Iranian Journal of Microbiology
Volume 5 Number 4 (December 2013) 345-349

Genetic evaluation of Locus of enterocyte effacement pathogenicity island (LEE) in Enteropathogenic *Escherichia coli* isolates (EPEC)

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Received: August 2013, **Accepted:** November 2013.

ABSTRACT

Background and Objectives: *Enteropathogenic Escherichia coli* (EPEC) divided into two groups typical and atypical (aspect). The main virulence genes are located in a pathogenicity island called LEE (*Locus of Enterocyte Effacement*). LEE frequently inserted in tRNA genes of *selC*, *pheU* and *pheV* in the bacterial chromosome. tEPEC and aEPEC strains have some differences in their pathogenicity. The purpose of this was to investigate the possible differences between tEPEC and aEPEC strains according to the virulence genes encoding by LEE and their relation to insertion sites.

Materials and Methods: In this study 130 *E. coli* isolates confirmed by biochemical analysis from diarrheal patients, were evaluated for EPEC pathotype by PCR. All EPEC strains tested for presence of some LEE encoded virulence genes and sites of LEE insertion by PCR method.

Results: Among 50 strains of EPEC 28 (56%) and 22 (44%) were typical and atypical strains respectively. 19 strains (30%) showed insertion in *selC*, 7 (14%) in *pheU*, 4 (8%) in *pheV*, 8 (16%) in *pheU* and *pheV*, 1 (2%) in *selC* and *pheU*, 6 (12%) in *pheV, pheU* and *selC* and 5 (10%) had no insertion in these sites. Moreover, *spa* (n = 8, 16%), *espB* (n = 16, 32%), *espD* (n = 18, 36%), *espF* (n = 8, 16%), *espG* (n = 13, 26%), *espH* (n = 12, 24%), *map* (n = 11, 32%) and *tir* (n = 4, 8%) were present among the strains.

Conclusion: Results showed that most of the virulence genes are present in tEPEC isolates. However, aEPEC isolates may acquire other virulence factors. The majority of tEPEC strains showed insertion at *selC* and aEPEC strains in *pheV* and *pheU*.

Keywords: EPEC, LEE, virulence genes, tRNA

INTRODUCTION

Enteropathogenic Escherichia coli (EPEC) is a leading cause of infantile diarrhea in developing countries (1, 2). The EPEC strains characterized by intestinal epithelium microvillus destruction which called attaching and effacing (A/E) lesions (3, 4). The A/E lesions is encoded by a 35 Kb pathogenicity island named locus of enterocyte effacement (LEE) (3, 5). LEE contains the genes encoding Intimin, a type III secretion system, a number of secreted (Esp.) proteins, and the translocated intimin receptor named Tir (5). Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene, is responsible for the intimate adherence between bacteria and enterocyte membranes (6, 7). Type III secretion system effectors interfere with diverse cell signaling processes (8, 9). EspA is the structural protein of a filamentous structure of the bacterial surface which interacts with the epithelial cell in the early stages of the A/E lesion. The EspB and EspD located at the distal end of the EspA filament, and suggested that these proteins involved in pore formation in the host cell membrane (8, 9, 11). Effector proteins such as Tir, EspB, EspG, EspF, EspH and Map translocated through this structure and translocation of these molecules into the host cells results in changes of the cytoskeleton

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of the underlying epithelial cells (8, 11, 12). Tir also acts as a receptor to intimin (9, 11). LEE PAI inserted in chromosome by insertion in tRNA genes (11, 13). LEE inserted at the *sel*C locus, encoding tRNA for synthesis of selenocysteine and at the *phe*U and *pheV* locus, encoding tRNA for synthesis of phenylalanine (11, 13, 14). EPEC is a pathotype with *eae* virulence gene but lacking shigatoxin (*stx*) (1). EPEC 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 (1, 15). pEAF encodes the bundle-forming pilus (BFP) a type IV pilus which interconnects bacteria within microcolonies, promoting their stabilization (1, 5). Recent epidemiological studies suggest an increasing identification of aEPEC in both developed and developing countries (1).

The aim of this study was to investigate the existence of differences between tEPEC and aEPEC strains according to the virulence genes encoding by LEE and their relation to insertion sites.

MATERIALS AND METHODS

Bacterial isolates. One hundred thirty isolates of *E. coli* from diarrheal cases were used in this study.

PCR assay. DNA from all isolates was extracted by phenol-chloroform method and then tested for presence of *eae* gene (Intimin) using primers designed by Zhang *et al*. (16) (Table 1). *E. coli* E2348/69 strain was used as a control strain. Presence of *stx* gene (Shigatoxin) was checked by primers designed by Toma *et al.* (17) and *E. coli* O157:H7 used as a control strain. For detection of *eaf* gene (Bundle forming pilli), primers designed by Kobayashi *et al*. (18) were used. The insertion site of the LEE PAI was determined by PCR using primers for *sel*C, *phe*U and *pheV* sites, and *E. coli K12* strain was used as control strain (Negative PCR results indicated insertion at tRNA sites under investigation). Presence of *spa*, *espB, espD, espF, espH, map* and *tir* genes were tested by PCR. The PCR conditions for amplification of the genes and expected size are presented in Table 1.

Statistical analysis. SPSS software version 16.0 was used and P values < 0.05 was considered as significant.

RESULTS

Of 130 isolates of *E. coli*, 50 were classified as EPEC based on the presence of *eae* and absence of the *stx*. Among all 50 EPEC isolates, 28 (56%) were positive for *eaf*, therefore were considered as tEPEC and other 22 (44%) were aEPEC. Determination of tRNA insertion site of LEE PAI revealed that *selC*, *pheU* and *pheV* (i.e. 24 (48%), 28 (56%) and 28 (56%), respectively)were site of insertion (negative PCR results indicated as insertion). In this study among 50 EPEC isolates *spa, espB, espD, espF, espG, espH*, *map* and *tir* detected in 8 (16%), 16 (32%), 18 (36%), 8 (16%), 13 (26%), 12 (24%), 11 (22%) and 4 (8%) isolates respectively. p value calculated for *spa* (0.05), *espB* (0.002), *espD* (0.014), *espG* (0.016), *espH* (0.029), *map* (0.001) which suggest significant correlation between type of EPEC with presence of these genes ($p < 0.05$). The *espF* (0.263) and *tir* (0.089) were not associated with type of EPEC. The presence of the virulence genes in tEPEC and aEPEC strains were listed in Table 2.

Also among 50 EPEC isolates, 19 strains (30%) showed insertion at *selC*, 7 (14%) in *pheU*, 4 (8%) in *pheV*, 8 (16%) in *pheU* and *pheV*, 1 (2%) in *selC* and *pheU*, 6 (12%) *in pheV, pheU* and *selC* and 5 (10%) had no insertion in these sites. In present study comparison of tEPEC and aEPEC isolates showed that the LEE insertion sites were more associated with *selC* ($p = 0.012$) at tEPECs, whereas in aEPECs *pheU* ($p = 0.05$) and *pheV* ($p = 0.007$) were sites of insertion (Table 3).

DISCUSSION

Enteropathogenic *E. coli* (EPEC) are organisms that cause diarrhea, especially in children in developing countries (20). EPEC contains a pathogenicity island called LEE which encodes several virulence factors that related with human enterocyte destruction (2, 20). There are many studies about presence of virulence genes in EPEC strains. Among 49 EPEC strains which was studied by Rodrigo *et al*. (21) *spa, espB* in 40 (81.6%) isolates and *espD* in 49 (100%), *espF* in 22 (42.8%) and *tir* in 45 (91.8%) were present. In a study by Kim *et al*. (22) in 10 EPEC isolates *spa* in 4(40%), *espB* and *espD* in 7 (70%) respectively were detected.

Contreras *et al.* (23) showed that *spa* (97.8%), *espB* (93.9%), *espD* (83.4%) and *tir* (92%) were present in

Table 1. Primers and PCR conditions.

Genes	Primers	PCR conditions	Size	References
eae	5'-CCGAATTCGGCACAAGCATAAGC-3' 5'- CCCGGATCCGTCTCGCCAGTATTCG-3'	94°C, 45s; 45°C, 45s; 72°C, 45s.	863 bp	[17]
stx	5'-GAGCGAAATAATTTATATGTG-3' 5'-TGATGATGGCAATTCAGTAT-3'	94°C, 45s; 52°C, 45s; 72°C, 45s.	518 bp	[18]
eaf	5'-CAGGGTAAAAGAAAGATGATAA-3' 5'-TATGGGGACCATGTATTATCA-3'	94°C, 45s; 55°C, 45s; 72°C, 45s.	399 bp	[19]
selC	5'-GAGCGAATATTCCGATATCTGGTT-3' 5'-CCTGCAAATAAACACGGCGCAT-3'	94°C, 45s; 60°C, 45s; 72°C, 45s.	527 bp	[15]
pheU	5'-CATCGGCTGGCGGAAGATAT-3' 5'-CGCTTAAATCGTGGCGTC-3'	94°C, 45s; 55°C, 45s; 72°C, 45s.	300 bp	[15]
pheV	5'-CTGGGTATTGCGGTATCGGTGA-3' 5'-GCTGGAGTTTGGACGGGGGTAA-3'	94°C, 45s; 55°C, 45s; 72°C, 45s.	604 bp	[3]
spa	5'-GCGGATCCATGGATACATCAACTACAG-3' 5'-GCAAGCTTTTATTTACCAAGGGATATTCC-3'	94°C, 45s; 60°C, 45s; 72°C, 45s.	639 bp	[8]
espB	5'-GCGGATCCATGAATACTATCGATAATAAC-3' 5'-GCGAATTCTTACCCAGCTAAGCGAGC-3'	94°C, 45s; 62°C, 45s; 72°C, 45s.	1111 bp	[8]
espD	5'-GCGGATCCATGGTTAATGTAAATAACG-3' 5'-GCGAATTCTTAAACTCGACCGCTGAC-3'	94°C, 45s; 61°C, 45s; 72°C, 45s.	1159 bp	[8]
espF	5'-GCGGATCCATGCTTAATGGAATTAGTAAC-3' 5'-GCGAATTCTTACCCTTTCTTCGATTGCTC-3'	94°C, 45s; 63°C, 45s; 72°C, 45s.	650 bp	[8]
espG	5'-GCGGATCCATGATACTTGTTGCCAAATTG-3' 5'-GCGAATTCTTAAGTGTTTTGTAAGTACG-3'	94°C, 45s; 61°C, 45s; 72°C, 45s.	1112 bp	[8]
espH	5'-GCGGATCCATGCGTTATATAGGGAGG-3' 5'-GCAAGCTTTTAAACTGTCACACCTG-3'	94°C, 45s; 61°C, 45s; 72°C, 45s.	506 bp	[8]
map	5'-GCTCTAGACATGTTTAGTCCAACGGCAATG-3' 5'-GCAAGCTTCTACAGCCGAGTATCCTG-3'	94°C, 45s; 66°C, 45s; 72°C, 45s.	629 bp	[8]
tir	5'-GCGGATCCATGCCTATTGGTAACCTTG-3' 5'-GCAAGCTTTTAAACGAAACGTACTGG-3'	94°C, 45s; 61°C, 45s; 72°C, 45s.	1669 bp	[8]

EPEC isolates. Mairena *et al.* (24) studied on LEE4 encoded virulence genes in tEPEC and aEPEC and detected *spa* (94%), *espB* (50%), *espD* (40%), *espF* (78%), *sepL* (90%) of all strains. They indicated that *spa* and *sepL* should be more conserved between EPECs, while *espB*, *espD*, and *espF* should be more diverse. Gartner *et al.* (25) compared tEPEC 2348/69 with aEPEC 3431 and O181 and indicated that LEE encoded virulence genes showed very variable pattern. Muller *et al.* (26) studied on LEE encoded virulence genes in aEPEC including *escV, bfpB, stx1, stx2, invE, elt, estIa, estIb, astA, aggR, pic , uidA, α-hly,e-hly, lifA(efa1)* and *ent* showed that virulence gene pattern is very different. Also demonstrated that aEPEC mostly inserted in *pheU* and *pheV.* Bouzari *et al*. (3) showed that in 17 EPEC isolates (8 tEPEC and 9 aEPEC) 6 (35%) showed insertion in *selC,* 7 (41%) in *pheU* and 2 (11.8%) in *pheV*. In 2 (11.8%) isolates no insertion at these sites were observed.

In other study, Vieira *et al*. (27) showed that potentially virulent aEPECs were associated with presence of PAI O122. Our study showed that the presence of the LEE encoded virulence genes that are essential for attaching & effacing are dominant in

Table 2. Distribution of virulence genes in tEPEC & aEPEC isolates.

Gene EPEC	*spa*	*espB*	*espD*	*espF*	*espG*	*espH*	*map*	*tir*
tEPEC (28 strains)	7 (25%)	14 (50%)	13 (46.4%)	7 (25%)	11 (39.2%)	10 (35.7%)	11 (39.2%)	4 (14.2%)
aEPEC (22 strains)	1 (4.5%)	2 (9%)	3 (13.6%)	3 (13.6%)	2 (9%)	2 (9%)	-	-

Table 3. tRNA insertion sites of tEPEC and aEPEC isolates.

Insertion sites EPEC	*selC*	*pheU*	*pheV*
tEPEC (28 isolates)	18 (64%)	8 (29%)	8 (29%)
aEPEC (22 isolates)	9 (40.9%)	13 (59.1%)	13 (59.1%)

tEPEC isolates. We have also found that the patterns of these genes are very variable. The majority of virulence genes were present in isolates that their LEE pathogenicity island was inserted at *selC* site. Also most of the tEPEC isolates had insertion in *selC*. These results have two conceptions: The aEPEC isolates have reduced virulence in compare to tEPEC isolates, or aEPECs possess other virulence factors than reported so far. Some of our isolates in this study also showed insertion in *selC*, *pheU* and *pheV* sites but no virulence genes could be detected. These results collectively could point at evolutionary differences of these isolates in regards to genome rearrangement due to horizontal gene transfer.

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