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Human isolates of *Aeromonas* possess Shiga toxin genes (*stx1* and *stx2*) highly similar to the most virulent gene variants of *Escherichia coli*

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Abstract

Strains producing Shiga toxins, encoded by *stx1* and *stx2* genes, can cause diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome. PCR screening of 80 clinical *Aeromonas* strains showed that 19 were *stx1*-positive and only one was positive for both *stx1* and *stx2*. PCR bands were very faint for some strains and negative results were obtained after subculturing. The

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obtained sequences of *Aeromonas* *stx*1 and *stx*2 genes were highly similar to those of the most virulent *stx* gene variants of Shiga toxin-producing *Escherichia coli*. These results may lead to a better understanding of the potential pathogenicity and virulence mechanisms of *Aeromonas*.

Keywords: *Aeromonas*, diarrhoea, HUS, *stx*1, *stx*2

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Shiga toxins (Stx1 and Stx2) are important virulence factors in the pathogenesis of gastroenteritis, hemorrhagic colitis and haemolytic-uremic syndrome (HUS) [1–3]. They are usually encoded in the genome of bacteriophages (highly mobile elements) that play a role in Stx expression and horizontal gene transfer, leading to the emergence of new *stx*-variants or *stx*-producing pathogens [4]. Several variants of *stx*1 and *stx*2 genes have been recognized [2,3], with *stx*2 (the prototype) and *stx*2c being considered the most important because they are more frequently associated with HUS than *stx*1 and $stx2d_{activatable}$ [2,3,5]. The *stx* genes show instability *in vivo* and *in vitro* as a result of the mobility of stx-phages [4,6], leading to the loss of *stx* genes after subculturing and/or infection [1,4,6–8].

Despite Shiga toxins being typical of *Shigella dysenteriae* and Shiga toxin-producing *E. coli* (STEC), they have also been described in species of other genera, including *Aeromonas* [7–12]. In *Aeromonas*, the presence of *stx*1 gene has only been detected in five strains [11,12] but never sequenced, and it is not known whether they have the *stx*2. Despite that, several cases of HUS associated with *Aeromonas* have been reported [13]. The present study investigates the *stx* genes (*stx*1 and *stx*2) in 80 human *Aeromonas* strains by PCR and sequencing.

All investigated strains (33 from extra-intestinal infections and 47 from diarrhoea) were genetically identified [14]. The strain of *Aeromonas veronii* bv. sobria associated with a case of HUS [13] was also tested. DNA was extracted from single colonies grown on sheep blood agar (24 h at 30°C) using InstaGene™ Matrix (Bio-Rad, Hercules, CA, USA). Separate PCRs for the detection of *stx*1 (350 bp) and *stx*2 (406 bp) with primers EVT-1&2 and EVS-1&2, respectively (TaKaRa Biomedicals, Tokyo, Japan) with positive and negative controls were employed [11]. PCR products were purified using GFX PCR DNA and the Gel Band Purification Kit (Amersham Biotech, Little Chalfont, UK) and sequenced on both strands using the TaKaRa primers (3.2 pmol/μL) in an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Using CLUSTAL W, the *stx*1 and *stx*2 obtained sequences were independently aligned with those available in the GenBank to establish their similarity. Their genetic relationships were graphically represented as phylogenetic trees using the neighbour-joining method, as described previously [14].

Nineteen of the 80 *Aeromonas* strains showed a band of the expected size (350 bp) for the *stx*1 and one of them also the band (406 bp) of *stx*2. However, in 15 of the 19 strains, the *stx*1 bands were faint as was also the *stx*2 band (Fig. 1a, b). The strain of *A. veronii* bv. sobria associated with a case of HUS [13] did not show even faint bands. To verify the results, the same DNA of the 19 *stx*1-positive strains was evaluated again under identical conditions and only four (875c, 883c, 885c and 887c) strains were positive for the *stx*1. Negative results corresponded with strains showing faint bands in the first analysis. The 19 *Aeromonas* strains were grown again from the glycerol stock and *stx*1 was amplified from a new DNA extraction. The results obtained reproduced the initial finding, with the appearance of the same 15 strains with faint *stx*1 bands and four with intense bands. The PCR for the *stx*2-positive strain (819c) was performed five times from four different DNA extractions from the same frozen stock and the expected band (406 bp) was only observed on two of five (40%) occasions as a faint band (Fig. 1c). These faint bands can be explained by the number of copies of *stx* genes in the template DNA [7] and their loss after subculture or infection agrees with previous findings [1,4,6–8], and may also explain the lack of amplification in the HUS isolate of *A. veronii* bv. sobria.

Snowden *et al.* [12] detected *stx*1 in only one environmental strain of *A. veronii* using other primers [15]. We evaluated the same primers, although the expected 121 bp band of *stx*1 could not be amplified in any of the 19 *stx*1-positive strains.

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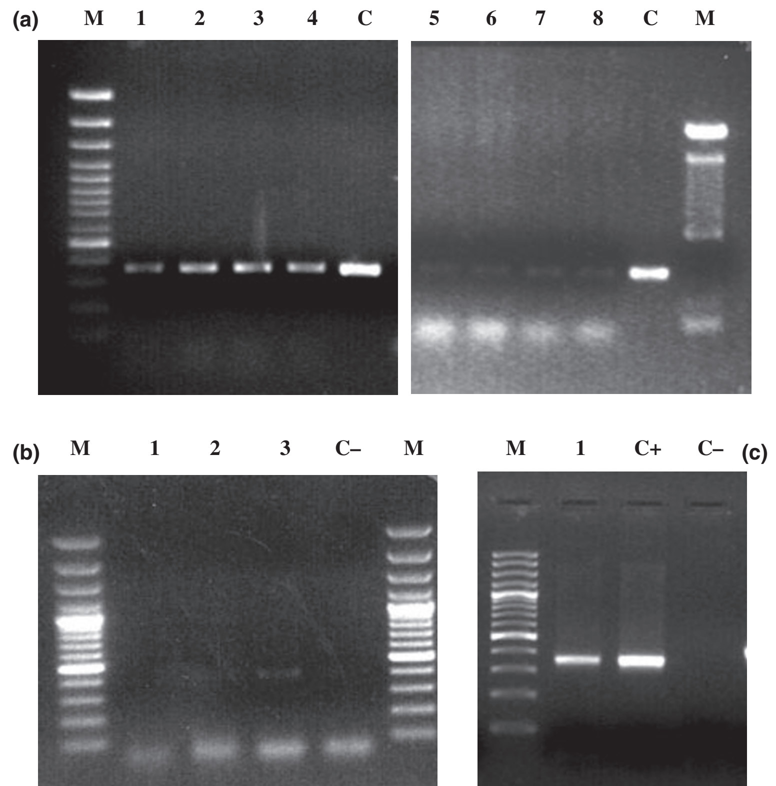

FIG. 1. Examples of PCR amplifications demonstrating the presence of *stx* genes in *Aeromonas* strains. 25 μL of PCR product electrophoresed in 1% agarose gel. (a) *stx*1 strong and faint bands. Lane 1, 875c; lane 2, 883c; lane 3, 885c; lane 4, 887c; lane 5, 194c; lane 6, 361c; lane 7, 191c; lane 8, 24c. (b) *stx*2 faint bands. Lane 1, 875c; lane 2, 819c; lane 3, 819c (duplicate PCR product). (c) *stx*1 amplified from plasmid DNA. Lane 1, 535c. Lane C+, positive control *Escherichia coli* O157:H (CECT 4076). Lane C–, negative control (Milli-Q water as template DNA; Millipore, Billerica, MA, USA). Lane M, Ladder 100 bp. *Aeromonas caviae* strains: 535c, 819c, 875c, 883c and 885c; *Aeromonas hydrophila* strains: 24c, 191c, 194c and 887c; *Aeromonas salmonicida* strain 361c.

We found more *stx*1-positive *Aeromonas* strains (23.75% 19/80) than in previous studies (10.25% 4/39) [11] and (9.1% 1/11) [12]. They were predominantly encountered in *Aeromonas caviae* (42.1% 8/19), *Aeromonas hydrophila* (31.6% 6/19) and *A. veronii* (10.5% 2/19), which are species commonly associated with gastroenteritis and even with a dysentery-like syndrome and HUS [13,16,17]. The only *stx*1-*stx*2-positive strain (819c) belonged to *A. caviae* and was isolated from a urine sample.

Sequences (350 bp) of *stx*1 of the four *Aeromonas* strains with intense bands were identical and showed 99.71% similarity (one different nucleotide) to the corresponding region of this gene of *E. coli* O157:H7**,** whereas a 99.51% similarity (two different nucleotides) was shown for the *stx*2 sequence of strain 819c (406 bp). The analysis showed that our *stx*1 sequences clustered with those from *S. dysenteriae*, *Shigella sonnei*, *E. coli* O157:H7 and other STEC human isolates (Fig. 2a), whereas *A. caviae stx*2 sequence clustered (Fig. 2b) with those of the most virulent human variants (*stx*2 prototype and *stx*2c) that are commonly associated with HUS [2,3]. This may suggest an equally potential role of both genes in *Aeromonas* and horizontal gene transfer [4] among those microorganisms, which is not surprising because they inhabit the same environments.

To determine whether the observed instability of *stx*1 was a result of its extra-chromosomal DNA location, this DNA, from strains 535c representative of strains with a faint band, and 887c with a strong band, was extracted using Ultraclean 6 minute mini plasmid prep kit (Mo Bio, Carlsbad, CA, USA). A single band (approximately 3.2 kbp) of extra-chromosomal DNA was obtained from both strains. This DNA was used as a template for *stx*1-PCR amplification [11] and a band of the expected size (350 bp) for *stx*1 as intense as the positive control (*E. coli* O157:H7) was only observed from strain 535c (Fig. 1c). This indicated that, at least in strain 535c and in the four strains reported by Haque *et al.* [11], the *stx*1 is not chromosomally encoded. These results corroborate the mobility of the *stx*-phages that can be lost by subculturing [4].

To determine the production of Stx1 and Stx2 toxins by the PCR positive strains, a rapid immunochromatographic test (Duopath Verotoxin test; Merck, Darmstadt, Germany) was used. Only strains 885c (which showed an intense *stx*1 band by PCR) and 535c (with a faint PCR band) showed a weak Stx1 signal, although none of the strains showed a Stx2 positive reaction. In conclusion, 10.53% (2/19) of the *Aeromonas* strains that possessed *stx*1 produced the Stx1 toxin. Similar values (using the same test) have been reported from STEC strains (6.7% produced Stx1 and 6–7% produced Stx2) from human wastewater [18]. However, the results obtained in the present study could be influenced by the fact that this test is specifically designed for STEC toxins.

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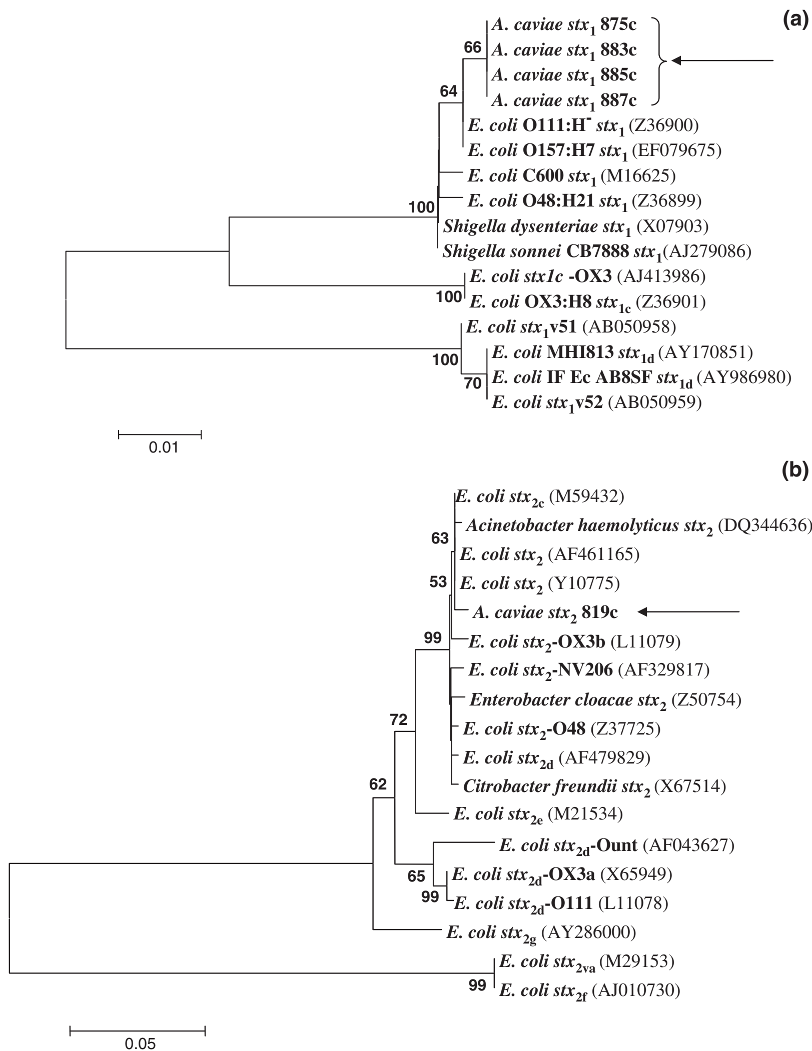

FIG. 2. Phylogenetic analysis of nucleotide sequence fragments (350 bp) of *stx*$_1$ genes (a) and (406-bp) of *stx*$_2$ genes (b) of *Aeromonas* and other enterobacteria. Phylogenetic trees were constructed with the neighbour-joining method using Kimura's two-parameter model. Bootstrap values greater than 50 from 1000 replicate trees are reported at the nodes. The scale bar indicates evolutionary distance of 0.01 and 0.05 nucleotide substitutions per site in (a) and (b), respectively. All numbers and accession numbers are given as cited in the GenBank database.

To our knowledge, this is the first report to provide *Aeromonas stx*1 and *stx*2 sequences that establish their high similarity with those of STEC. This is of particular importance because these genes have been poorly studied in *Aeromonas* and they may have a role in inducing diarrhoea and HUS.

The sequences of the *stx*1 and *stx*2 genes have been deposited in GenBank under accession numbers GU130282-GU130285 and GU130286, respectively.

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Transparency Declaration

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