Shiga Toxin 2-Producing Acinetobacter haemolyticus Associated with a Case of Bloody Diarrhea

Germin Grotiuz,† Alfredo Sirok,† Pilar Gadea, Gustavo Varela, and Felipe Schelotto*

Shiga Toxin-Producing E. coli Reference Laboratory, Department of Bacteriology and Virology, Institute of Hygiene, School of Medicine, Universidad de la República, Av. Dr. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay

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We report the first Shiga toxin 2-producing Acinetobacter haemolyticus strain that was isolated from the feces of a 3-month-old infant with bloody diarrhea. Usual enteropathogenic bacteria were not detected. This finding suggests that any Shiga toxin-producing microorganism capable of colonizing the human gut may have the potential to cause illness.

CASE REPORT

In November 2001, a 3-month-old male infant was admitted at Pereira Rossell Pediatric Hospital with bloody diarrhea of 12 h evolution without fever or other previous pathologies. The patient was treated empirically with intravenous ceftriaxone and hospitalized for 24 h.

Samples of feces were obtained before and after the patient was treated with antibiotics, and they were simultaneously studied at the Microbiology laboratory of the Pereira Rossell Pediatric Hospital and at the Reference laboratory for Shiga toxin-producing Escherichia coli. This last study, done in the context of an institutional program aimed at the regional surveillance of bloody diarrheas and hemolytic-uremic syndrome (HUS) etiology, is the focus of the present article.

The presence of Salmonella, Shigella, enteroinvasive E. coli, and enteropathogenic E. coli, Yersinia enterocolitica, and Campylobacter in fecal samples was studied using standard procedures, as previously described (18). The searching of Shiga toxin-producing E. coli (STEC) was done by selective enrichment protocol (9) on Trypticase soy broth (Bacto, France) and further isolation on MacConkey sorbitol (SMAC) plates (Oxoid, Hampshire, England).

The microscopic analysis of the two fecal samples showed a low number of leukocytes (5 to 10 per microscopic field) and did not reveal spiral bacteria that would suggest the presence of Campylobacter spp.

The cultures from the first fecal sample developed well in all inoculated media. From the second fecal sample, only 12 colonies (all sorbitol negative) were recovered on a directly inoculated SMAC plate after 48 h of culture. From the enrichment broth, we did not recover any microorganism on the SMAC plate.

The microbiological studies did not reveal the presence of usual enteropathogenic bacteria in any of the two samples.

Twenty sorbitol-positive colonies plus a lysate from the confluent zone in the SMAC plate of the first sample (nonfermenting colonies were not recovered) and the 12 sorbitol-negative colonies recovered from the second sample were analyzed by PCR, as previously described (13), to detect the presence of Stx1/Stx2-encoding organisms. This PCR was performed with primers VT1A-F (GAAGAGTCCGTGGGATT ACG) and VT1B-R (AGCGATGCAGCTATTAAATAA) for stx1 and with primers VT2A-F (TTAACCAACCCACGCAG) and VT2B-R (GCTCGGTGATCCTCTGGT) for stx2.

E. coli K-12 C600 E. coli K-12 C600 (F− thi-1 thr-1 leuB6 lacY1 tonA21 supE44 ΔΔΔ Δstx1 Δstx2) and Stx2/Stx1-producing E. coli STEC O157:H7 EDL933 were used as negative and positive controls, respectively, in all PCR assays (10).

Amplified DNA fragments were analyzed through 2% (wt/vol) agarose gel electrophoresis by staining with 0.5 μg/ml ethidium bromide.

The bacterial lysates derived from the first sample were stx1/stx2 negative, but in the second sample, we detected two strains (DS9B1 and DS9B2) carrying stx2-related sequences (presence of a weak band of 346 bp) (Fig. 1).

To obtain a best performance in the detection of the stx2-related sequences, an additional PCR was done using VT2A-F as the forward primer (13) and stxA1INT (TGCTGTCCGTT GTCATGGGA), which was especially designed for this study, as a universal reverse primer, allowing the amplification of an approximately 215-bp fragment of the stxA2 gene. The stxA1INT primer was designed using the consensus sequence derived from a multiple alignment of 54 stx2 homologous sequences (from position 385 to 403 of the stxA2 open reading frame). The reaction showed an intense 215-bp PCR product and confirmed the presence of stx2-related sequences (Fig. 1).

This PCR product was sequenced in the CTAG center (Technical Center for Genetic Analysis, School of Sciences, Montevideo, Uruguay). The sequence was first identified through a search at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and at local databases with the BLASTN search algorithm (1) and then analyzed using the European Molecular Biology Open Software Suite package (14) and CLUSTAL X multiple-sequence align-

* Corresponding author. Mailing address: Shiga Toxin-Producing E. coli Reference Laboratory. Department of Bacteriology and Virology, Institute of Hygiene, Av. Dr. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay. Phone: 598 2 4875795. Fax: 598 2 4873073. E-mail: baevir@higiene.edu.uy.
† G.G. and A.S. contributed equally to the experimental work and to the elaboration of this report.
The plasmid profiles were identical, showing a band of approximately 50 kb in 0.7% agarose gel electrophoresis by staining with ethidium bromide. Remarkably, this DNA molecule coincided with the lambdoid bacteriophage genome size.

The phage propagation was made from exponential cultures of stx2 PCR-positive colonies (optical density at 600 nm [OD600] = 0.5) in Luria-Bertani broth supplemented with 0.01 M MgSO4, 0.01 M CaCl2, and 0.2% maltose, induced with mitomycin C, and incubated at 37°C with shaking (200 rpm) long enough to show the lightening of the culture due to phage bacterial lysis. To precipitate the phage particles, the supernatants were cleaned by centrifugation, filtered (0.22-μm pore size), and supplemented with 2 M NaCl and then with 20% (wt/vol) polyethylene glycol 8000. The mixes were incubated for 1 h at 4°C. The particles were harvested by centrifugation (25,000 × g, 30 min, 4°C) and suspended in magnesium sulfate buffer. These crude stocks enriched with bacteriophages were then dropped onto double-layer plate agar cultures, as previously described (15), resulting in nontransparent, irregular lysis zones with shiny edges after 18 h at 37°C. We used E. coli K-12 K600 as the recipient strain (10).

These phage stocks were stx2 PCR positive. Double-layer agar plating assays were made to isolate individual phage plaques (15) and then obtain high-titer stocks. Approximately 50% of the enriched plaques analyzed via stx2 PCR revealed the presence of the target genes.

After RNase and protease K treatment (Sigma-Aldrich, Germany), the DNA phage genome was purified from stx2 PCR-positive high-titer pure stocks with phenol-chloroform and isopropanol precipitation, as previously described (15). It showed a DNA band of approximately 50 kb in 0.7% agarose gel electrophoresis stained with ethidium bromide. Remarkably, this DNA molecule coincided with the lambdoid bacteriophages genome DNA size.

In view of the similar molecular size of the phage genomic DNA obtained from high-titer phage stocks and the DNA recovered by plasmid extraction from the A. haemolyticus strains, we suspected that both genetic materials could be the
same. We studied by PCR the presence of the stx2-related sequences in these DNA samples, and we found that both types of sample were stx2 PCR positive (Fig. 1). We also observed that the colonies that lost the 50-kb DNA band after subculture became negative for stx2 PCR, for Vero cell cytotoxic assay, and for the presence of stx2-encoding bacteriophages.

*A. haemolyticus* has been described mainly as an environmental bacteria and an opportunistic, multiresistant intrahospital human pathogenic bacteria (7).

A few bacterial species and/or pathogenic clones can produce Shiga toxin (Stx) and have been associated with cases of bloody diarrhea, thrombocytopenic thrombotic purpura, and HUS in humans (12).

Stx belongs to a defined protein subfamily, the RNA N-glycosidases, that directly targets the cell ribosome machinery, inhibiting cell protein synthesis (5). These toxins can be classified into 2 antigenic groups, Stx-1 and Stx-2, that include (especially Stx-2) an important number of genotypic variants (12). In most cases, the Stx coding genes (*stxAB*) are included in the lysis cassette in the context of the lambdoid lysogenic bacteriophage genomic DNA (11).

This is the first report of a Shiga toxin-producing *Acinetobacter haemolyticus* (STAH) strain, and it is also the first report of its association with bloody diarrhea. As the isolated *A. haemolyticus* strain produces Shiga toxin, a relevant virulence factor, and as usual enteric bacterial pathogens associated with bloody diarrhea were not detected in this case, it is possible that this strain was the etiological agent. The production of Shiga toxin is directly implicated in the generation of bloody diarrheas, and the immunological condition of the patient (due to his age) could have made him especially susceptible to the infection. This finding suggests that any Shiga toxin-producing microorganism capable of colonizing the human gut may have the potential to cause human illness and life-threatening human diseases such as HUS. Although we do not detect the presence of intimin and enterohemolysin, we cannot discard the possibility that other virulence factors could be present in the STAH strain.

On the other hand, we think that there is a chance that another Shiga toxin-producing microorganism which we failed to isolate was implicated in the described case and it might be possible that *Acinetobacter haemolyticus* had acquired in vivo the Stx2-producing phenotype via horizontal transfer in the gut lumen of the patient. Extrachromosomal DNA analysis, isolation of stx2-encoding phage and purification of its genome, specially designed PCR tests, and the observed in vitro instability of the stx-related sequences suggested that the Shiga toxin genes of STAHAH are carried in the context of an infective bacteriophage. Nevertheless, to confirm the presence of a phage carrying the stx2 gene, we should make lisogenization assays with the purified bacteriophage to isolate an Stx2-producing recipient strain.

Lysogeny has been proven to occur in vivo (6), and it has important implications for the evolution of new pathogenic strains. Treatment with certain antibiotics can induce the lytic cycle of the lambdoid phage carrying stx genes and can subse-

quently cause the phage infection of formerly non-toxin-produc-
ing *E. coli* and other bacterial species. This may increase the production and release of Shiga toxin in the gut lumen, aggravating the disease. Therefore, we speculate that some conditions inherent to STAHAH strain bacterial fitness (such as tolerance to frequently used antibiotics, particularly cephalosporins) and stx2-encoding phage infection permissiveness may have promoted its survival and the subsequent acquisition in vivo of the Stx2-producing phenotype in the gut lumen of the treated patient.

This might be another argument against indiscriminate treatment of bloody diarrhea, especially in the cases that involve Shiga toxin-producing organisms.

**Nucleotide sequence accession number.** The sequence determined in this study was submitted to the GenBank database under accession number DQ344636.

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