

Cell vacuolation induced by *Haemophilus influenzae* supernatants in HEP-2 cells

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Haemophilus influenzae belongs to respiratory tract microbiota. We observed vacuoles formation in previous studies with *H. influenzae* culture supernatants, so in this work we characterised that cytotoxic effect. We observed an abundant production of acidic cytoplasmic vacuoles due to the presence of a “vacuolating factor” in *H. influenzae* supernatants which was characterised as thermolabile. Greatest vacuolating activity was observed when utilizing the fraction > 50 kDa. The presence of a large number of vacuoles in HEP-2 cells was verified by transmission electron microscopy and some vacuoles were identified with a double membrane and/or being surrounded by ribosomes. These results suggest similar behaviour to that of vacuolating effects described by autotransporter proteins an undescribed cytotoxic effect induced by *H. influenzae*.

Key words: *Haemophilus influenzae* - cell vacuolation - cytotoxicity assays

Haemophilus influenzae is a human pathogen; at present, none of its virulence factors has been described with vacuolating cytotoxic activity on host cells (Moxon 2009, Kostyanov & Sechanova 2012). Hap is an autotransporter protein of *H. influenzae* that shares features with vacuolating proteins, such as serine protease activity (Yen et al. 2008). Several authors had been worked on characterisations of bacterial supernatants and some proteins which possess vacuolating activity have been described, such as: Vat, Sat, Pet, PicU and EspC proteins of *Escherichia coli* (Guyer et al. 2002), vacuolating cytotoxic factor in *Aeromonas veronii* bt. *sobria* (Martins et al. 2007), Sh1A of *Serratia marcescens*, HlyA of *Vibrio cholerae* and VacA of *Helicobacter pylori* (Figueroa-Arredondo et al. 2001, Vidal et al. 2009). The aim of this study was to characterise the vacuolating effect induced by *H. influenzae* supernatants of ATCC strains, since this could be an undescribed pathogenic mechanism related with the vacuolating activity of certain autotransporter proteins, as a cell response against intracellular pathogens and also could be important for removing damaged organelles.

H. influenzae ATCC 10211, 49766, 49247 and 33930 strains were used. *E. coli* 933W and *E. coli* K12 strains were used as cytotoxicity controls. Brain heart infusion broth supplemented was employed as the culture medium and incubation was done for 24 h at 37°C. The ATCC CCL23 HEP-2 cell line (human larynx carcinoma) was grown as previously described by St Geme et al. (1993). We defined culture supernatants as the result of centrifuge the *H. influenzae* culture and separate it of the bacterial pellet and were obtained at incubation time periods, including 12 h, 15 h, 18 h, 21 h and 24 h, were filtered with 0.22-µm Millipore® nitrocellulose membranes. Protein concentration was measured by Bradford method and all the supernatants were adjusted to a concentration of 0.15-0.2 mg/mL.

Cytotoxicity assays were performed according to Guyer et al. (2002) and Arellano-Galindo et al. (2007), where *H. influenzae* supernatants were inoculated into a HEP-2 cell monolayer in a 24-well microplate and incubated 2 h at 37°C. Cell vacuolation was observed at 1 h, 2 h, 3.5 h and 24 h. Fifty percent cytotoxic dose (CD50%) was determined by the Probit analysis using SPSS 17.0 software and titre was defined as the reciprocal of the highest dilution that shows 50% vacuolation of HEP-2 cells. Cells were washed, fixed, stained with Giemsa dye and observed by optical microscope. Vacuolation induced was analysed by transmission electron microscopy (TEM). Cytotoxic effect was defined as any morphological change and vacuolating effect was defined as a cytoplasmic vacuoles formation, both after treatment.

The characterisation of “vacuolating factor” in supernatants included thermal stability assays in which supernatants were treated at several temperatures by 15 min, as described by Arellano-Galindo et al. (2007). Neutral red dye uptake of vacuoles was determined as described

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Figuroa-Arredondo et al. (2001). For the protease assay, *H. influenzae* ATCC 33930 supernatant was treated with 2U of Proteinase K Roche® at 37°C as described by Mitra et al. (2000); that culture supernatant was concentrated also, in order to estimate molecular size, utilizing the Amicon® Ultra centrifugal filter 50 kDa cut-off (Millipore®) according to Vidal and Navarro-García (2006). The vacuolating activity after each treatment was performed under the aforementioned conditions described. Mann Whitney *U* test ($p < 0.05$) was used to assess the differences in vacuolation between strains.

We found cytotoxic effects induced in HEP-2 cells using *H. influenzae* supernatants, such as cell vacuolation (Supplementary data), cytoplasm deformation, nucleus condensation and monolayer destruction. Vacuolation was observed at the highest percentage of cells (52–89%) compared with the others. Incubation time to obtain active culture supernatants was 21 h and it is agree with Wang et al. (1996), in which cytotoxin production depends on bacteria growth. Statistical analyses (Fig. 1) corroborated that cell vacuolation percentages showed significant differences comparing with negative control ($p < 0.05$). We observed HEP-2 cells cytolysis after continuous exposure to supernatants. It was proposed that cell vacuolation is a stage prior to cytolysis (Figuroa-Arredondo et al. 2001), which was evidenced when cells were detached from the monolayer. Therefore, this showed that continuous exposure to the *H. influenzae* “vacuolating factor” can result in cell death. Vacuolation comprises extensive cell injury that has been described for many bacteria (Vidal et al. 2009). Therefore, host-cell vacuolation could be considered as a conserved pathogenic mechanism among certain medically important bacteria, which could improve their pathogenicity and establishment in host cells. *H. influenzae* strains ATCC 49247 and ATCC 49766 had the highest CD50% value, 1:158.75 and 1:125.27 ($p < 0.1$), respectively; thus, they exhibited high vacuolating activity. These results demonstrated the variety of titres that could be obtained in the evaluation of the biological vacuolating activity of different bacterial supernatants. These data are in agreement with previous reports, which showed that some pathogens produce toxins that affect host-cell stability and that could be present in bacterial supernatants (Vidal et al. 2009) so we propose the presence of a “vacuolating factor” in *H. influenzae* supernatants.

TEM analysis was used to ratify the presence of cytoplasmic vacuoles elicited by *H. influenzae* supernatants. We observed no changes in cellular organelles, only some disruptions in the cytoplasmic cell membrane, a few plasma membrane projections and loose chromatin. TEM images showed that cells contained a large number of vacuoles and, in certain sections, the vacuoles appeared to be continuous with the cell membrane system or to show a double membrane. We did not find difference between morphology of vacuoles induced by all the supernatants (Fig. 2). Induced vacuoles occupied a considerable area within HEP-2 cytoplasm and, in comparison with vacuoles observed in autophagy (Kroemer & Levine 2008), appear to share some characteristics,

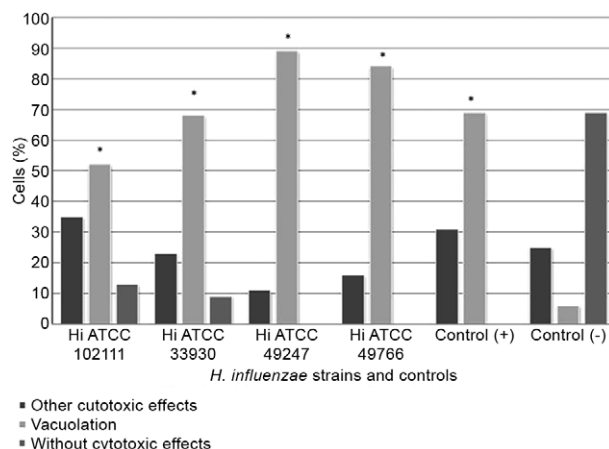


Fig. 1: cytotoxic vacuolating effect induced by *Haemophilus influenzae* (Hi) active supernatants on HEP-2 cells. “Other cytotoxic effects” means other cytotoxic effects different to vacuolation effect, as nuclear fragmentation, nucleus condensation and cytoplasm deformation. Asterisk means that the value has a statistically significant difference compared with the negative control ($p < 0.05$). Cytotoxicity percentages were determined by counting 100 cells in different fields of the sample.

such as double-membrane vacuoles. Ribosomes were identified in the vacuole periphery, suggesting a possible origin of rough endoplasmic reticulum; the intense cytoplasm vacuolation observed could be similar to the effect caused by aforementioned autotransporter proteins and autophagy has been observed occurring in cells intoxicated by some of them (Moal et al. 2011). According to our viewpoint, the images obtained suggest an autophagosome formation; while, TEM is a good method for screening (Eskelinen et al. 2011), is important to support these results with more specific techniques to elucidate whether the autophagic process is related with the cytotoxic damage caused by *H. influenzae* supernatants, because it has not yet been described as a mechanism of injury or as a response to *H. influenzae* infections, in which autophagy could play a protective role for host-cells attempting self-limit damage as well as promoting bacterium persistence (Wirawan et al. 2012).

The “vacuolating factor” was thermolabile, since vacuolating effect was lost at 65°C; this behaviour could be similar to that of some vacuolating cytotoxins, as cited Arellano-Galindo et al. (2007) and Martins et al. (2007). The majority of vacuoles induced by the *H. influenzae* “vacuolating factor” captured neutral red (acidic microenvironment) (Supplementary data), suggesting that they probably derived from late or pre-lysosomal endosomes, similar to the VacA protein (Figuroa-Arredondo et al. 2001). We did not observe residual activity of supernatants treated with proteinase, so we could suggest that “vacuolating factor” was proteinaceous. Similar results have been demonstrated with *V. cholerae* supernatants in order to recognise haemolysin as the responsible of vacuolating effects observed on that model (Mitra et al. 2000). Fractions of > 50 kDa of supernatant produced

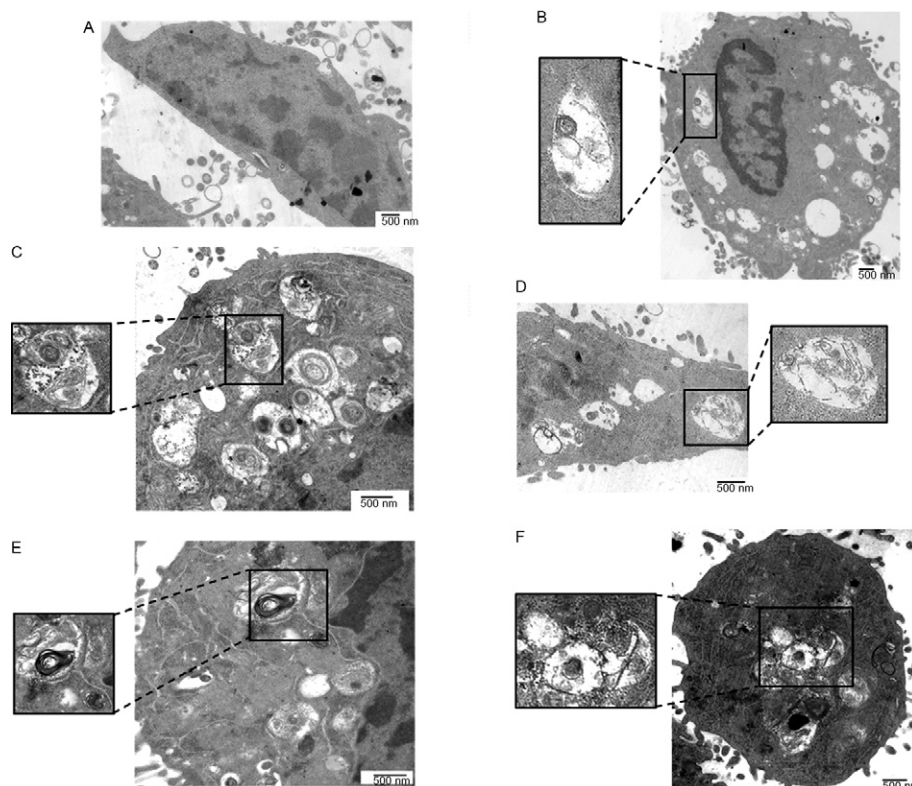


Fig. 2: transmission electron microscopy images presenting cytototoxic damage in HEp-2 cells induced by *Haemophilus influenzae* supernatants. A: *Escherichia coli* K12 negative control, magnification 25,000X; B: *H. influenzae* ATCC 49247, magnification 15,000X, 20,000X; C: *E. coli* 933W positive control, magnification 20,000X, 40,000X; D: *H. influenzae* ATCC 10211, magnification 25,000X, 40,000X; E: *H. influenzae* ATCC 49766, magnification 25,000X, 40,000X; F: *H. influenzae* ATCC 33930, magnification 20,000X, 25,000X.

intense cell vacuolation, with percentages ranging from 68-80%. Several autotransporter proteins possessing vacuolating activity were described in *E. coli*, in which their sizes were around 100 kDa and Hap_s protein has a size of 110 kDa (Yen et al. 2008); therefore, research on *H. influenzae* Hap_s residual activity would be important for understanding whether this virulence factor could be responsible for the cytotoxic effect observed. Structural similarity between VacA, *H. influenzae* IgA protease and Hap protein has been reported (Schmitt & Haas 1994, Kilian et al. 2002); therefore, it may be possible that Hap could share some functions with the VacA protein. Further experiments must be realised to elucidate if there is a relation between autotransporter protein family and vacuolating activity.

This paper could open a new way to understand which "vacuolating factor" is produced by *H. influenzae*. Several authors have commented the relevance of identify at first, cytotoxic and vacuolating activity in culture supernatants of bacterial strains, in order to characterise it after (Vidal et al. 2009).

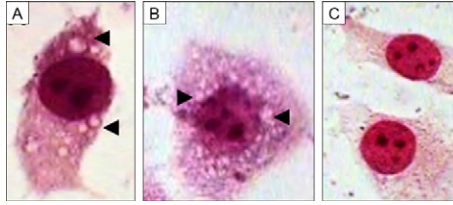
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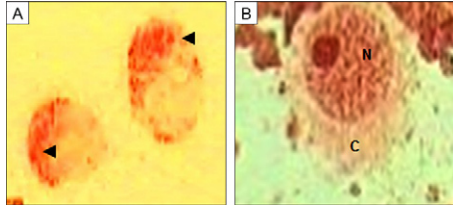
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Cytotoxic effects on HEP-2 cells induced by contact with *Haemophilus influenzae* supernatants obtained after 21 h of incubation. A: *H. influenzae* ATCC 10211; B: *H. influenzae* ATCC 49247; C: HEP-2 cell control. Arrowheads indicate cytoplasmic vacuoles. Magnification 100X.



Neutral red uptake in vacuoles induced by *Haemophilus influenzae* supernatants on HEP-2 cells. A: *H. influenzae* ATCC 33930; B: *Escherichia coli* K12 negative control; C: cytoplasm; N: nucleus. Arrowheads indicate acidic vacuoles. Magnification 100X.