J Bacteriol Parasitol 2016, 7:6 (Suppl)
http://dx.doi.org/10.4172/2155-9597.C1.026

The role of flagellar proteins in epidemic PCR-ribotype 027 (B1/NAP1) *Clostridium difficile* virulence

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Clostridium difficile is a major cause of healthcare-associated infection and inflicts a considerable financial burden on healthcare systems worldwide. Disease symptoms range from self-limiting diarrhea to fatal *pseudomembranous colitis*. Whilst *C. difficile* has two major virulence factors, toxin A and B, it is generally accepted that other virulence components of the bacterium contribute to disease. *C. difficile* colonizes the gut of humans and animals and hence the processes of adherence and colonization are essential for disease onset. Bacteria within biofilms are protected from multiple stresses including immune responses and antimicrobial agents. Increased antibiotic resistance and chronic recurrent infections have been attributed to the ability of bacterial pathogens to form biofilms. While biofilms have been well studied for several gut pathogens, little is known about biofilm formation by anaerobic gut species. We have limited understanding of how the causative bacterium *C. difficile* colonizes the host or how it can resist antibiotics and persist within the gut. While persistent infections have been previously linked to biofilm-formation by pathogens, biofilm development by *C. difficile* has not been characterized. Our work demonstrates the ability of this anaerobic pathogen to form complex biofilms, the involvement of important clostridial pathways in biofilm development and perhaps a connection between formation of spores which are believed to mediate persistence and biofilm formation. Importantly, we show that bacterial sensitivity to antibiotics is reduced in clostridial biofilms. Biofilm formation may be a mechanism employed by *C. difficile* to survive in hostile environments such as the human gut. Here we tested this hypothesis by comparing flagellated parental strains to strains in which flagella genes were inactivated using ClosTron technology. Our focus was on a UK-outbreak, PCR-ribotype 027 (B1/NAP1) strain, R20291. We compared the flagellated wild-type to a mutant with a paralyzed flagellum and also to mutants (*fliC, fliD and flgE*) that no longer produce flagella in vitro and in vivo. Our results with R20291 provide the first strong evidence that by disabling the motor of the flagellum, the structural components of the flagellum rather than active motility, is needed for adherence and colonization of the intestinal epithelium during infection. The R20291 flagellar mutants adhered less than the parental strain in cell adherence in vitro model. Finally we demonstrated that in strain R20291, flagella do play a role in colonization and adherence and that there are striking differences between *C. difficile* strains. In addition, we also demonstrate that clinical *C. difficile* hyper virulent strain R20291, form structured biofilms in vitro with R20291 accumulating substantially more biofilm. Microscopic analyses show multiple layers of bacteria encased in a proteinaceous biofilm matrix. Employing isogenic mutants, we show that virulence associated proteins, cwp84 and a putative quorum sensing regulator, *luxS* are all required for maximal biofilm formation by *C. difficile*. Interestingly, a mutant in *spo0A*, a transcription factor that controls spore formation was defective for biofilm formation indicating a possible link between sporulation and biofilm formation. Furthermore, we demonstrate that bacteria in clostridial biofilms are more resistant to high concentrations of vancomycin, a drug commonly used for treatment of CDI. Biofilm formation by *C. difficile* is a complex multifactorial process and may be a crucial mechanism for clostridial persistence in the host.

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