Evaluation of *Escherichia coli* STb toxin in an *in vitro* organ culture model (IVOC)

Avaliação de toxina STb de *Escherichia coli* em modelo de cultura de órgãos *in vitro* (IVOC)

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ABSTRACT
ETEC pathogenesis comprises adhesion to the small intestine, colonization and enterotoxin production. STb is one of the heat-stable toxins produced by ETEC. Previous reports, using culture supernatants of STb-positive ETEC strains, indicated loss of villous absorptive cells causing mild atrophy and microscopic alterations in jejunum mucosa. These culture supernatants contained many other compounds beside STb toxin and in addition the quantity of toxin in these supernatants was not determined. Thus, this study was undertaken to evaluate the effect of pure STb toxin on piglet jejunum explants in an *in vitro* organ culture (IVOC) model. Tissues of piglets of 11 weeks were used and put into culture. Morphometric analysis of tissues revealed that villous epithelial area was reduced in tissues treated with pure STb after 3, 4 and 5 hours ($p < 0.0001$) compared to untreated tissues. STb-treated tissues presented atrophic villi due to loss of villi tip. Our data shows that, in piglet jejunum explants, pure STb toxin can lead to cell modification and consequently to destruction, seen as villi atrophy. These changes result in a reduced absorptive area and could be partly responsible for the diarrhea observed in the animal following STb intoxication.

Keywords: *Escherichia coli*, ETEC, STb enterotoxin, IVOC model, piglet jejunum

RESUMO
A patogênese de ETEC compreende adesão ao intestino delgado, colonização e produção de enterotoxina. STb é uma das toxinas termolábeis produzidas por ETEC. Trabalhos prévios, usando sobrenadantes de cultura de cepas ETEC STb-positivas, indicaram perda de vilosidades de células absortivas causando atrofia e alterações microscópicas na mucosa jejunal. Estes sobrenadantes continham vários outros componentes além da toxina STb e, adicionalmente, a quantidade de toxina

nos sobrenadantes não foi determinada. Assim, este estudo foi delineado para avaliar o efeito da toxina STb pura em tecidos usando modelo de cultura de órgão *in vitro* (IVOC). Tecidos de leitões com 11 semanas de vida foram usados e adicionados em cultura. Análises morfométricas dos tecidos revelaram que a área do vilo epitelial foi reduzida em tecidos tratados com STb pura após 3, 4 e 5 horas ($p<0.0001$) comparado a tecidos não tratados. Tecidos tratados com STb apresentaram atrofia do vilo e perda da parte superior do mesmo. Nossos dados mostram que, em tecidos do jejuno de leitões, a toxina STb pura pode levar a modificações celulares e, consequentemente, destruição visualizada como atrofia do vilo. Estas mudanças resultam em área de absorção reduzida e podem ser parcialmente responsáveis pelo fenômeno de diarreia detectada em animais seguindo a intoxicação com STb.

Palavras-chave: *Escherichia coli*, ETEC, enterotoxina STb, modelo IVOC, jejuno de leitões

1 INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) cause traveler´s disease worldwide, principally in young children in developing countries (Kosek et al., 2003). ETEC can also cause infection in animals, with major concern in pigs and calves (Nagy and Fekete, 2005), affecting the economy of the industry with high animal morbidity and mortality (Zhang et al., 2007). Heat-labile and heat-stable enterotoxins have been implicated in ETEC pathogenesis in Man and animals. These enterotoxins are the main virulence factors causing diarrhea and can also play a role in tissue colonization (Zhang et al., 2006). ETEC pathogenesis comprises three finely orchestrated steps: i) adhesion to the small intestine, by fimbrial and afimbrial adhesins that enable bacteria to attach to receptors on the intestinal epithelium of the susceptible hosts; ii) tissue colonization and iii) toxin production and delivery.

ETEC can produce toxins that induce water and electrolyte secretion, resulting in severe dehydration and metabolic acidosis (Dubreuil, 2008). In swine, ETEC strains can express one or more of four enterotoxins, including heat labile (LT, *eltAB*), heat stable a (STa, *estA*), heat-stable b (STb, *estB*) and enteroaggregative *E. coli* heat-stable 1 (EAST1, *astA*) (Erume et al., 2008).

STb mature polypeptide comprises 48 amino acids containing four cysteine residues involved in disulfide bridge formation, with a Mr of 5,200 Da. Early studies (Burgess et al, 1978; Kennedy et al., 1984) reported that STb was active only in the piglet intestinal loop assay. Later on, it was shown that in mouse intestinal loops, purified toxin elicits a response after 30 min and fluid accumulation reaches a maximum after 3 h (Hitotsubashi et al., 1992). Some authors using culture supernatants of STb-positive isolates (Whipp et al., 1986, 1987; Rose et al., 1987) observed that STb-containing ETEC culture supernatants induced the loss of villous absorptive cells causing mild atrophy with microscopic alterations in pig jejunum mucosa.

The present study was undertaken to evaluate the effect of pure STb toxin on jejunum piglet explants in an IVOC model.

2 MATERIAL AND METHODS

STB TOXIN PRODUCTION AND PURIFICATION

STb toxin was produced according to the method of Gonçalves et al., (2007). An *E. coli* HB101 strain harboring the plasmid pMal-STb, which is responsible for the expression of a maltose-binding protein-mature STb fusion protein, was grown in Luria broth containing 50 μg of ampicillin/ml until the optical density (OD) at 600 nm reached 0.5. Then, 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added as an inducer of synthesis of the fusion protein. Three hours after induction, cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C. The pellet was gently resuspended in 500 ml of 30 mM Tris-HCl (pH 8.0) containing 20% (wt/vol) sucrose and 1 mM EDTA. After centrifugation at 8,000 × *g* for 10 min at 4°C, the cells were resuspended in 500 ml of 5 mM $MgSO_4$ and incubated at 4°C for 10 min. After centrifugation at 8,000 × *g* for 10 min at 4°C, the fusion protein was affinity purified from the supernatant representing the osmotic shock fluid by using an amylose resin (New England Biolabs, Pickering, Ontario, Canada). The cleaved material was loaded onto a C_8 reversed phase microbore column (Brownlee, Perkin Elmer, Shelton, CT, USA) using an Akta Purifier 10 system (GE Healthcare). STb was quantified spectrophotometrically at 214 nm using aprotinin as the reference protein. The purified STb toxin was lyophilized and kept at −20°C until use.

COLLECTION AND CULTURE OF PORCINE INTESTINAL EXPLANTS

Piglets of approximately 11 weeks were cared for in accordance with the Guidelines of the Canadian Council for Animal Care. The porcine intestinal IVOC model was used as previously described (Girard et al., 2005) with some modifications. Briefly, jejunum segments were obtained from colostrum-deprived young piglets from a conventional herd. Piglets were tranquilized with a mixture of 10 mg kg^{-1} of body weight of ketamine hydrochloride (Biomeda-MTC, Cambridge, Ontario, Canada) and 20 mg kg^{-1} of xylazine (Bayer, Toronto, Ontario, Canada) before being euthanized with an overdose of sodium pentobarbital (540 mg ml^{-1}; Faculté de Médecine Vétérinaire, Université de Montréal, Québec, Canada). The jejunum was carefully emptied of its content. From jejunum tissues, the serosa was carefully removed with a scalpel and mucus was gently removed with a sterile swab. Tissues were then cut into 10 mm^2 segments and placed mucosal side up onto biopsy foam pads (Curtin Matheson Scientific, Inc., Texas) in 24-well tissue culture plates (Sarstedt, Newton, NC). Complete RPMI 1640 medium containing 10% fetal bovine serum

(Gibco, NY), 0.25% lactalbumin hydrolysate, 0.2 μg ml^{-1} of hydrocortisone, 0.1 μg ml^{-1} of insulin, 1% D-mannose, 2 mM concentrations of L-glutamate and L-aspartate (Sigma Chemical Co., St. Louis, MO), and 1% penicillin/streptomycin (Gibco, CA, USA) was added to each well. Then, explants were treated with anti-trypsin (1 mg/mL in water) (Boehringer Mannheim). Non-treated tissues were included as controls.

Purified STb toxin (0,4 nM) was applied to the surface of mucosal jejunum explant. Non-treated tissues were used as negative controls. Then, tissues were incubated at 37 ^{o}C on a rocker platform in a 95% O_2/5% CO_2 atmosphere for 5 h. Then, the tissues were formalin-fixed, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin eosin (HE). Sections were examined using a light microscope. All experiments were done in triplicate. The data shown are the averages of the experiments conducted on three animals.

VILLI MEASUREMENT

A total of 25 well-oriented villi (per tissue block), taken at random, were observed at low magnification (10 x). The villi were measured (width, length and area) using an Axio Imager M1 with an Axio Cam HR microscope (Zeiss, Canada) coupled to the software program Axion Vision Rel 4.6.3. The photos from HE stained slides were taken using a charge-coupled device CoolSNAP camera (RS Photometrics, California).

STATISTICAL ANALYSIS

Statistical analyzes were done using a repeated linear model with tissue (treated versus non-treated) and time (4 levels) as within-subject factors. A priori contrasts were done to compare pairs of means. The average of the three measures for each individual animal at each time period was used. Statistical analyses were carried out with SAS v.9.2 (Cary, NC). Level of statistical significance was set at 0.05.

3 RESULTS

The potential of pure STb toxin to induce changes in pig jejunum in an IVOC model was assessed. This is the first description of the effect of pure toxin on jejunal piglet tissue in an *in vitro* explant model. Proteases already secreted into the intestines can be removed, to a certain extent, by washing (Hitotsubashi et al., 1992). Nevertheless, in our study, anti-trypsin was added, to assure inhibition of all trypsin-like activities that can be found in intestinal tissue. Addition of anti-trypsin did not alter the villi morphology (data not shown). However, under these conditions, we observed

modifications of STb-treated tissue explants when compared with non-treated ones (Figures 1, 2 and 3). This was not the case when anti-trypsin was omitted (data not shown).

The means length of treated and non-treated tissues did not differ at 0 hour (p=0.28) (Figure 1). For untreated villi, the length was the same as 0 hour over the time period retained for the experiment (p=0.13, p=0.39 and p=0.29 for 3, 4 and 5 hours, respectively). Treated and not treated tissues did not differ statistically at 0 (p=0.28) and 3 hours (p=0.053) but did at 4 and 5 hours (p=0.002).

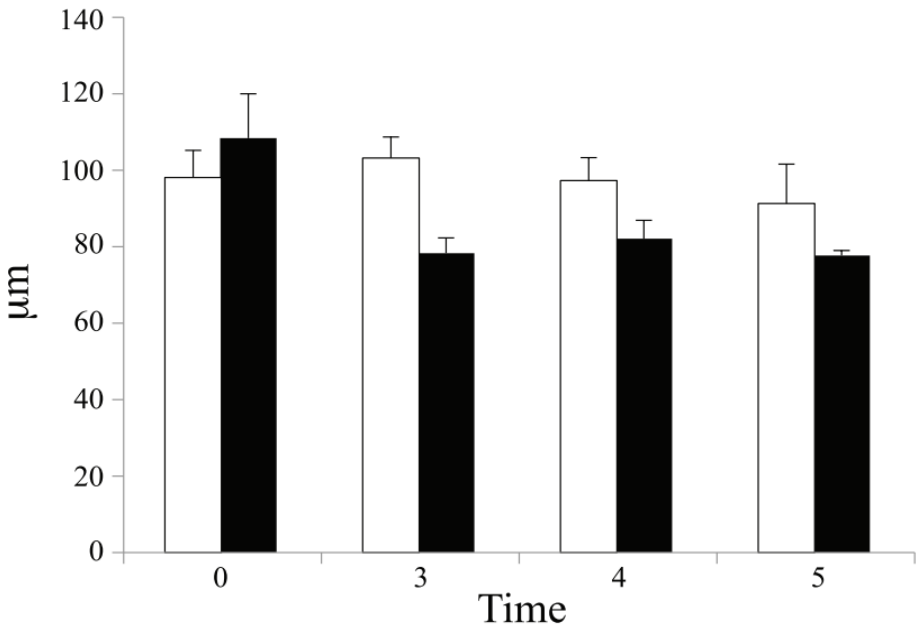

Figure 1. Effect of STb on pig jejunum villi length. Villi length of non-treated tissues (white box) and treated tissues (black box) are shown. A constant length for the non-treated tissues was observed over the 5 hours retained for the experiment. On the other hand, treated tissues presented a decrease in length from 3 to 5 hours post-treatment (p=0.002). Treated and not treated tissues did not differ statistically at 0 (p=0.28) and 3 hours (p=0.053) but did at 4 and 5 hours (p=0.002).

Width means of treated and non-treated tissues did not differ at 0 hour (p=0.24) (Figure 2). For non-treated tissues, the width did not differ significantly from 0 to 5 hours (p=0.40, p=0.43, p=0.29, for 3, 4 and 5 hours, respectively). For STb-treated tissue, the width differed significantly from 0 to 3 (p=0.01), 4 (p=0.02) and 5 hours (p=0.01). However, means of treated and untreated tissues did not differ statistically at 0 (p=0.24), 3 (p=0.21) and 5 hours (p=0.15) but differed at 4 hours (p=0.04).

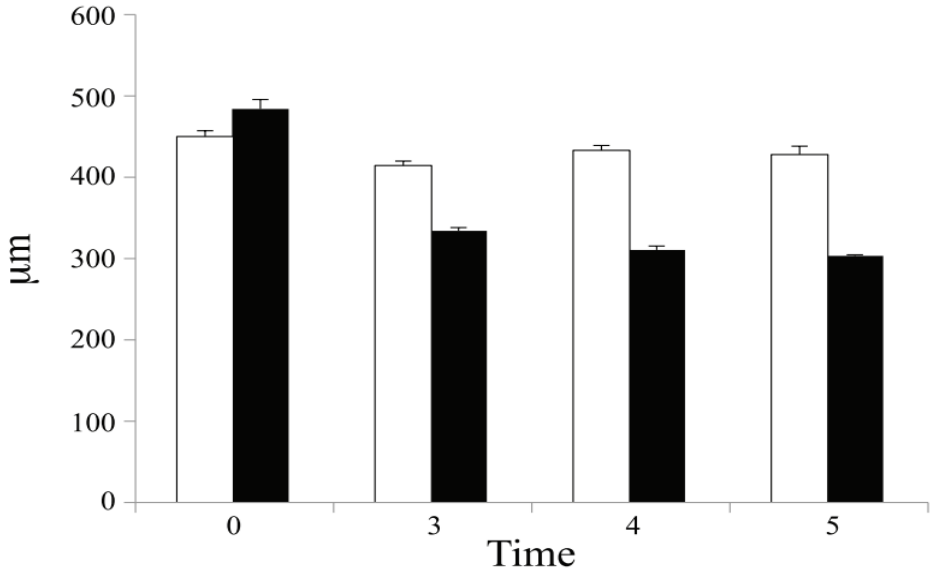

Figure 2. Effect of STb on pig jejunum villi width. Villi width of non-treated (white box) and treated tissue (black box) are shown. For non-treated tissues, the width did not differ significantly from 0 to 5 hours. For STb-treated tissue, the width differed significantly from 0 to 5 hours (p=0.01 at 3, p=0.02 at 4 and p=0.01 at 5 hours). However, means of treated and untreated tissues did not differ statistically at 0 (p=0.24), 3 (p=0.21) and 5 hours (p=0.15) but differed at 4 hours (p=0.04).

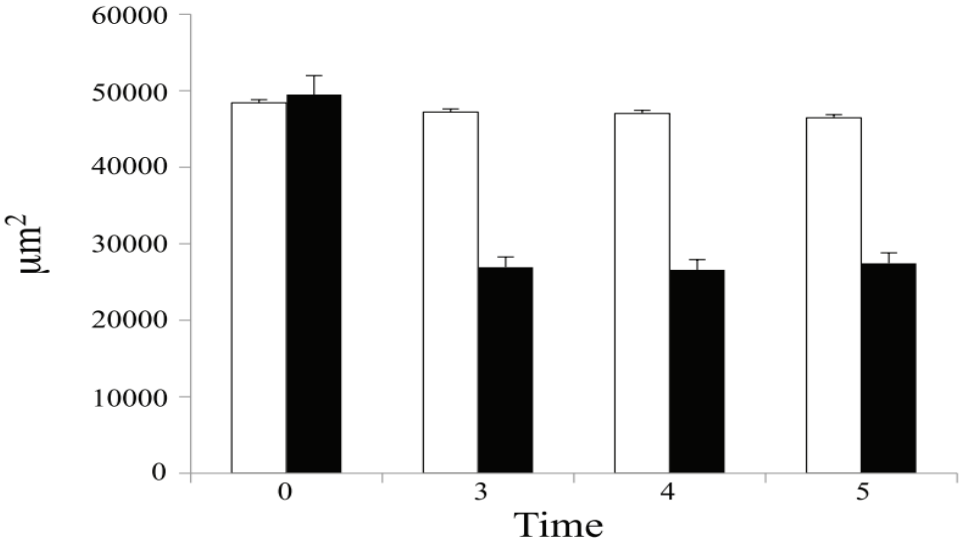

Figure 3. Effect of pure STb toxin on pig jejunum area. Villi area of non-treated (white box) and treated tissue (black box) are shown. Non-treated tissue showed constant villi area over 5 hours (p=0.29, 0.24 and 0.13, respectively for 3, 4 and 5 hours). On the other hand, treated tissues indicated a reduced area after 3 hours of treatment compared to 0 hour. Reduction in area was constant and significantly different after 3, 4 and 5 hours (p<0.0001).

Figure 3 shows that, for non-treated tissues, the area was similar to STb-treated tissue at time 0 (p=0.19). For non-treated tissues, the area was constant throughout the experiment (p=0.29, p=0.24 and p=0.13 for 3, 4 and 5 hours). After STb treatment, the villi area decreased after 3 hours and remains at a similar level till the end of the experiment (i.e. at 5 hours) (p<0.0001).

4 DISCUSSION

The present study shows, for the first time, that pure STb affect adversely pig jejunum villi in an IVOC model. Whipp et al. (1986) had shown that exposure of swine jejunum for 2 h to a culture supernatant containing STb induced microscopic alterations of the intestinal mucosa with loss of villous cells and partial atrophy of villi. These observations were later confirmed by other authors (Whipp et al., 1987, Rose et al., 1987). On the other hand, Hitotsubashi et al., (1992) observed that exposure of mouse jejunum to pure STb for 3 h caused a dilation of capillaries of the submucosa and a decrease in thickness of the lamina propria, but without indication of damage or inflammation. In our study, we observed villi atrophy of pig intestinal mucosa due to a loss of villous cells.

STb treatment was previously shown to induce morphological alterations associated with an apoptotic-like phenomenon (Gonçalves et al., 2007), such as an enlarged nucleus and membrane budding. Working with NIH-3T3 cells in culture, the authors, observed a progressive alteration of the cell physiological state and morphology, suggesting the induction of an apoptotic/mitoptotic-like process. In our study, no such changes could be observed (data not shown) and thus we cannot suggest a mechanism to explain how the villous cells are loss. However, we could observe a rapid alteration of the pig jejunum that can explain, at least in part, the fluid secretion and/or non-reabsorption by the intestinal tissue affected by STb toxin.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Authors´contributions and wrote the first draft of the manuscript. JDD interpreted the data and assisted with manuscript writing and editing. All authors have read and approved the final manuscript.

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