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Plesiomonas shigelloides exports a lethal cytotoxic-enterotoxin (LCE) by membrane vesicles



Marilucia Santos Ludovico^a, Luciano Moura Martins^b, Juares Ednaldo Romero Bianco^c, Célia Guadalupe Tardelli de Jesus Andrade^d, Rosabel Falcon^e, Paulo Pinto Joazeiro^c, Maria Silvia Viccari Gatti^a, Tomomasa Yano^{a,*}

^a Universidade Estadual de Campinas (UNICAMP), Departamento de Genética, Evolução e Bioagentes, Campinas, SP, Brazil

^b Instituto Adolfo Lutz Institute, Centro de Bacteriologia, São Paulo, SP, Brazil

^c Universidade Estadual de Campinas (UNICAMP), Departamento de Bioquímica e Biologia Tecidual, Campinas, SP, Brazil

^d Universidade Estadual de Londrina (UEL), Departamento de Biologia Celular, Londrina, PR, Brazil

^e Instituto de Medicina Tropical “Pedro Kouri” (IPK), Departamento de Microbiologia, La Habana, Cuba

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ABSTRACT

Plesiomonas shigelloides isolated from water in Brazil was previously described as a hemorrhagic heat-labile cytotoxic-enterotoxin producer. We purified this toxin from culture supernatants using ion metallic affinity chromatography (IMAC) followed by molecular exclusion chromatography. The pure toxin presented molecular mass of 50 kDa and isoelectric point (pI) around 6.9 by 2D electrophoresis. When injected intravenously, the purified cytotoxic-enterotoxin induced also severe spasms followed by sudden death of mice. Hence, we entitled it as lethal cytotoxic-enterotoxin (LCE). The presence of membrane vesicles (MVs) on cell surfaces of *P. shigelloides* was observed by scan electron microscopy (SEM). From these MVs the LCE toxin was extracted and confirmed by biological and serological assays. These data suggest that *P. shigelloides* also exports this cytotoxic-enterotoxin by membrane vesicles, a different mechanism of delivering extra cellular virulence factors, so far not described in this bacterium.

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Introduction

Plesiomonas shigelloides is an aquatic microorganism recognized as human and animal enteropathogen by epidemiological evidence.^{1,2} Despite the existence of several reports regarding the detection of enterotoxic and cytotoxic activities,

the virulence mechanisms related to the enteric infections are not yet completely defined.³⁻⁵ Sanyal et al.⁶ described enterotoxicity in live cultures and culture filtrates of *P. shigelloides* in rabbit ileal loop test, where the culture filtrates caused dislodgement of CHO and mouse adrenal tissue-culture cells. Its enterotoxicity was not affected when heated at 121 °C for 10 min. Gardner et al.⁷ described the production of a

* Corresponding author.

E-mail address: tyano@unicamp.br (T. Yano).

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heat-labile cholera enterotoxin-like protein and Okawa et al.⁸ described a thermostable cytotoxin, which is composed of three LPS-binding proteins, serologically related to the cholera enterotoxin. Falcón et al.² described that *P. shigelloides*, isolated from water, produced a heat-labile vacuolating activity over several cell line cultures, provoking enterotoxic activity, in suckling mouse test. The initial objective of this study was to understand the virulence factors produced by this strain (*P. shigelloides* 9P3-1).

It is known that bacteria outer membrane is not only a surface component that keep them in contact with the environment but it is also responsible for controlling the flux of nutrients what must enter or leave the microorganism cell. The outer membrane can release membrane vesicles (MVs) which are used as vehicle for or a virulence factor itself. These MVs are formed by the bulging in any part of the membrane structure, resulting in spherical fragments of different sizes, which are spread in the environment. More than presenting outer membrane components, as phospholipids, proteins, and lipopolysaccharides (LPS), they contain periplasmic contents.^{9,10}

A wide variety of Gram-negative bacteria are known to release MVs, which contain enzymes, toxins, adhesins, and DNA, mainly in some genera like *Escherichia*, *Bacteroides*, *Haemophilus*, *Pseudomonas*, and *Neisseria*.^{9,11-13} Hence, the formation of vesicles, which act as vehicles for carrying toxins, is thought to be an important virulence factor in these pathogens. STEC (Shiga toxin-producing *Escherichia coli*) releases MVs containing shigatoxin. Furthermore, MVs produced by *E. coli* O157:H7 strains may contain LPS, proteins, plasmids, linear DNA with virulence genes as *eae*, *stx1*, *stx2* and *uidA*, as well as antibiotic resistance genes, demonstrating that vesicles act delivering genetic material and toxins to other microorganisms.^{14,15}

In this work, we demonstrate that *P. shigelloides* (9P3-1) isolated from river water in Brazil exports a lethal cytotoxic-enterotoxin (LCE) by membrane vesicles. These results suggest that vesicles play an important role on the spreading of LCE to host cells during the bacterial infection.

Materials and methods

The following study was approved by the Institutional Committee for Ethics and Care in Animal Research (State University of Campinas – UNICAMP) which certifies that the protocol No. 919-1 is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA).

Bacteria and culture conditions

Among seven strains of *P. shigelloides* isolates from river water,¹⁶ the strain 9P3-1 (O4:H3) was selected for presenting the major reciprocal activity titer on Vero and CHO cells. For exotoxin production, *P. shigelloides* strains were grown in Luria Broth (LB, Difco Lab.) in a shaker at 150 rpm min⁻¹ (New Brunswick Scientific Co) for 14 h at 37 °C.

Purification of LCE (lethal cytotoxic-enterotoxin)

Culture supernatants were concentrated 6 × 10² times from its initial broth volume, using XM-300 and XM-50 ultra-filtration membranes (Amicon, Millipore). The concentrates were submitted to Cu²⁺ ion metallic affinity chromatography (IMAC) gel packed chromatography column (AKTA FPLC, GE-Amersham Healthcare), equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl, eluted with a 0–0.5 mM linear imidazole gradient.¹⁷ The chromatographic fractions were monitored by cytotoxic activity assays on Vero cells.² Fractions presenting cytotoxic activities were concentrated again by ultrafiltration membranes and applied to chromatography column Superdex 75 HR 10/30 (GE-Amersham Healthcare), equilibrated with 20 mM Tris-HCl buffer, pH 8.0 (Sigma) in a flow of 0.05 mL/min. The LCE purification was confirmed by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions¹⁸ with 10% polyacrylamide gels, using molecular-weight markers (Sigma) and by bidimensional (2D) SDS-PAGE analysis. Protein determination was measured using Bradford Bio-Rad Protein assays, according to manufacturer's instructions.

Cytotoxicity assays

Cytotoxic activity was tested according to the methodology described elsewhere.¹⁹ Vero (African green monkey kidney), CHO (Chinese hamster ovary) and CACO-2 (human epithelial intestinal cell line) (ATCC, Rockville, MD, USA) cell lineages were grown in Eagle's MEM (Nutricell, Campinas, SP, Brazil) supplemented with 10% (v/v) of calf serum (BCS).

Cellular viability assays

The viability assays were performed as previously described,²⁰ using neutral red as indicator. In this assay, the lethal cytotoxic dose (CD₅₀) was calculated as described by Welkos et al.²¹ Lactate dehydrogenase (LDH) assays for verification of cellular integrity were performed according to the methodology proposed by Mitchell et al.²² The kind of cell death induced by the pure cytotoxin was verified by flux cytometry analysis assay (annexin V-FITC Apoptosis Detection Kit I; BD Biosciences Pharmingen). Fluorescent Actin Staining (FAS) assay was performed as previously described using soluble Rodamine – FITC Labeled (Sigma) conjugated with Fluorescein.²³

Enterotoxic activities

Suckling mice test assay

Suckling mice (neonatal BALB/c 2–3 days-old) assay was performed for enterotoxin activity accordingly to a methodology described earlier.²⁴ After 3 h, mice were killed in a CO₂ chamber. Positive results were considered when ratios of intestinal weights (IW) to the remaining body weight (BW) was ≥0.08. Assays were conducted in quadruplicates.

Rabbit intestinal loop assay

The rabbit intestinal loop test was performed as described by Evans et al.²⁵ New Zealand white male rabbit weighting 1.5–2.0 kg were not fed for 24 h and sedated with Phen-tanyl (0.005 mg/kg) and Droperidol (1 mg/kg) (Janssen-Cilag

Table 1 – CEL lethal activity in mice.

Dose μg	Time/death Intraperitoneal	Dose μg	Time/death Intravenous
5.5	2 h	4.04	Instant
2.7	5 h	2.02	Instant
1.3	8 h	1.01	Instant
0.6	14 h	0.50	1 min
0.3	18 h	0.25	1.7 min
0.1	18–20 h	0.12	2.5 min
5.5 ^a	–	4.04 ^a	–
LB ^b	–	LB	–

^a CEL heated (56 °C for 15 min) did not cause death.
^b LB – culture medium as a control.

Farmacêutica Ltda) by intramuscular injection. Subsequently, the rabbits received an epidural analgesia with Morphine (0.1–0.3 mg/kg) (Lablesfal) and anesthetized by intramuscular injection with Zoletil anesthetic (20–30 mg/kg) (Virbac). Morphine maintained the animal under analgesia during all the experiment. Rabbits were kept for 18 h at room temperature and sacrificed thereafter. The fluid accumulation in the intestinal loop was measured as the ratio between the weight of the loop (in grams) and length (in centimeters). Ratios over 0.2 g/cm were regarded as positive. Assays were performed in triplicates.

Lethal activity

Groups of five adult females (5–6 week-old) BALB/c mice weighing ca. 20 g (CEMIB, UNICAMP, Brazil) were inoculated intraperitoneally and intravenously (Table 1) and were observed for periods of seven days (at every 15 min during the first hour of experiment and monitored periodically every 12 h afterwards). Assays were conducted in triplicates.

Serological analysis

Serum anti-LCE production

Specific anti-LCE antiserum was prepared from rabbits weighing ca. 3 kg. A 100 μg mL⁻¹ dose of purified LCE (neutralized by heating 100 °C during 15 min) was emulsified in an equal volume of Freund's complete adjuvant (Difco) and injected intramuscularly next to the popliteal ganglia. This procedure was repeated 15 days later and two other injections with the incomplete Freund's adjuvant administered at 45th and 60th day. Two weeks after the last injection, the animals were bled.

Serum neutralization tests

Serum neutralization tests were performed by adding a serial two-fold dilutions of anti-LCE to antisera anti-LT-I, anti-CT (kindly supplied by Dr. L.C. Ferreira, ICB-USP, SP, Brazil) and anti-Aerolisín.²⁶ All antisera were diluted in PBS (1/1024 maximum dilution) and applied to 96 wells in microtiter plates. The purified LCE (2 × CD₅₀) was added to the dilutions and after 60 min incubation at 37 °C, 0.1 mL of each mixture were transferred to the Vero cells monolayer grown in 96 wells microtiter plates. Plates were incubated at 37 °C and observed for morphological alterations for up to 72 h. The same tests

were conducted with the contents obtained from the bacterial membrane vesicles. All the assays were performed in triplicate.

Membrane vesicles (MV)

Isolation of membrane vesicles

MVs were obtained according to the methodology described by Kadurugamuwa.²⁷ *P. shigelloides* were cultured in 10 mL of Luria-Bertani Broth (Difco Lab., USA) with shaking at 150 rev min⁻¹ (New Brunswick Scientific Co.) for 24 h at 37 °C. Cultures were centrifuged at 7.700 × g for 15 min, at 4 °C. and supernatants were filtered through 0.22 μm polyvinylidene difluoride membranes (Millipore Co., Bedford, MA, USA) to remove residual cells and cellular debris. Then, filtrates were ultra-centrifuged at 150,000 rpm for 3 h at 4 °C with a 70 Ti rotor (Beckman Instruments, Inc., Fullerton, CA, USA) and MVs were then recovered from the pellet.

Polymyxin B treatment for membrane vesicles disruption

Pellets of MVs obtained by ultracentrifugation were suspended with 1 mL solution of phosphate buffer saline (PBS) with polymyxin B at a final concentration of 10,000 IU mL⁻¹. Suspensions were incubated at 37 °C for 1 h to disrupt membrane vesicles. After being centrifuged at 15,000 rpm for 15 min at 4 °C, the obtained supernatants were filtered using 0.22 μm membrane and applied to confluent monolayer Vero cells for cytotoxic activity, suckling mouse assay for enterotoxicity detection, and for mice lethal activity assay.

Electrophoresis SDS-PAGE of MVs contents

After ultracentrifugation, pellets containing MVs were suspended in 200 μL of 10 mM Tris-HCl buffer (pH 7.4) and the suspension was precipitated with 10% trichloroacetic acid (TCA). After this process, the suspension was centrifuged at 15,000 rpm for 15 min at 4 °C in order to obtain the supernatants. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE was performed with the obtained supernatant according to the methodology previously described using a 10% polyacrylamide (Bio-Rad Laboratories, Hercules, CA) and 0.1% SDS gel (Merck) under reducing conditions.¹⁸

Immunoblot analysis of LCE toxin obtained from MV

For immunoblot, the electrophoresed proteins were transferred to nitrocellulose membranes Hybond-ECL (GE Healthcare Bio-sciences, São Paulo-SP, Brazil) by the method proposed by Towbin.²⁸ Membranes were blocked in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.5) with 5% of non-fat milk for 2 h at room temperature and subsequently incubated with the same buffer with 1% of non-fat milk containing the antiserum anti-LCE (diluted 1:500 with TBS) for 2 h at 37 °C. The membrane was washed four times (10 min each) with TBS-T, and finally incubated with a secondary antibody (peroxidase-conjugated anti-rabbit IgG antibody) (Sigma) (1:1000) in TBS-T buffer with 1% non-fat milk for 90 min at 37 °C. After incubation, the membrane was washed four times with TBS-T. Peroxidase activity was

revealed by chemiluminescent substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Scan electron microscopy (SEM)

Vero cells were cultured on coverslips, infected with bacterial cultures of *P. shigelloides* and incubated at 37 °C for 30, 60 and 120 min, fixed in a solution containing 50% of 2.5% glutaraldehyde (Electron Microscope Science, USA) diluted in 10 mM Cacodylate buffer, pH 7.4 and 50% of cellular culture medium. After this first step, cells were fixed in a solution of 2.5% glutaraldehyde in 10 mM Cacodylate buffer, pH 7.4. Slides were dehydrated by graded ethanol series, dried in a critical point dryer CPD 030 (Bal-Tec Balzers) with CO₂ at 5 °C under 50 mbar pressure, covered with gold-palladium (25 nm) (40 mA – 98 s) in a Sputter coater – SCD 050 (Bal-Tec – Balzer). Preparations were observed using a Scan Electron Microscope SEM (FEI Quanta 200 – with X-ray probe).

Results

Purification of the LCE

The lethal cytotoxic-enterotoxin (LCE) produced by *P. shigelloides* (9P3-1) was purified from the culture supernatants by chromatographic processes through Cu²⁺ ion metallic affinity chromatography (IMAC) and a molecular exclusion column. Conventional SDS-PAGE and bidimensional SDS-PAGE (2D gel electrophoresis) resulted in a single protein band of 50 kDa. Based on electrophoresis 2D analysis the cytotoxic-enterotoxin presents an isoelectric point (pI) around 6.9 (Fig. 1).

Cytotoxicity assays

Culture supernatants and the purified LCE induced cytotoxic effects on CACO-2, CHO and Vero cells (Fig. 2). Cells showed an evident and extensive vacuolation, and a change from the normal spindle shape to a round configuration, with nuclear condensation; swelling, detachment and gradual destruction of the monolayer indicating, cells death (Fig. 2).

Cells viability assays

Fig. 3 shows the data obtained by the CD₅₀ cellular test assay, where 50% of death cells as determined via NR assay, corresponding to 0.7 µg mL⁻¹ of the pure LCE in a two-hour assay according to a methodology described previously.²¹ Loss of cells viability by the LDH assay (Fig. 4) revealed that the purified LCE damaged the cellular membranes. FAS assay (Fig. 5) showed the formation of stress fibers, characterized by the training and thickening of actin filaments beams, responsible for the rearrangement of cytoskeleton, as well as rounding of cells, and retraction of cytoplasmic contents and extension of matrix filaments. The flux cytometry analysis assay (Fig. 6) demonstrated the cellular death resulting from apoptosis.

Enterotoxigenic activities

Culture supernatants and the purified LCE caused a hemorrhagic fluid accumulation in the suckling mouse assay with enterotoxigenic ratios over 0.16; in the rabbit ileal loop test, an

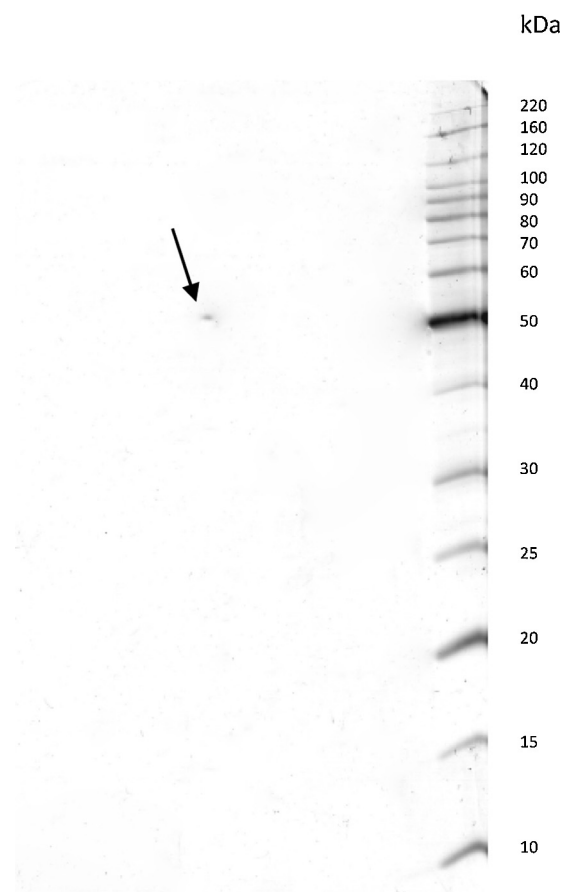


Fig. 1 – Bidimensional electrophoresis of *P. shigelloides* LCE. The revealed spot indicated by the arrow shows a protein of 50 kDa and isoelectric point (pI) of 6.8, indicating that this bacterium virulence factor can adequately be obtained and purified by chromatographic processes.

hemorrhagic mucus-like fluid was observed (25–30 mL). This activity was lost when the LCE was heated at 60 °C for 10 min, demonstrating that LCE acts as a thermolabile enterotoxin.

Lethal activity of LCE in mice

The purified LCE showed a potent lethal activity in four week-old mice, when received 5.5 µg mL⁻¹ injected intraperitoneally, causing death after 2 h (Table 1). Mice were prostrated, presenting bristly hair, and death. When LCE was injected intravenously, the lethal activity was very rapid, causing instantaneous death. The lethal dose determined in this test was 1.01 µg mL⁻¹ (Table 1).

Serological analysis

The cytotoxic activities of purified LCE were neutralized by homologous antiserum, presenting reciprocal titers of 1:1024, partially neutralized by anti-LT-1 produced by enterotoxigenic *E. coli* (ETEC) and antiserum anti-autolysin, with titers 1:256 and 1:32, respectively. However, no reaction was observed with anti-cholera toxin serum.

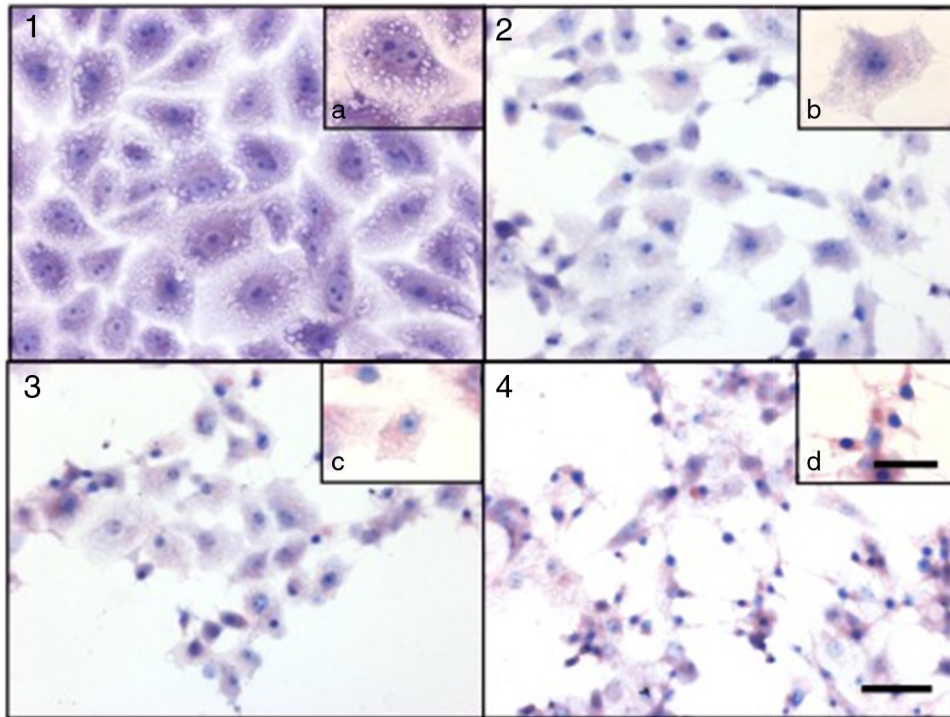


Fig. 2 – Cytotoxic effect on Vero cells evidenced by a May Grünwald-Giemsa stain. (1) Control cells; (2) 15 min of cytotoxic assay; (3) 30 min of cytotoxic assay; (4) 60 min of cytotoxic assay. Bars in the window 4.4 represent 40 μm and are valid for every big window (1-4) and 15 μm for the small windows (a-d).

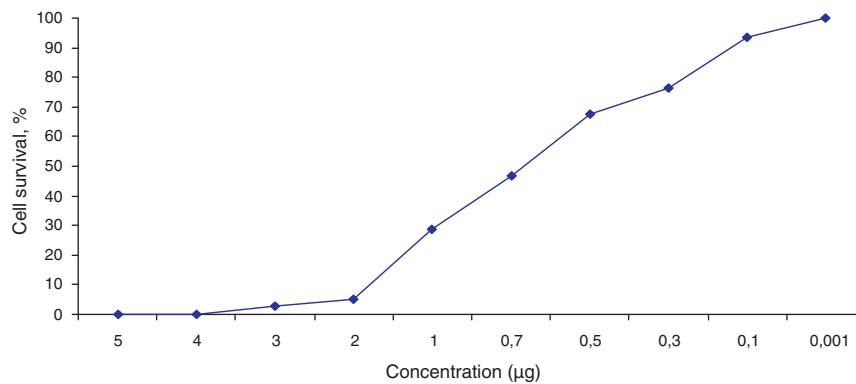


Fig. 3 – Cellular viability curve obtained through the NR assay with Vero cells treated with different cytotoxic protein concentration ($\mu\text{g mL}^{-1}$), during an exposure period of 2 h.

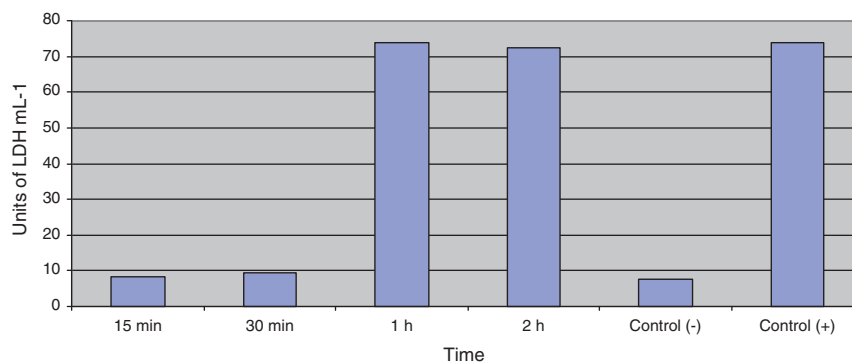


Fig. 4 – LDH activity kinetics as a function of time and exposure of Vero cells to LCE. Values presented were calculated from assay performed in triplicate.

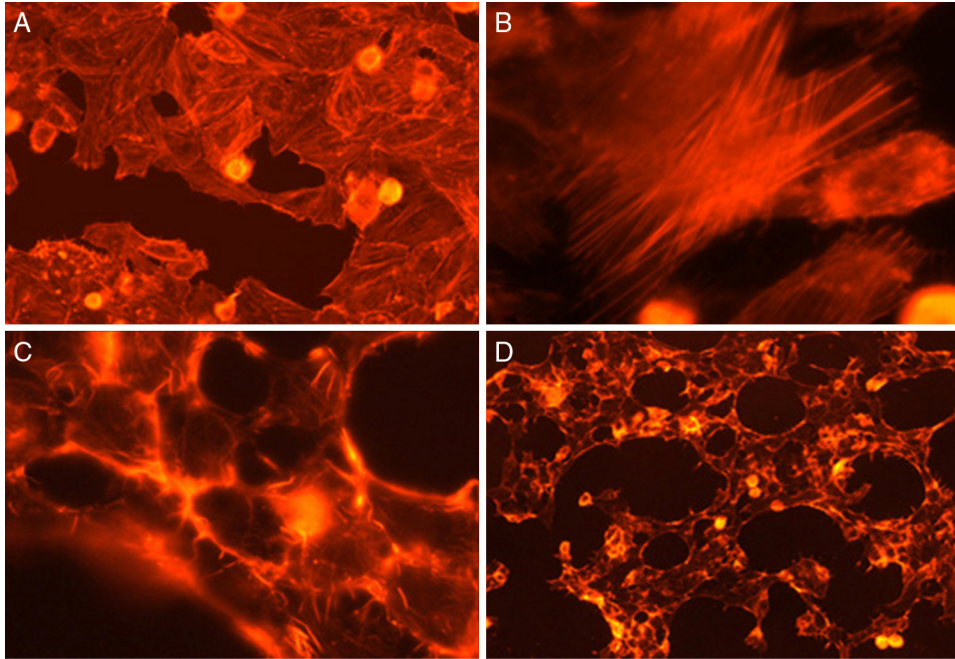


Fig. 5 – FAS assay on Vero cells show morphological and intracellular alterations. (A) Cell control; (B) formation of stress fibers; (C) rounding of cells; (D) dead cells.

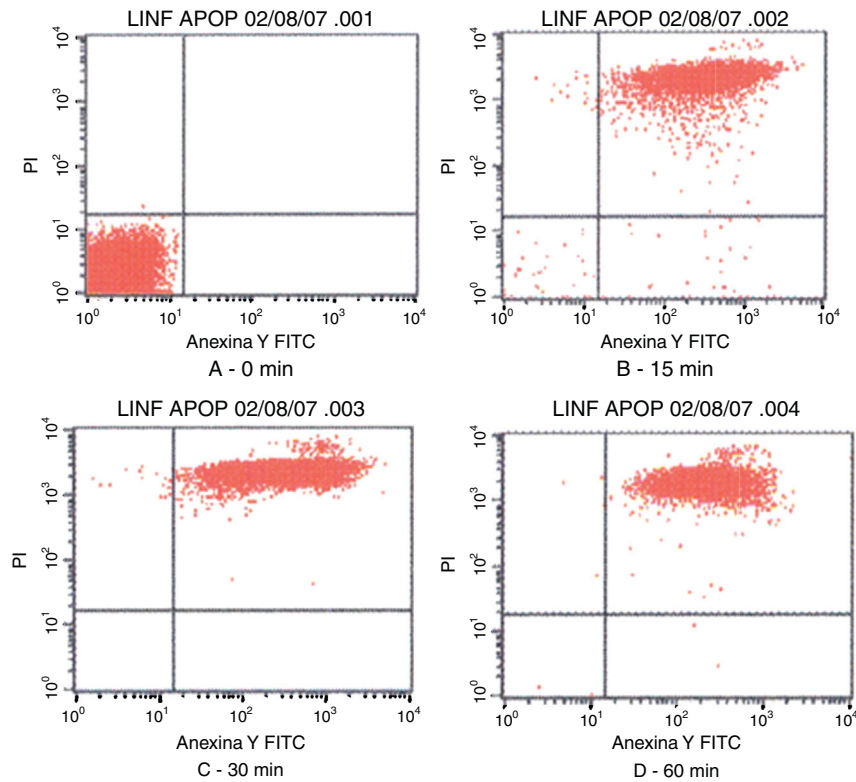


Fig. 6 – Contour diagram of Annexin V-FITC flux cytometry of human lymphocytes cultured by different time intervals (0, 15, 30 and 60 min) with LCF (5 μ g). The lower left quadrants of each panels show the viable cells, which are negative for PI and Annexin V-FITC binding. The upper left quadrants contain the non-viable, necrotic cells, Annexin V-FITC negative and PI positive. The lower right quadrants represent early phase of apoptotic cells Annexin V-FITC positive and PI negative. The upper right quadrants contain the non-viable, apoptosis cells, Annexin V-FITC positive and PI negative, indicating that they were in end stage apoptosis or already dead.

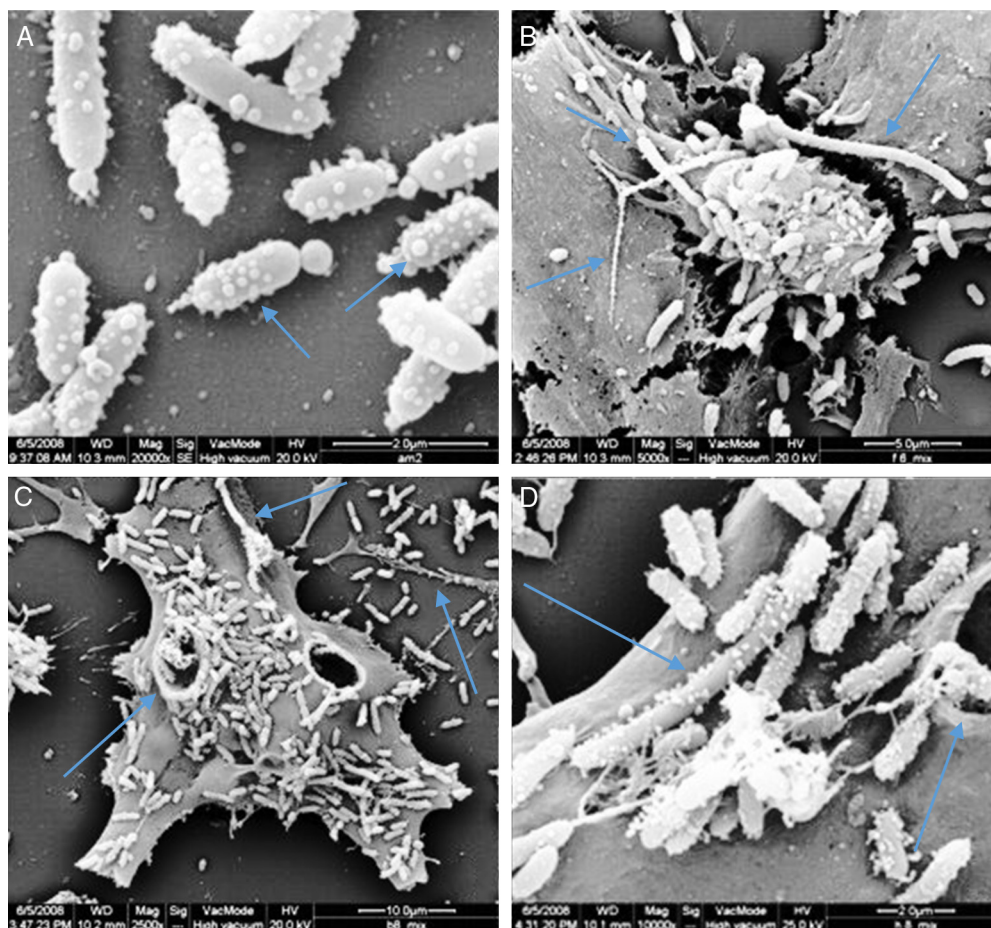


Fig. 7 – Scan electron micrograph shows many vesicles attached to the cells surfaces of *P. shigelloides* attached to Vero cells; blue arrow at A shows MVs forming several size; and the blue arrow in B shows the presence of super bacteria formation; the blue arrows at C shows bacteria invasion to Vero cells; the blue arrows at D shows super bacteria, invasion and MVs; yellow arrow at B and C shows MVs in strings.

Membrane vesicles (MVs) assays

Scan electron microscopy analyses showed the presence of many vesicles attached to the cell surface of *P. shigelloides*. Those vesicles presented spherical shape and diameters varying between 80 nm and 220 nm, predominantly the small ones. The presence of linked vesicles structuring a string of pearls-like were observed released by the bacterial cells (Fig. 7A). Some bacteria did not present signs of cellular division process, reaching 7.7 μm , similar to the described for *Proteus mirabilis*.²⁹

The SDS-PAGE analyses with MVs contents revealed the presence of many proteins, among them a 50 kDa protein, which was purified using the same methods applied for culture supernatants of *P. shigelloides*, presenting both cytotoxic and enterotoxic activities, confirming MVs as the origin of LCE.

Immunoblotting analysis of the MVs content revealed in presence of homologous rabbit antibody serum produced with the purified LCE. There was an immunological reaction against the 50 kDa structure corresponding to LCE protein band.

Discussion

Acute diarrhea is mainly caused by the increased intestinal secretion, commonly as a result of infection by enterotoxin-producing organisms like enterotoxigenic *E. coli* or *Vibrio cholerae*. Alternatively, it may result from decreased intestinal absorption due to infection by microorganisms, like enteropathogenic *E. coli*, *Shigella* sp. or *Salmonella* sp. that damage the intestinal epithelium.^{30,31} Studies have described *P. shigelloides* as an etiological agent in diarrheal diseases, and it has been demonstrated to produce various toxins that are detected free in culture supernatants.^{7,2}

The production of a hemorrhagic cytotoxic-enterotoxin by *P. shigelloides*, isolated from river water in Brazil,¹⁶ was previously studied by Falcón et al.² They reported fluid accumulation in the suckling mouse assay, vacuolating activity on cell culture assays, induced by thermolabile culture supernatant, since it lost activity after being heated at 60 °C for 15 min.

The LCE from culture supernatant of *P. shigelloides* was purified by chromatography using IMAC and a molecular exclusion

column. The 2D SDS-PAGE gel electrophoresis analysis determined that LCE presents an isoelectric point (IP) around 6.9 (Fig. 1). The purified LCE caused equal cytotoxic effects on CACO-2, CHO, and Vero cells (Fig. 2), by the NR assay presenting a CD_{50} of $0.7 \mu\text{g mL}^{-1}$ of pure LCE inducing the death of 50% of cells in 2-hour assay (Fig. 3) based in the methodology described earlier.²¹

Loss of cell viability by LDH assay (Fig. 4) was observed, revealing that LCE causes damage to cellular membranes. All cell assays displayed an evident and extensive vacuolation, followed by a change from the normal spindle shape to a round configuration and nuclear condensation; the cells became swollen and increased in degree of cell vacuolation followed by detachment and gradual destruction of the monolayer, and death (Fig. 2). In the last decades, some authors have related the cell vacuolation effect as indicative of bacterial virulence and pathogenicity.^{32,33} *V. cholerae* and *Helicobacter pylori* are known for their ability to produce vacuolating cytotoxins demonstrating the association between biological effects (vacuolation) and pathogenicity caused by these microorganisms.^{34,35}

For elucidating the pathogenic potential of the LCE, FAS assay was conducted to evidence the morphological and intracellular induced alterations by the formation of stress fibers, characterized by disorders over the actin filament beams, responsible for cytoskeleton rearrangement, cells rounding and retraction of cytoplasmic contents and extension of filaments (Fig. 5).

Flux cytometry analysis (Fig. 6) revealed that LCE not only induces cytoplasmatic vacuole formation but also provokes cellular death by apoptosis. Tsugawa et al.⁵ also observed the effect of cellular death by apoptosis of Caco-2 cells on cytotoxic assays.

Fluid accumulation in intestinal ileal loops occurs due to the presence of enterotoxins. Sanyal et al.⁶ demonstrated *P. shigelloides* ability to provoke enteritis in test with live cultures and animal, with positive results in rabbit intestinal loop with cultures filtrates.

Culture filtrates were also positive on the suckling mouse assays, and the activity persisted after heating the supernatant at 121°C for 15 min. However, Holmberg et al.³⁶ did not observe fluid accumulation in rabbit intestinal loops using culture filtrates from 31 clinical *P. shigelloides* isolates.

Our purified LCE of *P. shigelloides* caused a hemorrhagic fluid accumulation in the suckling mouse assay and hemorrhagic mucous-like fluid in the rabbit intestinal loop test (data not shown), an indication of the strong enterotoxigenicity of this protein. This activity was lost when the LCE was heated at 60°C for 10 min.

The purified LCE, when injected intraperitoneally, showed a potent lethal activity on four week-old mice, causing death after 3 h. Mice were initially prostrated and progressed to death; when intravenously injected the LCE caused death in a few seconds. The lethal dose of purified LCE was determined in the mouse model and calculated as $1 \mu\text{g mL}^{-1}$, inducing instantaneous and spasmodic death. Salvadori et al.³⁷ described a lethal toxin from the culture supernatant of *E. coli* isolated from chickens with swollen head syndrome, a lethal toxin similar to that of *Bacillus cereus*. In our study the

lethal activity was also heat-labile, since the LCE lost the lethal activity in mice after heating.

The cytotoxic activities of the LCE were neutralized by homologous antiserum but were not neutralized by the anti-cholera enterotoxin (CT) while it was partially neutralized by anti-LT-1 produced by enterotoxigenic *E. coli* (ETEC) (data not shown).

The LCE associated to MVs of *P. shigelloides* was observed after 24-hour incubation; however, the quantity of free culture supernatant toxin detectable was very low. Scan electron microscopy (SEM) analysis showed the presence of many vesicles attached to the cell surface of *P. shigelloides* (Fig. 7). The vesicles presented spherical shape and diameters varying between 80 nm and 220 nm, and the smaller vesicles were the most prevalent. Vesicles linked, as a string of pearls, were observed released from bacteria cells (Fig. 7A). We also observed structures known as super-bacteria (Fig. 7B-D) associated to bacterial growth, reaching maximum sizes of $7.7 \mu\text{m}$ without any sign of cellular division.

Vesicles production was a phenomenon observed in *P. shigelloides* grown under prolonged time conditions in adhesion assays, and were detected by SEM. The role attributed to the vesicles is a virulence mechanism similar to periplasmic enzyme, which is exported by STEC O157 and *Pseudomonas aeruginosa*^{11,15} or used for DNA transportation¹²⁻¹⁴ or as a bacterial virulence factor like adherence and evasion of the immune system,¹⁰ and also as a component for biofilms matrices.³⁸ Despite the data presented in some studies, the role of vesicles in transportation of different bacterial products and components has not yet been carefully analyzed. Nonetheless, these data suggest that vesicles formation by *P. shigelloides* represents an effective mechanism for the transport of toxin by this pathogen.

The hypothesis that the MVs are related to bacteria invasion to tissues cannot be ruled out. The bacterial invasion mechanisms into epithelial tissue are still not well understood. However, Grenier and Mayrand³⁸ mentioned in their studies that vesicles, with their high proteolytic activity, may possibly be implicated in the subsequent epithelium penetration by the bacteria. Our observations showed that MVs are released in a great number from the bacteria cell surfaces, and their small size would enable them to cross anatomical barriers that are, otherwise, impermeable to bacterial cells. MacDonald and Beveridge³⁹ in a study with *P. aeruginosa*, showed the importance of MVs attachment and predatory activities, and proposed that MVs play a key role in providing competitive advantage during bacteria growth.

SEM showed that most vesicles released from *P. shigelloides* are similar in size and morphology. The vesicles were observed to be close to spherical shape and ranging from 80 to 220 nm in size, in contrast to the vesicles observed by Dutta et al.⁴⁰ in *Shigella dysenteriae* type 1 strain after Mitomycin C addition to vesicles effectively released. Vesicles produced by *P. shigelloides* are comparable in size to those produced by other pathogens like *E. coli* O157:H7,¹⁴ *P. aeruginosa*,⁴¹ *Neisseria gonorrhoeae*⁴² and *Bacteroides gingivalis*.³⁸

Examined with SEM some *P. shigelloides* did not conclude the cellular division processes, reaching cellular sizes next to the ones presented by *P. mirabilis* (swarming) for example, which is considered a super bacterium.²⁸ Kadurugamuwa

and Beveridge,⁴¹ suggest that *P. aeruginosa* is producer of VMs that contain most of the metabolites necessary for cellular natural division (with autolysins). These observations showed that the deviation of such metabolites to MVs occur probably simultaneously, underlying periplasmic components (including autolysins) that would be trapped within the blebs. When the bacteria are supposed to adhere to host cells the production of an increased number of predatory MVs may be advantageous. Therefore, it would be a peak of cellular demand during adhesion not only for autolysins to be used in cell division, but also for predatory vesicles.⁴³

The SDS-PAGE analysis performed with the MVs content showed the presence of many proteins, including a 50 kDa protein, which was abundant in the MVs and was purified from the *P. shigelloides* culture supernatant, presenting in both cases cytotoxic and enterotoxic activities. This toxin showed to be different from the toxin reported by Okawa et al.,⁸ which was described as a complex of LPS binding proteins, with molecular masses higher than 600 kDa.

The immunoblotting analyses were conducted with the MVs contents and rabbit antibody serum produced with purified LCE obtained from cultured supernatants. The immunological reaction occurred against the 58 kDa structure. The band obtained suggests that the biological activities induced by the cytotoxic assays were due to the same enterotoxin, which can be exported from the bacteria cell and also by MVs. A possible consequence of this characteristic is that vesicles could evade antibodies action, thus avoiding the specific antibacterial host immune defenses.⁴⁴

To our knowledge, this is the first report showing that *P. shigelloides* released via membrane vesicles is a lethal cytotoxic enterotoxin. It is interesting to speculate which mechanisms are leading these bacteria cells to the formation of these MVs during cellular growth *in vitro* as well as the mechanisms for their action *in vivo*.

The LCE here described might be a putative intrinsic virulence factor of *P. shigelloides* isolated from river water in Brazil. Further studies are being conducted, in order to better understand the mechanisms of the enterotoxic, cytotoxic, and lethal activities induced by the LCE.

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Conflicts of interest

The authors declare no conflicts of interest.

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