

Faculty of Science

MATRIX ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY AS A NOVEL ASSAY FOR THE DETECTION OF *CLOSTRIDIUM DIFFICILE* TOXINS A AND B

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SPECTROMETRY AS A NOVEL ASSAY FOR THE DETECTION OF *CLOSTRIDIUM*
DIFFICILE TOXINS A AND B.**

by

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ABSTRACT

Clostridium difficile infection is a major cause of nosocomial diarrhoea, and can culminate in the life-threatening conditions pseudomembranous colitis and toxic megacolon. Disease is produced in *C. difficile* infection through the release of toxin A and toxin B. Current diagnostics rely on a combination of immunoassays, histopathological findings, and PCR. Immunoassays, though fast, lack sensitivity compared to more expensive and time-consuming histopathology methods. PCR, though sensitive, only indicates the presence of toxin genes and not whether they are being expressed. Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF) has been gaining popularity in medical laboratories as a means of identifying bacterial isolates due to high sensitivity and fast turnaround time. The purpose of this study is to investigate MALDI-TOF as a means of detecting the presence of *C. difficile* toxins A and B. The method developed was based on the proposed cellular processing mechanism of toxin A and B – both are large toxins (approximately 300 kDa) that are endocytosed and undergo a pH-induced conformation change in the early endosome. This causes the active domain and protease domain of the toxin to be translocated into the cytoplasm. Inositol hexakisphosphate, an abundant cytosolic signaling molecule, then binds to the protease domain of the toxin and induces the release of the 63 kDa active toxin. The active component of both toxin A and B glucosylates cellular Rho GTPases, which results in cytoskeleton derangement and cell death. The method developed required fecal material to be prepared as a 1:10 dilution in deionized water followed by filter sterilization. The filtrate volume was then divided into two equal volumes, and each volume was subjected to an acetonitrile precipitation. One pellet was resuspended in deionized water, and the other in sodium acetate buffer (100mM, pH 4.7) to mimic the conditions of the early endosome. The spectra produced from each condition were overlaid, which demonstrated the presence of a high-intensity peak at approximately 60 kDa in known toxin positive specimens. If this peak can be shown to be correlated to the presence of toxin in a larger sample set, this method may hold promise

as a means of detecting *C. difficile* toxins A and B. This protocol avoids the extensive time and labour associated with a mass-fingerprinting approach, while maintaining higher degree of specificity than simple intact protein detection due to the comparison of spectra between acid and neutral conditions.

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INTRODUCTION

Clostridium difficile is a Gram positive, sporegenous nosocomial pathogen of increasing concern due to the potential severity of disease produced, and because of the difficulty in controlling the spread of infection due to the persistence of spores in the environment. Infection is associated with antibiotic use, which is believed to sufficiently disrupt normal enteric flora to allow *C. difficile* overgrowth (Bartlett 2002). Pathologic effects are mediated entirely through the release of toxins, and only strains that possess the 19.6 kb pathogenicity locus are capable of producing disease (Braun et al 1996). Toxin A and toxin B are encoded by *tcdA* and *tcdB* found within the pathogenicity locus, and are classified as Large Clostridial Toxins (LCTs) because of their respective sizes, 308 and 270 kDa (von Eichel-Streiber et al 1992). Typically these toxins are both present in infection, though each is able to cause cytopathic effects individually with toxin B being approximately 1000 times as potent as toxin A (Chaves-Olarte et al 1997). Toxin A and toxin B have a homologous structure and are composed of four discrete domains, each with a distinct role in cellular processing – the A (active), B (binding), C (cutting) and D (delivery) domains (Jank and Aktories 2008).

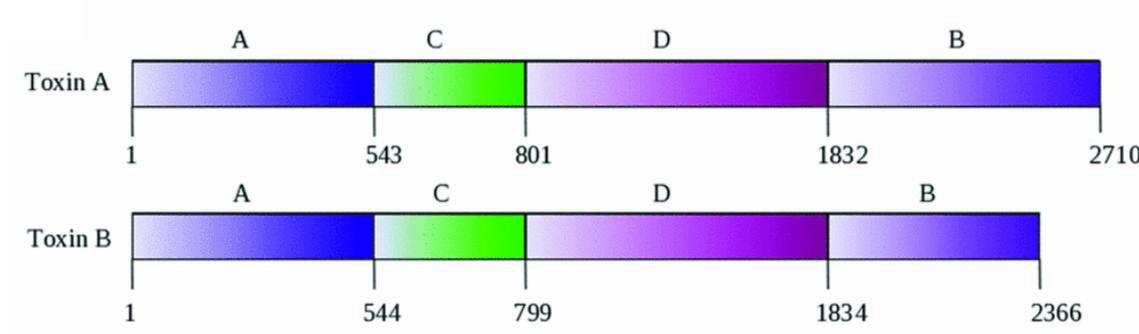


Figure 1. Domain organization of *Clostridium difficile* toxins A and B. Taken from Davies et al 2011.

The B (binding) domain of the toxin binds to epithelial cell surface carbohydrates, and the toxin is internalized (Tucker and Wilkins 1991; Florin and Thelestam 1983). Next, a drop in pH in the early endosome induces the toxin to insert the hydrophobic delivery domain (D) into the endosomal membrane to form a channel, which allows the active (A) and cysteine protease (C) domains to translocate across the endosomal membrane (Barth et al 2001; Qa'dan et al 2000). Cytosolic inositol hexakisphosphate (InsP6) then binds to the C domain, presumably as an allosteric switch, and induces autocleavage and release of the active toxin (Egerer et al 2009). The active toxin then acts as a Rho GTPase glucosyltransferase, which results in the depolymerization of actin fibers leading to cytoskeleton derangement and cell death (Just et al 1995). Severity of disease is dependent on host factors and on the strain of *C. difficile* involved, and can range from mild diarrhoea to the medical emergencies toxic megacolon and pseudomembranous colitis (Rupnik et al 2009).

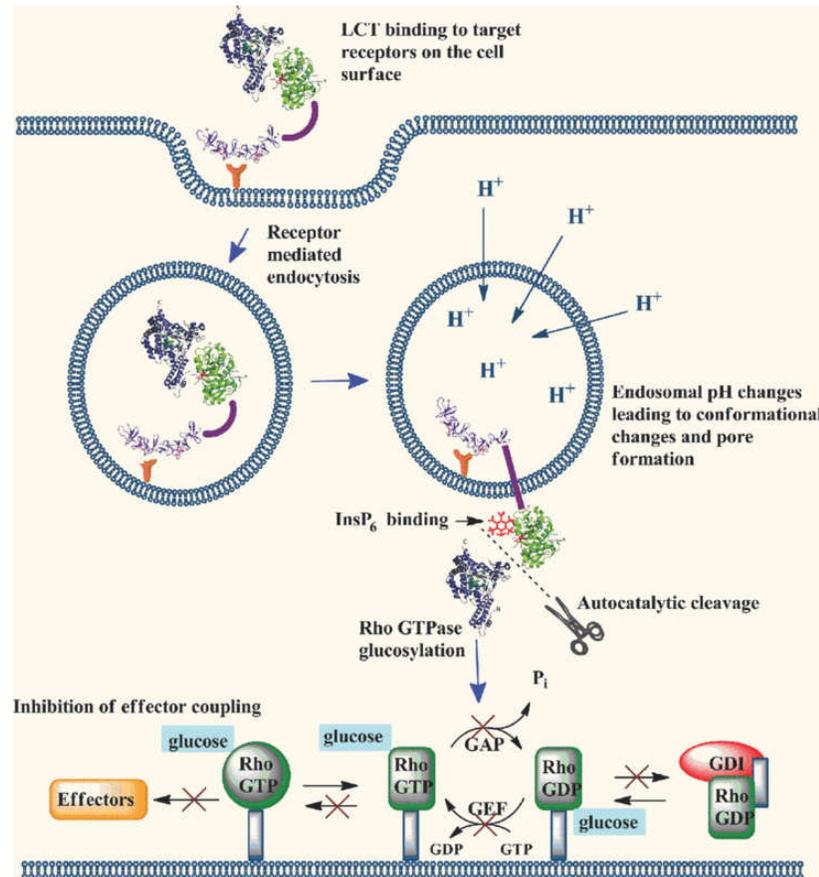


Figure 2. Cellular processing pathway of large clostridial toxins. Taken from Davies et al 2011.

Conventional diagnostic methods typically involve algorithms composed of immunoassays, histopathological findings and/or PCR. Protocols and assays used tend to vary considerably between different laboratories. Cytotoxic assays are the original gold standard of *C. difficile* toxin detection, and involve inoculating tissue monolayers with fecal supernatant and observing for cytopathic effects. Also used as a gold standard is cytotoxigenic culture, wherein fecal samples are selectively cultured for *C. difficile* and cultural supernatant is used to inoculate tissue monolayers. The major drawback to both gold standards is time (up to 48 hours for cytotoxic assays and up to 72 hours for cytotoxigenic culture) and expense (Eastwood et al 2009). As well, the exact protocol and cell lines used tend to vary between laboratories. Immunoassays specific for toxin A and/or toxin B have been widely adopted in clinical laboratories due to ease of use and relatively fast turnaround times, but they lack sensitivity compared to tissue culture standards. Rene et al (2012) evaluated eight commercial immunoassays and recorded sensitivities of 31.7 -55.2% compared to cytotoxic/cytotoxigenic standards. *C. difficile* antigen testing is performed in some laboratories, either as an initial screening test or as a combined antigen/toxin immunoassay. The antigen is a 46 kDa glutamate dehydrogenase (GDH) that typically exists as a high molecular weight aggregate, and is abundantly and constitutively expressed by both pathogenic and non-pathogenic *C. difficile* strains (Lyerly et al 1991). PCR has gained popularity as a diagnostic tool due to its high sensitivity and fast turnaround time. However, although PCR possesses high sensitivity, it has a somewhat lower positive predictive value – Novak-Weekley et al (2010) found PCR (Xpert *C. difficile* assay) to have a sensitivity of 94.4% and a positive predictive value of 84% when compared to toxigenic culture. The implication of these findings is that PCR is prone to the production of false positives – this is a predictable conclusion, due to the fact that PCR only detects the potential for toxin production (the presence of toxin genes) rather than the presence of the toxins. As well, *C. difficile* toxins are believed to be produced through a quorum sensing mechanism rather than constitutively, which lends further credence to the notion that the presence of toxin genes in enteric specimens is not a conclusive indication of *C. difficile* infection (Darkoh 2012). Early diagnosis of *C.*

difficile infection offers the best prognosis to the afflicted patient (Kelly&Lamont 1998), and so there is a need for an assay that demonstrates the presence or absence of toxin that combines the virtues of high sensitivity and fast-turnaround time.

Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) is a soft ionization technique that characterizes molecules based on their mass-to-charge ratio. Analyte molecules are cocrystallized in an organic acid matrix, which facilitates the ionization and desorption of the analyte when subjected to UV laser irradiation. The charged, gaseous analyte is then propelled by an electric field through a vacuum until it reaches a detector plate. The size of an ion is determined by the time it takes to reach the detector plate – the smaller the ion, the faster it will reach the detector plate. Proteins are typically identified with MALDI-TOF using mass fingerprinting, which consists of characterizing a protein based on the peptide set it produces when subjected to digestion. Since trypsin and other proteases used for this purpose cleave at specific residues, a characteristic set of fragments is produced that can be used to unambiguously identify the protein of interest (MALDI-TOF peptide and protein analysis is reviewed by Lewis et al 2000).

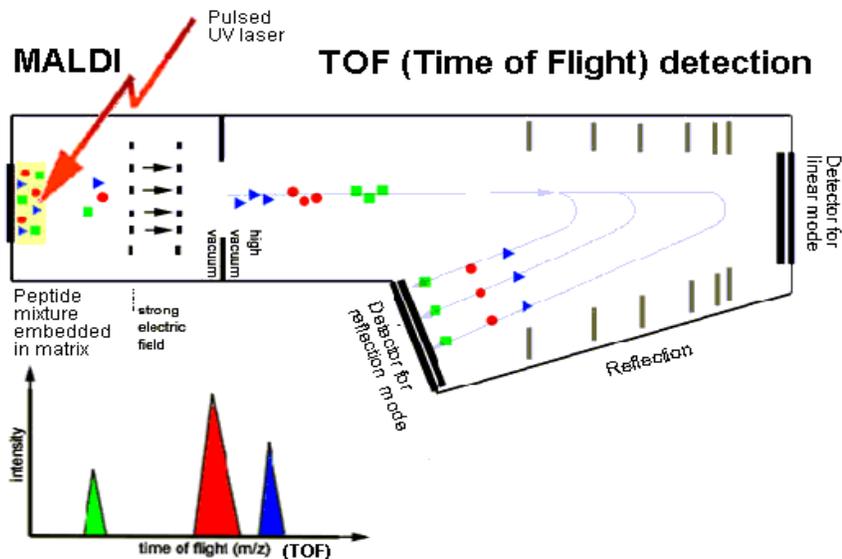


Figure 3. Schematic of the MALDI-TOF ionization and detection process. After laser irradiation and desorption, analyte molecules separate based on size as they travel towards the detector. Linear mode detection is used for larger (>20 kDa) proteins, and reflectron mode is used for smaller (<20 kDa) analyte molecules. Taken from the Radboud University Nijmegen website <http://www.ru.nl/science/gi/facilities/other-devices/maldi-tof/>.

This approach has been used successfully in the past to characterize LCTs (van Baar et al 2004). However, a mass fingerprinting approach has several drawbacks to large scale application in a clinical laboratory: first, a conventional tryptic digest takes approximately 12 hours to perform; second, preparation of samples from biological materials for digestion requires many steps to sufficiently purify and denature the protein of interest (van Baar et al 2004; Colquhoun et al 2006). Lastly, agents used to prepare the protein for the digestion process can interfere with the crystallization and ionization process and require removal before MALDI analysis. An alternative to mass fingerprinting is to simply detect the intact protein. Although this approach avoids the extensive preparation required for mass fingerprinting, it has two major shortcomings. One is that a protein cannot be unambiguously identified in this manner, since all that will be determined is the mass of the protein. The second is that MALDI is not well suited to detecting larger proteins (>100KDa) - larger proteins do not ionize as efficiently, and the peaks produced are much broader (van Remoortere et al 2010). These two problems can be addressed by taking advantage of the biology of *C. difficile* toxins; toxin A and B both undergo autocatalysis to produce a 63 kDa active toxin, which is more amenable to MALDI detection than the approximately 300 kDa holotoxins. As well, autocleavage occurs under specific conditions, which can be mimicked to add specificity to the method.

Inducing autocatalysis in toxin A and B has previously been performed using dithiothreitol (DTT), InsP6, or both (Egerer et al 2007; Egerer et al 2009; Reineke et al 2007). DTT is a well-known activator of cysteine proteases, and has a synergistic effect when used in combination with InsP6 on the autocleavage efficiency of *C. difficile* toxins (Egerer et al 2007). In previous studies, cleavage assays have been performed at a neutral pH. However, the change in conformation described by Qa'dan et al (2000) to a form presumably more conducive to autocleavage occurs at pH 4-5. In this study, it was theorized that adjusting the sample to pH 4-5 would produce conformational changes in the toxins that would enhance autocleavage upon the addition of InsP6, DTT, or a mixture of InsP6 and DTT.

Fecal material is arguably the most difficult biological fluid to analyze due to an abundance of microorganisms, intestinal cells, bile acids and other lipids, and undigested material that could interfere with protein analysis (Oleksiewicz et al 2005). However, despite the seemingly heterogeneous and complex nature of fecal material, Wu et al (2007) found that MALDI spectra were surprisingly simple and uniform between individuals in the 10-20kDa detection range (higher mass ranges were not examined in this study).

In this study, the efficacy of a variety of autocleavage conditions were tested on both pure toxin A as well as clinical fecal specimens to determine if the autocleavage event could be detected by comparing the region in the MALDI spectrum corresponding to the size of the active toxin (55-65 kDa) between samples before and after treatment.

MATERIALS AND METHODS

Biosafety considerations

Due to the sporogenous nature of *C. difficile*, special precautions were taken to prevent contamination of the work area. The study was carried out in a containment level 2 laboratory, and all work was conducted inside a biological safety cabinet using dedicated equipment. Pipette tips used to process clinical specimens were immersed in 10000 ppm Precept for at least ten minutes after use, and the biological safety cabinet was decontaminated with 2% Virkon after each use. The MALDI target plate was cleaned by first wiping with water and then propanol, followed by ultrasonication for ten minutes in propanol followed by TA30. Fecal material remaining after analysis and all disposable material used were autoclaved before disposal.

Materials

Acetylated Bovine Serum Albumin (BSA) was purchased from Promega, lyophilized pure *C. difficile* toxin A was purchased from List Biologicals, and sinipinnic acid matrix was purchased from Bruker Daltonics. Blunt fill needles, 0.22 micron filters, and 5 cc syringes were purchased from BD Canada. Reagents (sodium acetate, glacial acetic acid, anhydrous ethanol, trifluoroacetic acid, acetonitrile) were supplied by the Thompson Rivers University Chemistry and Biology departments.

Optimization of MALDI parameters and matrix selection

A saturated solution of sinapinnic acid in TA30 (3:7 HPLC grade acetonitrile:0.1% trifluoroacetic acid in water) prepared in the dried droplet method was used as a matrix, and a ground steel plate was used as a target. The dried droplet method simply involved placing 0.5 μL of matrix onto the target plate, followed by the addition of 0.5 μL of sample. The 1 μL volume was then mixed briefly by pipetting, and allowed to dry. Linear positron mode was used, and the detection range was 15-75 kDa. Laser intensity was set at 85%, and the laser was set to fire 100 shots in a random pattern. This was performed at five different locations on the target for a total of 500 shots per sample. BSA (0.1 $\mu\text{g}/\mu\text{L}$) was spotted with each batch of samples to ensure matrix integrity. Protein Standard II (Bruker Daltonics) was used as a calibrant, and was spotted onto the plate after each cleaning.

Induction of autocleavage in pure toxin

Pure toxin A (2 μg) was reconstituted in 100 μl of deionized water as per the specification sheet provided by List Biologicals. The pure toxin had been lyophilized with resuspension buffer components to form concentrations of 50mM NaCl and 50mM Tris when 100 μl of water was added. Four different conditions were applied to investigate which produced the desired autocatalytic effect, and were composed of combinations to produce final concentrations of DTT 2 mM and InsP6 5 mM. Conditions tested were deionized water, DTT, InsP6, and a

mixture of DTT and Insp6. All conditions were allowed to incubate for 5, 10, 20, and 60 minutes before being spotted.

Clinical Specimens

Stool specimens from suspected *C. difficile* infection cases were collected by Royal Inland Hospital (RIH) staff. After testing using the C.diff Quik Chek Complete® immunoassay (Techlab), the remainder of the specimen was stored at -80°C until the commencement of the project. Each specimen was assigned a number, and the immunoassay results for each specimen were recorded by RIH staff onto a master list (*C. difficile* antigen/toxin positive/negative). Upon receipt, specimens were allowed to thaw and immediately processed. Approximately 100µl of fecal specimen was diluted ten times in deionized water and filter sterilized with a 0.22 micron syringe filter in accordance with the method of fecal sample preparation for toxin detection outlined by O'Connor et al (2001). The filtrate was then split into two equal volumes. To each volume of filtrate one volume of HPLC grade acetonitrile was then added, vortexed for 5 seconds, and allowed to incubate at room temperature for 20 minutes. The sample pair was then centrifuged at 10000 rpm for 10 minutes at room temperature in a bench top centrifuge, and the supernatant poured off. One pellet was then resuspended in 200 µL deionized water, and the other in 200 µL sodium acetate (100 mM, pH 4.7). Spots were made from each suspension condition. The samples were then analyzed using MALDI-TOF.

RESULTS

Pure toxin

The attempted induction of autocatalysis in pure toxin A did not provide any clear signals. It was suspected that buffer contamination may have interfered with MALDI analysis, and so protein precipitation was attempted using 0.1% TFA in HPLC grade acetonitrile as per Hamrita

et al (2012). The mixture was allowed to incubate at room temperature for 30 minutes, and then was centrifuged at 10 000 rpm for 10 minutes. Autocatalysis conditions were once more tested, and no signals were produced.

Table 1. Effect of autocleavage conditions and incubation time on the presence of peaks in the 55-65k Da region for pure toxin A.

Autocleavage Condition	Peak between 55 and 65 kDa present/absent			
	5 minutes	10 minutes	20 minutes	60 minutes
DTT (2 mM)	absent	absent	absent	absent
InsP6 (5 mM)	absent	absent	absent	absent
DTT (2 mM) and Insp6 (5 mM)	absent	absent	absent	absent
Deionized water only	absent	absent	absent	absent

Clinical specimens

Initially, clinical specimens were diluted ten times in water and filtered through a 0.22 μm syringe filter. Matrices were prepared as a double layer as was suggested in the Bruker Daltonics user's manual for the detection of intact proteins. This involved spotting 0.5 μm of ethanol saturated with sinapinnic acid onto the target, allowing it to dry, and then spotting of a 1:1 mixture of TA30 saturated with sinapinnic acid and sample on top. The spectra produced were poor-quality, and were highly variable between spots from the same sample. Unsuitable matrix composition was suspected as a potential cause for lack of consistency between spots from the same sample, and so a dried droplet method was attempted instead. An aliquot of 0.5 μL of TA30 saturated with sinapinnic acid was spotted onto the target, to which 0.5 μL of sample was added and briefly mixed by pipetting. The dried droplet method produced more

consistent spectra between shots with less background noise. However, even with the dried droplet producing an improvement in the clarity of the spectra, peaks were still poorly resolved. It was theorized that lipid contamination may have been to blame, which can cause poor matrix cocrystallization (Wu et al 2007). The addition of one volume of acetonitrile followed by incubation for 20 minutes and precipitation via centrifugation at 10000 rpm on a benchtop centrifuge for 10 minutes was added to the protocol to remove hydrophobic contaminants, which drastically improved the quality of the spectra. Once intelligible spectra were produced, the induction of autocleavage was attempted. Precipitate was resuspended in both deionized water and sodium acetate (100 mM, pH 4.7). Baseline spots were taken, and DTT (2 mM final concentration) or a mixture of DTT and InsP6 (2 mM, 2 mM final concentration) were added to each resuspension condition. Samples were spotted after 30 minutes of incubation, and an intensity reading of greater than 500 was decided as indicating the presence of a peak due to the height of the baseline in raw spectra.

Table 2. Effect of clinical precipitate resuspension conditions on the presence of peaks in the 55-65kDa region.

Resuspension Condition	Peak between 55 and 65 kDa present/absent (intensity reading > 500)
Deionized water	absent
Deionized water, DTT (2 mM)	absent
Deionized water, DTT (2 mM), InsP6 (2 mM)	absent
Sodium acetate buffer (pH 4.7)	present
Sodium acetate buffer (pH 4.7), DTT (2 mM)	absent
Sodium acetate buffer (pH 4.7), DTT (2 mM), InsP6 (2 mM)	absent

The addition of DTT or DTT and InsP6 seemed to have decreased the quality of the spectra, and the presence of a peak corresponding to the size of the autocleavage product was not detected in the post-incubation spectra. However, when pre-incubation spectra from sodium acetate and water resuspension were overlaid from the same sample, there was a distinct, high-intensity peak present in the acidic solution not present in its water counterpart corresponding to a weight of approximately 56-60 kDa.

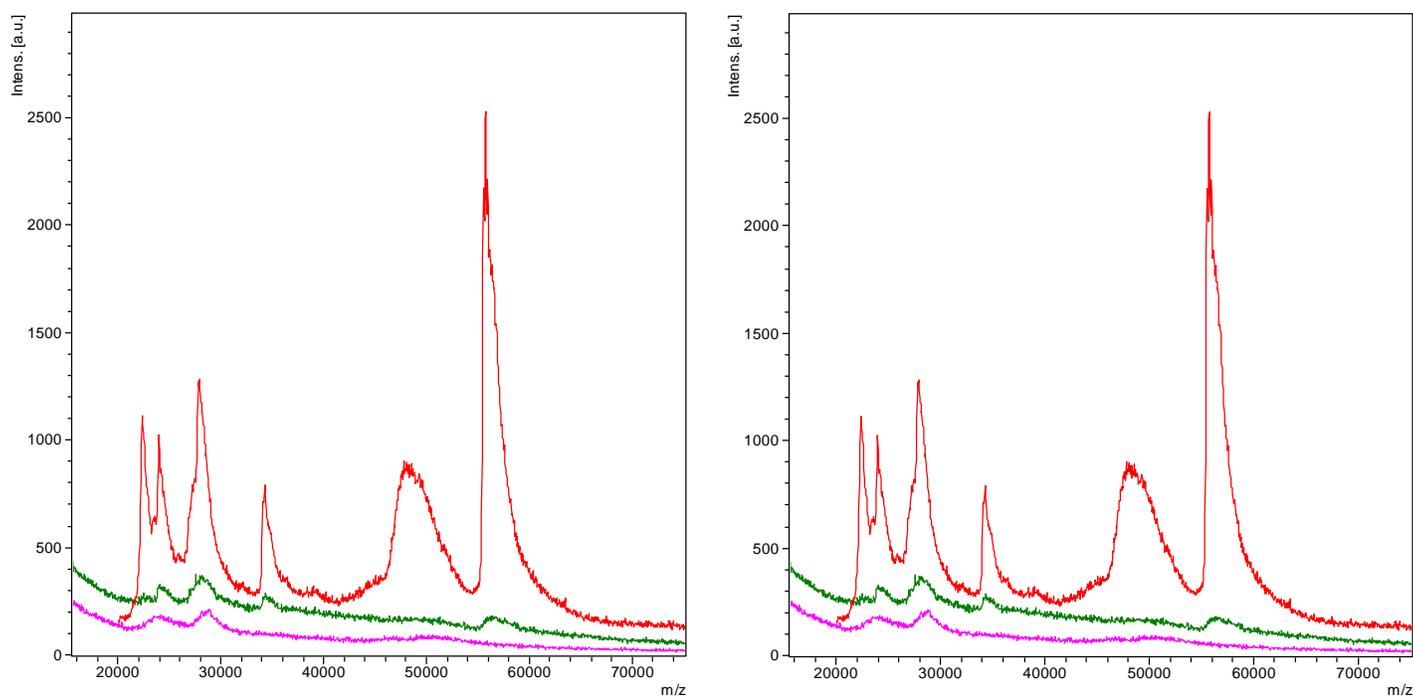


Figure 4. Comparison of known toxin (+) samples resuspended in 100 mM sodium acetate, pH 4.7 pre (red) and post-treatment with 2 mM DTT (green) and 2mM DTT+2mM InsP6 (pink). Two different samples shown.

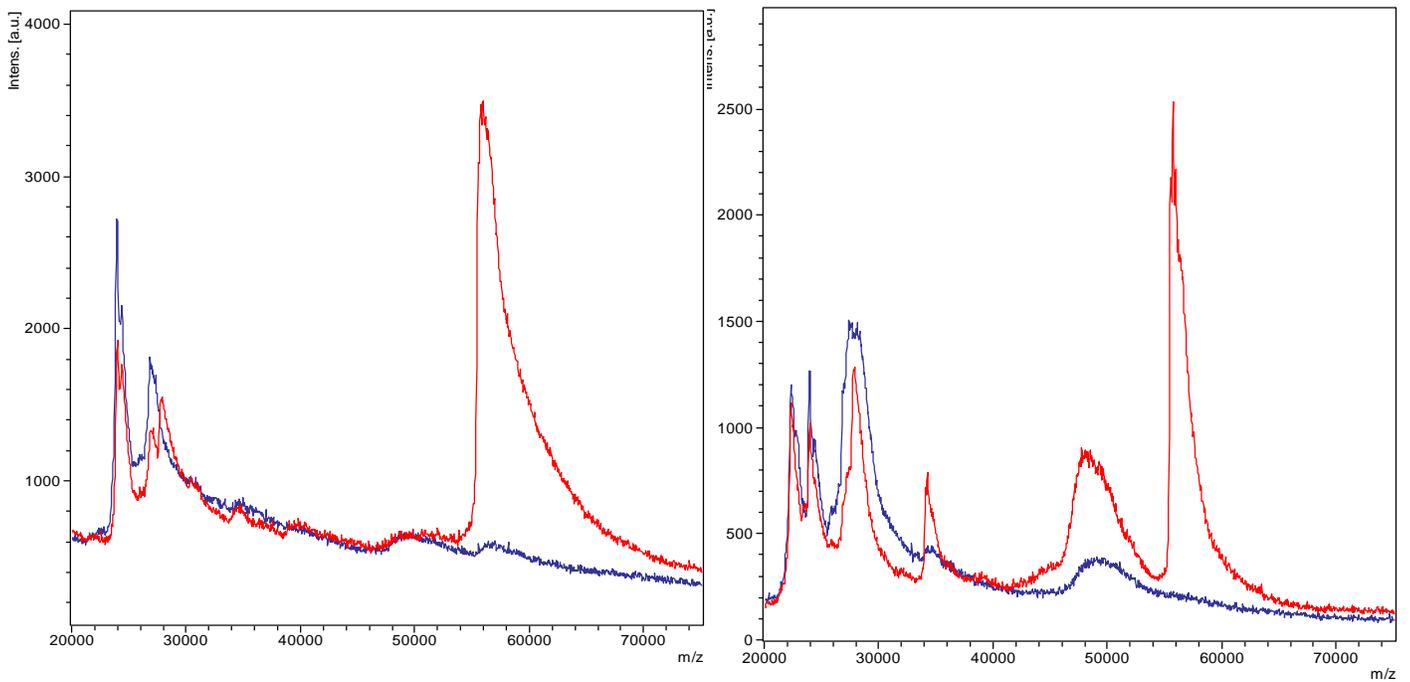


Figure 5. Comparison of known antigen(+)/toxin (+) samples resuspended in deionized water (blue) and 100 mM sodium acetate, pH 4.7 (red). Two different samples shown.

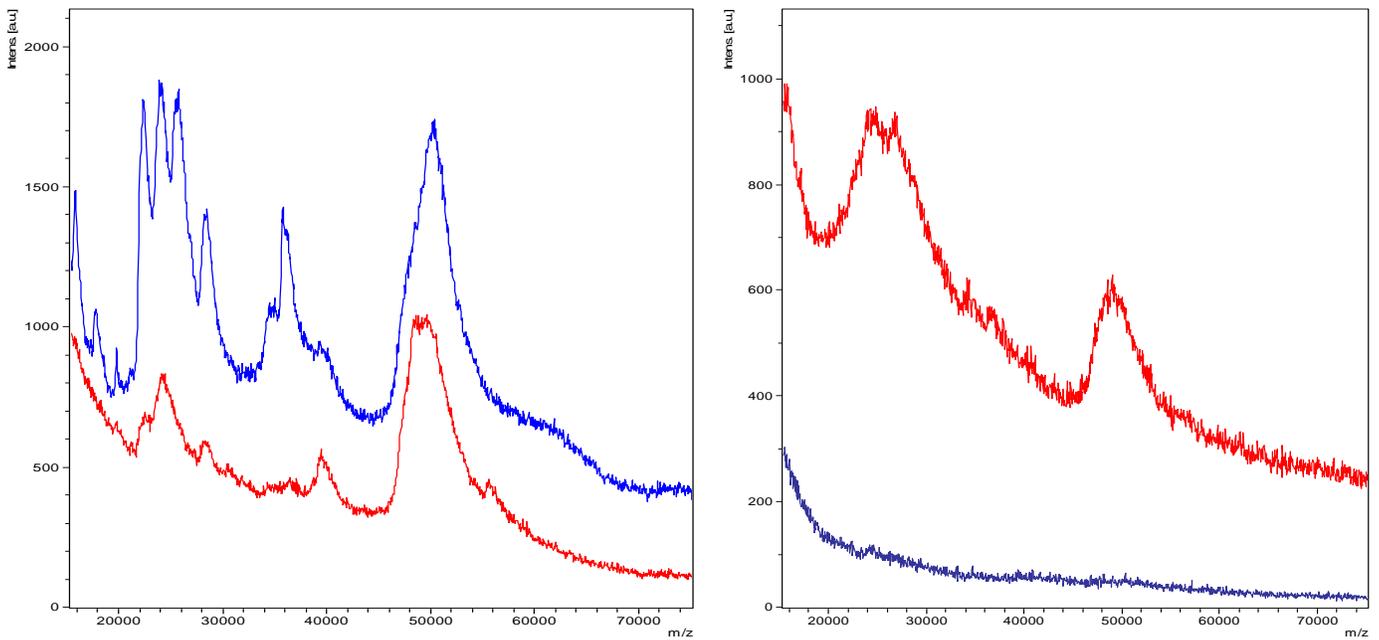


Figure 6. Comparison of known antigen (-)/toxin (-) samples resuspended in deionized water (blue) and 100 mM sodium acetate, pH 4.7 (red). Two different samples shown.

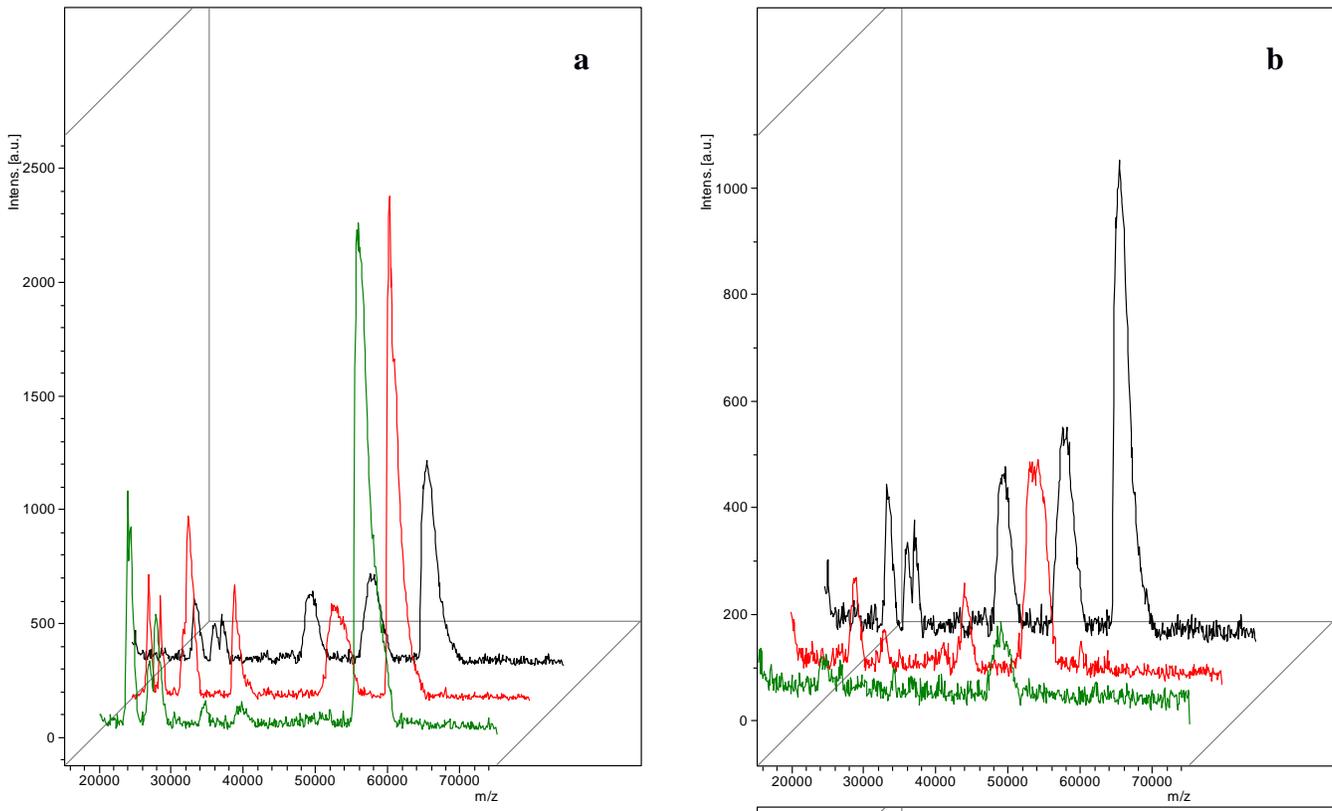


Figure 7a. Comparison between known antigen(+)/toxin (+) samples (red,green) and a known antigen (+) /toxin (-) sample (black). **7b.** Comparison between known antigen(-)/toxin(-) samples (red,green) and a known antigen (+)/toxin(-) sample (black).

The 56-60 kDa peak was not detected in known toxin-negative samples tested. However, one known antigen-positive/ toxin-negative sample was tested, which did demonstrate the presence of a peak between 56-60 kDa.

Despite the complex and heterogeneous nature of fecal material, spectra in the 15-75kDa range were remarkably similar between samples tested. This is similar to the findings of Wu et al (2007), wherein they examined the MALDI spectra in the 10-20 kDa range of feces spiked with haemoglobin from donors with unrestricted diets.

DISCUSSION

Initially, a mass fingerprinting approach was investigated as a means of demonstrating the presence of toxin A and B. As discussed previously, the time-consuming and labour intensive conventional mass fingerprinting approach was deemed impractical for use in the clinical laboratory, and so alternatives were sought to decrease time and effort required for sample identification. The longest step of the process, tryptic digestion, was targeted first. There are many modifications that can be used to decrease digestion time, though ultrasonic-assisted digestion appeared to be the most promising for several reasons: it is fast, with reports of complete digests being performed in as little as 40 seconds (Lopez-Ferrer et al 2005); it is easy to perform, and it is well suited to automation. The next modification investigated was abridgement of the conventional protein preparation procedure – typically, protein digestion involves protein denaturation with a chaotropic agent, disulfide bridge reduction, cysteine residue alkylation, and removal or dilution of chaotropic agents (van Baar et al 2004). These steps require several hours to complete, and so an alternative attempted was to dramatically simplify the procedure. Acetonitrile in water in a 4:1 ratio was used as a denaturant, as it was found by Strader et al (2005) to be an effective substitute for chaotropic agents and is compatible with MALDI analysis. As well, disulfide bond reduction and cysteine residue alkylation may not be required for digestion for all proteins (Lopez-Ferrer et al 2005). BSA was used to test digestion modifications, and a peptide set was produced that was identifiable as BSA using the simplified method with an overnight digestion in 4:1 acetonitrile in water with a 1:20 protease ratio. No successful digestions were produced using ultrasonic assistance along with a simplified protein preparation method. Mass fingerprinting was eventually abandoned as a means of protein identification due to the increasing number of expensive and time-consuming alterations that would be required to apply this method to analyzing fecal material, such as the use of immobilized trypsin, desalting filters, and potentially affinity purification. Though mass fingerprinting has been used to successfully identify proteins from pathogenic organisms in feces (Colquhoun et al 2006), the intention of this study was to

investigate the development of an assay comparable in turnaround time and ease of implementation to currently used enzyme immunoassays.

An autocleavage-based assay was investigated instead due to its relative simplicity and sturdiness compared to mass fingerprinting. Mass fingerprinting has many more steps, and so more potential for error in proper execution of each process. As well, a mass fingerprinting method is more prone to erroneous results due to contamination – in intact protein detection, a high-abundance contaminant would appear as a distinct peak; in mass fingerprinting, a high abundance contaminant would be digested alongside the protein of interest to produce a peptide spectra characteristic of neither. An advantage of a peptide mass fingerprinting is that it is considerably more sensitive than intact protein detection – larger mass proteins ionize with lower efficiency, and therefore must be present in higher quantities to be detected. The lowest limit of detection for intact proteins in blood samples for MALDI-TOF is typically $>1 \mu\text{mol/L}$, though this can vary considerably based on the size of the protein, its particular ionization efficiency, preparation methods, and signal suppression from the presence of other ions (Hortin 2006). The same may apply to fecal matter. The precipitation step in the method described in this study may help to partially compensate for reduced sensitivity at higher mass ranges. 100 μL of fecal material and 200 μL of water or sodium acetate for resuspension were arbitrarily chosen volumes – increasing the amount of fecal material used while decreasing the resuspension volume could concentrate the toxins, and increase the limits of sensitivity.

An omnipresent issue with MALDI is the effect of reagents and buffers on the cocrystallization process – final concentrations of reagents used were based on studies wherein successful pH induced conformation change/autocleavage of toxin had been induced (Qa'dan et al 2000; Egerer et al 2009; Reineke et al 2007), and were checked against the MALDI TOF buffer concentration tolerance chart

available on the UBC ChiBi website (see Appendix 1). Though the DTT concentration used was well below the suggested maximum concentration, a decrease in spectra quality was observed. This may have been due to this chart presumably applying to pure, low mass analysis, as this is typically what MALDI is used for – the ionization kinetics for high mass proteins in a complex sample may be sufficiently different to be affected by concentration of reagents considered reasonable for lower mass analysis. No information was found for suggested concentrations of InsP6 for MALDI-TOF analysis. The original design of this experiment involved comparing samples pre-and post-treatment; however, treatment seemed to significantly reduce the quality of the spectra. Instead, an alternative to this approach that arose during the course of this investigation was to compare precipitate resuspended in water to precipitate resuspended in sodium acetate. Not only does this method produce better quality spectra, but it is also faster than the previous method design. The peak produced corresponds to a smaller size than the expected autocleavage product (56-60 kDa compared to 63kDa) – it is possible that the active toxin may undergo a previously unidentified protein modification post-cleavage or predictable fragmentation during the ionization process, though this is just speculation. Unfortunately, no spectra were obtained from the attempted induction of autocleavage of pure toxin A to be used for comparison's sake. It is possible that buffer and experimental conditions interfered with the production of useable spectra, that the toxin had been degraded during storage or transport, or that autocleavage was not successfully induced. Interestingly, no peaks were present in the 55-65 kDa range for toxin negative samples, whereas the antigen positive/toxin negative sample demonstrated a peak neatly corresponding with the high intensity peak produced from toxin positive samples. It is possible that toxin was present in this sample, but was not detected with the immunoassay – if this were the case, then this protocol may hold potential as a higher sensitivity alternative to *C. difficile* toxin immunoassays.

Aside from the detection of toxin A and B, examining the 15-75 kDa mass range may allow the detection of a third *C. difficile* toxin (binary toxin) as well as other proteins secreted by *C. difficile* during toxin production. Although binary toxin alone is not suspected to cause disease, infection with strains possessing all three toxins has a higher case-fatality rate than infection with strains only capable of toxin A and B production, indicating that binary toxin may potentiate toxins A/B or act as a virulence marker (Bacci et al 2011). Binary toxin is composed of two independently produced proteins, with masses of 48 kDa and 94 kDa (Goncalves et al 2004). As well, Mukherjee et al (2002) demonstrated the secretion of a number of proteins by pathogenic *C. difficile* in the mass ranges of 24-50 kDa.

Despite the potential this assay presents, there are some shortcomings to consider. The method proposed does not lend itself well to quantification. The aliquot of fecal material used can only be roughly 100 μ L due to variations in consistency of fecal material, which also affects the final volume of filtrate. As well, MALDI is not inherently well suited to quantification studies due to matrix heterogeneity – that is, analyte molecules will crystallize in different concentrations in different parts of the matrix, and so signal intensity may vary considerably between shots (Szajli et al 2008). It is possible to create a standard curve for a given analyte of interest, and to take a sufficiently large number of shots to compensate for matrix heterogeneity. However, standard curves would be best applied to pure samples rather than complex ones – differences in fecal composition between individuals could foreseeably affect matrix cocrystallization, and therefore the relative intensities generated from a sample. Although technical hurdles exist to quantification, it is not a pressing concern due to the primarily qualitative nature of *C. difficile* infection diagnosis – all that is required is the detection of toxin coupled with clinical manifestations for the diagnosis of *C. difficile* infection (Bouza 2005).

The significance of the ~60 kDa peak present in precipitate resuspended under acidic conditions, along

with other peaks present in toxin positive samples in the 15-75 kDa range, needs further investigation. Many more samples need to be tested to determine if the presence of the peak is correlated to the presence of toxin. If there does turn out to be a correlation, the identity of the ~60 kDa peak would need to be confirmed through Western blotting.

If results obtained indicate that this method may be a viable assay, information such as sensitivity, specificity, accuracy, positive predictive value, and negative predictive value when compared to a gold standard will need to be determined. As well, appropriate storage conditions and testing timeframes for this assay need to be tested.

REFERENCES

1. Bacci, S., Molbak, K., Kjeldsen, M., & Olsen, K. (2011). Binary Toxin and Death after *Clostridium difficile* Infection. *Emerging Infectious Diseases*, *17*(6), 976–982.
2. Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R., & Aktories, K. (2001). Low pH-induced Formation of Ion Channels by *Clostridium difficile* Toxin B in Target Cells. *The Journal of Biological Chemistry*, *276*, 10670–10676.
3. Bartlett, J. G. (2002). Antibiotic-Associated Diarrhea. *New England Journal of Medicine*, *346*(5), 334–339.
4. Bouza, E., Muñoz, P., & Alonso, R. (2005). Clinical manifestations, treatment and control of infections caused by *Clostridium difficile*. *Clinical Microbiology and Infection*, *11*, 57–64.
5. Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M., & Eichel-Streiber, C. von. (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene*, *181*(1–2), 29 – 38.
6. Chaves-Olarte, E., Weidmann, M., Eichel-Streiber, C., & Thelestam, M. (1997). Toxins A and B from *Clostridium difficile* differ with respect to enzymatic potencies, cellular substrate specificities, and surface binding to cultured cells. *The Journal of Clinical Investigation*, *100*(7), 1734–1741.
7. Colquhoun, D., Schwab, K., Cole, R., Halden, R. (2006). Detection of norovirus capsid protein in authentic standards and in stool extracts by matrix-assisted laser desorption ionization and nanospray mass spectrometry. *Applied and Environmental Microbiology*, *72*(4), 2749-2755.

8. Darkoh, C. (2012). Regulation of toxin synthesis by *Clostridium difficile*. *University of Texas Dissertations and Theses*.
9. Davies, A., Roberts, A., Shone, C., & Acharya, K. (2011). Super toxins from a super bug: structure and function of *Clostridium difficile* toxins. *The Biochemical Journal*, 436(3), 517–526.
10. Eastwood, K., Else, P., Charlett, A., & Wilcox, M. (2009). Comparison of Nine Commercially Available *Clostridium difficile* Toxin Detection Assays, a Real-Time PCR Assay for *C. difficile* tcdB, and a Glutamate Dehydrogenase Detection Assay to Cytotoxin Testing and Cytotoxigenic Culture Methods. *Journal of Clinical Microbiology*, 47(10), 3211–3217.
11. Egerer, M, Gieseemann, T., Herrmann, C., & Aktories, K. (2009). Autocatalytic Processing of *Clostridium difficile* Toxin B: binding of inositol hexakisphosphate. *Journal of Biological Chemistry*, 284(6)3389–3395.
12. Egerer, Martina, Gieseemann, T., Jank, T., Satchell, K. J. F., & Aktories, K. (2007). Auto-catalytic Cleavage of *Clostridium difficile* Toxins A and B Depends on Cysteine Protease Activity. *Journal of Biological Chemistry*, 282(35), 25314–25321.
13. Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., & Sauerborn, M. (1992). Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Molecular and General Genetics MGG*, 233(1-2), 260–268.
14. Florin, I., & Thelestam, M. (1983). Internalization of *Clostridium difficile* cytotoxin into cultured human lung fibroblasts. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 763(4), 383 – 392.
15. Goncalves, C., Decre, D., Barbut, F., Burghoffer, B. Petit, J. (2004) Prevalence and characterization of a binary toxin (actin-specific ADP_ribosyltransferase) from *Clostridium difficile*. *Journal of Clinical Microbiology*, 42(5) 1933-1939.
16. Hamrita, B., ben Nasr, H., Remadi, S., Chaieb, A., Chahed, K. (2012). Enrichment methods for revealing lower molecular weight biomarkers in human breast plasma proteome. *African Journal of Biochemistry Research*, 6(1), 1-7.
17. Hortin, G. L. (2006). The MALDI-TOF Mass Spectrometric View of the Plasma Proteome and Peptidome. *Clinical Chemistry*, 52(7), 1223–1237.
18. Jank, T., & Aktories, K. (2008). Structure and mode of action of clostridial glucosylating toxins: the {ABCD} model. *Trends in Microbiology*, 16(5), 222 – 229.
19. Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., & Aktories, K. (1995). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature*, 375, 500–503.

20. Kelly, C. & Lamont, T. (1998). *Clostridium difficile* infection. *Annual Review of Medicine*, 49, 375-390.
21. Lewis, J., Wei, J., & Siuzdak, G. (2000). Matrix-assisted Laser Desorption/Ionization Mass Spectrometry in Peptide and Protein Analysis. In *Encyclopedia of Analytical Chemistry*. Chichester: John Wiley & Sons Ltd.
22. López-Ferrer, D., Capelo, J. L., & Vázquez, J. (2005). Ultra Fast Trypsin Digestion of Proteins by High Intensity Focused Ultrasound. *Journal of Proteome Research*, 4(5), 1569–1574.
23. Lyerly D., Barroso, T., Wilkins, T. (1991). Identification of the latex test-reactive protein of *Clostridium difficile* as glutamate dehydrogenase. *Journal of Clinical Microbiology*, 29(11), 2639-2642.
24. Mukherjee, K., Karlsson, S., Butman, L., Akerlund, T. (2002). Proteins released during high toxin production in *Clostridium difficile*. *Microbiology*, 148, 2245-2253.
25. Novak-Weekley, S. M., Marlowe, E. M., Miller, J. M., Cumpio, J., Nomura, J. H., Vance, P. H., & Weissfeld, A. (2010). *Clostridium difficile* Testing in the Clinical Laboratory by Use of Multiple Testing Algorithms. *Journal of Clinical Microbiology*, 48(3), 889–893.
26. O'Connor, D., Hynes, P., Cormican, M., Collins, E., Corbett-Feeney, G., Cassidy, M. (2001). Evaluation of Methods for Detection of Toxins in Specimens of Feces Submitted for Diagnosis of *Clostridium difficile*-Associated Diarrhea. *Journal of Clinical Microbiology*, 39(8), 2846-2849.
27. Oleksiewicz, M., Kjeldal, H., & T. G. Kleno. 2005. Identification of stool proteins in C57BL/6J mice by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. *Biomarkers*, 10(1), 29-40.
28. Qa'dan, M., Spyres, L., Ballard, J. (2000). pH-induced conformational changes in *Clostridium difficile* toxin B. *Infection and Immunity*, 68(5), 2470-2474.
29. Reineke, J., Tenzer, S., Rupnik, M., Koschinski, S. Hasselmayer, O. Schrattenholz, A. Schild, H., von Eichel-Streiber, C. (2007). Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature*, 446(7134), 415-419.
30. Rene, P., Frenette, C., Schiller, I., Dendukuri, N., Brassard, P., Fenn, S., & Loo, V. (2012). Comparison of eight commercial enzyme immunoassays for the detection of *Clostridium difficile* from stool samples and effect of strain type. *Diagnostic Microbiology and Infectious disease*, 73(1), 94–96.
31. Rupnik, M., Wilcox, M., & Gerding, D. (2009). *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nature*, 7(7), 526–536.

32. Strader, M. B., Tabb, D. L., Hervey, W. J., Pan, C., & Hurst, G. B. (2006). Efficient and Specific Trypsin Digestion of Microgram to Nanogram Quantities of Proteins in Organic–Aqueous Solvent Systems. *Analytical Chemistry*, 78(1), 125–134.
33. Szajli, E., Feher, T., & Medzihradsky K. (2008). Investigating the Quantitative Nature of MALDI-TOF MS. *Molecular & Cellular Proteomics*, 7, 2410-2418.
34. Tucker, K., & Wilkins, T. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. *Infection and Immunity*, 59(1), 73–78.
35. van Baar, B., Hulst, A. G., de Jong, A. L., & Wils, E. R. . (2004). Characterisation of botulinum toxins type C, D, E, and F by matrix-assisted laser desorption ionisation and electrospray mass spectrometry. *Journal of Chromatography A*, 1035(1), 97–114.
36. van Remoorte, A., van Zeijl, r., van den Oever, N., Franck, J., Longuespee, R., Wisztorski, M., Salzet, M., Deelder, A., Fournier, I., McDonnell, L. (2010) MALDI imaging and profiling MS of higher mass protein from tissue. *Journal of the American Society for Mass Spectrometry*, 21(11), 1922-1929.
37. von Eichel-Streiber, C., Laufenberg-Feldmann, R., Saringen, S., Schulze, J., Sauerborn, M. Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Molecular and General Genetics MMG*, 233(1-2), 260-268.
38. Wu, C., Tsai, C., Lu, C., Wu, P., Wu, D., Lin, S., Shiea, J. (2007). Diagnosis of occult blood in human feces using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. *Clinica Chimica Acta* 384, 86-92.

APPENDIX

Tolerances of MALDI
contaminants
Maximum Allowable

Component Maximum
Concentration

TOF MS to buffer components and
Concentration

Ammonium Acetate	500 mM
Ammonium Bicarbonate	250 mM
CAPS	200 mM
Dithiothreitol (Dtt)	500 mM
EPPS	250 mM
Glycerol	1% v/v
Glycine	500mM
Guanidine-HCl	500 mM
HEPES	100 mM
Imidazole	250 mM

Taken from the UBC Centre for High-Throughput Biology (CHiBi) website
(<http://www.chibi.ubc.ca>)