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MOLECULAR CHARACTERIZATION OF ENTEROINVASIVE *ESCHERICHIA COLI IPA* GENES BY PCR- RFLP ANALYSIS

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ABSTRACT

In this study, polymorphism in *ipa* genes was found in five out of nine EIEC serotypes studied. When *Sal*I and *Hin*dII were used in RFLP-PCR assays many EIEC serotypes showed polymorphism in *ipa*B and *ipa*D. On the other hand, no polymorphism was observed in *ipa*A and *ipa*C in these strains. The polymorphism present in EIEC strains is serotype-dependent, since restriction patterns were conserved amongst strains belonging to the same serotype. When IpaB deduced amino acid sequences of *S. flexneri* M90T and FBC124-13 were compared, ten amino acids changes could be observed mainly in the amino-terminal region. The deduced EIEC IpaD amino-acid sequence presented 91% similarity with the *Shigella* strain. In this case, amino acid changes were spread out through the whole structure, except in the carboxyl-terminal region.

Key words: *ipa*, enteroinvasive *Escherichia coli*, polymorphism, PCR-RFLP

INTRODUCTION

Escherichia coli plays an important role in maintaining intestinal physiology. However, there are pathogenic strains that cause distinct syndromes of diarrheal disease.

During the 60´s in XX century, it was found that *E. coli* strains isolated from patients with dysentery were also able to cause experimental keratoconjunctivitis in guinea pigs and caused a Shigella-like illness in humans, both children and adults (17,19,22). These *E. coli* strains are known as enteroinvasive *E. coli* (EIEC) and belong to the following O serotypes: O28ac:NM; O29:NM; O112ac:NM; O121:NM; O124:NM; O124:H30; O136:NM; O143:NM; O144:NM; O152:NM; O164:NM; O167:NM (13). Like *Shigella* spp., EIEC strains are frequently lactose negative, consistently lysine decarboxylase negative, and nonmotile, except the serotype O124:H30.

The role played by EIEC in endemic diarrheal disease has not been investigated extensively. However, some studies have indicated that EIEC can be isolated with a relatively high frequency depending on the population being investigated (1,2,9,14,16,20,21). In the city of Sao Paulo, in Brazil, EIEC has been found in 5-7% of children living in medium-income families and in 20% of children who live in a slum, in the outskirts of the city. Both groups studied were composed of children older than 2 years presenting acute diarrhea (21). A similar frequency has been reported by Echeverria *et al.* (2) in Bangkok. Outbreaks of food-borne infections due to EIEC have also been reported elsewhere (13).

Both EIEC and *Shigella* spp. strains cause bacillary dysentery in humans by invading and multiplying within epithelial cells of the colonic mucosa. This remarkable tropism results in an intense inflammatory response characterized by abscesses and ulcerations that damage the integrity of the epithelial cell lining of the colon, producing the pathognomonic symptoms of dysentery (5). The entry of *Shigella* and EIEC bacteria into susceptible host target cells requires the coordinated expression of numerous genes that are activated in response to signals of the microenvironment. The entire

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repertoire of genes required for entry into host cells is clustered in a 220 kb virulence-associated invasion plasmid present both in *Shigella* and EIEC strains (7,18). However, most of the genetic and structure-function studies regarding the biological interaction of these invasive bacteria with eukaryotic cells have been centered on *Shigella* rather than EIEC.

Expression of several plasmid-encoded proteins is required for the complete virulence phenotype of *Shigella* ssp. Ipa (invasion plasmid antigen) proteins A (71kDa), B (62 kDa), C (43kDa) and D (38 kDa) are encoded in the *ipa* operon (*ipa*A; *ipa*B; *ipa*C; *ipa*D) (15). Mutants of *ipa*B, *ipa*C, and *ipa*D were unable to induce actin polymerization at the site of attachment of bacteria to the cell membrane and were therefore unable to enter (8,12). Moreover, when internalized by macrophages, *ipa* mutants remain trapped in the phagosome and are not cytotoxic. This suggests that Ipa proteins are also involved in lysis of the endosome membrane upon entry to epithelial cells (25).

The cellular biology and genetics of entry have been investigated using mainly *S. flexneri*. However, most conclusions derived from these studies probably apply to other *Shigella* species, as well as to EIEC. Nevertheless, the precise pathogenesis of EIEC has yet to be elucidated.

Due to the important role that the *ipa* cluster plays in the entry of enteroinvasive bacteria to epithelial cells and to the lack of data available in literature regarding EIEC *ipa* genes, the aim of this work was to characterize EIEC *ipa* genes among several EIEC serotypes.

MATERIALS AND METHODS

Bacterial strains

Forty-two EIEC strains of different serotypes (Table 1) were isolated from the diarrheic stool of children, during the period running from 1964 to 1988, being 1 sample from Chile, 1 from South Vietnam, 2 from Japan and 38 from Brazil. These strains were Sèreny test positive when they were isolated, and they were previously identified by biochemical and serological methods (11). Only those clones that were positive for Congo red binding were used. *S. flexneri* M90T and *E. coli* DH5α were used as positive and negative controls, respectively.

Amplification of *ipa*A, *ipa*B, *ipa*C and *ipa*D genes

The *ipa* genes of EIEC strains were amplified by PCR with primers designed based on the known sequences of the NH_2-terminal and C-terminal regions of *ipa*A, *ipa*B, *ipa*C and *ipa*D of *S. flexneri* (M90T) (24). The primer sequences are listed in Table 2.

DNA cloning and sequencing

In order to proceed to the sequencing of the *ipa*B gene of the FBC124-13 O124:NM) and FBC167-8 (O168:NM) EIEC strains, the PCR products generated with primers ipaB-F and ipaB-R (Table 2) were cloned into a pUC18 cloning vector according to the manufacturer's instructions. Sequence analysis was carried out using two of the three recombinant plasmids obtained. In order to sequence the *ipa*D gene of EIEC strains FBC124-13 and FBC144-7 (O144:NM), three different products generated with primer set ipaD-F and ipaD-R (Table 2) were directly sequenced. An ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA-USA) and a BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems Foster City, CA-USA) were used according to the manufacturer's recommendations.

Polymorphism of *ipa* genes by endonuclease restriction

The amplified fragments of the *ipa*A, *ipa*B, *ipa*C and *ipa*D genes of EIEC and M90T strains were analyzed by RFLP. Restriction enzymes were chosen according to the nucleotide sequences described for *S. flexneri* (24), by consulting the URL: http://www.firstmarket.com/firstmarket/cutter. The restriction enzymes and the expected fragments are listed in Table 2. The fragment digestions were performed according to manufacturer's instructions (Invitrogen, Carlsbad, CA-USA).

RESULTS

PCR-RFLP analysis of the EIEC *ipa* genes, using different restriction endonuclease enzymes (Table 2), showed three distinct groups among EIEC serotypes: A, B, and C (Table 1). The first group (A) maintained the same restriction profile than that of *S. flexneri* M90T (serotypes O28:NM, O144:NM, O164:NM, and O167:NM) for all *ipa* genes. In group B, RFLP analysis demonstrated the occurrence of polymorphisms of the

Table 1. Strains tested and *ipa* RFLP polymorphism.

Serotypes	Nº of isolates tested*	Nº of isolates with the following RFLP polymorphism		
		A	B	C
O28ac:NM	4	4		
O29:NM	5		5	
O124:NM	6		6	
O136:NM	5		5	
O143:NM	4			4
O144:NM	5	5		
O152:NM	4		3	
O164:NM	4	4		
O167:NM	5	5		
Total	42	18	19	4

A – no occurrence of polymorphism;
B – occurrence of polymorphisms in the *ipa*B and *ipa*D genes;
C – occurrence of polymorphism in the *ipa*D;
* – all strains were Congo red test positive.

Table 2. Primers and restriction endonuclease enzymes used and size of restriction fragments of PCR products obtained from EIEC strains.

Gene	Oligonucleotide sequence 5´- 3´	Size of PCR products expected[4] (bp)	Restriction endonuclea enzymes	Size of restriction fragments expected[5] (bp)	Size of restriction obtained from EIECstrains (bp, approximately)
*ipa*A	ipaA-F ATGCATAATGTAAATAATACT ipaA-R ATTGATATTCTTTAATACTTT	1900	*Hae*III *Bgl*II	440, 650 and 800 680 and 1,200	440, 650 and 800 680 and 1,200
*ipa*B	ipaB-F[1]CAAGCTT-TGCACAACGTDAGCACCACCACCACC ipaB-R[2]CCGATATC-TATTTGTATCAAGCAGTAGTTTGTTG	1750	*Pvu*II *Dra*I *Sal*I	750 and 1,000 270, 700 and 800 200, 400 and 1,150	750 and 1,000 270, 700 and 800 600 and 1,150
*ipa*C	ipaC-F[3]CCCCCGGG-GGAAATTCAAAACACAAAACCAACC ipaC-R[1]CCAAGCTT-CGCACGAATATTACCCGCAATCTGACT	1100	*Bgl*I *Pvu*II	250and 850 430 and 680	250 and 850 430 and 680
*ipa*D	ATGAATATAACAACTCTGACT ATGGACAAAAAGTTTATCTGT	1000	*Eco*RV *Hin*dIII *Hin*dII	400 and 600 230 and 770 200, 380 and 420	400 and 600 230 and 770 200 and 800

1- *Hin*dIII endonuclease restriction site; 2- *Eco*RV endonuclease restriction site; 3- *Sma*I endonuclease restriction site; 4- Venkatesan, *et al.*, 2001; 5- http://www.firstmarket.com/firstmarket/cutter.

*ipa*B and *ipa*D genes in all strains belonging to the O29:NM, O124:NM; 0136:NM and O152:NM serotypes when *Sal*I (with generated fragments of 1,150 bp and 600 bp) and *Hind*II (with generated fragments 200 bp and 800 bp) were used, respectively (Figs. 1a,1b). The polymorphisms of *ipa*B and *ipa*D were conserved in these serotypes, except for one O152:NM serotype strain (FBC152-26), which showed polymorphism of IpaB, only. The third group (C) is represented by all strains belonging to serotype O143:NM, which showed polymorphism of *ipa*D, only. No serotypes showed polymorphism of *ipa*A or *ipa*C. The polymorphisms found in these EIEC serotypes appear to be conserved since all PCR fragments presented the same profile for those restriction endonuclease enzymes used.

Nucleotide sequences of *ipa*B and *ipa*D of EIEC proved to be highly similar to that of the *Shigella ipa* genes. The deduced amino acid sequences of IpaB and IpaD are shown in Figs. 2 and 3, respectively. The change at codon position 1,496 (A<u>C</u>A → A<u>A</u>A) resulted in a change in the deduced amino acid residue 520 position (T → K), leading to the loss of one restriction site in IpaB (Fig. 2). For IpaD, the base change at position 511

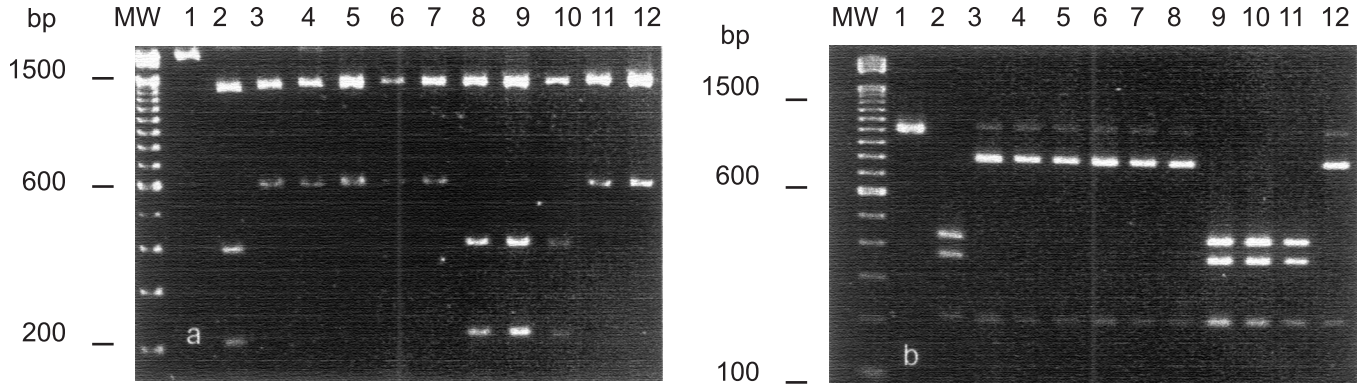

Figure 1. RFLP-PCR of the *ipa*B genes digested with *Sal*I (a) and *ipa*D with *Hin*dII (b) of different serotypes of EIEC strains. Lane MW, 100 bp DNA ladder (Gibco BRL); lane 1, PCR fragments of *ipa*B (**a**) and *ipa*D (**b**); lane 2, FBC28-1(O28:NM); lane 3, FBC29-7 (O29:NM); lane 4, FBC124-13 (O124:NM); lane 5, FBC124-33 (O124:NM); lane 6, FBC136-1 (O136:NM); lane 7, FBC136-37 (O136:NM); lane 8 FBC143-14 (O143:NM); lane 9, FBC144-7 (O144:NM); lane 10, M90T; lane 11, FBC152-26 (O152:NM); lane 12, FBC152-31 (O152:NM).

(AAC→AAA) abolished one of the *Hin*dII sites, resulting in a change in the deduced amino acid residue 193 position (N → K) (Fig. 3). These differences seemed to be conserved in the O29:NM, O124:NM; 0136:NM, and O152:NM serotypes, and for all O143:NM EIEC strains for IpaD.

The similarity between IpaB of the FBC124-13 EIEC strain and *S. flexneri* M90T was 97% and 41% with the SipB protein of *Salmonella enterica* serovar Typhimurium. When IpaB deduced amino acid sequences of *S. flexneri* M90T and FBC124-13 are compared, ten and seven different amino acids can be observed in the amino-terminal and carboxyl-terminal regions, respectively (Fig. 2). However, there is 100% identity between *Shigella* and EIEC in the hydrophobic region (amino acids 310-423), and 65% between EIEC and *Salmonella* (4,10).

S.flexneri	1	MHNVSTTTTGFPLAKILT STELGDNTIQAANDAANKLFSLTIADLTANQNINTTNAHSTS
EIEC 167-8	1	.....S…….....A.....A. .....
EIEC 124-13	1	…….IG.. .....L…….....A.....L.....K.....
S.flexneri	61	NILIPELKAPKSLNASSQLTLLIGNLIQILG EKSLTALTNKITAWKSQQQARQQKNLEFS
EIEC 167-8	59	.....
EIEC 124-13	61	.....
S.flexneri	121	DKINTLLSETEGLTRDYEKQINKLKNADSKIKDLENKINQIQTRL SNLDPESPEKKKLSR
EIEC 167-8	119	.....E……....
EIEC 124-13	121	.....E...D...N...R……....
S.flexneri	181	EEIQLTIKKDAAVKDRTLIEQKTLSIHSKLTDKSMQLEKEIDSFSAFSNTASAEQLSTQQ
EIEC 167-8	179	.....
EIEC 124-13	181	.....
S.flexneri	241	KSLTGLASVTQLMATFIQLVGKNNEESLKNDLALFQSLQESRKTEMERKSDEYAAEVRKA
EIEC 167-8	239	.....
EIEC 124-13	241	.....
S.flexneri	301	EELNRVMGCVGKILGALLTIVSVVAAAFSGGASLALAAVGLALMVTDAIVQAATGNSFME
EIEC 167-8	299	.....
EIEC 124-13	301	.....
S.flexneri	361	QALNPIMKAVIEPLIKLLSDAFTKMLEGLGVDSKKAKMIGSILGAIAGALVLVAAVVLVA
EIEC 167-8	359	.....
EIEC 124-13	361	.....
S.flexneri	241	TVGKQAAAKLAENIGKIIGKTLTDLIPKFLKNFSSQLDDLITNAVARLNKFLGAAGDEVI
EIEC 167-8	419	.....
EIEC 124-13	421	.....
S.flexneri	481	SKQIISTHLNQAVLLGESVNSATQAGGSVASAVFQNSAS**T**NLADLTLSKYQVEQLSKYIS
EIEC 167-8	479	.....
EIEC 124-13	481	.....N.....N..**K**.....G
S.flexneri	541	EAIEKFGQLQEVIADLLASM----------
EIEC 167-8	539	.....
EIEC 124-13	541	D.....G…..E….....

Figure 2. Alignment of *ipa*B amino acid sequence of the *S. flexneri, S. dysenteriae* and EIEC FBC167-8 (O167:NM) (without polymorphism) and FBC124-13 (O124:NM) (with polymorphism). The identical amino acids are indicated by dots. In the sample FBC124-13 occurred a mutation that abolished the *Sal*I site and caused an amino acid change from Thr (T) → Lys (K) (underlined).

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S.flexneri      1   MNITTLTNSISTSSFSPNNTNGSSTETVNSDIKTTTSSHPVSSLTMLNDTLHNIRTTNQA
EIEC 144-7      1   ----------..P.....
EIEC 124-13     1   ----------.DTH.N.PL.L.....

S.flexneri     61   LKKELSQKTLTKTSLEEIALHSSQISMDVNKSAQLLDILSRNEYPINKDARELLHSAPKE
EIEC 144-7     50   .....
EIEC 124-13    55   ...D.....KK.....

S.flexneri    121   AELDGDQMISHRELW AKIANSINDINEQYLKVYEHAVSSYTQMYQDFSAVLSSLAGWISP
EIEC 144-7    110   .....E.....
EIEC 124-13   115   .....YE.....D...K...N.....

S.flexneri    181   GGNDGNSVKLQVNSLKKALEELKEKYKDKPLYP ANNTVSQEQANKWLTELGGTIGKVSQK
EIEC 144-7    170   .....N.....K.
EIEC 124-13   175   .....K....DE.TK.....K.....E.

S.flexneri    241   NGGYVVSINMTPIDNMLKSLDNLGGNGEVVLDNAKYQAWNAGFSAEDETMK NNLQTLVQK
EIEC 144-7    230   .....N.....A.....N.....
EIEC 124-13   235   .....N.....

S.flexneri    301   YSNANSIFDNLVKVLSSTISSCTDTDKLFLHF
EIEC 144-7    390   .....HR...NG.. ----------
EIEC 124-13   395   .....KV... ----------
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Figure 3. Alignment of *ipa*D amino acid sequences of the *S. flexneri, S. dysenteriae*, and EIEC samples FBC144-7 (O144:NM) (without polymorphism) and FBC124-13 (O124:NM) (with polymorphism). The identical amino acids are indicated by dots. In the sample FBC124-13 a base change caused the amino acid change Asn (N) → Lys (K) (underlined).

The deduced amino acid sequence of IpaD with polymorphism showed a greater difference than the IpaB sequence of EIEC, when both were compared with *S. flexneri* M90T. The similarity between the FBC124-13 EIEC strain and *S. flexneri* M90T was 91% (Fig. 3), and 36% when compared to SipD of *Salmonella* serovar Typhimurium (4,10).

DISCUSSION

The interaction between facultative and obligate intracellular bacterial pathogens and host cells is a complex event that is accomplished by many different strategies (15). *Shigella* and EIEC are among a few bacteria which replicate freely in the cytoplasm of host cell. There are evidence that invasion plasmids of both bacteria hold high DNA homology, however, studies with *Eco*RV showed little similarity between profiles. (6,18). This events might provoke differences in some gene expression

The molecular characterization of *ipa*A, *ipa*B, *ipa*C, and *ipa*D of EIEC has been done by PCR-RFLP assay, and the determination of *ipa*B and *ipa*D nucleotide sequence. The amino-terminal and carboxyl-terminal regions of these genes show to be conserved, since *ipa* genes from nine EIEC serotypes were amplified by PCR assay using primer set designed from nucleotide sequence of *Shigella ipa* genes. All genes (*ipa*A, *ipa*B, *ipa*C, and *ipa*D) were amplified from all strains studied. The loss of one restriction site in EIEC IpaB sequence was due to the change of a single nucleotide. However, it is important to observe that this change is conserved in EIEC serotypes belonging to groups B and C. Therefore, these differences are a molecular characteristic of these bacteria. The IpaB deduced amino acid sequences of FBC124-13 strain differed from those of FBC O167-8 and *Shigella* strains by a small number of amino acids both in the amino-terminal and carboxyl-terminal regions.

The *S. flexneri ipa*B gene was divided into three regions, the amino-terminal, the carboxyl-terminal, and the middle region, which possesses two important domains (α-helix and hydrophobic). The amino-terminal region is very important for the expression and secretion of IpaB, while mutation in the carboxyl-terminal region do not affect these phenomena. Hydrophobic and α-helix domains are crucial for the invasion and for the induction of macrophage death, since mutations in these regions led to the construction of non-virulent strains (4). When IpaB deduced amino acid sequences of *S. flexneri*

M90T and FBC124-13 are compared, ten and seven different amino acids can be observed in the amino-terminal and carboxyl-terminal regions, respectively. However, the α-helix and hydrophobic regions did not show differences in their nucleotide sequences. The importance of differences found in the amino-terminal region must be verified, since this region is crucial for the expression and secretion of IpaB (Guichon, *et al.*, 2001). FBC124-13 was able to provoke guinea-pig keratoconjuntivitis only 5 days after the inoculum, while *Shigella* M90T did so after 24 hours, and FBC167-8, after 48 hours (data not shown).

The Ipa protein complex (IpaB-IpaD) has been proposed to occur in the outer membrane of *Shigella* and appears to play a role in modulating the transport of IpaC and IpaB (15). The IpaD carboxyl-terminal region may be involved in transport modulation, and the amino-terminal region would be involved in lysis and escape from host cell phagossomes (23). The deduced EIEC IpaD amino-acid sequence presented 91% of similarity with the *Shigella* strain. Amino acid changes occurred for whole protein structure, except in the carboxyl-terminal region, maybe because this region was not much exposed in the bacterial surface and selective environmental and immunological pressures could not contribute towards mutations (23).

Data shown here represent preliminary findings on the genetic differences between the EIEC and *Shigella* Ipa proteins. These differences are conserved among the EIEC serotypes and might influence bacterial pathogenicity. Further studies have to be carried out to elucidate whether the polymorphism presented for some EIEC serotypes may modify their pathogenicity.

The sequences described here have been assigned GenBank Accession numbers AY098990, AY098991, AY098992, and AF508305

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RESUMO

Caracterização molecular do gene *ipa* de *Escherichia coli* enteroinvasora pela análise de PCR- RFLP

No presente estudo, foi encontrado polimorfismo no gene *ipa* em cinco sorotipos de EIEC, de nove estudados. Quando enzimas de restrição *Sal*I e *Hin*dII foram utilizadas no ensaio de PCR-RFLP, amostras de EIEC apresentaram polimorfismo em *ipa*B e *ipa*D. Por outro lado, não foram observados polimorfismos nos genes *ipa*A e *ipa*C nestas cepas, quando diversas enzimas de restrição foram utilizadas. O polimorfismo presente em cepas de EIEC é sorotipo-dependente, uma vez que os padrões de restrição foram conservados entre as cepas pertencentes ao mesmo sorotipo. Quando a seqüência deduzida de aminoácidos de IpaB de *S. flexneri* M90T e FBC124-13 foram comparadas, mudanças foram observadas em dez aminoácidos na região amino-terminal. A seqüência deduzida de aminoácidos de IpaD de EIEC apresentou similaridade de 91% com a cepa de *Shigella*. Neste caso, mudanças de aminoácidos ocorreram em toda a estrutura da molécula de IpaD, exceto na região carboxi-terminal.

Palavras-chave: *ipa*, *Escherichia coli* enteroinvasora, polimorfismo, PCR-RFLP

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