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Investigation of Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) for detection of *Clostridium difficile* toxin A and B from stools

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Objective: Detection of *Clostridium difficile* toxins A and B from stools using matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI).
Methods: *C. difficile* toxin positive stool samples from Royal Inland Hospital were diluted 10-fold in deionized water or phosphate buffered saline (PBS) and vortexed to create a relatively homogeneous suspension. Samples were then centrifuged and the pellet removed. Proteins in the supernatant were precipitated with acetonitrile or ammonium sulfate and the solution was centrifuged again. The pellet was resuspended in deionized water or TA30 and spotted on a MALDI plate with SA (sinnapinic acid), SDHB (a mixture of 2,5-dihydroxybenzoic acid (2,5-DHB) and 2-hydroxy-5-methoxybenzoic acid), or CHCA (α-Cyano-4-hydroxycinnamic acid) as matrices.
Results: MALDI analysis showed no difference between samples diluted in deionized water and those diluted in PBS. Protein precipitation with acetonitrile produced higher quality mass spectra than protein precipitation with ammonium sulfate. Sample co-crystalization with SA provided higher quality spectra than SDHB or CHCA. No peaks were seen in the 63 kDa range in any of the samples. Autocleavage of a commercially purchased toxin A also failed to show the expected peak at 63 kDa.
Discussion: Further processing of stool samples is necessary for MALDI to successfully detect the 63 kDa active domain. No individual ion signals were detected between 60-65 kDa. This suggests that a clear mass window is available for the detection of the 63 kDa active domain, had MALDI analysis been successful.

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that is commonly the causative agent of antibiotic-associated diarrhea (1). It is found as part of the normal intestinal flora of 5% of adults and up to 70% of infants (2). It was first described by Hall and O'Toole in 1935 during their experiments involving the intestinal flora of newborn infants. In 1978, Bartlett et al. found *C. difficile* to be the causative agent of antibiotic-associated pseudomembranous colitis through tissue culture experiments using stool samples from affected patients.

These early studies contributed to our understanding of the importance of the indigenous microflora of the intestine (3). Antimicrobial therapies can result in the disruption of normal intestinal flora and allows for subsequent overgrowth of opportunistic *C. difficile,* which causes disease through toxin A and B production. These infections are of great concern due to the risk of resulting complications such as toxic megacolon. Additionally, a new, hypervirulent strain, NAP1/BI/027, was described in 2005 (4). This new strain showed increased resistance to fluoroquinolone antibiotics as well as various sized deletions and point mutations in the *tcdC* gene that encodes a protein thought to function as a negative regulator for toxin A and B production. The result is a new, hypervirulent *C. difficile* strain that is capable of increased toxin production. In 2002, it was estimated that each case of *C. difficile* infection (CDI) in the US resulted in more than $3,600 in additional health care costs and these costs are estimated to exceed $1.1 billion per year (5).

Both toxins A and B, 308 and 269 kDa proteins, respectively, must undergo processing within the target cell before the 63 kDa active domain is released and able to produce its toxigenic effects (Figure 1) (6). The whole 308 or 269 kDa toxin is first taken into the host cell through receptor mediated endocytosis. This brings the toxin into the cell and inside endosomes, which become acidic, causing the toxin to refold. Toxin refolding exposes hydrophobic domains within the toxin, allowing it to penetrate and insert itself into the membrane of the endosome. This results in the active domain being translocated outside of the endosome while still attached to the remainder of the toxin located inside the endosome. Cytosolic inositol hexakisphosphate (Ins_6P) is then able to cleave the active domain free from the rest of the toxin. This frees the 63 kDa active domain, which possess glucosyltransferase activity capable of inhibiting ADP-ribosylation of the GTP-binding protein Rho. The cell is then unable to regulate the microfilament cytoskeleton, causing subsequent depolymerization of actin fibers, cytoskeleton instability, and cell death (7, 8).

C. difficile is frequently a nosocomial pathogen that is difficult to control due to its ability to produce spores (http://www.cdc.gov/HAI/organisms/cdiff/Cdiff_faqs_HCP.html#a2). Therefore, it is critical that a clinically and user friendly method of diagnosis is available for reliable and

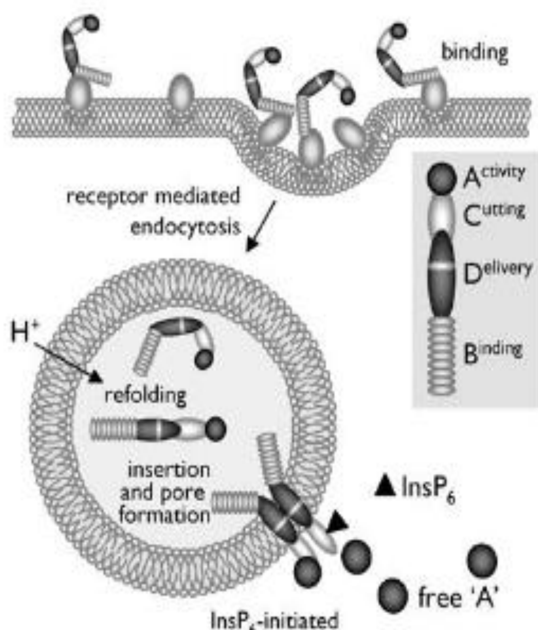

FIG 1. Model for the uptake of toxins A and B into the host cell for the release of the 63 kDa active domain. The toxin is first brought into the cell and into endosomes through receptor mediated endocytosis. As the endosome acidifies, the toxin refolds to expose hydrophobic surfaces. This allows for the toxin to insert itself through the endosome membrane. The "cutting" and "activity" domains of the toxin are translocated outside of the endosome while the rest of the toxin remains inside. Cytosolic Ins_6P is then able to cleave the toxin at the "cutting" domain, releasing the 63 kDa active domain into the host cells. (Taken from Giesemann et al. 2008 (6))

early detection of CDI's. Many recent studies have used matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS or simply MALDI), for the identification of bacteria and yeasts as it has been shown to be a fast, accurate, and cost-effective technique (9).

MALDI has many advantages over other analytical tools used in proteomics as it is easy to operate, requires relatively inexpensive matrices for sample preparation, and can be automated to allow for easy screening large numbers of samples (10). It is also able to tolerate much higher salt concentrations than liquid chromatography/mass spectrometry, meaning proteins can be detected directly from biological samples without the need to desalt the sample first. Because of these advantages, we believe MALDI to be a viable diagnostic tool for identifying infectious microorganisms by their protein biomarkers.

The present study attempts to develop a protocol for the use of MALDI to diagnose CDI's. Our protocol relies on the detection of the 63 kDa active domain from clinical stool samples. This 63 kDa active domain is released by both toxin A and B through autocleavage induced by host cell Ins_6P during infection (6). Detection of this 63 kDa active domain therefore indicates CDI.

MATERIALS AND METHODS

Induction of autocleavage in commercially purchased toxin A and B. Toxin A and B was purchased from List Biologicals (Campbell, California, USA) and attempts were made to induce autocleavage in order to show that the 63 kDa active domain can be detected with MALDI and to investigate the limits of detection by spiking the toxin into weighed stool. Toxin A and B were purchased in lyophilized form and reconstituted in deionized water according to the specification sheet provided by the manufacturer. As the toxin had been lyophilized with resuspension buffer, reconstitution of the toxin yielded a resuspension buffer consisting of 50 mM Tris, pH 7.5, 50 mM NaCI, and 0.1 % trehalose.

Autocleavage of toxins A and B were attempted separately using varying concentrations of dithiothreitol (DTT) and Ins_6P, ranging from 2 to 10 mM and 5 to 20 mM, respectively (11). Each reaction was adjusted to pH 4.5 with sodium acetate and a total volume of 26 µL with deionized water. Incubation times ranged from 0.5- 72 h both at room temperature and at 37°C (Table 1).

Samples were taken before and after incubation and analyzed using MALDI and SDS-PAGE in an attempt to view the 63 kDa active domain known to be released through autocleavage (6).

TABLE 1. Concentrations and volumes of DTT and Ins_6P used to induce autocleavage in 1 µg of toxin A. Adjusted to pH 4.5 with sodium acetate and a total volume of 26 µL with deionized water.

Toxin A or B (µg)	DTT	Ins_6P	Total volume (µL)
1	2 mM, 6 µL	5 mM, 6 µL	26
1	4 mM, 13 µL	8 mM, 11 µL	26
1	6 mM, 10 µL	12 mM, 8 µL	26
1	8 mM, 13 µL	15 mM, 10 µL	26

Clinical stool sample preparation. Known toxin and antigen positive and negative stool samples from Royal Inland Hospital (RIH) were stored at -80°C and transported to Thompson Rivers University, where they were stored at 4°C (12). Samples were either-confirmed toxin and antigen positive or negative by RIH through enzymatic immunoassay (EIA) using Alere C. DIFF QUIK CHEK COMPLETE. Those that tested negative for *C. difficile* toxins but positive for the antigen were subsequently tesed by PCR to detect toxin producing genes. Approximately 100 µL of each sample was diluted in 1000 µL deionized water or phosphate buffered saline (PBS). Diluted samples were then vortexed for up to 5 minutes until a relatively homogeneous mixture was achieved. Samples were then centrifuged at 1000 x g for 20 seconds and the pellet removed or filter sterilized with 0.22 micron syringe filters. An equal volume of acetonitrile was added and the solution incubated for 30-60 minutes at room temperature to precipitate the proteins from solution. Protein precipitation was also attempted by adding equal volumes of ammonium sulfate to the supernatant and incubating at 4°C overnight. After incubation, all samples were centrifuged at 18,000 x g for 10 minutes at room temperature and the supernatant removed.

The pellet was then suspended in 10-400 µL of deionized water or TA30 (3:7 HPLC grade acetonitrile: 0.1% trifluoroacetic acid in deionized water) and analysed with MALDI.

MALDI parameters and matrix selection. Samples were spotted on a ground steel MALDI target plate in triplicate and allowed to co-crystallize with either a SA (sinnapinic acid), SDHB (a mixture of 2,5-dihydroxybenzoic acid (2,5-DHB) and 2-hydroxy-5-methoxybenzoic acid), or CHCA (α-Cyano-4-hydroxycinnamic acid) matrix using the dried droplet method.

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This consisted of adding 1 μL (0.02 μg) of sample to 1 μL of matrix, briefly mixing by pipetting up and down, and spotting 1 μL of this mixture on the target plate. The spots were then allowed to air dry. Spots were analyzed using a microflex series MALDI-TOF-MS mass spectrometer set to linear positron mode with a laser intensity ranging from 10-100%.

If a peak at 63 kDa is detected, the presence of the toxin will be confirmed using SDS-PAGE and protein sequencing.

RESULTS

Induction of autocleavage in commercially purchased toxin A and B. MALDI analysis was unable to detect the presence of proteins in the 63 kDa range, suggesting that the attempts to induce autocleavage had failed. The results of the autocleavage experiments were also visualized using SDS-PAGE (10% polyacrylamide gel at 100 V for one hour), however, no band in the 63 kDa range was seen (data not shown).

Clinical stool samples. MALDI analysis of clinical stool samples showed no peaks at 63 kDa, however peaks at 56 kDa was seen in 10 samples (Table 2 and Figure 2). No differences in MALDI spectra quality were seen between samples diluted in deionized water and samples diluted in PBS. Replacing the filter sterilization step with a centrifugation step proved to be time and effort saving, while still providing the same quality spectra. Protein precipitation with acetonitrile produced higher quality spectra compared to protein precipitation with ammonium sulfate and sample co-crystallization with a SA matrix produced higher quality spectra than samples co-crystallized with a SDHB or CHCA matrix (data not shown). No difference in MALDI spectra was seen between pellets resuspended in deionized water compared to those resuspended in TA30. Pellets resuspended in 50 - 200 μL of deionized water or TA30 produced the highest quality spectra.

TABLE 2. Summary of stool samples used showing EIA, PCR, and MALDI results. EIA testing for C. difficile toxin A/B and antigen. Only samples with inconclusive EIA results were subjected to PCR analysis. A=antigen, T=toxin. No peak seen at 63 kDa in any samples.

Sample type	Number of samples	PCR	Number of samples with signal at 56 kDa
A+ T+	26	N/A	5
A+ T-	18	11	5
A- T+	0	N/A	0
A- T-	8	N/A	0

DISCUSSION

In general, stool is a complex combination of 75% water and 25% solid matter (13). The solid matter is composed of dead intestinal, blood, and bacterial cells, undigested food, steroids, bile acids, lipids, inorganic matter, and proteins. Given the complex composition of stool, MALDI mass spectra were expected to be complicated and show vast differences between samples. Unexpectedly, the MALDI mass spectra were relatively similar between samples, showing a hump containing unresolved complex mixture in the mass range m/z 20,000 - 60,000 (Figure 2a). No individual ion signals were seen in the 63 kDa range in any of the samples, regardless of their being toxin and antigen positive or negative. This suggests that there is an open mass window for unambiguous detection of the 63 kDa active domain. Although further work is necessary to create a protocol for processing *C. difficile* toxin positive stools in such a way that the 63 kDa active domain can be detected with MALDI, this open mass window is encouraging for the idea of using the 63 kDa active domain as a biomarker detected by MALDI to diagnose CDI's.

A peak was seen at 56 kDa in ten samples (Table 2). The identity of this peak was briefly considered to be a metabolite of *C. difficile* toxins. However, failure to reproduce this peak in other toxin positive samples led to the conclusion that this 56 kDa peak would not serve well as a biomarker.

Failure to consistently detect the 56 kDa peak between trials of same sample shows one of the flaws inherent to MALDI: only a very small amount of sample can be analysed at a time. Although the necessity for small sample volumes can be an advantage in instances where limited sample is available, it is a disadvantage when analysing heterogenous samples such as stool. Depending on the protocol used, only 0.5 – 1 μL of a sample-matrix solution can be deposited on each spot on a MALDI target plate. Although all samples are spotted in triplicate and efforts were made to form a homogenous solution after diluting samples in deionized water or TA30, it is difficult obtain a representative sample with such small volumes. This is evident from the inconsistency of the 56 kDa peak between trials.

A similar study has found MALDI to be a potential tool for detecting fecal occult blood (FOB) in stool, a warning sign for gastrointestinal diseases and colorectal cancer (13). Stool samples were spiked with different amounts of blood and processed similar to the methods presented here. The results showed MALDI to be approximately 10-100 times more sensitive in detecting FOB compared to conventional chemical analysis, the estimated detection limit being between 1 and 0.1 μg/mg feces. It is thought that the ion suppression effect, where signals of lowly abundant ions are suppressed by abundant ions during desorption/ionization and ion transmission, worked to the researchers' advantage in the study. The presence of large quantities of Hb-related α and β chains may have prevented the mass spectrometric detection of other molecules in the solution. It is speculated that the ion suppression effect worked against us in the present study, overpowering signals from the 63 kDa active domain that was sought out. The success of detecting FOB in stool shows that MALDI can be used to detect a single kind of protein from a crude protein extract.

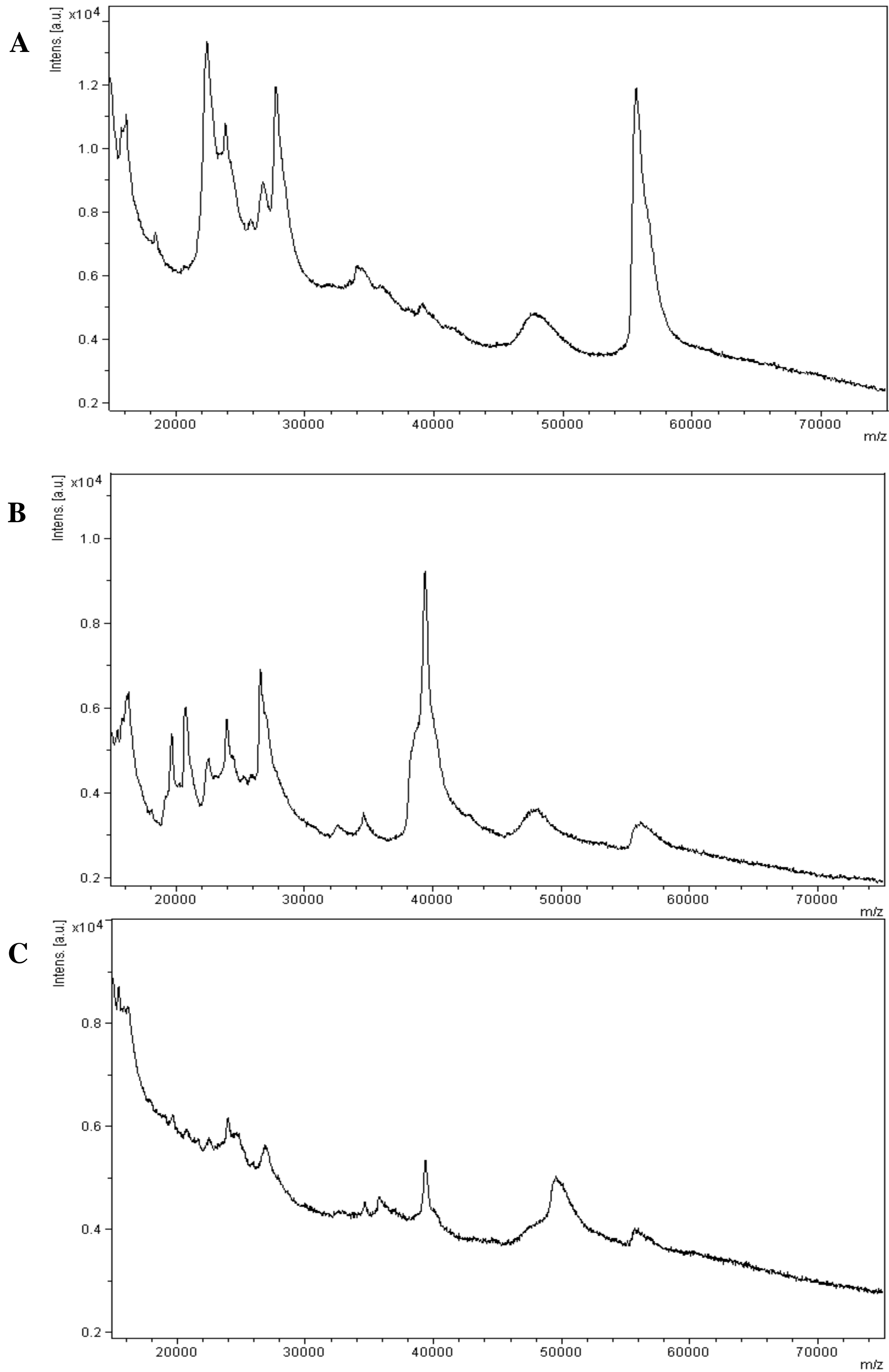

FIG 2. MALDI mass spectra of three *C. difficile* toxin and antigen positive stool samples all showing a hump containing an unresolved complex mixture in the mass range *m/z* 20,000- 60,000. (a) Shows a relatively high intensity peak at 56 kDa, while (b) and (c) do not.

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We were unable to use MALDI to directly detect *C. difficile* toxins A and B from crude stool protein extracts and commercially purchased toxin A. Further studies would be required to ascertain the possibility of using this technological tool to detect C. difficile toxin as an alternative method of diagnosis to the tests currently available. Although inconclusive, this study is a starting point for the direct use of MALDI as a diagnostic tool for CDI's.

FUTURE DIRECTIONS

Other research has taken advantage of toxin-specific substrates when determining the presence of toxins using MALDI (14). The possibility of using 4-nitrophenyl-beta-D-glucopyranoside (PNPG) as a substrate for *C. difficile* toxins A and B in stool samples was briefly explored in this study (15). However, this idea was discarded since the β-glucuronidase activity of *Escherichia coli* cleaves PNPG in much the same way *C. difficile* toxins do (16). Furthermore, it is unlikely that MALDI would have been able to detect the products of PNPG produced by *C. difficile* toxins due to their small sizes. Designing a substrate or an immunoglobulin specific to *C. difficile* toxins that could be easily detected by MALDI is an area of research we hope to further explore.

Protein-immunoglobulin complexes have been shown to be detectable with MALDI under pH neutral conditions using a SA matrix (17). A 150 kDa immunoglobulin complexed with the 63 kDa active domain would yield a protein complex over 200 kDa, above the ideal range of detection for MALDI as proteins over 100 kDa do not ionized as efficiently as smaller proteins (18). However, it has been shown that immunoglobulins complexed with four beta-lactoglobulins, resulting in a mass of over 200 kDa, can be detected with MALDI (17). This gives promise for our large protein-immunoglobulin complex to also be detectable.

It is speculated that failure to induce autocleavage in the commercially purchased toxins A and B was the result of the 0.1 % trehalose present in the buffer, which acts as a protein stabilizer (19). Further studies involving these commercially purchased toxins would require purification prior to attempts to induce autocleavage, possibly using dialysis (Kirstin Brown, personal communication, December 1, 2015). Purifying toxins A and B from a culture of *C. difficile* grown in the lab was considered, however, lack of access to an anaerobic chamber did not allow for this option for our laboratory.

This research would have been greatly strengthened had the 63 kDa active domain in its purified form been attained. It would then have been possible to determine the detectability of the protein in minimally processed stool using MALDI as well as its limits of detection in stool, by spiking stool from healthy patients with the protein. Once the limit of detection is determined, a western blot could be performed in order to determine whether or not CDI's produce enough of the toxin to be detected using MALDI (Kirstin Brown, personal communication, December 1, 2015).

It may also be beneficial to use other biomarkers for CDI's such as fecal inflammatory cytokines or fecal calprotectin (20, 21). However, these markers would not distinguish between patients with CDI's and those with other causes of diarrhea, only between patients with asymptomatic *C. difficile* colonization and those with CDI's. It is possible that an abundant fecal biomarker for CDI's that is efficiently ionized by MALDI can be found by carefully comparing the MALDI spectra from CDI positive stool with CDI negative stool.

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