Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* (ETEC) strains isolated in Rio de Janeiro city, Brazil

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) strains have been implicated as important etiological agents of diarrheal disease, especially in developing countries. This group of microorganisms has been associated with a diverse range of genotypic and phenotypic markers. In the present study, 21 ETEC isolates previously defined according to the toxigenic genotypes, were characterized on the basis of O:H typing, cell adherence patterns, and colonization factors (CFs) antigens. Genetic diversity was investigated by random amplification polymorphic DNA (RAPD-PCR), pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE). LT-I probe-positive isolates belonged to serotypes ONT:HNT, O7:H24, O48:H21, O88:H25, O148:H28, O159:H17 and O159:H21. ST-h probe-positive isolates belonged to serotypes O159:H17, O148:H28 and O6:H-. Serotypes O148:H28, O159:H17 and O6:H- were associated with the CS6, CFA/I and CS1 CS3 antigens, respectively. Most ETEC strains exhibited a diffuse pattern of adherence to cultured epithelial cells. In general, phenotypic and genotypic characteristics correlated well. RAPD-PCR, PFGE and MLEE showed reproducibility and good discriminatory potential. The application of molecular typing systems allowed the detection of significant diversity among the isolates, indicating a non-clonal origin and revealing intra-serotype variation overlooked by classical epidemiological approaches. The phenotypic and genotypic diversity observed lead us to recommend the use of different typing systems in order to elucidate the epidemiology of ETEC infection.
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Keywords: Enterotoxigenic *Escherichia coli*; Molecular characterization; ETEC serotyping; Cell adherence; Colonization factor antigen

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are an important worldwide cause of diarrheal disease in humans, affecting especially children in developing areas and visitors from developed countries [1–4]. Two major virulence determinants have been identified in ETEC strains: enterotoxins and colonization factors antigens (CFAs), which are often encoded by plasmids that also encode heat-stable (ST) and/or heat-labile (LT) enterotoxins [5,6]. Distinct types of CFAs have been described in human ETEC isolates and they are mainly constituted by protein fimbriae [7]. A considerable proportion of ETEC strains do not

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appear to express known CFA. Once attached to the enterocytes, the bacteria produce either LT or ST or both, leading to diarrhea [5].

While not specifically implicated as virulence determinants, some O and H antigens [8] have typically been associated with strains recovered from patients with infection and harboring certain virulence traits. Such associations suggested that ETEC strains may represent stable cell lineages and led to the formulation of a clonal origin hypothesis [5,9,10]. Studies on the clonal structure have contributed to the assessment of the pathogenic diversity of *E. coli* by providing extensive data for understanding the relationship between phenotypic and genotypic properties [11,12]. Phenotype-based subtyping methods including serotyping, biotyping and outer membrane protein (OMP) or lipopolysaccharide (LPS) profiles are in most cases not considered as reliable indicators of ETEC genetic relatedness, requiring the investigation of additional stable markers [5,6,11–13]. The analysis of the clonal structure performed by multilocus enzyme electrophoresis (MLEE) has revealed that classical enteropathogenic groups of *E. coli* are composed of a variety of clones or clone complexes that harbor distinct combinations of virulence determinants [14,15].

Recently, several molecular biology epidemiological methods have been successfully applied for typing purposes [16]. The genetic characterization of ETEC strains by analyses of plasmid profiles, pulsed-field gel electrophoresis (PFGE) and ribotyping [17,18] has been considered a valuable tool for the investigation of clonal diversity allowing to determine the relatedness of multiple isolates and to identify epidemiological markers. However, in some instances, these techniques show limitations in relation to the discriminatory power, resolution, reproducibility, cost and interpretation of typing data. A PCR-based technique, the randomly amplified polymorphic DNA (RAPD-PCR) technology, has received considerable attention as an alternative typing approach for ETEC strains due to its simplicity, relatively low cost, flexibility and sensitivity in revealing inter- and intra-serotype specific variations [11,19].

The aim of the present study was to investigate the relatedness among ETEC strains isolated in Rio de Janeiro, Brazil, by use of phenotypic and genotypic typing systems. Serotype analysis, patterns of adherence to epithelial culture cells, expression of CFAs and the genetic diversity assessed by RAPD-PCR, PFGE and MLEE methods were evaluated.

2. Materials and methods

2.1. Bacterial strains

A total of 21 ETEC isolates were included in the study. They were recovered during an investigation on the occur-

rence of different categories of diarrheagenic *E. coli* (DEC) in children of less than 3 years of age, with and without diarrhea, seeking medical assistance at a Pediatric Hospital [20]. The investigation was carried out from August 1994 to May 1995 in Rio de Janeiro city, Brazil. Isolates were classified as ETEC on the basis of hybridization assays with specific probes for the ST-p, ST-h and LT-I toxin genes. Isolates were kept frozen at −70°C in Trypticase Soy Broth (TSB; BBL, Microbiology Systems, Cockeysville, Maryland, USA) containing 20% glycerol. Prior to use, each isolate was streaked onto Trypticase Soy Agar (TSA; BBL) and grown overnight at 37°C.

2.2. Serotyping

E. coli isolates grown on nutrient agar were used for the identification of somatic (O) and flagellar (H) antigens by standard agglutination methods [21] using specific antisera O1 to O173 and H1 to H56 prepared in the Bacteriology Section of Instituto Adolfo Lutz (São Paulo, Brazil).

2.3. Cell adherence tests

ETEC isolates were tested for adherence to HEp-2 cells (50 to 70% confluence) and to CaCo-2 cells (confluent monolayers) as described previously [22]. Samples (35 μl) of each bacterial culture grown overnight in TSB were incubated with the cell monolayer in a single infection period of 3 h at 37°C in a humid 5% CO_2 atmosphere (3-h assay). Weakly adherent or non-adherent isolates were retested by the same procedure with an additional incubation period of 3 h (6-h assay). HEp-2 cells (ATCC CCL23) were grown in Eagle's minimal essential medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% fetal calf serum (FCS; Gibco BRL, Rockville, MD, USA). CaCo-2 cell line was kindly provided by A. Zweinbaum (INSERM Unité 178, Villejuif, France) and was grown in Dulbecco's modified Eagle's minimum essential medium (Sigma) supplemented with 10% FCS, 1% non-essential amino acids and 1% L-glutamine. The following *E. coli* strains were used as controls for the adherence tests: E2348/69 (localized adherence, LA), C1845 (diffuse adherence, DA) and 0431-4/85 (aggregative adherence, AA).

2.4. Random amplification of polymorphic DNA

RAPD-PCR analysis was carried out with three arbitrary 10-mer primers: 1254 (CCGCAGCCAA), 1253 (GTTTCCGCCC) and 1290 (GTGGATGCGA) [11]. Reaction products were analyzed after electrophoresis in 1.5% agarose gels and staining with ethidium bromide. RAPD-PCR profiles were inspected visually and defined according to the presence or absence, and intensity of polymorphic bands. A 1-kb DNA ladder (Gibco BRL) was used as a molecular mass marker.

2.5. PFGE

The preparation of DNA for PFGE analysis was performed as described previously [23] with some modifications. Bacterial cells grown overnight on TSA agar plates were resuspended in PIV buffer (1 M NaCl, 10 mM Tris–HCl, pH 7.6) and embedded in 1% low-melting point agarose (NuSieve GTG Agarose; FMC Bioproducts, Rockland, MA, USA). Lysis of bacterial cells was carried out by placing plugs in a lysis solution containing 1 mg of lysozyme per ml followed by two steps of deproteination by washing with ESP solution for 18–24 h at 50°C. The plugs were then washed seven times (four times for 1 h each and three times for 2 h each time) with TE buffer at 37°C. For restriction endonuclease digestion, DNA in the plugs was incubated with 20 U *Xba*I for 18–24 h at 37°C according to the manufacturer's instructions (Boehringer Mannheim Corporation, Indianapolis, IN, USA). Electrophoresis was performed in 1% agarose gels (Molecular Biology Certified Agarose, Bio-Rad Laboratories, Richmond, CA, USA) in 0.5× Tris-borate-EDTA buffer by using the CHEF DR-III system (Bio-Rad). The parameters were: pulse time ranging from 5 to 50 s for 23 h at 11°C, in a voltage gradient of 6 V cm^{-1}. A 50–1000 kb DNA ladder (Sigma) was used as a molecular mass marker. PFGE profiles were compared by visual inspection and by automated analysis using the Molecular Analyst Fingerprint Plus software (Bio-Rad), version 1.12. Cluster analysis was done by the unweighted pair group method with arithmetic averages (UPGMA) of the Image Analysis System. The percentages of similarity were estimated by the Dice coefficient.

2.6. MLEE

In order to analyze enzyme electrophoresis variation, bacterial cell lysates were prepared as previously described and characterized for the allelic variation at 10 enzyme-encoding loci [24,25]: aconitase, E.C.4.2.1.3 (ACO); adenylate kinase, E.C.2.7.4.3 (ADK); alcohol dehydrogenase, E.C.1.1.1.1 (ADH); glucose-6-phosphate, E.C.1.1.1.49 (G6PDH); glucose-6-phosphate dehydrogenase, E.C.1.1.1.44 (PGD); glucose phosphate isomerase, E.C.5.3.1.9 (GPI); isocitrate dehydrogenase, E.C.1.1.1.42 (IDH); malate dehydrogenase, E.C.1.1.1.3.7 (MDH); phenylalanyl-leucine peptidase, E.C.3.4.11.1 (PEP-2); phosphoglucomutase, E.C.2.7.5.1 (PGM). Numerical analysis of MLEE data was performed using the NTSYS-pc version software package (F. James Rolf, Exeter Software) and affinities among strains were calculated using the Dice coefficient. The similarity matrix was transformed into a phenogram by using the UPGMA algorithm [26]. Isolates that lacked detectable enzyme activity, were designated a null allele state at the locus in question.

Isolates recovered from a single child displaying identical RAPD-PCR, PFGE and/or MLEE types were considered to be identical; if isolates were found identical, only one of such isolates was selected for the detection of CFAs.

2.7. Detection of CFA

The presence of CFs was investigated by dot blot enzyme-linked immunosorbent assay [27] with polyclonal antisera against CFA/I, and CS6 [28], and CS18 (kindly provided by Gloria Viboud [29]) or with monoclonal antibodies against CS1, CS2, CS3, CS5, CS7, CS12, CS14, and CS17 kindly provided by Ann-Mari Svennerholm [27,30]. Heated bacterial extracts prepared in CFA–BSA (bovine serum albumin) agar [28] were dotted on nitrocellulose filters and incubated at room temperature for 30 min. The filters were blocked overnight with 0.01 M phosphate-buffered saline (PBS) pH 7.2, containing 1% BSA. Then antiserum, diluted in 0.05% Tween 20, was added in PBS–BSA followed by an incubation period of 90 min at room temperature. Filters were washed in PBS–Tween and CFA-expressing strains were detected after being incubated with anti-rabbit or anti-mouse immunoglobulin conjugated with horseradish peroxidase for 90 min at room temperature after which 4-chloro-1-naphthol was added.

3. Results

The characteristics of the 21 ETEC isolates included in this study are presented in Table 1. The isolates sharing genotypic and phenotypic properties defined a strain. Thus, altogether, they comprised 11 different ETEC strains. In general, *E. coli* isolates from a single child displayed identical profiles and they were assumed to represent the same strain. Eight distinct serotypes were identified in the ETEC group. LT-I-probe-positive strains detected among diarrheal children belonged to serotypes ONT:HNT, O7:H24, O88:H25, O148:H28, O159:H21, while serotypes O48:H21 and O159:H17 were recovered from children without diarrhea. ST-h-probe-positive strains isolated from symptomatic children belonged to serotypes O148:H28 and O159:H7, and serotype O6:H- was associated with non-diarrheal children. Considering the toxin-serotype associations, all strains of serotype O159:H21 hybridized only with the LT probe, while serotypes O148:H28 and O159:H17 were associated with ST-h and LT-I genes. The remaining serotypes were limited to a few strains.

CFs were identified in four ETEC strains (Table 1), and were more frequently observed among the ST-probe positive group. ST-h+ strains isolated from symptomatic children were related to serotypes O148:H28 and O159:H17 presenting the CS6 and CFA/I antigens, respectively. The antigenic complex CS1CS3 was related to asymptomatic infections and to serotype O6:H. Among the LT-I probe positive strains CFs occurred only in the diarrheal group

Table 1
Phenotypic and genotypic characteristics of ETEC isolates obtained from children with and without diarrhea, in Rio de Janeiro City, Brazil

Isolates	Toxigenic genotype	Electrophoretic profiles[a]					CFA[b]	Serotype[c]	Adherence pattern[d]			
		MLEE	PFGE	RAPD					HEp-2		CaCO-2	
				1254	1253	1290			3-h	6-h	3-h	6-h
36III	LT-I	1	1	4	4	4	–	O159:H21	NA	DA	DA	–
36IV	LT-I	1	1	4	5	4	–	O159:H21	DA	–	–	–
136I	LT-I	2	4	7	8	6	–	O88:H25	NA	NA	NA	NA
136II	LT-I	2	4	7	8	6	ND	O88:H25	NA	NA	NA	NA
136III	LT-I	2	4	7	8	6	ND	O88:H25	NA	NA	NA	NA
187V	LT-I	3	5	4	9	7	–	O159:H21	DA	–	DA	–
214III	LT-I	4	6	8	10	8	–	ONT:HNT	NA	NA	NA	NA
225II	LT-I	5	7	9	11	9	–	O7:H24	NA	DA	DA	–
225IV	LT-I	6	D	3	11	3	CS6	O148:H28	DA	–	NA	DA
63V	LT-I	7	2	5	6	1	–	O159:H17	DA	–	DA	–
121I	LT-I	8	3	6	7	5	–	O48:H21	NA	NA	DA	–
108I	ST-h	9	9	2	2	2	CFA/I	O159:H17	DA	–	DA	–
108II	ST-h	9	9	2	2	2	ND	O159:H17	DA	–	DA	–
108IV	ST-h	9	9	2	2	2	ND	O159:H17	DA	–	DA	–
108V	ST-h	9	9	2	2	2	ND	O159:H17	DA	–	DA	–
245I	ST-h	2	D	3	3	3	CS6	O148:H28	DA	–	DA	–
245III	ST-h	2	D	3	3	3	ND	O148:H28	DA	–	DA	–
245IV	ST-h	2	D	3	3	3	ND	O148:H28	DA	–	DA	–
245V	ST-h	2	D	3	3	3	ND	O148:H28	DA	–	DA	–
46II	ST-h	10	8	1	1	1	CS1 CS3	O6:NM	NA	DA	DA	–
46V	ST-h	10	8	1	1	1	ND	O6:NM	NA	DA	DA	–

[a] D, degraded.
[b] –, negative for the CFs tested.
[c] NT, non-typable; NM, non-motile; ND, not determined.
[d] DA, diffuse adherence; NA, low or non-adherent.

and were associated with one strain of serotype O148:H18 that expressed CS6.

In general, most ETEC isolates were weakly to moderately adherent showing a diffuse adherence pattern, which could be detected in a 3-h assay using the HEp-2 and CaCo-2 cell lines or in a 6-h assay. Serotypes O159:H17 and O148:H28, isolated from diarrheaic children and expressing CFA/I and CS6 antigens, respectively, were strongly adherent displaying a typical diffuse phenotype clearly discernible in a 3-h assay. Serotypes O159:H21 and O7:H24 with no detectable CF were also strongly adherent, but the incubation period for defining the phenotype varied among strains. Strains of serotypes ONT:HNT and O88:H25, as well as those isolated from asymptomatic children, did not express known CFs, and showed an undefined pattern or were weakly or non-adherent to both cell lines. An exception was detected with serotype O6:H- expressing the CS1 CS3 antigens, which showed moderate adherence with a diffuse phenotype.

The genetic relatedness among ETEC strains was investigated by RAPD-PCR, PFGE and MLEE techniques. Representative RAPD profiles determined with primers 1254, 1253 and 1290 are shown in Fig. 1. Amplification reactions generated informative profiles composed of two to 15 bands ranging from approximately 300 to 3,000 bp. Reactions performed with primers 1254 and 1253 (70% G+C) and 1290 (60% G+C) resulted in nine, 11 and

nine different RAPD profiles, respectively. The total number of polymorphic bands were 15, 15 and 7 for primers 1254, 1253 and 1290, respectively. There was a direct correlation between higher G+C contents of the primer and the ability to detect polymorphism, reproducibility and stability of reactions; however, no direct correlation was observed with their discriminatory potential. All three primers used showed considerable ability to detect intra-serotype variations generating distinctive RAPD patterns. These variations were often correlated with the degree and frequency of the adherence phenotype.

PFGE analysis using XbaI endonuclease generated nine distinct electrophoretic profiles composed of 13 to 18 polymorphic bands ranging in size from 50 to 580 kb (Fig. 2). Same as in RAPD-PCR, PFGE typing revealed intra-serotype variations. Although generating stable, reproducible and discriminatory banding profiles for most of the tested isolates, PFGE analysis could not be performed on strains belonging to the serotype O148:H28 due to the DNA degradation. These isolates were, in turn, typed and discriminated by RAPD-PCR.

The genetic similarity estimated from the allele profiles is shown in Fig. 3. All of the 10 loci investigated by MLEE were polymorphic with the number of allelic states ranging from two at PGM, ACO, PEP-2, G6P to five at PGD. Comparisons of the allelic profiles revealed 10 ETs (enzyme genotypes) arranged as single branches or clus-
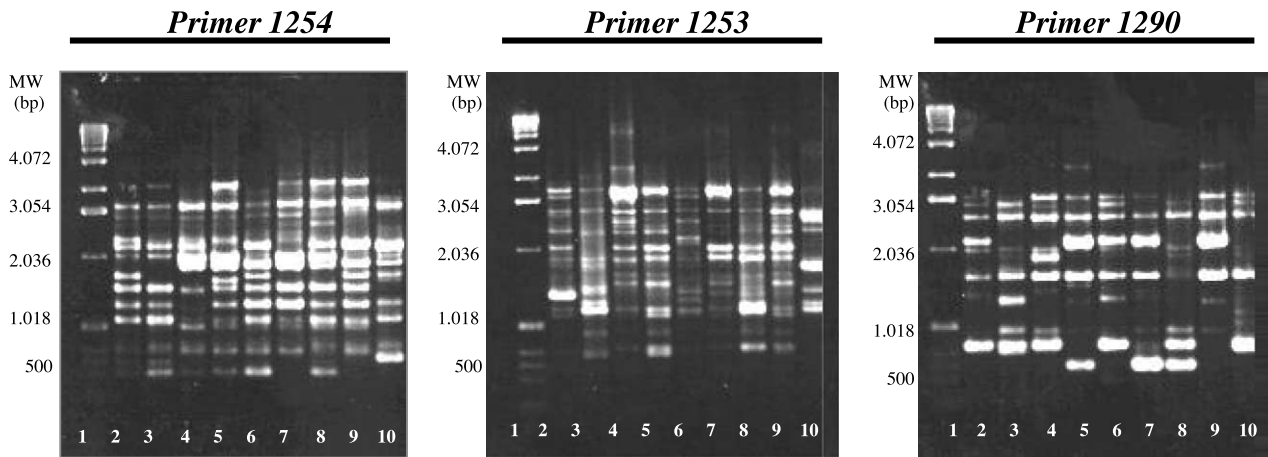
Fig. 1. RAPD-PCR profiles of representative ETEC isolates obtained by use of three different primers (1254, 1253 and 1290). Lane 1, 1-kb DNA ladder; lane 2, isolate 46II; lane 3, isolate 108I; lane 4, isolate 245I; lane 5, isolate 36III; lane 6, isolate 63V; lane 7, isolate 121I; lane 8, isolate 136I; lane 9, isolate 214III; lane 10, isolate 225II.

tered into small not closely related clonal groups and exhibited distinct virulence markers. Null alleles were detected at GPI, PGM, MDH and IDH loci related to one isolate belonging to serotype O48:H28. In general, identical allele profiles were observed among isolates belonging to the same serotype; otherwise, a common ET (ET2 type) was detected in both serotypes O88:H25 and O148:H28.

4. Discussion

ETEC is responsible for significant rates of morbidity and mortality among children, particularly in developing areas [5,6]. Detection of virulence markers by molecular approaches and immunological assays has been widely used for the identification and precise characterization of ETEC strains in epidemiological studies [3–6,29,31–33].

ETEC is a highly diverse group in relation to potential combinations among serotypes, toxins types and CFs [8].

Despite the diversity, which complicates the development of a broadly protective vaccine, specific associations of characteristics have been consistently implicated in ETEC diarrhea [8]. In the present study, the frequency of distinct O:H types among ETEC was diverse; however, specific toxin-serotype associations were observed, particularly among serotypes O6:H-, O88:H25 and O148:H28, corroborating with the results of previous observations [8,33].

CF-negative ETEC strains corresponded to 63.6% of the isolates analyzed. Studies in different parts of the world have shown that a considerable number of strains do not possess any known CFs suggesting the presence of as yet undescribed fimbrial or afimbrial antigens [5,6,28]. The association of specific O and H antigens with strains lacking a defined CF has been already described [8] and, similarly to our results, mainly related to LT strains. CFs were mainly identified in ST+ strains, as also reported in other studies including Brazilian isolates [29,34]. This find-
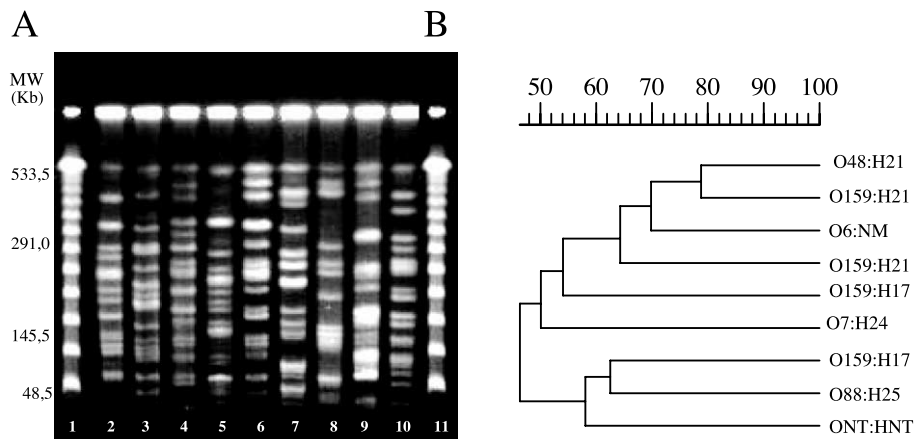

Fig. 2. A: Representative PFGE profiles of *Xba*I-digested DNA of ETEC strains; B: Dendrogram resulting from computer-assisted analysis based on PFGE profiles similarity. Lanes 1 and 11, molecular mass markers expressed in kilobases (λDNA concatemers ranging from 48.5 to 1018.5 kb); lane 2, isolate 63V (O159:H17); lane 3, isolate 136III (O88:H25); lane 4, isolate 108IV (O159:H17); lane 5, isolate 214III (ONT:HNT); lane 6, isolate 121 I (O48:H21); lane 7, isolate 187V (O159:H21); lane 8, isolate 46V (O6:NM); lane 9, isolate 36I (O159:H21); lane 10, isolate 225II (O7:H24).
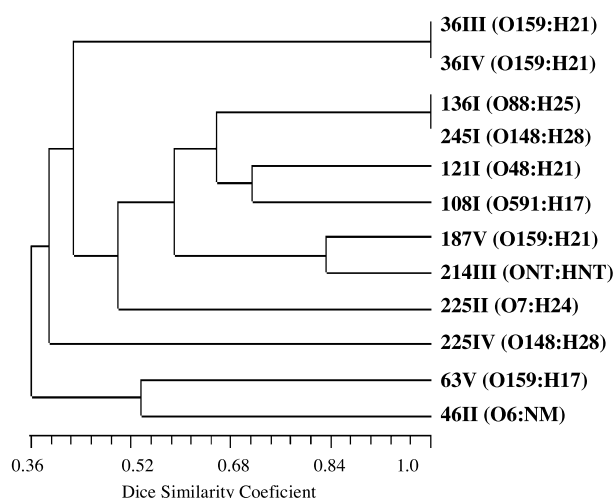
Fig. 3. Genetic relationship among multilocus enzyme genotypes (ETs) of ETEC strains used in this study. *E. coli* strain designation and serotype are given at the right.

ing is consistent with the fact that plasmids coding for such CFs usually harbor ST [35,36]. Particular CFs types have been associated with the adherence properties of these strains. ETEC strains harboring known CF were isolated from diarrheal children and were shown strongly adherent. Otherwise, CFs have also been detected among strains from asymptomatic child, as observed for the antigen complex CS1 CS3, which was found weakly adherent. ETEC strains expressing specific CFs, such as the CFA/I antigen, have been reported to adhere only to CaCo-2 cells [34]. In our study, adherence to both cell lines (CaCo-2 and HEp-2) was observed reinforcing the hypothesis for the co-expression of others adhesins or specific molecules. The evaluation of several cell culture assays to test the adherence ability of ETEC strains revealed that CaCo-2 cells is the most suitable cell line for such purpose [34]. Our data confirmed this observation showing that CaCo-2 cells were more sensitive to evaluate the adhesion ability of ETEC strains by allowing the detection of the adherence phenotype in a shorter time and by promoting this property to non-adherent strains in HEp-2 cells assays.

In this study we used three different techniques to assess the genetic polymorphism among ETEC strains. RAPD-PCR, PFGE and MLEE analysis revealed the genetic diversity among ETEC strains showing a high level of polymorphism and indicating the non-clonal nature of the bacterial population studied. Results of previous investigations on the clonal origin of clinically relevant ETEC strains showed that O:H antigens could precisely group genetically related strains [37]. Therefore, the genetic heterogeneity detected in the present study either by PFGE, RAPD-PCR and MLEE was probably due to the diversity of serotypes found.

The discriminatory power of the methodologies used to evaluate the genetic diversity was in agreement with the phenotypic variations detected, indicating their relevance

as typing systems in epidemiological investigations. In well-standardized experimental conditions, RAPD-PCR showed a consistent correlation with PFGE and MLEE analysis. It was fast, reproducible and stable, and can be considered as an informative tool for the genetic characterization of ETEC strains [19].

While the results of molecular approaches used to investigate the genetic relatedness among ETEC strains were in general agreement, in a few cases, ETEC clustering by comparing the three techniques was not congruent. These results could be expected since these methods, despite being widely used for common purposes, rely on the analysis of distinct targets, i.e. PFGE and RAPD-PCR are current methods based on variations that evolve very rapidly and MLEE makes use of variations that are slowly accumulated [16,24,38]. Despite the high polymorphism detected by PFGE and RAPD, genetic exchanges of virulence determinants in the studied population seem to occur among phylogenetically related *E. coli* strains as revealed by the MLEE clustering linkage. The observed diversity could be explained if we consider that these strains were isolated from children living in different geographic areas in the same city over a relatively long period of time. Additionally, it should be considered that the virulence gene markers for this diarrheagenic category are mainly encoded on genetic mobile elements, which makes the horizontal transfer of such sequences possible, favoring the emergence of heterogeneity among clonal complex groups.

Although a small number of isolates was included in the present investigation, they comprised all the ETEC recovered during a previous investigation on the occurrence of the different categories of DEC in children, representing 12.7% of the DEC isolates [20]. The results indicate that the ETEC isolates in this particular study exhibited a wide range of phenotypic and genotypic characteristics, which in some cases, were distinct from those of isolates recovered in other Brazilian locations [6,39]. This information is of special value since the available data on ETEC infections in the studied community remains scarce. Considering that ETEC is a major cause of diarrheal disease in many developing countries and the importance of environmental factors in facilitating the emergence of infectious agents, efficient diagnostic methods are required to precisely identify and characterize such microorganisms. Therefore, we recommend the application of different methods in order to better elucidate the epidemiology of ETEC infections and to establish the relatedness among virulent lineages circulating in a given community.

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