Characterization and Epidemiologic Subtyping of Shiga Toxin–Producing *Escherichia coli* Strains Isolated from Hemolytic Uremic Syndrome and Diarrhea Cases in Argentina

M. RIVAS,1 E. MILIWEBsky,1 I. CHINEN,1 C.D. ROLDÁN,2 L. BALBI,3 B. GARCÍA,3 G. FIORILLI,2 S. SOSA-ESTANI,4 J. KINCAID,5 J. RANGEL,5 P.M. GRIFFIN,5 and the Case-Control Study Group6

**ABSTRACT**

Argentina has a high incidence of hemolytic uremic syndrome (HUS); 12.2 cases per 100,000 children younger than 5 years old were reported in 2002. Shiga toxin (Stx)–producing *Escherichia coli* (STEC) is the primary etiologic agent of HUS, and STEC O157 is the predominant serogroup isolated. The main objective of the present work was to establish the phenotypic and genotypic characteristics of the STEC strains in general isolated from Argentine children during a prospective study and the clonal relatedness of STEC O157:H7 strains using subtyping techniques. One hundred and three STEC strains isolated from 99 children were included. The phenotypic and genotypic features were established, and a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was performed to determine stx2 variants. The clonal relatedness of *E. coli* O157 isolates was established by phage typing and pulsed-field gel electrophoresis (PFGE). The 103 STEC strains belonged to 18 different serotypes, and 59% were of serotype O157:H7. Stx2 was identified in 90.3%, and stx1 in 9.7%. Among the 61 STEC O157 strains, 93.4% harbored the stx2/stx2vh-a genes; PT4 (39.3%) and PT2 (29.5%) were the predominant phage types. Using PFGE with the enzyme *Xba*I, a total of 41 patterns with at least 80% similarity were identified, and seven clusters with identical profiles were established. Some of the clusters were further split by PFGE using *Bla*I as the second enzyme. Isolates with indistinguishable PFGE patterns were with one exception also indistinguishable by phage typing and stx genotyping. These findings confirmed that some isolates were genetically related. However, no epidemiological linkages were identified. STEC strains with different genotypes and belonging to diverse serotypes were isolated in Argentina. Some STEC O157 strains could not be distinguished by applying subtyping techniques such as PFGE and phage typing.

1Servicio Fisiopatología, Instituto Nacional de Enfermedades Infecciosas (INEI)–ANLIS “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina.
2Hospital Nacional de Pediatría “Prof. Dr. Juan P. Garrahan,” Buenos Aires, Argentina.
3Hospital Pediátrico “Dr. Humberto Notti,” Mendoza, Argentina.
4Centro Nacional de Endemopatías (CeNDIE)–ANLIS “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina.
5Foodborne and Diarrheal Disease Branch, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.
6Case-Control Study Group: G. Chillemi, A. Baschker, E. Manfredi, INEI–ANLIS “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina; M.G. Caletti, D. Amoedo, S. Martín, Hospital Nacional de Pediatria “Prof. Dr. Juan P. Garrahan,” Buenos Aires, Argentina; P. Valls, P. Lo Giudice, S. Pesle, I. Principi, M. Peralta, Hospital Pediátrico “Dr. Humberto Notti,” Mendoza, Argentina; M.C. Marsano de Mollar, Departamento de Epidemiología, Ministerio de Desarrollo Social y Salud, Mendoza, Argentina; R.M. Hoekstra, Biostatistics and Information Management Branch, CDC, Atlanta, Georgia.
Molecular Epidemiology of E. coli O157:H7

Introduction

Shiga toxin (Stx)-producing Escherichia coli (STEC) cause uncomplicated diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Griffin and Tauxe, 1991). HUS, a life-threatening complication that occurs in 5–10% of patients, is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure (Boyce et al., 1995; Slutsker et al., 1998). The dominant STEC serotype is O157:H7; it is the serotype most commonly involved in large outbreaks (Paton and Paton, 1998).

In Argentina, where HUS is endemic, approximately 350 cases are reported annually. The estimated annual rate was 10.4 and 12.2 cases per 100,000 children under 5 years old in 2001 and 2002, respectively (Rivas et al., 2003; Ministry of Health, Argentina, 2003, data unpublished), a rate that is 10 times higher than in other industrialized countries (Mead and Griffin, 1998). More than 7,000 cases of HUS have been reported since 1965 (Comité de Neftrología, 1995). Recently, evidence of STEC infection was found in 59% of Argentine HUS cases, and O157 was the predominant serogroup isolated (Rivas et al., 1998; Miliwebsky et al., 1999).

The ability of STEC strains to cause severe disease in humans is related to their capacity to secrete Stx1, Stx2, and their variant toxins (Pradel et al., 2001; Beutin et al., 2004), encoded by lysogenic bacteriophages. Another virulence factor of STEC is a 94-kDa outer-membrane protein, called intimin. It is encoded by the gene, eae, on a 34-kb chromosomal pathogenicity island, termed the “locus of enterocyte effacement” (LEE). This locus is associated with intimate adherence to epithelial cells, initiation of host signal transduction pathways, and formation of attaching-and-effacing intestinal lesions (McDaniel et al., 1997).

Some STEC strains produce enterohemolysin (EHEC-Hly), encoded by a large plasmid-borne (90-kb) ehxA gene, which has been associated with severe clinical disease in humans (Schmidt et al., 1995).

Cattle appear to be the main reservoir of STEC strains (Armstrong et al., 1996; Parma et al., 2000; Cobbold and Desmarchelier, 2001), which were recovered from fecal samples of 39% of healthy animals in a recent Argentine survey (Meichtri et al., 2004). STEC is transmitted to humans through contaminated foods (WHO, 1997), water (Swedlow et al., 1992; Keene et al., 1994; Friedman et al. 1999), and direct contact with infected persons (Belongia et al., 1993) or animals (Renwick et al., 1993; Crump et al., 2002).

Genetic fingerprinting is used to establish relatedness of E. coli O157 isolates. Pulsed-field gel electrophoresis (PFGE) is the “gold standard” of genetic fingerprinting methods for E. coli O157 and has frequently been used in epidemiological investigations of outbreaks or sporadic cases in order to establish relatedness between human clinical isolates and isolates from suspect foods or animals (Bell et al., 1994; CDC, 1997). PFGE forms the basis for national and international molecular subtyping networks for foodborne disease surveillance such as PulseNet (Swaminathan et al., 2001).

The aim of this study was to characterize the STEC strains isolated from Argentine children during a prospective study, and to estimate the genetic relatedness and molecular epidemiology of the strains.

Materials and Methods

Bacterial strains

One hundred and three STEC strains were isolated from 99 children included in a prospective case-control study conducted in Argentina to evaluate risk factors for sporadic STEC infection. Patients were enrolled from January 2001 through December 2002 in the public tertiary care pediatric hospitals, Hospital “Dr. Humberto Notti” in Mendoza, serving an urban and semi-rural area, and Hospital “Prof. Dr. Juan P. Garran” in Buenos Aires City, serving an urban area. STEC were sought in stool samples of all children with diarrhea or HUS; all strains isolated were included in the study.

Phenotypic and genotypic characteristics of isolates

The presence of stx1, stx2, and rfbO157 was identified by a multiplex polymerase chain re-
RFLP assay, using the primers and conditions described by Ramachandran et al. (2003). Phage typing was performed by the method originally described by Ahmed et al. (1987) and extended by Khakhria et al. (1990). The E. coli O157:H7 typing phages used were provided by R. Ahmed, of the National Microbiology Laboratory, Canadian Centre for Human and Animal Health, Winnipeg, Manitoba, Canada.

Macrorestriction fragment analysis by PFGE was performed using the 24-h PulseNet standardized PFGE protocol for E. coli O157:H7 (CDC, 2004) with minor modifications. Restriction digestion of DNA embedded in plugs was carried out with 25 U of XbaI (Promega Corp., Madison, WI) at 37°C overnight. BlnI (Amersham Biosciences Corp., Piscataway, NJ) was used as a second enzyme when it was required, with 30 U at 37°C overnight. The PulseNet size standard strain used was E. coli O157:H7 G5244 (provided by CDC). DNA fragments were resolved in 1% pulsed-field certified agarose (Bio-Rad Laboratories, Hercules, CA) in 0.5 M Tris borate EDTA electrophoresis buffer at 14°C. PFGE was performed using CHEF DR-III system (Bio-Rad Laboratories), using a linear pulse ramp of 2.2–54.2 sec with a run length of 18 h and a constant voltage of 200 V. Analysis of the TIFF images was carried out by the BioNumerics software package (Applied Maths, Belgium) using the Dice coefficient and UPGMA to generate dendrograms with 1.5% tolerance values. Clusters were confirmed visually.

RESULTS

Among the 99 children included, 13 had HUS, 76 had bloody diarrhea (BD), and 10 had non-bloody diarrhea (NBD). Fourteen BD cases developed HUS during follow-up. Among the 96 patients with a single STEC isolated, 58 (60%) were O157:H7, and 38 (40%) were non-O157. Three patients had E. coli O157:H7 plus one or more other STEC isolated: O26:H11 in one, O145:NM in one, and all three serotypes in another.

Characterization of isolates

The 103 STEC isolates were of 18 different serotypes, comprising 16 O serogroups and eight

Subtyping of isolates

Genotyping of stx2 variants was done by a restriction fragment length polymorphism (RFLP) analysis of the B-subunit-encoding DNA fragments obtained by PCR (Tyler et al., 1991). The reference E. coli strains 92-3580 O157:H7 (stx2v-a) and 93-016 O113:H21 (stx2v-b) were kindly provided by Dr. D. Woodward, National Microbiology Laboratory, Canadian Centre for Human and Animal Health (Winnipeg, Canada).

Subtyping of eae was performed by a PCR–RFLP assay, using the primers and conditions described by Ramachandran et al. (2003).
Molecular Epidemiology of E. coli O157:H7

H serogroups. Seventeen strains were non-motile. The serotype frequency was O157:H7 (59.2%), O145:NM (12.6%), O26:H11 (5.8%), O113:H21 (3.9%), O174:H21 (2.9%), O8:H19 (1.9%), O145:H25 (1.9%), ONT:NM (1.9%), and one each (0.9%) of O2:H11, O15:H27, O25:NM, O58:H40, O91:H7, O103:H2, O103:H25, O111:NM, O121:H19, and O171:H2. Genes for Stx2 were identified in 93 (90.3%) STEC strains, and Stx1 genes in 10 (9.7%).

All 61 STEC O157 strains had genes encoding the intimin gene and enterohemolysin. Among the 42 non-O157 STEC strains, 29 (69%) had genes encoding intimin and enterohemolysin, five (11.9%) had the genes for only enterohemolysin, and eight (19.0%) had none of the genes for the accessory virulence markers. The ehxA sequence was identified in all the STEC strains studied that presented the enterohemolytic phenotype. The associated syndromes and the characteristics of the 103 STEC isolates studied are summarized in Table 1.

Ninety-seven (94.2%) STEC strains were susceptible to all antimicrobial agents assayed. Three STEC O157:H7 strains were resistant to ampicillin, one to tetracycline, and another to tetracycline and trimethoprim-sulfamethoxazole. One O103:H2 strain was resistant to streptomycin and tetracycline.

Typing of STEC isolates

Among the 61 STEC O157 strains, the PCR-RFLP genotyping method showed that 57 (93.4%) harbored the stx2 and stx2vh-a, three (4.9%) carried only stx2, and one had only a stx2vh-a sequence. Among the 42 non-O157 STEC strains, five different stx genotypes were identified. The stx2 genotype was the most common (50%), followed by stx1 (23.8%), stx2vh-b (16.7%), stx2 and stx2vh-a (7.1%), and

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Syndromes</th>
<th>No. of strains</th>
<th>stx genotype</th>
<th>Virulence markers</th>
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<tbody>
<tr>
<td>O157:H7</td>
<td>HUS(^a)</td>
<td>9</td>
<td>stx2/stx2vh-a (9)</td>
<td>Int-(\gamma), ehxA (9)</td>
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<td>BD(^b)</td>
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<td>stx2/stx2vh-a (46), stx2 (3), stx2vh-a (1)</td>
<td>Int-(\gamma), ehxA (50)</td>
</tr>
<tr>
<td>O145:NM</td>
<td>HUS(^a)</td>
<td>4</td>
<td>stx2 (4)</td>
<td>Int-(\gamma), ehxA (4)</td>
</tr>
<tr>
<td></td>
<td>BD(^c)</td>
<td>9</td>
<td>stx2 (9)</td>
<td>Int-(\gamma), ehxA (9)</td>
</tr>
<tr>
<td>O26:H11</td>
<td>BD(^c)</td>
<td>4</td>
<td>stx1 (3), stx2/stx2vh-a (1)</td>
<td>Int-(\beta), ehxA (4)</td>
</tr>
<tr>
<td></td>
<td>NBD(^d)</td>
<td>2</td>
<td>stx1 (2)</td>
<td>Int-(\beta), ehxA (2)</td>
</tr>
<tr>
<td>O113:H21</td>
<td>BD</td>
<td>2</td>
<td>stx2vh-b (2)</td>
<td>ehxA (2)</td>
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<td>2</td>
<td>stx2vh-b (2)</td>
<td>ehxA (2)</td>
</tr>
<tr>
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<td>stx2vh-b (1)</td>
<td>ehxA (2)</td>
</tr>
<tr>
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<td>2</td>
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<td>ehxA (2)</td>
</tr>
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<td>2</td>
<td>stx2 (2)</td>
<td>ehxA (2)</td>
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<td>ehxA (2)</td>
</tr>
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<td>stx1</td>
<td>ehxA (2)</td>
</tr>
<tr>
<td>O15:H27</td>
<td>BD</td>
<td>1</td>
<td>stx2vh-b</td>
<td>ehxA (2)</td>
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<td>1</td>
<td>stx2</td>
<td>ehxA (2)</td>
</tr>
<tr>
<td>O58:H40</td>
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<td>1</td>
<td>stx1</td>
<td>ehxA (2)</td>
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<tr>
<td>O91:H7</td>
<td>NBD</td>
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<td>ehxA (2)</td>
</tr>
<tr>
<td>O103:H2</td>
<td>BD</td>
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<td>stx1</td>
<td>ehxA (2)</td>
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<tr>
<td>O103:H25</td>
<td>HUS</td>
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<td>ehxA (2)</td>
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<td>1</td>
<td>stx2vh-a</td>
<td>ehxA (2)</td>
</tr>
<tr>
<td>ONT:NM</td>
<td>BD</td>
<td>2</td>
<td>stx2 (2)</td>
<td>ehxA (2)</td>
</tr>
</tbody>
</table>

\(^a\)One HUS case associated with mixed O145:NM and O157:H7 infection.
\(^b\)Thirteen BD cases associated with O157:H7 infection developed HUS during follow-up.
\(^c\)One BD case associated with a mixed O26:H11, O145:NM and O157:H7 infection developed HUS during follow-up.
\(^d\)One NBD case associated with a mixed O26:H11 and O157:H7 infection.

HUS, hemolytic uremic syndrome; BD, bloody diarrhea; NBD, non-bloody diarrhea; STEC, Shiga toxin–producing Escherichia coli.
only stx2vh-a (2.4%). The stx-PCR results were in agreement with the data obtained with the Vero cell cytotoxicity assay.

The intimin subtypes β (serotypes O2:H11, O26:H11, O145:H25, ONT:NM), γ (O145:NM, O157:H7), ε (O25:NM, O103:H2, O121:H19), and θ (O103:H25, O111:NM) were identified.

The phage types most frequently identified among the STEC O157 strains were PT4 (24, 39.3%) and PT2 (18, 29.5%), followed by PT49 (11, 18%), PT14 (three, 4.9%), PT47 (two, 3.3%), and PT8, PT32, and PT54 (one strain each, 1.6%).

By XbaI-PFGE, a total of 41 different patterns were identified among the 61 E. coli O157 strains within at least 80% similarity. Twenty-one patterns were identified among 32 strains isolated in Buenos Aires, and 25 patterns were identified among 29 strains isolated in Mendoza. Five XbaI-PFGE patterns—AREXHX01-0011, AREXHX01-0022, AREXHX01-0045, AREXHX01-0057, and AREXHX01-0102—were identified in both regions during the 2 years of this study.

Seven clusters of isolates with indistinguishable XbaI profiles were found: cluster I (10 strains), cluster II (two strains), cluster III (three strains), cluster IV (two strains), cluster V (four strains), cluster VI (four strains), and cluster VII (two strains). Some clusters were split by PFGE using the second enzyme BlnI. Strains that were indistinguishable by PFGE using two enzymes were also indistinguishable by phage typing and stx genotyping with the exception of two isolates in cluster I (Table 2).

In cluster I, all 10 strains belonged to PT4; 9 carried stx2 and stx2vh-a, and one carried only stx2. In Buenos Aires, two strains with identical BlnI (AREXHA26-0003) patterns were isolated from BD cases in April 2002; two strains isolated from HUS and BD cases in February 2001, and another recovered from a BD case in February 2002, presented the same AREXHA26-0004 pattern. In Mendoza, two strains with identical BlnI (AREXHA26-0002) patterns were isolated from BD cases over a 14-month interval. The three remaining strains of this cluster differed in the BlnI-PFGE pattern, the stx genotype, or the antibiotic susceptibility pattern.

The two strains in cluster II, isolated from an HUS case in Buenos Aires in 2001, and from a BD case in Mendoza in 2002, showed the same PT2 but differed in BlnI-PFGE pattern.

The two strains grouped in cluster IV belonged to pattern AREXHA26-0006, were of PT2, and carried the stx2 and stx2vh-a. They were isolated in Mendoza from BD and NBD cases during January and May 2002, respectively.

### Table 2. Discrimination of Escherichia coli O157 Strains Within XbaI-PFGE Cluster by Other Subtyping Techniques

<table>
<thead>
<tr>
<th>Cluster/no. of XbaI-PFGE</th>
<th>No. of strains</th>
<th>BlnI-PFGE pattern no.</th>
<th>Phage type</th>
<th>stx genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (AREXHX01-0011)</td>
<td>1</td>
<td>AREXHA26-0001</td>
<td>4</td>
<td>stx2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>AREXHA26-0001</td>
<td>4</td>
<td>stx2/stx2vh-a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>AREXHA26-0002</td>
<td>4</td>
<td>stx2/stx2vh-a</td>
</tr>
<tr>
<td></td>
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<td>AREXHA26-0003</td>
<td>4</td>
<td>stx2/stx2vh-a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>AREXHA26-0004</td>
<td>4</td>
<td>stx2/stx2vh-a</td>
</tr>
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<td>1</td>
<td>AREXHA26-0005</td>
<td>4</td>
<td>stx2/stx2vh-a</td>
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<tr>
<td>II (AREXHX01-0102)</td>
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<td>AREXHA26-0006</td>
<td>2</td>
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<tr>
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<td>1</td>
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<td>2</td>
<td>stx2/stx2vh-a</td>
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<tr>
<td>III (AREXHX01-0057)</td>
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<td>AREXHA26-0001</td>
<td>4</td>
<td>stx2</td>
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<td>AREXHA26-0007</td>
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<td></td>
<td>1</td>
<td>AREXHA26-0011</td>
<td>14</td>
<td>stx2/stx2vh-a</td>
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<tr>
<td>IV (AREXHX01-0076)</td>
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<td>AREXHA26-0006</td>
<td>2</td>
<td>stx2/stx2vh-a</td>
</tr>
<tr>
<td>V (AREXHX01-0045)</td>
<td>4</td>
<td>AREXHA26-0006</td>
<td>2</td>
<td>stx2/stx2vh-a</td>
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<tr>
<td>VI (AREXHX01-0022)</td>
<td>3</td>
<td>AREXHA26-0008</td>
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<td>stx2/stx2vh-a</td>
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<td>VII (AREXHX01-0195)</td>
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<td>2</td>
<td>stx2/stx2vh-a</td>
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<td>1</td>
<td>AREXHA26-0008</td>
<td>49</td>
<td>stx2/stx2vh-a</td>
</tr>
</tbody>
</table>

*Two strains resistant to ampicillin.
The four strains included in cluster V, all with PFGE pattern AREXHA26-0006, carried stx2 and stx2vh-a, and were PT2. They were isolated from HUS and BD cases in both Mendoza and Buenos Aires. One strain was ampicillin resistant.

Three strains in cluster VI, all with the AREXHA26-0008 pattern, stx2 and stx2vh-a genotype and PT49, were isolated from BD cases in January 2001, and January and February 2002 in Buenos Aires. Another strain of this cluster, isolated also from a BD case in March 2002 in Mendoza, showed a different BlnI-PFGE, AREXHA26-0010, which belonged to PT14.

The strains included in cluster III and in cluster VII could be differentiated by BlnI-PFGE and phage typing.

The remaining 34 E. coli O157 strains yielded unique XbaI-PFGE patterns.

**DISCUSSION**

One hundred and three STEC strains were isolated from 99 children included in the case-control study. STEC O157:H7, a Stx2 and Stx2vh-a producer, harboring eae (Int-γ) and ehxA, was the most common serotype, isolated from 62% of the patients. STEC O157:H7 is estimated to cause >60% (Miliwebsky et al., 1999) and >80% (Reller and Griffin, 2004) of cases of post-diarrheal HUS in Argentina and the United States, respectively. Moreover, most patients with HUS in Canada, the United Kingdom, Germany, and in central Europe are also infected with E. coli O157 (Reller and Griffin, 2004). In Chile, E. coli serogroup O157 that expressed both Stx1 and Stx2 has been reported to be the pathogen most frequently isolated from children with HUS (Ríos et al., 1999). None of the isolates in the current study contained both Stx1 and Stx2. The prevalence of Stx2-producing STEC O157 strains was similar to the situation observed in other countries (van de Kar et al., 1996; Werber et al., 2003).

Stx2 was identified in 90.3% of the 103 STEC strains. Recently, Boerlin et al. (1999) demonstrated an interaction between eae and stx2 genes. Moreover, they observed a strong statistical association between the presence of the stx2 genotype and the severity of human disease, including the development of HUS and bloody diarrhea. Our findings supported the hypothesis of synergism between the adhesin intimin and Stx2 gene because all STEC strains isolated from HUS cases were eae-positive and Stx2 producers.

PFGE was used to genetically subtype patient isolates, which demonstrated great diversity because 41 different PFGE patterns occurred among the 61 STEC O157 strains. Seven clusters with identical XbaI-PFGE patterns were identified and some isolates were also indistinguishable when tested with a second enzyme (BlnI). However, no obvious epidemiological linkages could be demonstrated between strains.

The three XbaI-PFGE patterns identified most frequently—AREXHX01-0011, AREXHX01-0022, and AREXHX01-0045—comprised 30% of the E. coli O157 isolates. These PFGE patterns are common patterns within the Argentine database and have been detected by our laboratory in different regions of Argentina for more than 10 years.

In general, there was a good correlation between the phage typing and PFGE results, but PFGE had a higher discriminatory power. All strains in PFGE cluster I (AREXHX01-0011) belonged to PT4; all strains in clusters II (AREXHX01-0102), IV (AREXHX01-0076), and V (AREXHX01-0045) were of PT2. Moreover, all strains in PFGE cluster VI (AREXHX01-0022) were of PT49. However, the PFGE clusters III (AREXHX01-0057) and VII (AREXHX01-0195) contained isolates of more than one PT, similar to findings reported by Krause et al. (1996).

PFGE has been used successfully in many investigations of foodborne illness, where the evidence implicating a food or other exposure has been considerably strengthened by finding indistinguishable PFGE patterns between the clinical and food or environmental isolates. A sporadic HUS case in Buenos Aires City was linked to the consumption of home-prepared hamburger contaminated with E. coli O157:H7. Patient and meat isolates harbored stx2 and stx2vh-a, eae, and ehxA, belonged to PT4, and showed the same XbaI (AREXHX01-0011) and BlnI-PFGE patterns (AREXHA26-0003) (Rivas et al., 2003).
A cluster of STEC O157:H7 strains (pattern AREXHX01-0011) was indistinguishable from a common pattern within the U.S. national database (pattern EXHX01.0047). This is the second most common pattern in the U.S. database, representing approximately 4.6% of the database. The most recent multi-state outbreak with this pattern occurred in 2003, and was linked to beef; investigation of this outbreak resulted in a recall of beef (Laine et al., 2005). Of patterns of *E. coli* O157 isolates from meat submitted to the PulseNet database by the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA-FSIS), this pattern makes up 5% and is also the second most common pattern in food isolates. STEC O157 strains with this pattern have been isolated from ground beef, steak, and cattle.

**CONCLUSION**

This study shows that STEC strains with different genotypes and belonging to diverse serotypes were isolated in Argentina. Moreover, among the 61 *E. coli* O157 isolates, some could not be distinguished by PFGE typing alone or with phage typing. These may be clonal groups. As in other countries, epidemiologic evaluation of PFGE clusters in Argentina may detect multi-location, diffuse outbreaks. Good-quality epidemiological investigations are needed to derive the public health benefit of genetic fingerprinting.

**REFERENCES**


Address reprint requests to:
Dr. M. Rivas
Servicio Fisiopatogenia
Instituto Nacional de Enfermedades Infecciosas–ANLIS “Dr. Carlos G. Malbrán”
Av. Vélez Sarsfield 563
(1281) Buenos Aires, Argentina

E-mail: mrivas@anlis.gov.ar