EHEC are Shiga-toxins (*Stx*) that induce cytotoxic effects on the microvascular endothelial cells.¹⁰ The PCR technique of Muller et al.²⁰ used in the present study is a novel single MPCR that allows for the specific differential detection of LEE-harboring EPEC, ATEC, and STEC isolates and also identifies LEE-negative STEC strains in a straightforward and robust reaction. The eight primers developed by Muller et al.²⁰ proved to be specific for the corresponding four genes and, its evaluation with large number of clinical *E. coli* isolates demonstrated that the MPCR is highly specific and reliable. It was concluded that the MPCR is a specific method for the identification and differentiation of EPEC, ATEC, STEC, and LEE-negative STEC strains and might be as routine diagnostic tool. In our study none of the isolates were found to be *Stx* producer, this could be due to absence of *Stx* producer isolates from our collection or they are *Stx*-producing *E. coli* (STEC) that have lost the toxin-encoding prophage. Although the epidemiological association of aEPEC with diarrhea is still controversial, its high prevalence worldwide and the involvement of some strains with diarrheal outbreaks support the concept that some aEPEC strains are diarrheagenic. However the findings of Tennant et al.²⁶ indicated that clinical isolates of aEPEC obtained from patients in Australia or New Zealand are not derived from tEPEC or STEC and they probably carry other virulence determinants that could compensate for their lack of *BFP*. Although in a study conducted by Alikhani et al.²⁷ atypical EPEC isolation was reported from diarrheal cases. Two hundred and twelve fecal samples collected from 62 chickens, 50 ducks and 100 pigeons were investigated for the presence of *Stx1*, *Stx2*, *eae* and *ehxA* virulence genes by multiplex PCR. All EPEC isolates were atypical as they lacked *bfpA* and atypical EPEC were from poultry.²⁸ However, the 14 isolates detected as (*escv+*, *bfpA-*) atypical

EPEC by this PCR assay in our study, could either represent LEE-positive (*escv+*) atypical isolates or tEPEC that have lost the genes for *bfp* (*bfpA*-). Comparing the isolates detected by PCR (8.2%) with O typed strains (7.4%) and O:H typed strains (5.2%); it is obvious that PCR is more appropriate technique especially when large numbers of isolates are considered for detection. Overall the results obtained in the present study revealed that the multiplex PCR assay is useful tool for the rapid screening of the large number of isolates. It is very appropriate technique in an epidemic situation where the rapid diagnosis is required. However use of serotyping is necessary in surveillance studies when more information regarding the isolates is required.

ACKNOWLEDGEMENTS

This project was financially supported by Pasteur Institute of Iran (grant no: 268).

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