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ISOLATION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) SEROTYPE O91:H21 FROM A CHILD WITH DIARRHEA IN PORTO ALEGRE CITY, RS, BRAZIL

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SHORT COMMUNICATION

ABSTRACT

We describe the isolation of one strain of Shiga toxin-producing *Escherichia coli* O91:H21 from a child with diarrhea in the city of Porto Alegre, RS, Brazil. Considering the pathogenic potential of STEC, these organisms should be looked for more carefully among our population.

Key words: enterohemorrhagic *E. coli,* STEC, enteroaggregative *E. coli,* enteropathogenic *E. coli,* diarrhea

Enterophatogenic *Escherichia coli* (EPEC) was the first category of *E. coli* to be implicated in gastrointestinal diseases (1). In the 70's two other categories of diarrheagenic *E. coli* were described: enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) (19). During the 80's, the demonstration that EPEC strains could adhere to epithelial cells in culture (4) resulted in the description of two new categories of *E. coli*: enteroaggregative *E. coli* (EAEC or EAggEC) and diffuse adhering *E. coli* (DAEC) (20, 24). The diarrheagenic potential of EAEC and DAEC was a matter of controversies, however, both categories were recently reported to cause diarrheal diseases, and EAEC is now considered an emerging pathogen (19). EAEC was significantly associated with persistent diarrhea (≥14 days) and growth retardation in the Northeast of Brazil (27). In another report, EAEC was isolated only from patients in similar frequency for acute and persistent diarrhea (23). EAEC may also represent a potential cause of diarrhea among AIDS patients (31).

Shiga toxin-producing *E. coli* (STEC) is another category of organisms with the potential to cause diarrheal disease. The major virulence factor produced by these organisms is Shiga toxin (Stx1 and Stx2, also called verocytotoxins), which seems to be directly responsible for the hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) developed by some patients following STEC infection. HUS may result in permanent renal failure, and sometimes leads the patient to death (13). Subsets of STEC that are able to cause attaching and effacing (A/E) lesions similar to EPEC organisms are termed enterohemorrhagic *E. coli* (EHEC, or typical EHEC) (19). Even though EHEC serotype O157:H7 is the organisms most often implicated in large outbreaks, others serotypes have been reported to cause a considerable number of cases of HUS in many countries, specially in the southern hemisphere (Reviewed in ref. 11). STEC from various serotypes, including serotype O157:H7, were isolated in high frequency from cattle in Rio de Janeiro State, Brazil (3). A previous epidemiological study conducted in Rio de Janeiro, however, detected no STEC organisms from urban children less than 2 years old (22).

Both EHEC and EPEC possess a large chromosomal region called LEE (locus of enterocyte effacement) (9). This region contains genes involved in adherence, such as *eae* (for intimin) and *tir* (for translocated intimin receptor), as well as genes involved in the production and delivery of virulence factors into the infected intestinal cells (For details see ref. 5, and 10). STEC strains that do not posses *eae* gene have been isolated from patients in sporadic cases of HC and HUS, as well as

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outbreaks (6). STEC strains isolated from HUS patients that produce Stx2 and adhere to cells in culture in an aggregative pattern have also been described (6,17). These strains were negative for the presence of *eae* gene and may possess distinct adherence factors (17).

The use of serology alone to differentiate enteropathogenic strains of *E. coli* may be misleading, since within a certain "O" serogroup different categories of *E. coli* may be present (29). Recently, molecular methods such as "Polymerase Chain Reaction" (PCR) and molecular probes have been extensively used to detect *E. coli* involved in gastrointestinal infections (19).

In this report we further characterized a group of sixteen *E. coli* that agglutinated with anti-EPEC polyvalent antiserum during screening for EPEC serogroups. The *E.* coli strains were isolates from patient's fecal samples submitted to our laboratory during the period from November 1995 to June 1996. During this period, a total of 1756 fecal cultures were processed, among them 375 were from children less than 2 years old and were screened for the presence of possible EPEC organisms in the absence of another enteropathogen, such as *Salmonella* sp., *Shigella* sp. or *Campylobacter* sp.

Three to six lactose-fermenting and two to three lactose-negative colonies from a MacConkey agar plate were biochemically confirmed to be *E. coli*, and then screened using anti-EPEC polyvalent antisera (Probac, Brazil) as directed by the manufacturer. The polyvalent antisera consist of three separated pools, being able to react with the following serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158. Sixteen strains from different patients, named Br1 to Br16, agglutinated with the EPEC polyvalent antiserum and were further tested with monovalent antisera (Denka Seiken, Japan), following exactly the manufacturer's instructions. Serotyping of strain Br12 was kindly performed by Dr. Teizo Tsukamoto at the Osaka Prefectural Institute of Public Health, Osaka, Japan.

Control strains were used in all the experiments. For positive controls, the following strains from the Research Institute for Microbial Diseases (RIMD) bacterial culture collection were used: RIMD 0509829, a typical EPEC strain belonging to serotype O111:H2, and RIMD 0509952, an EHEC strain serotype O157:H7 isolated in Osaka, Japan, that secretes Shiga toxins (Stx1 and Stx2); and the ETEC strain RIMD0509266 that produces both heat-labile (LT) and heat-stable toxins (ST1). Non-pathogenic *E. coli* strain HB101 was used as a negative control. Adhesion assay and the Fluorescent Actin Staining (FAS) assay were performed using HeLa cells (Riken, Japan), essentially as described by Cravioto *et al.* and Knutton *et al.* (4,15), respectively. For both tests, the infected cells were washed three times with phosphate buffered saline (PBS) 3 h post infection, then fresh tissue culture medium was added (Dulbecco's Modified Eagle's Media, DMEM, Sigma Chemical Co., St. Louis, Mo.) and the infection was allowed to proceed for a total of 6 h. The FAS assay was performed using fluorescent rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, Ore., USA) to stain the infected cell monolayers. Primers EAE1 (5'-CCC GGC ACA AGC ATA AGC TAA-3') and EAE2 (5'-ATG ACT CAT GCC AGC CGC TCA-3') were used to amplify a 920 bp segment of the *eae* gene of both EPEC and STEC (this study). The following PCR primers were also used in this study. pCVD432/start (5'-CTG GCG AAA GAC TGT ATC AT-3') and pCVD432/stop (5'-CAA TGT ATA GAA ATC CGC TGT T-3') to detect EAEC (25); for ETEC heat-labile toxin (LT), the upstream primer TW20 (5'-GGC GAC AGA TTA TAC CGT GC-3') and the downstream primer JW11 (5'-CGG TCT CTA TAT TCC CTG TT-3') were used (26); for ETEC heat-stable toxin (STI), the degenerated upstream primer JW14 (5'-ATT TTT MTT TCT GTA TTR TCT T-3'; M = A or C and R = A or G) and the downstream primer JW7 (5'-CAC CCG GTA CAR GCA GGA TT-3') were used to amplify both the STh and STp gene sequences (26). *Stx*1 and *Stx*2 genes were simultaneously detected with a single primer pair: MK1 (5'-TTT ACG ATA GAC TTC AC-3') and MK2 (5'-CAC ATA TAA ATT ATT TCG CTC-3') (12), and positive samples were confirmed using primers specific for *Stx*1, SLTI-5 (5'-AGC TGA AGC TTT ACG TTT TCG G-3') and SLTI-3 (5'-TTT GCG CAC TGA GAA GAA GAG A-3'), and *Stx*2 genes, SLTII-5 (5'-TTT CCA TGA CAA CGG ACA GCA GTT-3') and SLTII-3 (5'-ATC CTC ATT ATA CTT GGA AAA CTC A-3') (30). Bacterial DNA from samples and controls was prepared from 200 µl of an overnight culture in Luria broth (LB), centrifuged and resuspended in 500 µl of deionized water and boiled for 5 min, then kept in a deep freezer until use. Amplification of each gene target was carried out in separate tubes using the *Ex Taq* polymerase kit (Takara Shuzo Co. Japan) following the instructions provided by the manufacturer. Reaction mixtures contained 5 µl of the bacterial DNA in a final volume of 25 µl. For *eae*, the reaction mixtures were heated to 95°C for 5 min and were then subjected to 30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s, with a final extension performed at 72°C for 5 min. Cycling conditions for the others PCR reactions were the same as described in the references cited for each primer set. PCR products were examined by agarose gel electrophoresis comparing samples and controls.

A summary with the characteristics of the 16 strains is shown in Table 1. Strain Br4 displayed a typical localized adherence (LA) (Fig. 1A), and was also able to induce actin accumulation beneath the bacterial adherence site, as demonstrated by the positive FAS assay. This strain belonged to serogroup O142, and was confirmed by PCR to possess the *eae* gene. Three strains belonging to serogroup O111, one strain from serogroup O91, and seven strains from non-identified serogroups were classified as EAEC by the typical stacked-bricks adherence pattern to cultured HeLa cells (Fig 1C). PCR for EAEC was positive for only two of the eleven strains initially classified as

Table 1. Characteristic of *Escherichia coli* strains analyzed in this study.

Strain	Serogroup or Serotype	Cell Adherence	FAS assay	PCR for:			
				EAE	EAEC	Stx	ST1/LT
Br1	ND	AA	-	-	-	-	-
Br2	ND	AA	-	-	-	-	-
Br3	ND	AA	-	-	-	-	-
Br4	O142	LA	POS	POS	-	-	-
Br5	ND	AA	-	-	-	-	-
Br6	0111	AA	-	-	-	-	-
Br7	ND	AA	-	-	-	-	-
Br8	O111	AA	-	-	POS	-	-
Br9	ND	AA	-	-	-	-	-
Br10	ND	NA	-	-	-	-	-
Br11	ND	NA	-	-	-	-	-
Br12	O91:H21	AA	-	-	-	Stx2	-
Br13	ND	NA	-	-	-	-	-
Br14	O111	AA	-	-	POS	-	-
Br15	ND	DA	-	-	-	-	-
Br16	ND	AA	-	-	-	-	-

ND, No agglutination with monovalent antiserum against classical EPEC serogroups; AA, aggregative adherence; LA, localized adherence; DA, diffuse adherence; NA, No significant adherence; POS, positive reaction; See text for details.

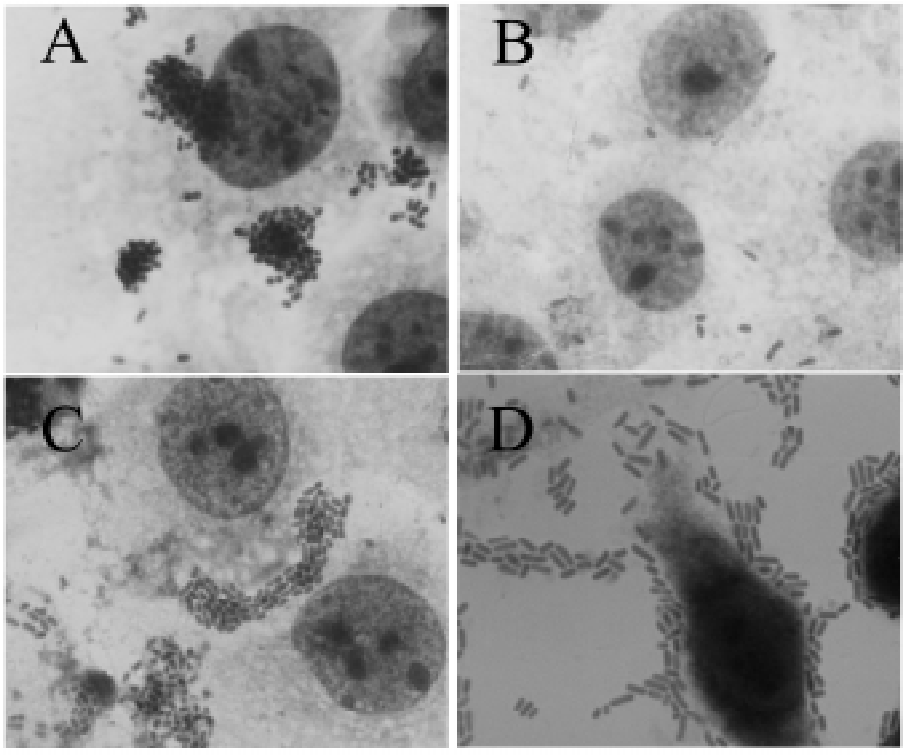

Figure 1. Adherence pattern of *Escherichia coli* strains isolated in Porto Alegre, RS, Brazil to HeLa cells. Localized adherence (LA) of EPEC strain Br4 (A), aggregative adherence (AA) of EAEC strain Br8 (C), and aggregative adherence of STEC strain Br12 (D). A non-adherent strain (Br10) is also shown (B).

EAEC by the typical cell adherence pattern. This fact was not unexpected, since it was reported that some EAEC strains do not harbor the gene sequence recognized by this primer set (25).

PCR was also performed for all sixteen strains to detect the genes for production of STl and LT by ETEC, and Stx1 and Stx2 by STEC. None of the sixteen strains produced ST1 or LT (Table 1). Strain Br12 was positive for *stx*2, and the secretion of Stx2 was confirmed by cytotoxicity to Vero and HeLa cells, and by reverse passive latex agglutination test (Verotox-F, Denka Seiken, Japan) (data not shown). This STEC strain was classified as serotype O91:H21, and confirmed to be negative for *eae* and the FAS test (Table 1). This strain was able to adhere to HeLa cells in culture displaying an aggregative pattern of attachment (Fig.1D).

To the best of our knowledge, this is the first description of an STEC strain isolated from a patient in the south of Brazil (Porto Alegre, RS). The patient, a two years old child, suffered from diarrhea and no other pathogen could be implicated as the cause of the gastrointestinal disease. The disease did not progressed to HC or HUS. STEC strain belonging to serotype O91:H21 was reported to be isolated from a patient with HUS (7), and from a patient with bloody diarrhea (14). The reason why strain B12 agglutinated with polyvalent antisera is not known, since serogroup O91 is not included among the serogroups recognized by the polyvalent anti-EPEC antiserum employed for initial screening of possible EPEC organisms. A total of eleven other strains of unknown serogroups were also positive with the polyvalent antisera used. It is known, however, that strains tested positive by polyvalent antisera should be confirmed using monovalent antisera, since false positive reactions do occur (2).

Even though *eae*-positive EHEC strains are more often involved in outbreaks, several cases of HUS have been attributed to *eae*-negative STEC strains (21, 28). It is possible that the concentration of efforts directed to isolate strains of EHEC serotype O157:H7 may cause other STEC strains to be overlooked. Considering that two countries that border with the south of Brazil, Argentina and Uruguay, reported several cases of HUS due to non-O157 strains (16), it should be advisable to look for STEC organisms in stool samples submitted for culture, particularly in those Brazilian States where consumption of beef is high. Contrary to the typical EHEC strains, like O157:H7, the majority of STEC strains do ferment sorbitol, so detection of these organisms is best accomplished by molecular methods, such as PCR, designed to reveal the presence of *stx* genes.

In conclusion, our pilot study suggests that true EPEC strains may be uncommon among the patients cared for in our laboratory. EAEC organisms appear to be much more prevalent among our patients. The reason why we found a very low prevalence of true EPEC (0.2%) is possibly related to the higher socio-economic level of the population attended by our institution. EPEC infection is a common cause of diarrhea in low-income populations (8,22), but its relative importance decreases dramatically when this pathogen is searched for in populations of better socio-economic levels, such as in developed parts of the world (18,19). Since our study was restricted to a group of 16 *E. coli* that initially agglutinated with anti-EPEC polyvalent antisera, the true prevalence of EAEC or STEC among our population is probably underestimated. Finally, considering the life-threatening potential of STEC, the real incidence of these organisms among Brazilian population deserves further studies. We are currently planning a more detailed study to evaluate the prevalence of STEC and other diarrheagenic *E. coli* among our population in the south of Brazil.

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RESUMO

Isolamento de *Escherichia coli* O91:H21 produtora de toxina Shiga (STEC) de uma criança com diarréia em Porto Alegre, RS, Brasil

É descrito o isolamento de uma cepa de *Escherichia coli* O91:H21 produtora de toxina Shiga (STEC) em associação com diarréia em uma criança da cidade de Porto Alegre, RS, Brasil. Dado o potencial para causar complicações graves, infecções por STECs deveriam ser melhor avaliadas em nossa população.

Palavras-chave: *Escherichia coli* enterohemorrágica, STEC, *Escherichia coli* enteroagregativa, *Escherichia coli* enteropatogênica, diarréia

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