Prevalence of the stx2 Gene in Coliform Populations from Aquatic Environments

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Shiga toxin-producing Escherichia coli strains are human pathogens linked to hemorrhagic colitis and hemolytic uremic syndrome. The major virulence factors of these strains are Shiga toxins Stx1 and Stx2. The majority of the genes coding for these toxins are borne by bacteriophages. Free Stx2-encoding bacteriophages have been found in aquatic environments, but there is limited information about the lysogenic strains and bacteria present in the environment that are susceptible to phage infection. The aim of this work was to study the prevalence and the distribution of the stx2 gene in coliform bacteria in sewage samples of different origins. The presence of the stx2 gene was monitored every 2 weeks over a 1-year period in a municipal sewage treatment plant. A mean value of 10^5 genes/ml was observed without significant variation during the study period. This concentration was of the same order of magnitude in raw municipal sewage of various origins and in animal wastewater from several slaughterhouses. A total of 138 strains carrying the stx2 gene were isolated by colony hybridization. This procedure detected approximately 1 gene-carrying colony per 1,000 fecal coliform colonies in municipal sewage and around 1 gene-carrying colony per 100 fecal coliform colonies in animal wastewaters. Most of the isolates belonged to E. coli serotypes other than E. coli O157, suggesting a low prevalence of strains of this serotype carrying the stx2 gene in the wastewater studied.

Shiga toxin-producing Escherichia coli (STEC) strains are important human pathogens linked to the development of hemorrhagic colitis and hemolytic uremic syndrome worldwide. The major virulence characteristics of STEC strains are the production of Shiga toxins and the ability to colonize the bowel through the adhesin protein intimin, which is responsible for attaching and effacing lesions in the intestinal mucosa (18). However, not all pathogenic STEC strains have been shown to produce intimin, suggesting that other factors could contribute to human disease (30). There are two main groups of Shiga toxins (Stx1 and Stx2) and several Stx2 variants (c, d, e, and f) (2, 19, 35, 44). The majority of stx genes are bacteriophage borne (24, 27, 36, 38, 40), which may be important for the spread of STEC strains. Recent studies reported significant numbers of free stx2-bearing bacteriophages in the environment (24, 25), which may maintain the gene and infect new bacteria. Some studies have already shown that the transduction of stx-bearing bacteriophages to new bacterial hosts occurs in vivo (1) and in vitro (34).

E. coli O157:H7 was the first serotype associated with hemorrhagic colitis (33, 45), although more than 100 STEC serotypes have since been isolated from different sources, including contaminated food (22) and recreational (14) and drinking (21, 28, 39) water.

Usually, the isolation of pathogens from the environment is difficult due to the low proportion of pathogens compared with the generally high microbial concentration. Consequently, the confirmation of a presumptive causative agent involved in an outbreak is not always achieved (5). Moreover, the detection of non-O157 STEC strains is particularly difficult due to the lack of common phenotypic characteristics such as those presented by typical E. coli O157:H7 strains (delayed fermentation of sorbitol and the absence of β-glucuronidase activity). As a result, they cannot be identified with selective culture media such as sorbitol-MacConkey agar supplemented with cefixime and tellurite (46) or Rainbow agar (3), which are recommended for E. coli O157:H7 isolation. Some molecular methods based on the detection of Shiga toxins have been described because these are the unique common features among non-O157 strains (46). However, most of them are expensive and time-consuming and do not allow the isolation of clones. Furthermore, they are not able to detect strains carrying stx genes that do not express the toxin.

Recently, a new molecular method suitable for the screening and isolation of potential Shiga toxin-producing strains has been developed based on the detection of the stx2 gene (4). This method has allowed the detection of stx2 gene-carrying bacteria among coliform bacteria grown on Chromocult coliform agar (Merck, Darmstadt, Germany) by using a specific probe to detect a fragment of 378 bp of the A subunit of the stx2 gene. The advantage of this method is that individual colonies may be isolated, and then the clone can be isolated further characterized; this is important to a better understanding of the ecology of the stx2 gene in bacterial populations.

This study reports the prevalence and the isolation of bacteria carrying the stx2 gene in several samples of raw human sewage from different treatment plants and wastewater from different slaughterhouses by the most probable number (MPN) method combined with a nested PCR and the colony hybridization method (4). Additionally, the effects of secondary and
tertiary treatments on the bacterial indicators (numbers of total coliforms (TC), fecal coliforms (FC), and *E. coli* organisms) in relation to the *stx*2 gene-carrying bacteria were also determined for samples from two sewage treatment plants. The population of *stx*2 gene-carrying bacteria was also monitored over a 1-year period to assess seasonal changes in STEC shedding, which has previously been described for *E. coli* O157:H7 (10). Finally, *stx*2 gene-carrying strains were phenotypically characterized.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *E. coli* O157:H7 strain ATCC 43889, which produces *Stx*2, was used as a positive control. *E. coli* O157:H7 strain ATCC 43888 and *E. coli* C600, which produce neither *Stx*1 nor *Stx*2 and do not possess the genes for these toxins, were used as negative controls. These bacteria were grown on tryptic soy agar (TSA) (Difco, Le Pont de Claix, France) overnight at 37 °C and then stored at -80 °C for 10 min, and immediately transferred to ice-cold absolute ethanol. Samples were then centrifuged at 16,000 × g for 5 min, and 2 μl of the supernatant was used as a template for nested-PCR amplification.

**Detection and quantification of *stx*2 gene-carrying bacteria.** The detection and quantification of *stx*2 gene-carrying bacteria were performed by a combination of the MPN technique and nested PCR and by colony hybridization. The MPN of bacteria carrying the *stx*2 gene was determined after the nested-PCR amplification (performed as described below) of DNA extracted from 10-fold dilutions of each sample. All PCR assays were performed in 25-μl volumes containing 2 mM MgCl2, 10% bovine serum (provided by the manufacturer (Eppendorf, Hamburg, Germany), 200 μM deoxynucleoside triphosphate, 2 U of Taq DNA polymerase (Eppendorf), a 0.3 μM concentration of each primer, and 2 μl of the extracted DNA, and the final volume was adjusted with sterile double-distilled water. External and internal reverse and forward primers used in this study were described previously (24). First, PCR and nested-amplification products of 378 and 169 bp, respectively, were resolved on a 2% agarose gel and stained with ethidium bromide. Nested-PCR amplification products were confirmed by dot blotting using an internal digoxigenin-labeled probe consisting of 26 bp of the A subunit of the *stx*2 gene (24), according to previously established protocols (4). The MPN was calculated by counting the obtained bands corresponding to positive amplification. The sensitivity of this quantification method was calculated by analyzing 10-fold dilutions of a culture of *E. coli* O157:H7 ATCC 43889 grown in tryptic soy broth at 37 °C for 6 h by MPN-PCR method and by plating replicate dilutions on TSA.

The enumeration of bacteria carrying the *stx*2 gene by colony hybridization was performed as follows. Tenfold dilutions of each sample were prepared with Ringer 1/4 Solution (Oxoid, Basingstoke, England). Aliquots of 250 μl were spread onto 140-mm-diameter Chromocult coliform agar plates and incubated at 37 °C for 24 h. The counting of blue and red colonies was performed for the enumeration of TC and *E. coli* organisms, respectively. Later, plates from dilutions presenting heavy but nonconfluent colony growth were selected for colony transfer, which was performed by carefully placing a nylon membrane onto the surface of the agar and quickly peeling it from the plate. Later, the bacterial cells on the membranes were lysed and fixed as described previously (26). The membranes were washed twice with a solution consisting of 0.1% sodium dodecyl sulfate and 3% SSC (20 °C for 15 min) and prehybridized with standard prehybridization solution at 68 °C for 1 h 30 min and prehybridized with standard prehybridization solution at 68 °C for 2 h. The membranes were then hybridized with the specific probe at 65 °C. The external PCR primers for the *stx*2 gene that were described previously (24) were used to prepare the *stx*-specific probe used in the colony hybridization.

The DNA extracted from temperate bacteriophages induced from *stx*2-positive *E. coli* strains was used as the template for the PCR as previously described (4). The 378-bp amplimer obtained was labeled with digoxigenin to be used later as a specific probe according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Labeling was performed by the incorporation of digoxigenin-11-deoxyuridine-triphosphate during the PCR as described previously (23). The specificity of the probe was evaluated by using the collection strains *E. coli* 43889, *E. coli* C600, *E. coli* CN33, and *E. coli* DH5α. The digoxigenin-DNA luminescent detection kit (Roche Diagnostics) was used to detect positive hybridization by placing membranes in contact with X-ray films according to the

### Table 1. Characteristics of the municipal sewage treatment plants and animal slaughterhouses and enumeration of *stx*2 gene-carrying bacteria and bacterial indicators analyzed in this study

<table>
<thead>
<tr>
<th>Plant Characteristics of the sewage of the treatment plant or slaughterhouse</th>
<th>No. of samples</th>
<th><em>stx</em>2 gene-carrying bacteria [log10 (CFU + 1)/ml]</th>
<th>E. coli Organisms [log10 (CFU + 1)/ml]</th>
<th>FC [log10 (CFU + 1)/ml]</th>
<th>TC [log10 (CFU + 1)/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 H; 400,000 inhabitants</td>
<td>31</td>
<td>1.6 (0.3)</td>
<td>5.1 (0.2)</td>
<td>5.4 (0.3)</td>
<td>6.1 (0.2)</td>
</tr>
<tr>
<td>2 H; 5,000 inhabitants</td>
<td>13</td>
<td>2.0 (0.4)</td>
<td>5.1 (0.3)</td>
<td>5.3 (0.2)</td>
<td>5.8 (0.3)</td>
</tr>
<tr>
<td>3 H; 1,400,000 inhabitants</td>
<td>7</td>
<td>1.9 (0.4)</td>
<td>4.9 (0.1)</td>
<td>5.3 (0.2)</td>
<td>5.9 (0.1)</td>
</tr>
<tr>
<td>4 H; 1,500 inhabitants</td>
<td>2</td>
<td>2.3 (0.5)</td>
<td>4.8 (0.2)</td>
<td>5.1 (0.1)</td>
<td>6.2 (0.1)</td>
</tr>
<tr>
<td>5 H; 1,500 inhabitants</td>
<td>3</td>
<td>1.2 (0.2)</td>
<td>4.6 (0.1)</td>
<td>4.7 (0.1)</td>
<td>5.5 (0.2)</td>
</tr>
<tr>
<td>6 A; pig slaughterhouse</td>
<td>3</td>
<td>3.0 (0.4)</td>
<td>6.0 (0.2)</td>
<td>6.0 (0.3)</td>
<td>6.3 (0.1)</td>
</tr>
<tr>
<td>7 A; cattle slaughterhouse</td>
<td>6</td>
<td>2.6 (0.9)</td>
<td>4.5 (0.7)</td>
<td>4.6 (0.7)</td>
<td>4.6 (0.6)</td>
</tr>
<tr>
<td>8 M; mixed origin</td>
<td>2</td>
<td>2.0 (0.4)</td>
<td>5.0 (0.1)</td>
<td>5.0 (0.1)</td>
<td>5.4 (0.2)</td>
</tr>
</tbody>
</table>

*H, human origin; A, animal origin; M, mixed-animal origin (cattle, goat, lamb, and poultry). The percentage of samples that tested positive for *stx*2 detection was 100%.*
manufacturer’s instructions. Colonies showing a positive hybridization signal were counted. Then, \( stx_2 \)-positive strains were isolated on TSA from the original Chromocult coliform agar plate, and the presence of the \( stx_2 \) gene was confirmed by specific PCR as described below.

**Sequencing.** Some of the fragments obtained in the nested PCR were sequenced with the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Madrid, Spain) and the ABI PRISM 3700 DNA sequencer (Applied Biosystems) according to the manufacturer’s instructions. The samples were sequenced in both the reverse and forward orientations with the same primers used in the nested PCR.

**Phenotypic characterization.** The strains carrying the \( stx_2 \) gene were biochemically phenotyped using the Phene-Plate system with 96-well PhP-RE microplates (Ph-Plate Microplate Technique AB, Stockholm, Sweden) developed for FC phenotyping analyses. They were used according to the manufacturer’s instructions. Additionally, the presence of \( \beta \)-glucuronidase activity and the ability to ferment sorbitol in 24 h were assessed. The sorbitol fermentation test was performed by inoculating purple bromocresol broth tubes containing 1% sorbitol (Sigma, St. Louis, Mo.) with the strain to be tested and incubating them at 37°C for 24 h. The \( \beta \)-glucuronidase activity was assayed by incubating the strain in 250 μl of phosphate-buffered saline with a \( \beta \)-glucuronidase tablet (Diatabs, Rosko, Denmark) and incubating it for 4 h or overnight at 37°C. The expression of the \( E. coli \) O157 serotype was tested by Western blot analysis of the lipopolysaccharide (LPS) with specific anti-O157 antibodies (Oxoid) as described below. Strains from different samples showing different PhP phenotypes and \( stx_2 \)-carrying strains was found among FC and H9262 strains.

**RESULTS**

**Presence of the \( stx_2 \) gene in raw sewage.** The MPN–nested-PCR method was efficient for the enumeration of \( stx_2 \) gene-carrying bacteria (Fig. 1). Bacteria carrying the \( stx_2 \) gene were detected in all of the 67 sewage samples analyzed, independently of the human or animal origin of the samples. The values obtained with the MPN-nested PCR in the urban wastewater treatment plants ranged from 1.2 to 2.3 \( \log_{10} \) units (MPN + 1) per ml. For all of the municipal sewage samples (human origin), the ratios of \( stx_2 \) gene-carrying bacteria to bacterial indicators were of the same order of magnitude. These proportions ranged from 1:10,000 to 1:100,000 (Table 1). However, a higher variability in the presence of \( stx_2 \) gene-carrying bacteria was observed in animal wastewater samples. In the cattle samples (the most numerous of our samples), the proportion of the numbers of \( E. coli \) organisms, FC, and TC that carried \( stx_2 \) was 1:100, 1 log unit higher than in human sewage samples. A similar ratio of \( stx_2 \)-carrying strains was found among FC and E. coli organisms in wastewater samples from other animal origins, although the number of samples is too low to consider these data to be any more than a mere indication. However, the ratio of \( stx_2 \)-carrying strains to TC for any wastewater sample was slightly lower than that for human sewage samples. The lowest proportion was observed in wastewater samples from the cattle slaughterhouse (Table 1).

**Seasonal distribution of the \( stx_2 \) gene.** The values obtained for \( stx_2 \) gene-carrying bacteria, \( E. coli \) organisms, and TC over a 1-year period of monitoring plant 1 were similar throughout the period, showing only minor variations. MPNs up to 100/ml were found for \( stx_2 \) gene-carrying bacteria, while TC, FC, and \( E. coli \) were present in quantities up to \( 10^5 \), \( 10^6 \), and \( 10^5 \) CFU/ml, respectively (Fig. 2). The proportions of each of these bacterial populations that carried \( stx_2 \) were similar to those indicated above. No seasonal differences were observed for any of the measured parameters, as indicated by an analysis of variance test (\( P < 0.05 \)).

**Reduction of numbers of \( stx_2 \) gene-carrying bacteria in the effluents of sewage treatment plants.** There was a reduction in the numbers of all of the studied bacterial indicators and \( stx_2 \) gene-carrying bacteria in the effluents of the two sewage treatment plants analyzed. There were some differences between values from the activated-sludge treatment plus lagooning system (plant 5) and the lagooning treatment (plant 4) (Table 2). At plant 4, there was a reduction of 2 \( \log_{10} \) units of \( stx_2 \) gene-carrying bacteria and of around 1 \( \log_{10} \) unit of TC, FC, and \( E. coli \) organisms between raw sewage and the most distant lagoon effluent. At plant 5, the reduction of the numbers of TC, FC, and \( E. coli \) organisms in the tertiary effluent ranged from 3.5 to 4.5 \( \log_{10} \) units. However, the exact reduction in the number of \( stx_2 \) gene-carrying bacteria, which must be greater than 1 \( \log_{10} \) unit, could not be determined, since \( stx_2 \) gene-carrying bacteria were not detected in the effluents.

**Isolation and characterization of \( stx_2 \) gene-carrying bacteria.** One hundred thirty-eight coliform strains were isolated from the different samples by using the colony hybridization method. The presence of the \( stx_2 \) gene was confirmed by spe-
cific PCR for all of these isolates with the exception of five strains that apparently lost the gene upon subcultivation. Some of the 378-bp amplimers were sequenced, and they showed a high homology to the stx\(_2\) gene sequence from the 933W phage. A total of 125 isolates showed the expected pattern of typical E. coli on Chromocult coliform agar (blue-colored colonies indicating \(\beta\)-D-glucuronidase activity).

According to the LPS pattern and the phenotype obtained with the PhP system, 59 representative strains were selected for further characterization. A total of 28 different LPS patterns and 44 different Phene system phenotypes with an identity level of 0.975 were observed. Strains from different samples with different LPS patterns and phenotypes were considered representatives of each sample. Later, these strains were identified with the API 20E system. Fifty-five of these isolates were classified as E. coli (all of these, with one exception, were indole positive), while the others could not be identified with the API 20E system. The majority of the isolated strains was able to ferment sorbitol within 24 h and had \(\beta\)-D-glucuronidase activity (46). Only five strains were unable to ferment sorbitol within 24 h, of which only one belonged to the E. coli O157:H\(^-\) serotype. This serotype was also negative for the \(\beta\)-D-glucuronidase test.

Production of Shiga toxins. Despite the fact that all of these strains produced the PCR products of the stx\(_2\) gene, only 23 isolates (39%) produced the toxin protein Stx2, 4 isolates (7%) produced Stx1 but failed to produce Stx2, and only 1 isolate (2%) produced Stx1 and Stx2 (Table 3). The last isolate corresponded to the E. coli O157 isolate. There was a higher proportion of bacteria that carried the stx\(_2\) gene and were not capable of toxin production among municipal sewage isolates than among animal wastewater isolates: 94% of the bacteria from municipal sewage versus 43% from animal wastewater samples.

**DISCUSSION**

Since E. coli O157:H7 was first isolated from water in 1989 (21), numerous waterborne-STE C outbreaks have been reported (14, 28, 39). The low dose and the difficulty of isolating these microorganisms in aquatic environments increase the need for the development of sensitive and specific methods for STEC detection. A method suitable for the enumeration of stx\(_2\) gene-carrying bacteria in sewage samples has been applied in this study to analyze their presence in environmental samples. In recent years, the use of quantitative PCR for the enumer-

<table>
<thead>
<tr>
<th>Sample source and type(^{a})</th>
<th>Mean no. of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC [log (CFU + 1)/ml]</td>
</tr>
<tr>
<td>Plant 4</td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>6.1</td>
</tr>
<tr>
<td>LE2</td>
<td>5.3</td>
</tr>
<tr>
<td>LE3</td>
<td>5.1</td>
</tr>
<tr>
<td>Plant 5</td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>5.4</td>
</tr>
<tr>
<td>SE</td>
<td>2.4</td>
</tr>
<tr>
<td>TE</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^{a}\) RS, raw sewage; SE, secondary effluent; TE, tertiary effluent; LE2, lagooning effluent 2 (sewage effluent between lagoons 2 and 3); LE3, lagooning effluent 3 (sewage effluent between lagoons 3 and 4).

\(^{b}\) The number of bacteria was below the limit of detection.
TABLE 3. Toxin production and distribution of stx1 and stx2 genes between the different representative strains isolated

<table>
<thead>
<tr>
<th>Origin</th>
<th>Toxin productiona</th>
<th>stx1 gene</th>
<th>stx2 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stx1 only</td>
<td>Stx2 only</td>
<td>Stx1 and Stx2</td>
</tr>
<tr>
<td>Animal</td>
<td>1</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Human</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a The numbers of strains were 59.

The analysis of microorganisms, particularly for *E. coli* O157:H7 (37), has become widely extended. However, the combined use of nested PCR and the MPN method in this study allowed the MPN detection of 10 to 100 of the targeted bacteria per ml (4). The colony hybridization method allowed the detection of 1 stx2 gene-carrying colony among approximately $10^6$ total colonies on 140-mm-diameter agar plates. The results obtained with the MPN–nested-PCR method were consistent with the quantification of strains isolated by using the colony hybridization method. The data revealed high levels of stx3 gene-carrying bacteria in raw human sewage and wastewater containing fecal wastes from different animals, independently of the origins of the samples. The ratio of stx2 gene-carrying organisms to FC was approximately 1:1,000 in all the samples with the exception of cattle wastewater, which showed a higher proportion of stx3 gene-carrying organisms (1:100), probably because cattle are the major reservoir of STEC (9). We observed some variations in the concentrations of different microbial indicators and stx3 gene-carrying bacteria among the different human sewage treatment plants, which could be related to the different kinds of wastewater that they receive. Although some results were obtained from a small number of samples, significant values were obtained from plants which supplied high numbers of samples. These data are representative, since the incoming raw sewage of those treatment plants has been widely studied, showing very constant values for the analyzed bacterial indicators (7, 20).

Unlike what happens with cattle shedding of *E. coli* O157:H7 (9), there were no significant seasonal differences in the levels of shedding of stx3 gene-carrying bacteria in the monitored human wastewater treatment plant. These results are in agreement with those from studies performed with contaminated river waters in Japan, which found stx3 gene-carrying bacteria at densities between $10^2$ and $10^4$ cells per ml independently of the season (16). The high diversity of *E. coli* serotypes and other enterobacteria which may carry the stx3 gene may explain such a difference, because the MPN–nested-PCR method analyzes all these bacterial groups at the same time. There was only a minor decrease in the number of stx3 gene-carrying bacteria for two samples obtained during the winter. Data from this study and the fact that *E. coli* O157:H7 cells have been demonstrated to survive for long periods in manure (15, 43) and in water (5, 32, 42) suggest that human sewage and animal wastewater, in addition to cattle and other, wild animals, should be regarded as reservoirs of STEC.

There was a reduction in the number of stx3 gene-carrying strains after treatment in both treatment plants analyzed. In treatment plant 4, there was a larger reduction in the number of stx3 gene-carrying strains than in the number of bacterial indicators, suggesting that STEC strains are not specifically resistant to the lagooning treatment. It was not possible to compare this treatment with the activated-sludge treatment plus lagooning system (plant 5), because after secondary treatment, the numbers of stx3 gene-carrying bacteria were already under the limit of detection. Values of reduction could not be calculated; it can be stated only that the log$_{10}$ reduction was greater than 1.2 log$_{10}$ units. However, the activated-sludge treatment plus lagooning system was also observed to be efficient for the reduction in the number of stx3 gene-carrying bacteria. The values obtained in treatment plant 4 confirm previous results showing a small reduction in the number of FC during the winter season, which is the period in which the results reported here were obtained (7).

The specific colony hybridization method used in this study enabled the isolation of 138 coliform strains carrying the stx2 gene. The expected PCR band of 378 bp corresponding to the stx2 gene amplimer was produced by all strains, with the exception of five strains that appeared to lose the gene after subcultivation. This phenomenon is consistent with the results of previous studies of clinical samples that showed the loss of stx genes upon subcultivation (13; J. C. Paton and A. W. Paton, Letter, J. Clin. Microbiol. 35:1917, 1997). Only one strain belonged to the serotype O157:H7, which suggests a low prevalence of this serotype in stx2-carrying strains in the analyzed samples.

This study shows that the stx2 gene is commonly present in municipal sewage and animal wastewaters of different origins, with a wide distribution range among coliform bacteria. Some of these bacteria not only harbor the gene but also are able to produce the toxins and consequently may represent a potential health risk, which must be taken into account. Most of the analyzed strains that expressed the Stx2 toxin arose from animal samples. Stx1 toxin production was also detected in some strains (4 of 59), usually arising from samples of human origin. It should be noted that in isolates from human wastewater and wastewater of animal origin, the gene is not always expressed. The toxin is produced in approximately 50% of the strains from animal wastewater, while of the strains from human sewage, although all of them carried the gene, less than 10% showed expression of the protein toxin. Hence, the animal strains seemed to maintain the characteristic, while the human strains have acquired the gene in a nonfunctional way, since they do not produce Stx2.

The presence of the stx1 gene evaluated in bacterial populations in this study has also been observed in the genomes of free bacteriophages infectious for *E. coli* or *Shigella* spp. present in sewage (8, 24). It is well known that the stx2 gene is carried in the genomes of temperate bacteriophages, which may be the origin of free phages detected in sewage. Although the presence of inducible phages in the strains analyzed in this study has not been evaluated (this topic will be the subject of future studies), the potential contribution of phages to the mobility of the stx3 gene should not be underestimated. The presence of the stx2 gene in populations detected in sewage (phages or bacteria) indicates an exchange of this gene between these populations. More information is needed to understand the ecology of the stx2 gene in water environments and its involvement in the pathogenicity of STEC strains.
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