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Adhesion and invasion of *Clostridium perfringens* type A into epithelial cells

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ABSTRACT

Clostridium perfringens is the causative agent for necrotic enteritis. It secretes the major virulence factors, and α- and NetB-toxins that are responsible for intestinal lesions. The TpeL toxin affects cell morphology by producing myonecrosis, but its role in the pathogenesis of necrotic enteritis is unclear. In this study, the presence of *netB* and *tpeL* genes in *C. perfringens* type A strains isolated from chickens with necrotic enteritis, their cytotoxic effects and role in adhesion and invasion of epithelial cells were evaluated. Six (27.3%) of the 22 *C. perfringens* type A strains were harboring the *tpeL* gene and produced morphological alterations in Vero cells after 6 h of incubation. Strains *tpeL* (–) induced strong cell rounding after 6 h of incubation and produced cell enlargement. None of the 22 strains harbored *netB* gene. All the six *tpeL* (+) gene strains were able to adhere to HEp-2 cells; however, only four of them (66.6%) were invasive. Thus, these results suggest that the presence of *tpeL* gene or TpeL toxin might be required for the adherence of bacteria to HEp-2 cells; however, it could not have any role in the invasion process.

Clostridium perfringens spore-forming gram-positive bacterium is the causative agent of gaseous gangrene in humans, enterotoxaemia in cattle, and necrotic enteritis in chicken. Based on the type of potent and lethal toxins produced, they are classified into five toxinotypes: from A to E.[1–3]

Large clostridial cytotoxins (LCTs) comprise *C. difficile* toxin A (TcdA) and toxin B (TcdB), *C. sordellii* lethal toxin (TcsL) and hemorrhagic toxin (TcsH), *C. novyi* alpha toxin (TcnA), and toxin *C. perfringens* large cytotoxin (TpeL), are important virulence factors in pathogenesis of myonecrosis and intestinal diseases.[4–6]

C. perfringens is able to produces other potent toxins and enzymes, including NetB related to human and veterinary diseases. However, the role of the NetB-toxin in the necrotic

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enteritis is still controversial because it has been detected in both healthy and sick animals.[7]

TpeL toxin was initially detected in *C. perfringens* type C isolated from swine and in *C. perfringens* type A strains isolated from chicken. It is lethal to mice and cytotoxic to Vero cells, causing an enlargement, rounded cells, and forming aggregates, eventually detached cells from the plate.[4,8,9]

In early study, chickens inoculated with *C. perfringens* TpeL (+) showed higher capacity to cause severe intestinal lesions and an earlier onset of symptoms than chickens not inoculated with TpeL.[10] This suggests that TpeL may potentiate or contribute to the pathogenesis of necrotic enteritis caused by *C. perfringens* type A strains.[9]

Adhesion process to epithelial cells is the first step to the bacterial colonization, and this is mediated by fimbriae or non-fimbriae appendices.[11] *C. prefringens* is able to produce biofilm on cell surfaces and the presence of pili as well as the production of sialidase can collaborate with this process.[12] McClane[13] showed that adherent *C. perfringens* strains increase the neuraminidase and toxin production.

In this study, the presence of *netB* and *tpeL* genes in *C. perfringens* type A strains isolated from chickens with necrotic enteritis and their cytotoxic effects and role in the adhesion and invasion of the epithelial cells were evaluated.

Chickens belonged to five Brazilian states, Ceará (CE, 1 chicken), Sao Paulo (SP, 2 chickens), Paraná (PR, 3 f chicken), Santa Catarina (SC, 2 chicken s), and Rio Grande do Sul (RS, 1 chicken). Twenty-two *C. perfringens* strains previously isolated from the intestinal samples of nine chickens with necrotic enteritis, identified and toxinotyped as *C. perfringens* type A by biochemical tests. The presence of the *cpa* gene codifying the toxin α in all *C. perfringens* strains was also evaluated by PCR. This study was approved by the Ethics Committee of the Biomedical Sciences Institute, University of Sao Paulo (No. 104/CEEA).

Bacterial DNA was obtained in accordance with Sambrook et al.[14] Briefly, the bacteria grown into 5 mL brain heart infusion (BHI) were harvested by centrifugation (14,000 × *g*, 10 min), and the aliquots of supernatant were maintained at −80 °C until use. The pellets were washed twice with 0.1 M phosphate-buffered saline (PBS, pH 7.2), and incubated with 10 mg/mL lysozyme at 37 °C for 3 h. Then, 20% SDS and 20 mg/mL proteinase K were added and incubated at 55 °C for 2 h. The DNA was extracted by using equal volumes of phenol–chloroform and eluted in 100 μL of TE. PCR assays were performed to detect *netB* and *tpeL* genes with final reaction volumes of 25 μL containing 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), 0.4 mM of each primer[5,15] and 1 ng of DNA. Thermocycler (PE Applied Biosystems Gene Amp PCR System 9700) was programmed to: initial denaturation at 94 °C (5 min), followed by 30 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (1 min), and a final extension at 72 °C (7 min). The PCR products were analyzed on 1% agarose gel stained with ethidium bromide (0.5 mg/mL) and photographed by using Kodak Digital System DC-120. DNA from *C. perfringens* JGS 5369 strain was used as control. DNA was sequenced using MegaBACE 1000 system. Sequencing data were analyzed by using Nucleotide BLAST (NCBI, Bethesda, Maryland, USA).

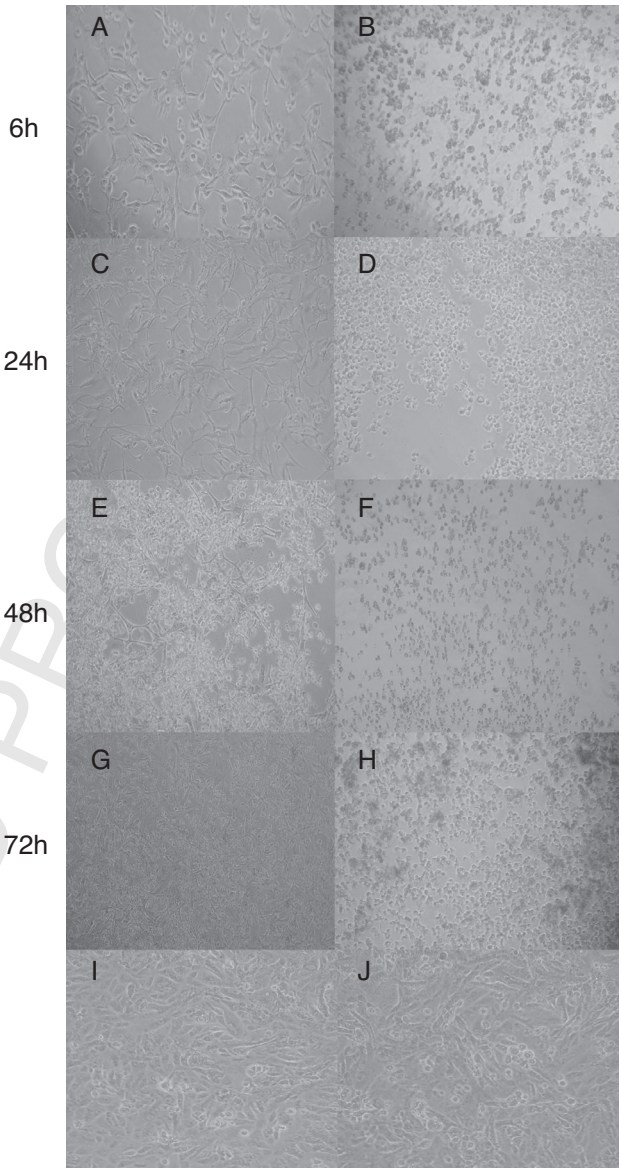

Fig. 1 – Cytotoxic of *C. perfringens* type A strain on Vero cells in different times. (A, C, E, G) *C. perfringens tpeL* (+) gene; (B, D, F, H) *C. perfringens tpeL* (–) gene; (I) Vero cells (control); (J) Vero cells and BHI. Times of Incubation: A and B, 6 h; C and D, 24 h; E and F, 48 h; G and H, 72 h. Magnification: 1000×.

The cytotoxic effect at different times (6, 24, 48, and 72 h) was evaluated on Vero African Green Monkey kidney cell line (Vero, ATCC CRL-81) cultured in Dulbecco's modified Eagle's medium (DMEM; Vitrocell, Embriolife, SP, Brazil) supplemented with 2% (v/v) fetal bovine serum (FBS). In each well of a 24-well microplate, 1 mL of cell suspension (1×10^5 cells/mL) was dispensed, and incubated at 37 °C in 5% CO_2 for 24 h. The Vero cells were treated with the supernatant of the bacteria cultured in BHI for 24 h. Morphological alterations in cells were assessed by using a light microscope. All assays were independently performed twice in duplicate.

The bacterial adherence assays were performed by using HEp-2 cells (1×10^5 cells/mL), as previously described by Nakano et al.[8] Briefly, 900 μL of DMEM supplemented with 2%