Title: Clostridium difficile virulence mechanism study (CDVM study)

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1. Research background and objectives

Clostridium difficile is an anaerobic, gram-positive, sporulating bacterium that can give rise to a spectrum of gastrointestinal presentations, including diarrhoea, pseudomembraneous colitis and toxic megacolon. C difficile infection (CDI) is the most common cause of hospital-associated gastrointestinal infection. It accounted for approximately half a million infections and 29,000 deaths in the United States in 2011 (Lessa et al., 2012), and it also represented a major healthcare burden in Canada and across Europe (Bauer et al., 2011). Meanwhile, the hyper-virulent ribotype 027 (BI/NAP1) strain has caused major outbreaks and deaths in North America and Europe. In Asia, the hyper-virulent ribotype 027 was only found sporadically; rather, the ribotype 002 was common in Asia. Our recent work showed that the ribotype 002 was the most common ribotype in Hong Kong (22.8%), and was associated with increased mortality compared to other ribotypes (47.6% versus 12.7%) (Wong et al., 2016). Despite its importance in this locality, there is a paucity of data regarding the virulence mechanism of this ribotype.

This project aims (1) to investigate the virulence mechanisms of C. difficile ribotype 002, by analysing its growth, cytotoxicity, and metabolite production in vitro and in vivo. We also aim (2) to compare these virulence mechanisms with other common ribotypes in Asia, and (3) to correlate these with important clinical outcomes. The results of this study will have a major significance to the medical and scientific community. The mechanistic data will point to important molecular pathways that can be targeted for novel therapies; whereas the phenotypic data will inform us of its virulence and therefore will be useful for disease surveillance and public health interventions. The identified toxin(s) and metabolite(s) may be useful as prognostic biomarkers to stratify patients with different outcomes, as patients with a worse outcome may require more aggressive therapy. Given the prevalence and severity of this ribotype, our

results will be useful for gastroenterologists, microbiologists, infectious diseases physicians and public health specialists in the Asia Pacific region.

2. Methodology

Study subjects and sample collection

Stool samples will be collected from the in-patients in the Prince of Wales Hospital. Patients newly diagnosed with Clostridium difficile infection will be invited to participate in this study. All participants will sign an informed consent form to be recruited into this study. Clinical data regarding patients' age, gender, medical history, drug history and laboratory test results will be recorded. A fresh stool samples will be collected from the subjects using a 30-mL universal sample container with spoon cap. The stool samples will be delivered to laboratory and stoed at -80 degrees Celsius freezers. All research samples will be de-identified after collection and processing. The study will be conducted in compliance with the Declaration of Helsinki guideline.

Patients must meet the following criteria in order to be enrolled in the study:

1. Patients with a confirmed diagnosis of CDI, as documented by diarrheal symptoms and positive stool test result for C. difficile toxin or toxigenic C. difficile, or colonoscopic findings of pseudomembranous colitis (PMC).

2. Patients aged over or equal to 18 years old.

3. Patients able and willing to provide informed consent.

Patients will be excluded if they meet the following criteria:

1. Patients with concomitant infection by other microbes such as Salmonella, Campylobacter, Vibrio, Shigella, and Escherichia coli.
2. Patients under 18 years old.
3. Patients who cannot give consent.

Laboratory and statistical methods

The collected stool samples will be stored at -80C refrigerator. Only one tube of stool sample will be collected from each patient.

Stool filtrate will be used to test for the presence of toxins, including toxin A and toxin B. There are some tests that can be used, including enzyme linked immunosorbent assay (ELISA) and cell cytotoxicity neutralization assay (CCNA). Commercial kits will be used for these assays. Stool DNA extraction can be done to obtain information about toxin A and toxin B through polymerase chain reaction (PCR).Strain typing will also be done to identify the ribotypes of the stool samples collected. This is to also determine which ribotype is the most common ribotype among others.

The statistical software SPSS 13.0 (SPSS, Chicago, IL, USA) and the R Project for Statistical Computing will be used for all statistical analyses. For the microbiome data, the assembly and taxonomical assignment will be performed using the mothur software.

Sample size

Assuming a power of 70% to detect a significant difference at 5% false positive rate, 227 samples will be required at an effect size (Cohen's d) of 0.195.

3. Conclusion

This proposed research aims to collect clinical stool samples to validate virulence mechanisms of different ribotypes of C. difficile being investigated in laboratory work. The study does not involve any invasive or harmful procedure. This important study will inform the characteristics of common ribotypes in Hong Kong to benefit the community.

References

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