Active Shiga-Like Toxin Produced by Some Aeromonas spp., Isolated in Mexico City

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Shiga-like toxins (Stx) represent a group of bacterial toxins involved in human and animal diseases. Stx is produced by enterohemorrhagic Escherichia coli, Shigella dysenteriae type 1, Citrobacter freundii, and Aeromonas spp.; Stx is an important cause of bloody diarrhea and hemolytic uremic syndrome (HUS). The aim of this study was to identify the stx1/stx2 genes in clinical strains and outer membrane vesicles (OMVs) of Aeromonas spp., 66 strains were isolated from children who live in Mexico City, and Stx effects were evaluated in Vero cell cultures. The capacity to express active Stx1 and Stx2 toxins was determined in Vero cell cultures and the concentration of Stx was evaluated by 50% lethal dose (LD₅₀) assays, observing inhibition of damaged cells by specific monoclonal antibodies. The results obtained in this study support the hypothesis that the stx gene is another putative virulence factor of Aeromonas, and since this gene can be transferred horizontally through OMVs this genus should be included as a possible causal agents of gastroenteritis and it should be reported as part of standard health surveillance procedures. Furthermore, these results indicate that the Aeromonas genus might be a potential causative agent of HUS.

Keywords: Shiga-like toxin, Aeromonas spp., diarrhea, uremic hemolytic syndrome, outer membrane vesicles

INTRODUCTION

For several years, the significance of Aeromonas spp., as a human diarrhea-causing agent was controversial; several studies demonstrated that the pathogenic mechanism of Aeromonas is multifactorial because many virulence factors are involved, including the production of cytotoxins (Castro-Escarpulli et al., 2002, 2003). These toxins can cause diarrhea or hemorrhagic colitis, and may play a major role in the hemolytic-uremic syndrome (HUS) and TTP development (Bogdanović et al., 1991; Fang et al., 1999; Monforte-Cirac et al., 2010).

Abbreviations: Ab, Antibody; HUS, Hemolytic uremic syndrome; INP, Instituto Nacional de Pediatría Mexico; LD₅₀, Median lethal dose; OMVs, Outer membrane vesicles; PCR, Polymerase chain reaction; STEC, Shiga toxin-producing E. coli; Stx, Shiga-like toxin; TTP, Thrombotic thrombocytopenic purpura.
The cytotoxins implicated in these diseases include Shiga toxin and the closely related Stx. Stx variants are expressed in Shigella, Enterobacter, Citrobacter, Acinetobacter, Campylobacter, and Hamiltonella bacterial species (Mauro and Koudelka, 2011). Alperi and Figueras (2010) described the presence of Stx1 and Stx2 in clinical isolates of Aeromonas spp., associated with gastroenteritis, hemorrhagic colitis, and HUS. Genes encoding these toxins are located in different lambdoid bacteriophages that lysogenize this strain. In addition, the genus Aeromonas has a zero-secretion system named OMVs. OMVs could be a means by which some proteins, RNA, periplasmic space components and other components associated with virulence, may be transferred horizontally to other genera; therefore, it is believed that OMVs play an important role in pathogenicity (Guerrero-Mandujano et al., 2015a,b).

For this reason, the aim of this study was to evaluate the damage caused by the production of Stx by strains isolated from Mexico City children in Vero cell cultures.

MATERIALS AND METHODS

Strains
This study included 66 clinical isolates from the INP, 54 obtained from intestinal and 12 from extra-intestinal infections. Strains were isolated from specimens obtained for routine testing at the mentioned hospital; therefore, no informed consent was required from parents or legal guardians of children. All strains were genetically identified by 16S rDNA-RFLP (Hernández-Cortez et al., 2011). The typed strain for Escherichia coli O157:H7 CECT 4076 was used as the positive control and E. coli K12 strain (5512 ENCB) from the collection of the Medical Bacteriology Laboratory (Escuela Nacional de Ciencias Biológicas, IPN) was used as the negative control for toxin production. The strains were maintained for short periods at room temperature on blood agar based slants; for longer storage, these toxins are located in different lambdoid bacteriophages that lysogenize this strain. In addition, the genus Aeromonas has a zero-secretion system named OMVs. OMVs could be a means by which some proteins, RNA, periplasmic space components and other components associated with virulence, may be transferred horizontally to other genera; therefore, it is believed that OMVs play an important role in pathogenicity (Guerrero-Mandujano et al., 2015a,b).

For this reason, the aim of this study was to evaluate the damage caused by the production of Stx by strains isolated from Mexico City children in Vero cell cultures.

DNA Sequencing
Polymerase chain reaction products were purified using a PureLink Quick Gel Extraction Kit (Invitrogen®, Mexico) according to manufacturer’s instructions. The products were directly sequenced on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the forward and reverse primers used for PCR, according to manufacturer’s instructions. Sequencing was performed at the Instituto de Biología, UNAM (Mexico). Sequence analysis was performed with the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI).

Microplate Vero Cells Preparations
This procedure was performed in 96-well microplates with Vero (ATCC CCL81) cell monolayer with 80% confluence, adding minimal essential medium (MEM; Invitro®, Mexico) supplemented with 10% v/v fetal bovine serum (FBS; Invitro®, Mexico).

The cell suspension was homogenized and adjusted to 10⁵–10⁶ cells/mL using a Neubauer chamber. After adjusting, the suspension was deposited in 200-µL well. The microplates were incubated at 37°C under 5% CO₂ for 24 h (CO₂ Incubator, VWR Scientific, USA) (Giono-Cerezo et al., 1994).

Cell-Free Bacterial Preparations
Five colonies from each blood agar plate were inoculated into 3 mL of Craig medium (0.4% yeast extract, 3% casamino acids, 0.05% K₂HPO₄). These were incubated for 24 h at 37°C and the optical density of the bacterial culture was used to adjust the culture to 0.25 at 600 nm. Cell-free preparations were made by centrifuging the cultures at 14,000 g for 10 min at 4°C, followed by filtration of the supernatant through a membrane filter (pore size 0.45 pm, Sartorius Minisart NML). Cell-free supernatants were stored at −20°C. A total of 66 cell-free bacterial preparations were obtained in this way; the positive control (E. coli O157:H7) and the negative control (E. coli K12) were obtained also in the same way (Giono-Cerezo et al., 1994).

Cytotoxic Assay and LD₅₀ Determination
The cell-free filtrate (20 µL) was inoculated into wells containing cells and the respective growth medium without antibiotics. Inoculated cells were incubated for 96 h at 37°C with 5% CO₂ and observed every 24 h on the inverted microscope. The cytotoxic effect was expected to appear as rounding and shrinkage of cells with thick granulation and, finally, progressive and irreversible destruction of the monolayer. All tests were performed in duplicate; viability controls with MEM and Craig medium were performed also (Giono-Cerezo et al., 1994).

The LD₅₀ was determined in Aeromonas strains detected as positive for causing cytotoxic damage in Vero cells. A standard 96-well microplate with Vero cells was prepared as indicated in 2.6, but the medium was changed to 100 µL of MEM with 1% BFS. This preparation was exposed to 100 µL of cell -free bacterial preparation, and serial dilutions were done on the whole row of plates. After 24 h of incubation at 37°C with 5% CO₂, the LD₅₀ was determined, under the inverted microscope. LD₅₀ was
assigned to the well in which 50% of Vero cells were damaged and 50% un-damaged (Marques et al., 1986).

**Blockade and Toxin Neutralization Assay**
The toxin neutralization assay was performed in the strains that induced cytotoxic damage in Vero cells because this damage is indicative of Stx. For this test, a microplate was prepared with Vero cell grown to a confluence of 90–100% with the LD$_{50}$ of each cell-free bacterial preparation. To perform the neutralization assay to show cell damage produced as a consequence of Stx action, two monoclonal antibodies (Universidad Nacional Autónoma de México) obtained from E. coli O157:H7 were used.

For each Ab a Bradford protein quantitation was done according to manufacturer’s instructions (Biorad), with a result of 78.2 and 74.8 µg/µL, respectively, of anti-Stx1 and anti-Stx2 A. The concentrated Ab was worked and a double dilution of the Ab. Then, 10 µL of the Ab (anti-STX1 or anti-STX2) was incubated with 190 µL of the cell-free supernatant at a concentration of LD$_{50}$ for 1 h at 37°C. Following this, 200 µL of the latter was inoculated into Vero cells at 90% of confluence and incubated for 24 h at 37°C with 5% CO$_2$ (Marques et al., 1986).

**OMVs Procurement and DNA Extraction from OMVs**
Outer membrane vesicles were obtained from Aeromonas hydrophila F-0050. The protocol was performed as previously described by Guerrero-Mandujano et al. (2015a).

The OMVs’ DNA was obtained through InstaGene Matrix (BioRad®️, Mexico) according to the instructions provided by the manufacturer. Then, the OMVs’ DNA was purified with the DNA extraction phenol-chloroform technique (Guerrero-Mandujano et al., 2015b).

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**FIGURE 1 | Cytotoxicity assay and LD$_{50}$ determination performed in Vero cell cultures.** (A) Vero cell culture with Minimal Essential Medium (Viability test). (B) Vero cell culture with Craig Medium (Viability test). (C) Negative control with E. coli K12. (D) Positive control with E. coli O157: H7. Cytotoxic damage is shown (arrows) by destruction of the cell monolayer and “old lace” effect. (E) Cytotoxic damage of A. hydrophila F-0050 strain. (F) Cytotoxic damage of A. caviae 421423 strain. (G) Graphical representation of the LD$_{50}$ titer of all strains.
RESULTS

**stx1 and stx2 Gene PCR Amplifications**
Polymerase chain reaction screening of 66 clinical Aeromonas strains showed that 22/66 (33.3%) strains contained the stx1 gene, 42/66 (63.6%) strains contained both genes, no strains contained only the stx2 gene, and 2/66 (3%) strains were negative for both genes.

**DNA Sequencing**
BLASTn analysis showed a 79 to 99% similarity and an expected value of \(3 \times 10^{-16}/2 \times 10^{-97}\) between the stx-1/stx-2 genes of E. coli O157:H7 and the amplicon from Aeromonas spp., strains.

**Cytotoxic Assay and LD50 Determination**
The cytotoxicity test performed in Vero cell cultures showed that 17/66 (25.7%) cell-free bacterial preparations caused cytotoxic

| Cell free bacterial preparation from strain | LD50 | Inhibition with anti-Stx1 Ab | Inhibition with anti-Stx2 Ab |
|-------------------------------------------|------|------------------------------|-----------------------------|
| E. coli O157:H7                           |      |                              |                             |
| A. hydrophila 377218                       |      |                              |                             |
| A. bestiarum 380264                        |      |                              |                             |
| A. hydrophila F-4093                       |      |                              |                             |
| A. hydrophila 455681                       |      |                              |                             |

**FIGURE 2** | Inhibition of cytotoxic effect induced in Vero cells culture by cell-free bacterial preparations of Aeromonas spp. strains bearing the stx1 or stx2 gene.
damage, suggesting production of an active Stx (Figure 1), revealed by the characteristic damage caused by Stx.

The results for determining the LD$_{50}$ titer were as follows: 6/17 (35.2%) cell-free bacterial preparations presented a titer of 1:16; 5/17 (29.4%) cell-free bacterial preparations depicted a titer of 1:8; 3/17 (17.6%) cell-free bacterial preparations had a titer of 1:64; 2/17 (11.7%) cell-free bacterial preparations with a titer of 1:32; and 1/17 (5.8%) cell-free bacterial preparations with a titer of 1:256 (Figure 1G).

**DISCUSSION**

The genus *Aeromonas*, as a producer of Stx, could be an emergent pathogen that causes diarrhea mainly in pediatric patients (Hernández-Cortez et al., 2011; Figueras and Baez-Hidalgo, 2014). Nevertheless, this genus has become more relevant in the medical area, as it is a pathogen causing HUS (San Joaquín and Pickett, 1988; Bogdanović et al., 1991; Robson et al., 1992; Fang et al., 1999; Monforte-Cirac et al., 2010).

In this study, we found, by PCR amplification of the *stx1* and *stx2* genes, that 22/66 (33.3%) of the aeromonad strains contained the *stx1* gene. 46/66 (63.6%) strains contained both genes, and there were no strains containing *stx2* alone. This can lead us to suggest that this gene is widely distributed in the *Aeromonas* strains isolated from pediatric patients and that these patients can be at risk for developing HUS.

There is only one previous report in which the *stx* genes were studied in *Aeromonas* strains isolated from different hospitals in Spain; the results obtained were that 19/80 (23.7%) strains had the *stx1* gene and only 1/80 (1.25%) strain was positive for both genes (Alperi and Figueras, 2010). These results convey that there is a greater distribution of the gene in Mexican strains; however, we should take into account that, in Spain, the *Aeromonas* genus is a known gastrointestinal pathogen isolated routinely in patients with a gastrointestinal profile, therefore, an adequate treatment is given to confront this genus.

In Mexico, this genus is not included in the list of pathogens causing diarrhea and it is not isolated routinely; as a result, it is difficult to give any treatment and good quality of life to patients; moreover, there are no statistics about its prevalence. In addition, the genus becomes more relevant due to the socioeconomic conditions of the Mexican population, which include water shortage; this facilitates its transmission and increases the possibility of HUS becoming a public health problem.

Sequencing of the amplicons was carried out and a Blast search was performed, which showed 79–99% similarity and an expectancy value of 3e–16/2e–97 between genes *stx1/stx2* as compared to *E. coli* O157:H7.

The LD$_{50}$ was determined to evaluate the cytotoxic damage in Vero cell culture; with this parameter and based on Marques et al. (1986), the strains can be grouped in low (2 × 10$^3$ to 6 × 10$^4$), moderate (10$^3$–10$^4$), and high (10$^2$–10$^3$) production (Figure 1G). Marques et al. (1986) carried out a study in which they made a similar assay to that of the present study, but with different strains
of STEC, they obtained that 262/400 (63%) strains were grouped as low Stx producers, and 48% produced cytotoxic damage; nevertheless, in 40% of these strains the cytotoxic damage was inhibited with anti-Stx antibodies.

In the present study, 25.7% of bacteria-free preparations caused cytotoxic damage characteristic of Stx in Vero cells, suggesting production of active Stx, all Aeromonas strains were grouped as low producers of Stx; however, in 50–70% of the cell-free supernatants, the cellular damage was inhibited, and in 20–30% of cell-free supernatants the cellular damage was reduced (Figure 3). The cell-free supernatants in which cellular damage was reduced this could have been because of two variants in the toxin present in the supernatant, consequently, as they are different immunologically, the Stx could not be totally inhibited and the cellular damage remained (Marques et al., 1986).

Two previous studies (Haque et al., 1996; Alperi and Figueras, 2010) obtained 10.2% and 10.53% of Stx1-producing Aeromonas strains, respectively. The reason for a higher percentage of Aeromonas strains producing active Stx could be due to the high availability of the Stx bacteriophage inside the culture as some STEC strains carriers of the Stx bacteriophage have been isolated from urban wastewater from treatment plants, wastewater from slaughterhouses and cattle stools (García-Aljaro et al., 2004, 2009). Similarly transduction studies have been carried out in vivo with Stx phages from E. coli, which can infect intestinal microbiota bacteria, giving the toxigenic characteristic to strains that were not infected before (Acheson et al., 1998; Schmidt et al., 1999; Gamage et al., 2003). The same could happen with the genus Aeromonas when inducing a gastrointestinal profile, followed by the fact that the genus Aeromonas has the ability to capture and integrate virulence factors in its genome, and one of these is the Stx-encoding gene.

The rest of the strains caused cytotoxic damage in the cell, possibly suggesting production of different toxins other than Stx, since Aeromonas is a genus capable of producing two cytotoxic enterotoxins, thermostable (AST) and thermodlabile (ALT) (Figueras and Baez-Hidalgo, 2014), that can cause cytotoxic damage in Vero cells; another possibility is that the Aeromonas strains have more than one bacteriophage inserted in their genome, as it has been demonstrated that STEC strains with double lysogeny are able to regulate bacteriophages to inhibit the capacity of lytic cycle induction, which results in a decrease of Stx production but without losing the gene (García-Aljaro et al., 2004; Imamovic and Muniesa, 2011). Therefore, it is necessary to establish how many bacteriophages do Aeromonas strains contain, including in the present study, to be able to correlate the presence of Stx bacteriophages and the amount produced of Stx.

On the other hand, the presence of Stx bacteriophages in inducible Aeromonas represents a horizontal transfer mechanism of the stx gene, but it is not the only one. In more recent studies, it has been determined that Aeromonas are capable of producing OMVs (Guerrero-Mandujano et al., 2015a) and these OMVs are capable of transporting integrated DNA (Guerrero-Mandujano et al., 2015b). In the present study, we amplified the stxl gene from the DNA extracted from the OMVs of one A. hydrophila strain of clinical origin, which induced cytotoxic damage in Vero cells, and this damage was inhibited with anti-Stx antibodies. This indicates that the Stx bacteriophage is the transport mechanism of the stx gene; furthermore, the OMVs and the recently called Transport System Type 0 are capable of spreading the stx gene interspecies or intraspecies. Nevertheless, it is required to confirm if the gene contained in the OMVs is complete. The results obtained in this study support the hypothesis that the stx gene is another putative virulence factor of Aeromonas that might be transferred through OMVs. It could represent another mechanism for horizontal transport of the stx gene to other Aeromonas strains and/or other bacterial genera.

For this reason, this genus should be included as possible causative agent of gastroenteritis when trying to identify causal agents and should be reported as part of standard health surveillance procedures.

AUTHOR CONTRIBUTIONS
IP-M and AG-M performed the experiments and MR-R performed the bioinformatics analyses and drafted the manuscript; CH-C designed the primer; VB-G, and JM-L critically commented and revised the manuscript; GC-E and VB-G received support from Estímulos al Desempeño en Investigación and Comisión y Fomento de Actividades Académicas (Instituto Politécnico Nacional) and Sistema Nacional de Investigadores (SNI, CONACyT). This study was funded by the Secretaría de Investigación and Posgrado del Instituto Politécnico Nacional (SIP 20160609). The SIP-IPN was not involved in the development of the study design, the collection, analysis, and interpretation of data, in the writing of the report nor in the decision to submit the paper for publication. IP-M and AG-M held a scholarship from CONACyT and BEIFI.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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