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DIARRHEA OUTBREAK IN PERNAMBUCO, BRAZIL, ASSOCIATED WITH A HEAT-STABLE CYTOTOXIC ENTEROTOXIN PRODUCED BY *Aeromonas caviae*

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SUMMARY

In the present study enterotoxic and cytotoxic activities of twenty *Aeromonas caviae* strains were examined. They originated from fecal specimens of patients with acute diarrhea during an outbreak in Brazil in 2004. Culture supernatants of fourteen strains (70%) caused fluid accumulation in rabbit ileal intestinal loops and in suckling mice assays, and also showed a cytotoxic activity in Vero and Caco-2 cells. The enterotoxic and cytotoxic factors were heat-stable after culture supernatants treatment at 100 ºC. The results revealed that *A. caviae* strains produce a putative diarrheagenic virulence factor, a heat-stable cytotoxic enterotoxin that could be linked to the diarrhea outbreak that took place in Brazil.

KEYWORDS: Diarrhea outbreak; *Aeromonas caviae;* Cytotoxic enterotoxin; Virulence factors.

INTRODUCTION

Aeromonas spp. are Gram-negative rods that belong to the *Aeromonadaceae* family, which are widely spread across nature and can be found in a great variety of places including salt and drinking water, soil, sewage systems and uncooked or refrigerated foods[6]. *Aeromonas* is considered to be a common pathogen of fishes and following the 1970s these microorganisms have also been considered to be human pathogens[8,13]. Acute diarrheal disease after the ingestion of contaminated water and food is common in many countries[9]. The virulence factors produced by these species, such as enterotoxins, hemolysins, cytotoxins, and adhesins, are the main determinants of human enteropathological processes[6].

In 2004, there was an outbreak of acute diarrhea in Sao Bento do Una, Pernambuco State, Brazil[5], in which *V. cholerae* O1 and *Aeromonas* ssp. in ISR 16S-23S and RAPD amplification were recovered; all *V. cholerae* revealed homogeneous profiles and presence of potential virulence genes, however, it was observed that *Aeromonas* spp. were highly heterogeneous**.** It was concluded, then, that *V. cholerae* O1 was probably the responsible agent for the diarrhea outbreak. Nevertheless, the participation of *Aeromonas* was not ruled out[11].

Therefore, the present study aimed to verify the possible diarrheagenic virulence factors of *A. caviae*, because it is the most prevalent specie (ca. 41%) of all the isolated *Aeromonas* spp.[11].

Twenty *A. caviae* were cultivated in Müller Hinton broth (Difco Lab. USA) with the aid of a shaker, at 37 ºC for 16 h. The culture supernatants were analyzed for enterotoxic activity by the suckling mice assay[1]**.** Three neonatal Balb/C mice, two to four- days-old, were used for each sample. A 50 µL volume of each sample was administered to each mouse, intragastrically. After three hours, the mice were executed following recommended procedures and the whole intestines were removed. Both the intestines and remaining corpse were weighed to calculate the ratio between the intestines (I) and the remaining corpse (B): I/B. Assays were performed in sets of three. Results were considered positive when the ratio results were ≥ 0.09[1].

Additionally, a rabbit intestinal loop test[2] was conducted using white adult male New Zealand rabbits weighing 1.5-2.0 kg. The intestinal lumen was rinsed three times with saline solution, then, series of intestines were exteriorized through a midline incision; ligated intestinal segments (loops) of about 5 cm of length, separated by a 1 to 2 cm interloop. Each loop was filled with 1 mL of the sample solution. A control loop injected with sterile culture medium was included. Rabbits were maintained for 18 h at room temperature following euthanasia. The fluid accumulation in the intestinal loops was measured as the ratio between the weight of the loop (in grams) and length (in centimeters). Ratio results > 0.2 were regarded as a positive response[2]. The assays were performed in sets of three. Sterile culture medium (Müller Hinton broth) was used as a negative control loop and the positive control was the cultured supernatant of *A. veronii* biovar *sobria* AS 31[10].

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

List of Abbreviations: *A. caviae, Aeromonas caviae; A. hydrophila, Aeromonas hydrophila; A. veronii, Aeromonas veronii; V. cholerae, Vibrio cholerae;* MEM, minimum essential medium; FBS, fetal bovine serum.

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The culture supernatants that were positive in the suckling mice assay, meaning 70% (14 of 20), were also positive in the rabbit intestinal loop test[2], confirming the enterotoxic activity of *A. caviae* isolates.

The cytotoxic activity of *A. caviae* culture supernatants was detected as described[7] in Vero (African green monkey kidney), Caco-2 (human intestinal carcinoma), HeLa (human uterus carcinoma) and HEp-2 (human larynx carcinoma) (ATCC, Rockville, MD, USA) cell lines cultivated in tissue culture flasks with Eagle's minimum essential medium (MEM, Nutricell, Campinas, SP, Brazil) supplemented with 10% (v/v) of fetal bovine serum (FBS). The filter-sterilized culture supernatants were inoculated in a confluent monolayer (serial two fold dilutions). Then, the plates were incubated at 37 ˚C in a 5% CO_2 chamber. The culture filtrates of *A. sobria* AS-69, a non cytotoxin-producing strain[10], and MEM medium were used as negative controls. Cell monolayer morphology was observed using an inverted microscope.

The same *A. caviae* culture supernatants, in which enterotoxic activity was observed, induced morphological alterations in Vero and Caco-2 cells after three hours of culture filtrates inoculation, inducing cellular elongation, rounding, loosening of intercellular junctions and detachment were observed, leading to cellular death (Fig.1). These observations resemble those previously described[4], in which bacterial suspensions of *A. hydrophila*, *A. veronii* biovar *sobria* and *A. caviae* were assayed in cultured Vero and CHO cells. Despite the similarities of the cytotoxic effects, injuries and destruction induced on cells, we observed that the cytotoxin present in our samples was found free in the cultured supernatants.

The cytotoxic and enterotoxin activities of the *A. caviae* culture supernatants were not affected by heat treatment at 60 ºC and 100 ºC for 15 min. These assays demonstrated that the cytotoxic-enterototoxic activity produced by these *A. caviae* is heat-stable.

The description of a heat-stable cytotoxic activity produced by *A. caviae* in HEp-2 cells has already been described[12], but our culture supernatants did not exhibit cytotoxic effects on HEp-2 or HeLa cells (data not shown). The most intense effects were observed in Caco-2 cells, a human intestinal cell lineage, reinforcing its specific activity in intestinal cells in these *in vitro* assays.

Therefore, this work reveals the expression of a heat-stable cytotoxic

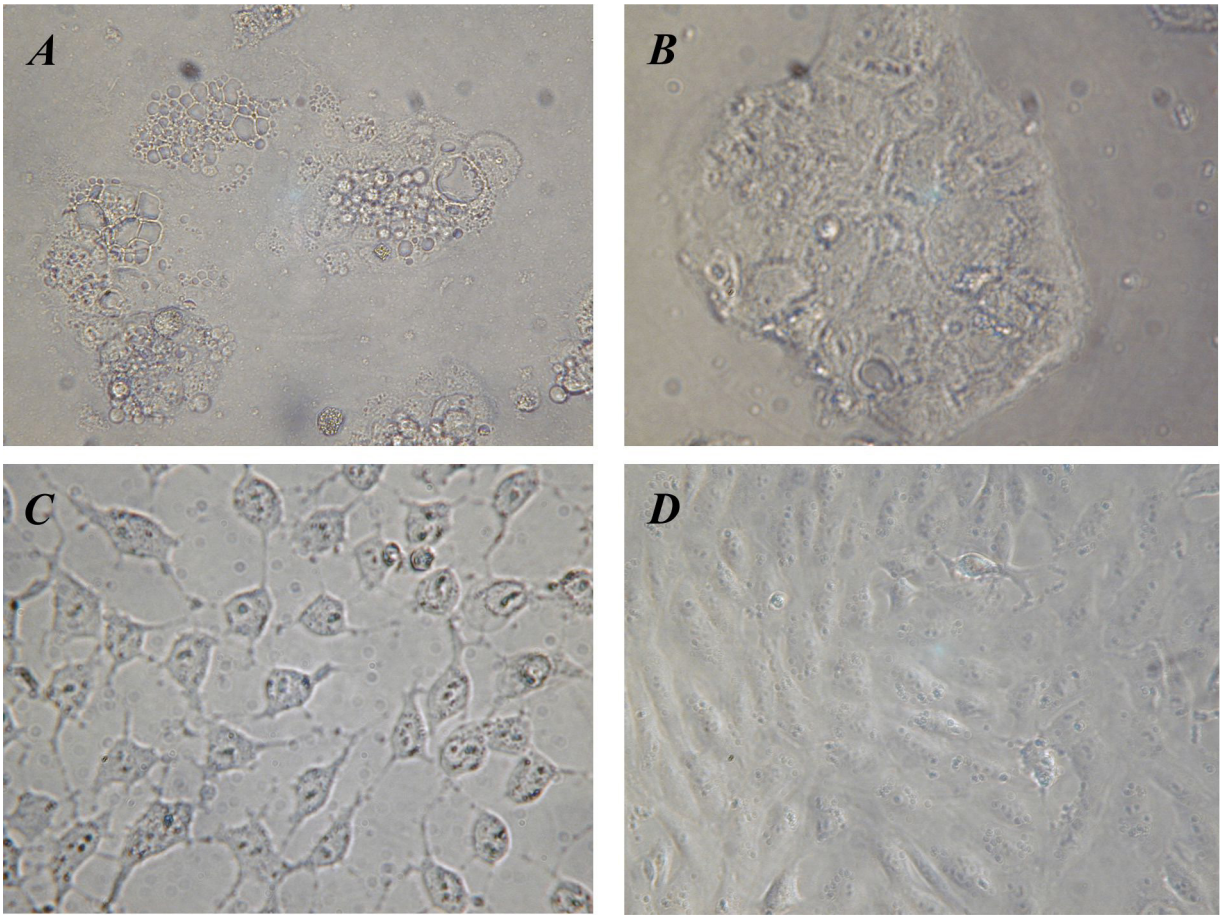

Fig. 1 - Aspects of the cytotoxic effects of *A. caviae* cultured supernatants submitted to heat-stability treatment in Caco-2 and Vero cells. **A -** Cytotoxic assay with heat-treated culture supernatants of *Aeromonas caviae* in Caco-2 human intestinal cells, indicating cellular vacuolation and monolayer destruction after 20 min. After 20 h, the monolayer was completely destroyed and detached. **B -** Caco-2 control cells. **C -** Vero cells treated with the heat stable enterotoxic cytotoxin exhibited rounding, cell-to-cell leakage of cellular membrane junctions and nuclear condensation. **D -** Vero control cells. Magnifications 430X.

enterotoxin by *A. caviae* that could be associated with the diarrhea outbreak that took place in Pernambuco, Brazil. It could be a new diarrheagenic virulence factor not yet described among the *Aeromonas* species. The purification of this heat-stable cytotoxic enterotoxin is currently being conducted to identify its chemical nature, aiming to elucidate its role in the enteropathogenic activities induced by *A. caviae*.

RESUMO

O surto de diarreia em Pernambuco, Brasil é associado com uma enterotoxina citotóxica termo-estável produzida por *Aeromonas caviae*

Em 2004 ocorreu um surto de diarreia aguda no Estado de Pernambuco, Brasil. Setenta por cento (14 dos 20) dos sobrenadantes de cultura de *Aeromonas caviae,* isoladas neste episódio induziram acúmulo de líquido em testes de alça ligada de intestino de coelhos, assim como em teste em camundongos recém-nascidos. Os mesmos sobrenadantes mostraram também atividade citotóxica em células de Vero e Caco-2, mas não em células HeLa e HEp2. As atividades enterotóxicas e citotóxicas mantiveram-se mesmo após o aquecimento a 100 ºC dos sobrenadantes de cultura. Este trabalho revela a expressão de um provável fator diarreiogênico: uma enterotoxina-citotóxica termo-estável, produzida por *A. caviae* que pode ser associada ao surto de diarreia ocorrido no Brasil. Atualmente estamos purificando esta enterotoxina termo-estável, com o objetivo de elucidar seu papel como fator de virulência na diarreia causada por *A. caviae.*

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DISCLOSURE

The authors have no conflicts of interest to declare.

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