Perspectives on Shiga-like Toxin Infections in Argentina

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ABSTRACT

Argentina has the highest frequency of hemolytic uremic syndrome (HUS) in the world (300 cases/year). The risk of HUS in children from 6 to 48 months old is approximately 22/100,000 in Buenos Aires. In Argentina, HUS is the most frequent cause of acute renal damage and the second cause of chronic renal injury in children. We have shown that during the spring/summer season, the incidence of Shiga-like toxin (SLT)–associated bloody diarrhea in children less than 5 years old is 30 to 39%. The risk of HUS in SLT-associated bloody diarrhea is about 4 to 5%; 14% of children with SLT diarrhea developed incomplete HUS. Household contacts of children with HUS are commonly colonized with SLT-producing Escherichia coli (39%), and seroconversion occurs in 42% of these. No evidence of free fecal SLTs was observed in healthy children.

In Argentina E. coli serotype O157:H7 has been associated with only 2 to 18% of HUS patients and in 4.5 to 7% of children with bloody diarrhea. Other serotypes were also recognized. About 20% of Argentine children start to eat meat at 5 months old, and 80% of them have meat in their diets at least three times a week. Eighty percent of the meat consumed is undercooked. Few data about the incidence of SLT-producing E. coli in cows in our country are available. E. coli O157:H7 was isolated in only 7.7% of calves aged 1 to 3 weeks with E. coli bacillosis from different farms in Argentina. Preliminary data show that SLT-producing E. coli were also present in stools from healthy animals and in fresh retail ground beef, determined by polymerase chain reaction.

Key words: Shiga-like toxins, hemolytic uremic syndrome, disease-reservoirs

Argentina has the highest frequency of hemolytic uremic syndrome (HUS) in the world (approximately 250–300 cases/year) (13, 14, 17). The risk of HUS in children from 6 to 48 months old is approximately 22/100,000 in Buenos Aires. On the basis of census data and cases diagnosed in Buenos Aires, the incidence of the disease appears to be 7 to 10 times greater than reported from "high-risk" areas in the world (13, 17, 23) (Figure 1). Ninety-two percent of patients with HUS have a history of diarrhea, usually grossly bloody stools (85%), in the preceding 2 weeks.

Because of this exceptionally high frequency of HUS in Argentina, we sought to define prospectively the role of verotoxins or Shiga-like toxins (SLTs) in patients with HUS and diarrhea patients during spring/summer seasons. To determine probable sources of (EHEC) enterohemorrhagic E. coli infections in humans, we decided to study the presence of SLT-producing E. coli in bovines.

METHODS USED FOR DIAGNOSIS OF EHEC INFECTIONS

We used the same procedures for the identification of SLTs or verotoxin-producing E. coli in human samples and in bovine and retail meat samples.

Bacteriological procedures

Stool specimens were cultured as early in the course of disease as possible. A clinical microbiology laboratory (Research Laboratory of Infectious Diseases, Unit 9, Hospital de Niños “Ricardo Gutiérrez,” Buenos Aires, Argentina) screened for E. coli strains on MacConkey–sorbitol medium for detection of nonsorbitol fermenters (25). Ten to 20 E. coli strains were saved from each sample to perform the latex agglutination test for E. coli O157:H7, DNA hybridization, and polymerase chain reaction (PCR). SLT-producing E. coli were sent to a reference laboratory (Escherichia coli Reference Center, Pennsylvania State University, USA.) for serotyping.

Shiga-like toxins were detected with either of two methods.
1. Fecal cytotoxin assay. This was performed using a modification of a method previously described (2, 5). Aliquots of fecal samples were mixed with nonimmune rabbit serum, rabbit antiserum to Shiga toxin, or a monoclonal antibody to SLT-II B subunit (BC5BB12) and 4 h at 37°C. Verotoxin cells in [H]thymidine medium were added to the fecal extract–antibody mixture. After 18 to 22 h of incubation at 37°C, the cells were lysed and the thymidine incorporated counted in a β-scintillation counter. Neutralization was determined by comparing the percentage of surviving cells with normal rabbit serum to the percentage surviving with specific antibodies. A sample was interpreted as positive for SLT-I or SLT-II if there was a significant difference between cell survival with toxin alone versus with toxin plus antibody.
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2. SLT receptor enzyme-linked immunosorbent assay (ELISA). A rapid method to detect SLTs was developed based on specific binding of them to their natural receptor, Gb3 (12, 15, 19, 20, 37), which was coated onto microdilution plates (1). Bound toxin was then detected by ELISA with monoclonal antibodies anti-SLT-I and SLT-II. The sensitivity of the Gb3 ELISA was 0.2 ng (2 ng/ml) of purified toxin. The assay was very specific in that no cross-reactivity was noted with purified cholera toxin, E. coli heat-labile and heat-stable enterotoxins, and Clostridium difficile cytotoxin, or sonicate extracts of other cytotoxin-producing organisms such as other shigellae, pathogenic and nonpathogenic E. coli, Salmonella spp., Campylobacter spp., and Aeromonas spp.

To detect SLT genes in E. coli isolates,

1. DNA hybridization assay was performed with colony lysates as previously described using digoxigenin oligonucleotide probes synthesized by (PCR) (4, 18, 26, 27). Nitrocellulose filters with colony lysates were hybridized for 12 to 15 h at 65 to 68°C with 50 ng/ml of specific digoxigenin probe for SLT-I or SLT-II. The final washing step was made in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) (high stringency). Positive colonies were detected using Fab fragment antidigoxigenin monoclonal antibody conjugated with alkaline phosphatase and the corresponding substrates according to the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN) (26).

2. Polymerase chain reaction was performed on DNA of E. coli strains isolated from patients' stool samples. The PCR primers were designed to flank the region of the A subunit of SLT genes containing the codon corresponding to glutamic acid 166 in SLT-I and aminoacid 167 in SLT-II (6, 16, 33, 38). Thirty-five cycles of 1 min at 94°C, 40 sec at 60°C, and 40 sec at 62°C were used for amplification of the SLT genes. Products of the samples and positive and negative controls as well as a molecular mass marker were analyzed by electrophoresis in agarose gels containing ethidium bromide and were photographed under ultraviolet illumination (4).

Another useful diagnostic aid was the detection of antibodies to SLT-I and SLT-II, serum antibodies studies. To determine the presence of serum antibodies to SLT-I and SLT-II, neutralization assays were performed in HUS patients, patients with bloody diarrhea, and healthy children. Serial dilutions of patient serum samples were mixed with 2 to 10 median cytotoxic doses (CD50) of purified Shiga toxin or a sonicate of E. coli C 600 (933 w).

Appropriate positive (purified Shiga toxin or E. coli 933 w sonicate with antibodies to SLT-I or SLT-II) and negative (medium) controls were included. The end point for neutralization was the last dilution of serum that significantly increased cells' survival compared with that of toxin without serum. Fifty-four percent of HUS patients and 27% of bloody diarrhea patients had seroconversion. Twenty percent of healthy children had low titers of antibodies to SLT-I and or SLT-II in their serum samples (23).

SPECTRUM OF EHEC DISEASE IN HUMANS

Hemolytic uremic syndrome

During a period of 10 years, from 1982 to 1991, 528 patients were treated for HUS in Hospital de Niños “Ricardo Gutiérrez,” a pediatric reference center of Buenos Aires. The mean age of the patients was 20.4 months (range, 3 to 84 mo); 54.1% of them were female. Although HUS occurs throughout the year, there has been a striking variation in seasonal onset, with the numbers beginning to increase in spring and remaining high until the middle of fall (24).

Classically HUS consists of microangiopathic hemolytic anemia with thrombocytopenia and acute renal injury (11). Oliguria is present in nearly all HUS patients, and the duration of anuria is a mean of 8.5 days (maximum 28 days). Proteinuria and hematuria, usually microscopic, are present in all our patients. Azotemia is detected on admission (mean BUN 278 ± 103 mg/dl). Heart failure and hypertension commonly occur (24).

Central nervous system findings such as irritability, tremor, or ataxia are present in all our patients. Other important but less common findings include seizures (15 to 35%), stupor (20%), and decerebrate rigidity, retinal hemorrhages, or coma (11%) (24).

The main sequelae of HUS are renal and neurological. HUS is the most frequent cause of acute renal damage in Argentina and the second cause of chronic renal injury in children. Fifty percent of patients remain with important renal sequelae: one third of them with proteinuria, 15% with hypertension, and 8 to 10% with chronic renal failure in a 5-year period. Follow-up studies have also demonstrated irreversible neurological sequelae in 7% of patients who survive (35).

In those children with complete sample collections available (stool culture, DNA hybridization, and PCR of isolated E. coli for toxin genes, stool for toxin neutralization assay, and paired serologies for antibodies to SLT-I and/or SLT-II), HUS was associated with SLT-producing E. coli 96% of the time. No causes other than SLT-producing E. coli were found in these patients (23).

SLT-associated diarrhea

Ninety-two percent of the children with HUS have a history of diarrhea in the preceding 3 to 8 days. Prospectives studies in children with watery diarrhea and with grossly bloody diarrhea were carried out during HUS season. One hundred watery diarrhea patients, mean age 14.6 months (±8.7 mo), 46% female, and 254 patients with grossly bloody diarrhea, mean age 18.8 months (±12.5 mo), 45.3% female, were included in these studies. In the watery
diarrhea group, 67% of the patients had regular water supply, 85% ate meat, 63% were breast-fed, and 12% received antibiotics during the diarrhea episode. In the bloody diarrhea group 53.9% had regular water supply, 80.3% ate meat, 16.1% were breast-fed ($P < 0.0001$), and 37.5% received antibiotics. Free verotoxins in the stool specimens or DNA-probe-positive isolates were present in 39% of the 254 patients with bloody diarrhea, and in 21 (21%) patients with watery diarrhea ($P = 0.002$). This very high incidence of SLT-associated enteritis is more than 10-fold greater than in other parts of the world, where these organisms have been sought systematically ($21, 30, 31, 36$) (Figure 2).

### Incomplete HUS

While we were studying the frequency of development of classic HUS in patients with grossly bloody diarrhea, we were struck by the frequent finding of what appeared to be incomplete forms of HUS occuring in these patients. During the course of bloody diarrhea, 14/254 patients had abnormalities typically seen in HUS without fulfilling the definition of classic HUS. Anemia was found in all 14 patients; 11 of them (78.6%) also had thrombocytopenia. Serum haptoglobin levels were decreased during the acute phase of the disease in all of these children. In 6 patients schistocytes were also detected. The first platelet count was $135 \pm 74 \times 10^9/L$ ($\pm SD$), and the second value was $203 \pm 75 \times 10^9/L$ ($P = 0.02$); the first blood sample was obtained 4.2 ± 1.8 days after onset of diarrhea, and the second sample was obtained 13.8 ± 5.8 days after onset. Hematuria or proteinuria with anemia but without azotemia or thrombocytopenia was found in 3 patients. Four of the patients also had transient hypertension ($\geq 2$ SD above the mean for age) during the follow-up period, although no treatment was required. None of the children had isolated thrombocytopenia or isolated renal injury. Eight of 90 patients (8.9%) with SLT bloody diarrhea versus 6/155 (3.9%) with non-SLT bloody diarrhea had incomplete HUS; 9 patients with toxin-associated diarrhea did not return for follow-up of their illness.

It is likely that these complications of toxin-associated diarrhea have the same pathophysiological basis, namely, toxin-induced vascular endothelial injury. Twenty-two percent of the bloody diarrhea patients were treated with antibiotics during the illness episode. No significant difference was observed in frequency of developed HUS or incomplete HUS related to this therapy. However, patients who received cotrimoxazole tended to have HUS and/or incomplete HUS more often than children who did not receive this drug ($P = 0.08$) ($21$).

### Epidemiology

Different EHEC serotypes are described in relation with hemorrhagic colitis and HUS. Although $E. coli$ O157:H7 is the most common serotype isolated in outbreaks in the United States and Canada ($10$), in Argentina it was isolated in only 2 to 18% of the HUS patients and in 4.5 to 7% of SLT-associated bloody diarrhea patients. Other serotypes, such as O111, O15, O2, O1, O25, and O75, are described in our country. Based on these results, it is important to design screening methods not only for the identification of $E. coli$ O157:H7 but for the identification of other EHEC serotypes as well.

### Person-to-person transmission

We conducted a prospective study in 87 household contacts of 51 children with HUS to determine the frequency of infection with SLT-producing bacteria ($22$). Free fecal toxin was detected in 39% of the household members. Free fecal toxin was more frequently detected in the mothers (16/30) and the siblings (5/9) than in the fathers (4/25), $P < 0.05$. The fact that fathers had evidence of infection significantly less often than siblings or mothers of index cases is consistent with person-to-person transmission to those in closest contact with the index case.

Thirty-three percent of the HUS families in which two or more members were studied had more than one household member with free fecal toxin in stool. Serum samples were available in 77 household contacts; 42% of them had evidence of seroconversion. In summary, family contacts of children with HUS in Argentina are often asymptomatically infected with the SLT-producing organisms.

### Healthy children

None of the 103 healthy children included in the prospective studies as controls had free fecal cytotoxin, positive culture for O157:H7, or DNA probe/PCR-positive organisms; 20% of them had low serum titers of antibodies to Shiga-like toxins ($23$). These serum titers would have been produced early in life because of mild or asymptomatic infection with SLT-producing $E. coli$, and they could protect these children from developing HUS in the future.

### Bovine reservoir

EHEC has a bovine reservoir and generally is transmitted by undercooked meat, unpasteurized milk, or food or
water contaminated with bovine feces (3, 7–9, 29, 34, 39). There are few previous data about the incidence of SLT-producing *E. coli* in cattle in our country. *E. coli* O157:H7 was isolated in only 3/39 (7.7%) of strains of calves aged 1 to 3 weeks with *E. coli* bacillosis from different farms in Argentina (28). Preliminary data show that SLT-producing *E. coli* were also present in stools from healthy animals and in fresh retail ground beef, determined by PCR.

Our studies have identified SLT-producing *E. coli* in fecal samples of cattle that are used for milk and meat production. *E. coli* strains were isolated from fecal samples collected from 244 bovines (healthy or with diarrhea) at slaughterhouses or farms for identification of SLT-producing *E. coli*. Eighty bovines were positive for SLT-I and/or SLT-II in PCR assays. Only two of them were non-sorbitol fermenters and none of them (sorbitol positive and negative) were O157 according to the latex agglutination test (32).

Consumption of raw or undercooked ground beef is recognized as a risk factor for EHEC infections. We’ve studied 686 *E. coli* strains isolated from raw ground beef and frozen and homemade hamburgers (2% of bovine meat consumed in Argentina) from different manufacturers and butcher’s shops. They were analyzed by Gb3 ELISA and PCR techniques for SLTs. One hundred and forty-six of 686 (21.3%) *E. coli* strains were positive for SLT-I and/or SLT-II. Only one strain was a sorbitol nonfermenter, and it was confirmed to be *E. coli* O157:H7 by the latex agglutination test (1.6%). These are preliminary data; further studies are ongoing in order to obtain more.

**CONCLUSIONS**

1. Argentina has an exceptionally high frequency of HUS (22/100,000 children less than 5 years old).
2. The high incidence of SLT-associated grossly bloody diarrhea (30 to 39%) presumably explains the unusual frequency of HUS in Argentina.
3. There is a significant difference between the incidence of SLT-associated bloody diarrhea (39%) and SLT-associated watery diarrhea (21%) in Buenos Aires (*P* = 0.02).
4. The risk of HUS in SLT-associated bloody diarrhea is about 4 to 5%, and 14% of children with SLT-diarrhea develop incomplete HUS.
5. Twenty percent of healthy children have low serum titers of antibodies to SLTs, showing probable asymptomatic infections early in their lives.
6. Evidence of SLT-producing *E. coli* in fecal samples of cattle that are used for milk and meat production could explain the role of the animals as a source of EHEC human infections.
7. The incidence of *E. coli* O157:H7 is low in HUS patients (2 to 18%) and bloody diarrhea patients (4.5 to 7%) in Argentina.
8. Perhaps HUS is the tip of “an iceberg” of a broader spectrum of disease, in which SLT-induced injury of the vascular endothelium is probably the central event (Figure 3).

**REFERENCES**


