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Genetic Heterogeneity of Shiga Toxin-Producing *Escherichia coli* Strains Isolated in São Paulo, Brazil, from 1976 through 2003, as Revealed by Pulsed-Field Gel Electrophoresis

Tânia Mara I. Vaz,[1,2] Kinue Irino,[1] Lucilia S. Nishimura,[2] Maria Cecília Cergole-Novella,[2] and Beatriz Ernestina C. Guth[2]*

Instituto Adolfo Lutz, São Paulo,[1] and Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo,[2] Brazil

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The pulsed-field gel electrophoresis (PFGE) patterns of 46 Shiga toxin-producing *Escherichia coli* (STEC) strains isolated in São Paulo, Brazil, during the period from 1976 to 2003 were compared with those found among 30 non-STEC strains that carried *eae* and that belonged to the same serogroups as the STEC strains. All except two of the STEC and non-STEC strains of human origin were from sporadic and unrelated cases of infection; two O111 strains originated from the same patient. Multiple PFGE patterns were found among STEC strains of distinct serotypes. Moreover, the PFGE restriction patterns of STEC strains differed substantially from those observed among non-STEC strains of the same serogroup except serotype O26 strains. Based on the indistinguishable PFGE pattern for two O157:H7 STEC strains isolated in the same geographic area at an interval of approximately 15 days and toxin profile data, the first occurrence of an O157:H7 outbreak in Brazil during that period can be suggested. In general, a close relationship between types of intimin, serotypes, and diarrheagenic groups of *E. coli* was observed. This is the first time that a large collection of STEC strains from Brazil has been analyzed, and a great genetic diversity was shown among O157:H7 and non-O157:H7 STEC strains isolated in São Paulo, Brazil.

Shiga toxin-producing *Escherichia coli* (STEC) strains are important food-borne pathogens associated with a broad spectrum of human infections, ranging from mild diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (17, 32, 40).

Although O157:H7 is reported worldwide to be the most important STEC serotype due to its association with severe diseases and with many outbreaks of diarrhea and HUS, STEC strains belonging to a larger number of serotypes have already been isolated from human infections. In fact, in some geographic areas STEC strains other than O157:H7 are more commonly isolated from humans, and increasing numbers of reports have documented its occurrence in association with sporadic cases of diarrhea and HUS in many countries (4, 5, 7, 8, 36). In Brazil, non-O157:H7 STEC strains have also been more frequently isolated from human infections; and serotypes O26:H11, O103:H2, and O111:H8(H^-) (where H^- is nonmotile) accounted for most of the cases (19, 20, 42; K. Irino et al., unpublished data).

Shiga toxin (Stx) represents the main virulence factor in STEC strains, and two distinct toxins (Stx1 and Stx2) similar in biological activity but immunologically distinct were recognized (29). Several subtypes of Stx2 have been identified on the basis of sequence homology and immunological cross-reactivity. Studies have indicated that Stx2 and Stx2c, encoded by different genotypic subtypes of stx_2, have been associated with more severe human diseases, while the stx_{2d} genotype is apparently of lesser clinical significance (14).

Several other virulence factors involved in the pathogenicity of STEC have been described. The intimin encoded by the *eae* gene, located in the chromosomal locus of enterocyte effacement, is responsible for the intimate adherence of bacteria to enterocytes, which causes attaching and effacing lesions (29). Distinct types of intimin have been described in STEC strains, based on the heterogeneity of amino acid sequence in the C-terminal end (1, 30). However, some STEC strains involved in severe disease do not carry the genetic information encoding intimin (9, 26, 31). Additionally, a plasmid-encoded enterohemolysin (Ehx) may play a role in pathogenesis (29).

Currently, molecular biology-based methods are used for epidemiological investigations of outbreaks and for the control and monitoring of the spread of potential pathogens. Pulsed-field gel electrophoresis (PFGE) is the most common molecular biology-based method used for the subtyping of STEC strains. This method is considered the "gold standard" for the subtyping of STEC strains, due to its high power of discrimination and reproducibility, and has been successfully used all over the world (10, 11, 16, 24). Moreover, the standardization of PFGE analysis for epidemiological purposes has been achieved in the United States to facilitate the subtyping of bacterial food-borne pathogens among different laboratories (39).

Even though the occurrence of STEC strains has been demonstrated in Brazil since 1976 (19, 22, 42), the analysis of the genetic diversity of STEC strains belonging to different serotypes has not been carried out before. Thus, in the present study, stx_2 and the intimin subtypes of O157:H7 and several non-O157 STEC strains isolated from human infections in previous studies conducted in São Paulo, as well as a few STEC strains isolated from environmental sources and belonging to the same serotypes as the human strains, were characterized

* Corresponding author. Mailing address: Disciplina de Microbiologia, Universidade Federal de São Paulo, Rua Botucatu 862/3 andar, São Paulo, SP CEP 04023-062, Brazil. Phone: 55-11-5576-4537. Fax: 55-11-5572-4711. E-mail: becguth@ecb.epm.br.

TABLE 1. Serotypes and sources of STEC strains isolated in São Paulo, Brazil, during the period from 1976 to 2003

Serotype	No. of strains					Total
	Human sources		Non-human sources			
	Diarrhea	HUS	Cattle	Food	Water	
O26:H11	4	1				5
O55:H19	1					1
O77:H18	1		2			3
O103:H2	2[a]					2
O93:H19	1			1		2
O111:H8(H$^-$)	22		1			23
O111:H11	1					1
O118:H16	2					2
O157:H7	4[b]		1		1	6
ONT:H2	1					1
Total	39	1	4	1	1	46

[a] One strain was isolated from a child with hemolytic anemia.
[b] One strain was isolated from a patient with bloody diarrhea.

and their genetic diversity was analyzed by PFGE. Moreover, non-STEC strains belonging to serogroups O26, O111, and O157, which are commonly found among STEC strains and which were isolated from children with diarrhea in our community, were also analyzed for some virulence properties and their PFGE types, and these properties and types were compared to those of STEC strains.

MATERIALS AND METHODS

Bacterial strains. A total of 76 *E. coli* strains isolated in São Paulo, Brazil, during the period from 1976 to 2003 were included in this study. Among them 46 were STEC strains (Table 1) isolated from human infections ($n = 40$) (22, 42; Irino et al., unpublished), dairy cattle ($n = 4$) (23), meat ($n = 1$) (2), and a water supply ($n = 1$). The human STEC strains were isolated from sporadic cases; and some of them were isolated from patients with bloody diarrhea (22), hemolytic anemia (20), and HUS (19) (Table 1). The remaining 30 strains were selected from the collection of the Instituto Adolfo Lutz, a central public health laboratory in São Paulo, because they were isolated from children with diarrhea; carried *eae* (*eae*$^+$); were devoid of *stx*; and belonged to serogroups O26, O111, and O157. Moreover, none of these strains carried the *bfp* (bundle-forming pilus) or enteropathogenic *E. coli* (EPEC) adherence factor sequence, as previously determined by hybridization assays with specific DNA probes (21). These 30 *eae*$^+$ non-STEC strains belonged to serotypes O26:H11 ($n = 14$), O26:H$^-$ ($n = 4$), O111:H2 ($n = 2$), O111:H11 ($n = 1$), O111:H$^-$ ($n = 3$), O111:H8 ($n = 1$), O111:HNT (where NT is nontypeable) ($n = 1$), and O157:H$^-$ ($n = 4$) (Table 2). All the strains studied except one O111:H$^-$ STEC strain (strain 256/03) and one O111:H8 non-STEC strain (strain 95/03), which originated from the same patient, were isolated from sporadic and unrelated cases,. The serotypes and enterohemolytic phenotypes of the STEC strains were identified previously (22, 42; Irino et al., unpublished). The enterohemolysin production of the non-STEC strains was determined as described by Beutin et al. (3).

Biochemical characterization. Rhamnose and dulcitol fermentation tests were performed by standard methods (13).

Subtyping of *stx*$_2$ variants. For the differentiation of *stx*$_2$ variants, the genotyping method of Tyler et al. (41) was extended to include the primers and

TABLE 2. Virulence markers presented by the STEC and non-STEC strains studied

Strain group and serotype	Origin[a]	No. of strains	Presence of the following virulence markers:				
			ehx	*stx*$_1$	*stx*$_2$[b]	*eae*	Intimin
STEC							
O26:H11	HD, HUS	5	+	+	−	+	β
O55:H19	HD	1	−	+	−	−	−
O77:H18	HD	1	+	+	+ (2c)[d]	−	−
	Bovine	2	+ (1)[c]	+	+ (2)	−	−
O93:H19	HD	1	+	+	+ (2c)	−	−
	Ground meat	1	−	+	+ (2)	−	−
O103:H2	HD, HA	2	+	+	−	+	ε
O111:H8	HD	8	+ (3)	+	−	+	θ
O111:H11	HD	1	−	+	−	+	θ
O111:H$^-$	HD	14	+ (3)	+	−	+	θ
	DC	1	+	+	+ (2)	+	θ
O118:H16	HD	2	+	+	−	+	β
ONT:H2	HD	1	+	+	−	+	ε
O157:H7	HD, BD	4	+	−	+ (2/2c)	+	γ
	Bovine	1	+	−	+ (2c)	+	γ
	Water	1	+	−	+ (2)	+	γ
Non-STEC							
O26:H11	HD	14	+ (11)	−	−	+	β (14)[c]
O26:H$^-$	HD	4	+ (2)	−	−	+	β (2), NT (2)
O111:H2	HD	2	−	−	−	+	β
O111:H11	HD	1	−	−	−	+	β
O111:H$^-$	HD	3	−	−	−	+	α (1), β (1), γ (1)
O111:H8	HD	1	−	−	−	+	θ
O111:HNT	HD	1	−	−	−	+	α
O157:H$^-$	HD	4	−	−	−	+	NT (4)
Total		76					

[a] HD, human diarrhea; HA, hemolytic anemia; BD, bloody diarrhea; DC, diarrheic calf.
[b] *stx*$_{2c}$ includes *stx*$_{2vh\text{-}a}$ and *stx*$_{2vh\text{-}b}$.
[c] Values in parentheses in this column represent number of strains.
[d] Values in parentheses in this column represent subtypes.

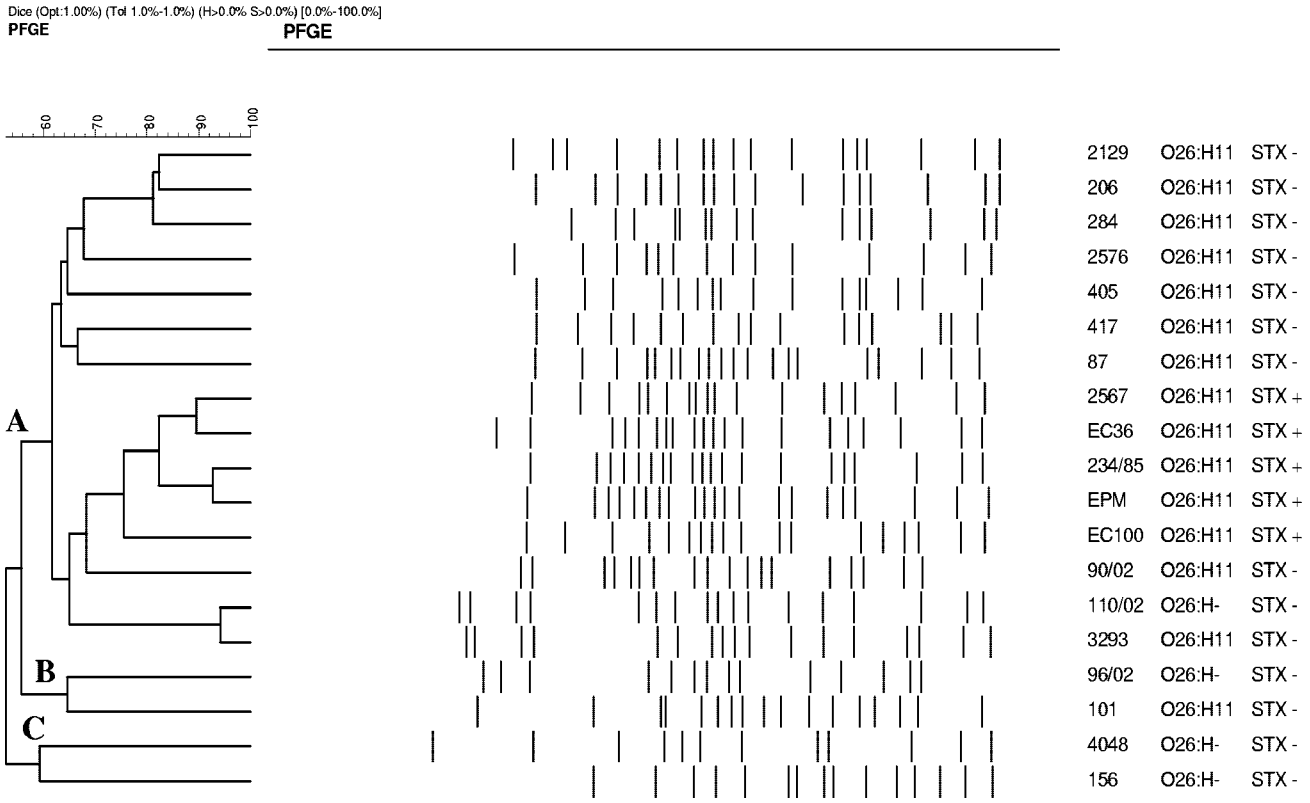

FIG. 1. Dendrogram outlining the relationship of O26 STEC and non-STEC strains, determined by macrorestriction analysis of genomic DNA with XbaI.

restriction enzymes described by Pièrard et al. (34). According to this method, stx_{2c} includes $stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$. Reference *E. coli* strains C600W (stx_2), 92-3580 ($stx_{2vh\text{-}a}$), 93-016 ($stx_{2vh\text{-}b}$), EH250 (stx_{2d}), and K12H5 (negative control) were used as described previously (18).

Intimin typing. The PCR primers and the cycling conditions used for the subtyping of the *eae* genes into intimins α, β, and γ were those described by Adu-Bobie et al. (1); and the PCR primers and the cycling conditions used for the subtyping of the *eae* genes into intimin types ε and θ were those described by Oswald et al. (30) and Zhang et al. (44), respectively.

PFGE. We essentially followed the PFGE method described by Gautom (15), with some modifications. The digestion time was extended to 16 h, and PFGE was performed on a CHEF-DRIII PFGE apparatus (Bio-Rad). The pulse time was increased from 5 to 50 s over a 20-h period. For O26 strains, the pulse ramp time was that described by Zhang et al. (45). The band patterns were analyzed by using the GelCompar II program, and the similarity between PFGE patterns was evaluated by using the Dice coefficient similarity (tolerance, 1%). Strains were considered to have the same PFGE patterns when all bands were identical. *E. coli* O157 strains EDL 933 and G5244 (Centers for Disease Control and Prevention reference strain) were used as controls.

RESULTS

Biochemical characteristics of STEC and non-STEC strains. In general, most of the STEC and non-STEC strains studied belonged to different serotypes and fermented rhamnose and dulcitol. The inability to ferment these carbohydrates (rhamnose and dulcitol negative [Rha^- Dul^-]) was associated only with STEC strains of serogroups O26 and O118, as reported previously (42). Among the non-STEC strains analyzed, the Rha^- Dul^- phenotype was identified only in serotype O26:H11 (eight strains) and in serotypes O26:H^- and O111:H11 (one strain each).

Virulence markers. Different subtypes of stx_2 were identified among STEC strains belonging to serotypes O77:H18, O93:H19, and O157:H7 (Table 2). A close correlation between the intimin type and STEC and non-STEC strains of serogroups O111 and O157 was observed. Intimin type θ was identified among O111 STEC strains and in one *stx*-negative O111 strain (strain 95/03), while among the other non-STEC strains, intimin type α or β prevailed. All O157:H7 STEC strains possessed intimin type γ, whereas the intimins of non-STEC O157 strains were nontypeable with the set of primers used. In contrast, no differences were observed among strains belonging to serogroup O26 (including STEC and non-STEC strains), which presented intimin type β. Additionally, this same intimin occurred in serotypes O118:H16 (STEC) and O111:H2 (non-STEC). The presence of the enterohemolysin (*ehx*) sequence among non-STEC strains was identified only in O26:H11(H^-) strains (Table 2).

PFGE patterns and clonal analysis. The banding patterns were defined for 43 of the 46 STEC strains and the 26 non-STEC strains studied. One STEC strain of human origin belonging to serotype O77:H18 and two O93:H19 strains isolated from a human and food could not be analyzed by PFGE due to incomplete digestion. In general, in each of the serogroups analyzed, distinct clusters were formed by STEC and non-STEC strains (Fig. 1 to 3). Among the O26 strains, one cluster (cluster A) comprised all five isolates of STEC strains, with a high degree of similarity (80 to 98%) among four of them; and some non-STEC strains were also included in cluster A

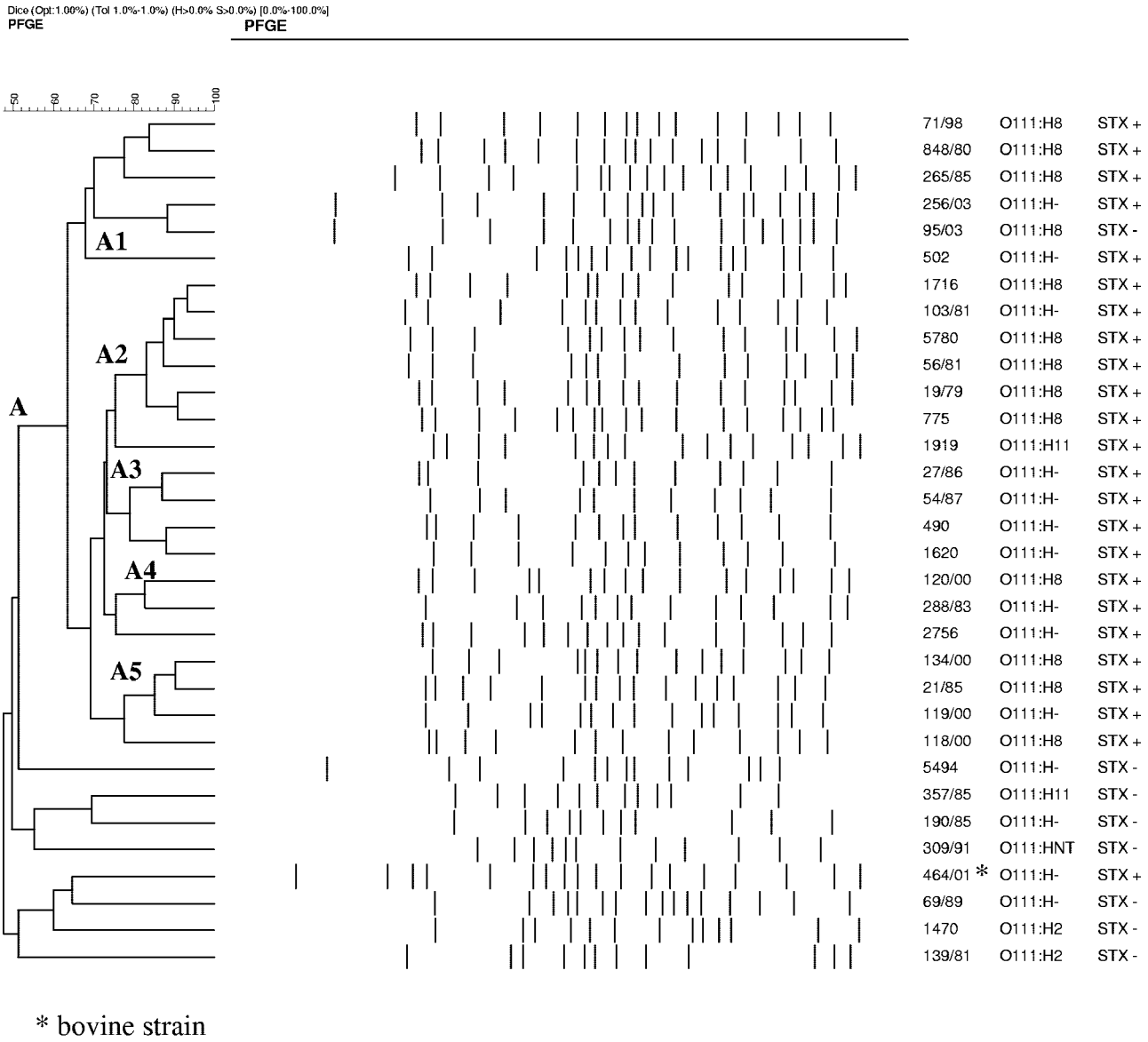

FIG. 2. Dendrogram outlining the relationship of O111 STEC and non-STEC strains, determined by macrorestriction analysis of genomic DNA with XbaI.

(Fig. 1). Regarding serogroup O111, all *stx*-positive strains and one *stx*-negative strain (strain 95/03) from human infections were grouped in the same cluster, which presented five subgroups. The remaining s*tx*-negative O111 strains were distantly related to O111 *stx*-positive strains (less than 50% of similarity); and the only O111 *stx*-positive strain, isolated from a diahrreic calf (strain 464/01), was more related to *stx*-negative strains (Fig. 2). Regardless of the origins, all O157:H7 STEC strains were grouped in the same cluster, and two strains (strains 337 and 385) presented identical profiles. All O157 non-STEC strains formed a distant cluster (less than 50% similarity) from the *stx*-positive strains (Fig. 3). STEC strains isolated from human infections and belonging to serotypes O103:H2, ONT:H2, and O118:H16 each exhibited distinct PFGE patterns (data not shown). On the other hand, two strains belonging to O77:H18 and isolated from cattle presented identical profiles (data not shown).

DISCUSSION

The several studies conducted in the last few years in Brazil have demonstrated the occurrence of important STEC strains and have demonstrated that they are the cause of human disease, including bloody diarrhea, hemolytic anemia, and HUS (19, 20, 22, 42; Irino et al., unpublished). The prevalence of STEC strains in the animal reservoir, especially bovines, has also been demonstrated (23, 27, 33). However, the genetic relatedness of the STEC strains isolated in Brazil has not been analyzed before.

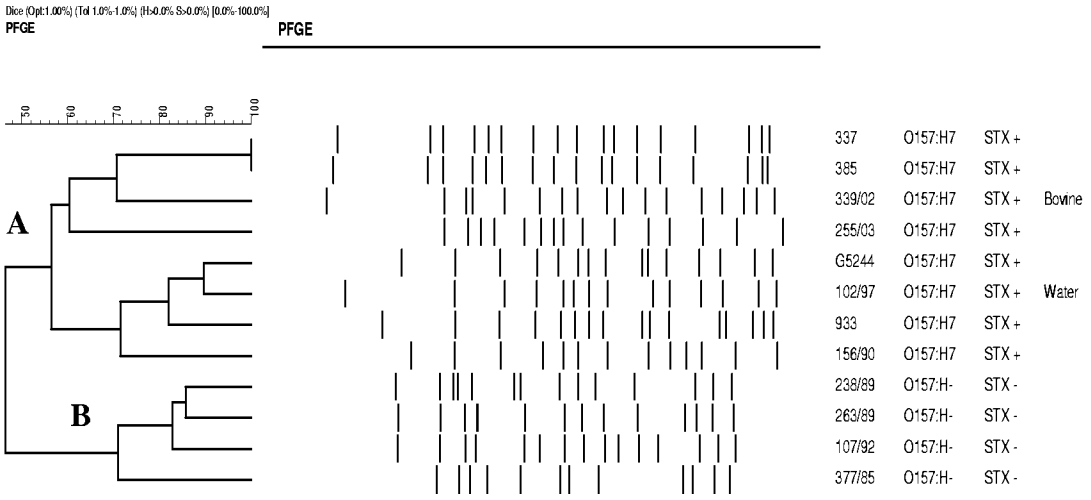

FIG. 3. Dendrogram outlining the relationship of O157 STEC and non-STEC strains, determined by macrorestriction analysis of genomic DNA with XbaI.

In this study a large variety of PFGE patterns was identified among the STEC strains that were of different serotypes and that were isolated in São Paulo between 1976 and 2003. Moreover, despite the distinct PFGE patterns presented by the O26 strains, a higher degree of similarity was observed among them in comparison to the degree of similarity observed among strains belonging to serogroups O111 and O157. The results obtained in the present study were similar to those reported by other investigators (12, 35, 38, 43). Eklund et al. (12) also identified several PFGE subtypes among STEC strains in Finland and showed a high degree of similarity among O26:H11 STEC strains (62 to 85%). Besides the similarities of the PFGE profiles, the O26 STEC and some non-STEC strains that were grouped in cluster A in our study also displayed the same phenotypic characteristics, such as the presence of enterohemolysin (Table 2) and the inability to ferment rhamnose and dulcitol (Rha^- Dul^-). On the other hand, the O26 non-STEC strains from clusters B and C, which were all rhamnose and dulcitol fermenters, were less related to STEC strains. These results are in agreement with recent observations made by two different groups in Germany that have also pointed out that *E. coli* O26 Rha^- Dul^- strains are distributed into two pathotypes, STEC and atypical EPEC (6, 27).

The analysis of serogroup O111, which comprised the majority of our strains, showed that all O111 STEC strains isolated from human infections formed a separate cluster that was subdivided into several related groups (groups A1 to A5; 70 to 78% similarity). These strains were distinct from non-STEC strains. It was interesting that the O111 STEC strain isolated from a bovine (strain 464/03) was more closely related to non-STEC strains than to human STEC strains. In addition, the only non-STEC strain which was included in the STEC cluster was an O111:H8 eae^+ strain (strain 95/03) isolated from the patient who also shed an O111:H^- STEC strain (strain 256/03). These strains displayed very similar PFGE patterns (89%); and although they presented the same number of bands, curiously, minor PFGE pattern changes were observed. One can suggest that these differences may probably be due to the presence or the absence of the *stx* bacteriophage. The lack of a bacteriophage that originated a distinct PFGE pattern was reported by other investigators in O26 and O157:H7 STEC strains (25, 28). Similarly to serogroup O111, PFGE patterns clearly classified Brazilian O157 strains in two main groups. One group corresponded to six STEC O157:H7 strains, and the other group was composed of non-STEC O157 strains. It should be stressed that two STEC O157:H7 strains isolated from different patients living in the same city over an interval of approximately 15 days had indistinguishable PFGE patterns and the same toxin gene profile, and other virulence-associated markers of the two strains were the same. These results may be interpreted as an indication of the first possible O157:H7 outbreak in Brazil. This underlines the importance and usefulness of active laboratory surveillance of STEC infections by the use of PFGE and should be recommended.

It has been sugested that the clinical outcome of STEC infection depends not only on the *stx* genotype but also on the stx_2 subtype of the infecting strain. The stx_1 and stx_1 stx_2 genotypes are more prevalent among STEC strains isolated from patients with uncomplicated infections (8), whereas the progress to HUS has been significantly associated with the presence of the stx_2 and/or the stx_{2c} genotype but not with the stx_{2d} genotype (8, 14). In the present study, it was interesting that the stx_2 and/or stx_{2c} genotype occurred only among the O157:H7 strains isolated from humans with infections, including patients with severe and bloody diarrhea, and from environmental sources. On the other hand, all the other STEC strains carried stx_1 or stx_1 stx_2; and although most of them were isolated from uncomplicated diarrheal cases, one O26:H11 strain and one O103:H2 strain harboring stx_1 were isolated from patients with HUS and hemolytic anemia, respectively.

Although we have not tested all the different intimin types and subtypes described in the literature (5, 7, 30), a close relationship between the intimin type, serotype, and diarrheagenic group of *E. coli* could be observed in the present study. As has also been reported by other investigators, we showed that strains belonging to serogroup O26 were associated with intimin β, irrespective of its group (7, 30, 37). Interestingly, intimin type γ related to O157:H7 STEC strains was also iden-

tified in one non-STEC strain belonging to serotype O111:H$^-$. Although intimin α has been described in a limited number of *E. coli* strains belonging to classical EPEC serotypes O55:H6, O127:H$^-$, O127:H6, O157:H$^-$, and O157:H45 (30, 37), in the present study we identified intimin α in two atypical EPEC strains (O111:H$^-$ and O111:HNT).

In conclusion, PFGE patterns showed that substantial genetic heterogeneity exists among O157:H7 and non-O157:H7 STEC strains isolated in São Paulo, Brazil, suggesting the establishment of distinct clones over time in our setting. In contrast to the O111 and O157 strains, whose serotypes and virulence factors are associated with diarrheagenic groups, nondistinct features other than *stx* genes will distinguish STEC and non-STEC O26 strains.

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