Title: Characterization of Membrane Vesicles of *Clostridioides difficile*

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Abstract:
Bacteria are able to secrete extracellular vesicles, a feature usually associated with gram–negative bacteria. Recently, the production of this structure was describe in gram-positive bacteria. Even though their budding mechanism remains elusive since these organisms possess a thick cell wall. These vesicles are capable of carrying several molecules, such as proteins and DNA. Recently, these structures were describe in the anaerobic pathogen *Clostridioides difficile*. This study proposes to investigate these vesicles in two different strains of *C. difficile* (R20291 and HU29) and to characterize their molecular profile to determinate the pathogenic and immunogenic potential. R20291 strain was examined by Transmission and Scanning electronic microscopy (TEM and SEM). Vesicle purification were made with both strains, R20291 (hipervirulent) and Hu29 (non-toxigenic) from stationary phase cultures. The methodology includes centrifugation, ultra-filtration and differential ultracentrifugation without and with density gradient. The obtained fractions were analyzed by TEM and dynamic light scattering. The protein profile was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), western blotting (WB) for toxin detection and multi-enzymatic digestion, followed by mass spectrometry. We observed bacterial cells with budding vesicles in TEM e SEM, including possible sites with budding. The isolated vesicles preparations show high quantity of vesicles, with a diameter between 20 and 400 nm, and average of 165 nm. The size of the vesicles is similar to reports in the literature. The presence of budding vesicles in bacteria demonstrate probable process to thin the cell wall. SDS-PAGE demonstrates different protein profile group of each EV fractions. Toxin A was detected in WB of the toxigenic strain. The result from R20291 mass spectrometry detected a total of 194 vesicle-associated proteins, including A and B toxins. Besides, proteins such RNA polymerase, flagelin, ribosomal proteins, Spo0A, SecA and others. Most proteins were from cytoplasm and organic metabolism process. Furthermore, the presence of both toxins in vesicles was never described in *C. difficile*. These results suggest that vesicles may have pathogenic function. Others experiments are being conducted to determine the protein profile from Hu29 strain, as well as TEM and SEM. Cytotoxic assays with purified vesicles will be made with Vero and HT29 cells.

Keywords: *Clostridioides difficile*, membrane vesicle, electronic microscopy, proteomic

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