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PROTOCOL DEVELOPMENT FOR THE USE OF MATRIX ASSISTED LASER DESORPTION/IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF-MS) FOR DETECTION OF CLOSTRIDIUM DIFFICILE TOXIN A AND B FROM STOOL SAMPLES

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by

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ABSTRACT

Background: *Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacillus. *C. difficile* is important in the medical community because it is the most common cause of antibiotic-associated diarrhea and infections can lead to serious complications such as toxic megacolon and pseudomembranous colitis. Infection is mediated by toxins A and B, both of which are proteins that undergo processing within the target cell to yield a 63 kDa active domain.

Objective: This study investigates the use of MALDI-TOF-MS to detect the active domains of *C. difficile* toxins A and B from stool samples to explore the possibility of using MALDI as a diagnosite tool for *C. difficile* infections.

Methods: Known toxin and antigen positive and negative stool samples sent 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 a sinnapinic acid (SA), 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 co-crystalized.

Results and Discussion: MALDI analysis showed no difference between samples diluted in deionized water and those diluted in PBS. Protein precipitation with acetonitrile produced higher quality spectra than protein precipitation with ammonium sulfate. Sample cocrystalization with a SA matrix provided higher quality spectra than sample co-crystalization with a SDHB or CHCA matrix. MALDI analysis showed no peaks in the 63 kDa range in any of the samples. Because stool is a complex combination of materials, MALDI mass spectra were expected to be complicated and show vast differences between samples. Surprisingly, all ten MALDI spectra acquired were relatively similar. Similar individual ion signals were seen between 20 and 60 kDa and above 70 kDa. 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. **Conclusion:** We were unable to use MALDI to detect the 63 kDa active domains of *C*. *difficile* toxins A and B from crude stool protein extracts. Further studies would be required to ascertain the possibility of using this technological tool to detect *C*. *difficile* toxins as an alternative method of diagnosis to the tests currently available. Although inconclusive, this study is a starting point for the investigation of MALDI as a diagnostic tool in a clinical setting.

Thesis supervisor: Associate Professor Naowarat Cheeptham

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TABLE OF CONTENTS

Titlei
Abstractii
Acknowledgementsiv
List of Figuresvi
List of Tablesviii
Introduction
Background1
Clostridium difficile toxins A and B1
Current diagnostic tools
MALDI as a diagnostic tool
Objective
Materials and Methods
Induction of autocleavage in commercially purchased toxin A9
Clinical stool sample preparation10
MALDI parameters and matrix selection11
PNPG as a toxin-specific substrate
Results
Induction of autocleavage in commercially purchased toxin A12
Clinical stool samples
PNPG as a toxin-specific substrate16
Discussion17
Literature cited
Appendix A

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Background

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that is commonly the causative agent of antibiotic-associated diarrhea (CDC 2012; Hookman et al. 2009). It is found as part of the normal intestinal flora of 5% of adults and up to 70% of infants (Vaishnavi 2010). It was first described by Hall and O'Toole in 1935 during their experiments involving the intestinal flora of new born 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 (Johnson et al. 1998). Antimicrobial therapies can result in the disruption of normal intestinal flora and cause subsequent overgrowth of opportunistic *C. difficile*. These infections are of great concern due to the risk of resulting complications such as toxic megacolon and pseudomembranous colitis. Additionally, a new, hypervirulent strain, NAP1/BI/027, was described by McDonald et al (2005). This new strain showed increased resistance to flouroquinolone 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 United States resulted in more than \$3600 in additional health care costs and these costs are estimated to exceed \$1.1 billion per year (Kyne et al. 2002). In 2011 there was an estimated half a million CDIs in the United States and 29,000 people died within 30 days of diagnosis (CDC 2015).

Clostridium difficile toxins A and B

Pathogenicity is mediated by toxin A and B production, encoded by the *tcdA* and *tcdB* genes, respectively (Cohen et al. 2000). These genes are part of a 19.6 kb pathogenicity locus (PaLoc) that is only present in toxigenic strains. Early studies looking into the mechanisms of action of toxin A and B found that both inhibit ADP-ribosylation of the GTP-binding protein

Rho, rendering it inactive and unable to regulate the microfilament cytoskeleton (Just et al. 1994; Just et al. 1995). These toxins produce their effects by inactivating Rho proteins, resulting in depolymerization of actin fibers, cytoskeleton instability, and cell death. Binary toxin is also produced by certain strains of *C. difficile*. Although its role in pathogenicity remains unknown, it has been found that hypervirulent strains of *C. difficile* produce this toxin in addition to toxin A and B (Papatheodorou et al. 2013).

Both toxins A and B, 308 and 269 kDa in size, respectively, must undergo processing within the target cell before a 63 kDa active domain is released and able to produce its toxigenic effects (Figures 1 and 2) (Pruitt et al. 2012; Giesemann et al. 2008). The whole 308 or 269 kDa toxin is first taken into the target cell through receptor mediated endocytosis (Figure 2). 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 rest of the toxin located inside the endosome. Cytosolic inositol hexakisphosphate (Ins6P) induces autocleavage at the "cutting domain", releasing the active domain into the target cell. The free 63 kDa active domain possesses glucosyltransferase activity and inactivate Rho, Rac, and Cdc42 within the target cell (Voth et al. 2005). The target cell becomes unable to regulate the microfilament cytoskeleton, causing subsequent depolymerization of actin fibers, cytoskeleton instability, and cell death (Just et al. 1994; Just et al. 1995). The presence of this 63 kDa active domain in stool therefore indicates CDI.



Figure 1. Molecular protein structure of *C. difficile* toxins A (TxA) and B (TxB), showing homologous catalytic domains with glucosyltransferase activity (black), autocleavage domains (circles), hydrophobic regions which allow the toxins to be inserted into the target cell's endosomal membrane during processing (triangles), and COOH-terminal repeats with receptor binding domains (white). (Taken from Pruitt et al. 2012)



Figure 2. 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 Ins6P 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)

Current diagnostic tools

As in any bacterial infection, early diagnosis enables early treatment and prevention of complications. Current methods for the diagnosis of CDI are less than ideal for clinical use as they are either time consuming, relatively insensitive, or require expensive and specialized equipment (CDC 2012; Kelly et al. 1998). Stool cultures are slow to yield results and only confirm the presence of the bacteria, not necessarily infectious toxin producing bacteria. PCR assays can confirm the potential for disease but does not confirm the expression of the genes responsible for toxin production. Tissue culture cytotoxicity assay detects toxin B only, is difficult to perform, is costly, and requires up to two days for results. Enzyme immunoassay, although easy to perform, are relatively insensitive and may give false results. The glutamate dehydrogenase (GDH) detection assay tests for the presence of the enzyme glutamate dehydrogenase and is relatively sensitive and specific for C. difficile (Eastwood et al. 2014). However, GDH detection assay is only able to confirm the presence of *C. difficile* and does not indicate toxin production. In combination, these methods allow for more reliable results, however the time sensitive nature of CDI treatment remains unaddressed. To add to these difficulties, toxins A and B are very unstable, degrading at room temperature and becoming difficult to detect only a few hours after stool sample collection (CDC 2012).

Currently, the Interior Health Authority uses a combination of toxin and antigen screening with TechLab C. DIFF QUIK CHEK COMPLETE, which screens for GDH as well as *C. difficile* toxins A and B (Dr. Prenilla Naidu, Sue Whitehead, and Dr. Cheryl Millar personal communication, 2015). If screening yields inconclusive results (e.g., antigen positive but toxin negative), subsequent PCR analysis is performed to confirm the presence of the PaLoc (Figure 3). Again, these methods are flawed in that the presence of the PaLoc does not confirm toxin production, only the potential for disease. Many clinical laboratories in Canada have seen that a shocking 40% of toxin and antigen screenings yield inconclusive results (Dr. Prenilla Naidu, personal communication, 2015). Of this 40%, 20-50% are PCR positive for PaLoc.



Figure 3. Diagram showing current diagnostic steps used by The Interior Health Authority to diagnose CDIs. Stool samples with suspected CDIs are screened with C.DIFF QUIK CHEK from TechLab, which screens for both GDH and *C. difficile* toxins A and B. If the sample tests negative for both, there is no CDI diagnosis. If the sample tests positive for both, a CDI is diagnosed. If the test is inconclusive, samples are sent for PCR testing for PaLoc. If negative, no CDI is diagnosed. If positive, a CDI is diagnosed.

MALDI as a diagnostic tool

C. difficile is frequently a nosocomial pathogen that is difficult to control due to its ability to produce spores (CDC 2012). Therefore, it is critical that a more clinically useful method of diagnosis is available for reliable and early detection of CDIs. Many recent studies

have involved the identification of bacteria with the use of matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS or MALDI), as it has been shown to be a fast, accurate, and cost-effective technique (Bohme et al. 2010).

During MALDI analysis, the analyte is co-crystalized with an organic matrix and the analyte-matrix mixture is subjected to laser irradiation (Lewis et al. 2000). The laser light energy is absorbed by the matrix, which is vapourized and indirectly causes the analyte to vapourize as well. MALDI-TOF refers to the time-of-flight mass analyzer used with the MALDI ionization source. TOF analysis propels ions towards a detector plate and the size of the ion is determined based on the time it takes to reach the detector plate. Since ions have the same energy but different masses, smaller ions reach the detector plate faster than larger ions. In MALDI-TOF-MS results are given in the form of a mass spectrum, a plot of the intensity of the ion signal as a function of the mass-to-charge ratio.

MALDI has many advantages over other analytical tools used in proteomics as it is easy to operate and can be automated to allow for easy screening of large sample numbers (Cho et al. 2013). 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. MALDI is highly sensitive, has a fast turnaround time, and tests are relatively inexpensive to run, all of which are characteristics highly sought after in a diagnostic tool (Lin et al. 2007). The toxin and antigen screenings currently in use by the Interior Health Authority costs \$13 per run with an additional \$45 for PCR analysis if the results are inconclusive B (Dr. Prenilla Naidu personal communication, 2015). Because extensive sample preparation is normally not necessary with MALDI, the cost to diagnose *C. difficile* infections with MALDI would be cents per specimen. Furthermore, our 63 kDa protein of interest falls within the mass range MALDI is capable detecting, as proteins larger than 100 kDa do not ionize as efficiently (van Remoortere et al. 2010). Because of these advantages, we believe MALDI to be a viable diagnostic tool for identifying CDIs using the 63 kDa active domain as a biomarker.

Previous studies in our laboratory have explored the possibility of using MALDI to detect the active domains of *C. difficile* toxins A and B in stool samples with no success (Koeck et al. 2013; McClean et al. 2014, Lam et al. 2014). This research will explore

different protein extraction methods as well as different MALDI matrices to optimize *C*. *difficile* toxin detection.

The concern that the 63 kDa active domain may not be present in stool in high enough concentrations for detection with MALDI to be successful should be addressed. Although MALDI is sensitive enough to detect femtomoles of analyte (Lin et al. 2007), our analyte will not consist of purified C. difficile toxins. Because we aim to reduce laborious sample preparation in the interest of creating a protocol that will be quick to yield results, our analyte will consist of the 63 kDa protein of interest as well as a mixture of proteins found in stool. The ion suppression effect, where the ion signals from high abundance ions suppress the signals from low abundance ions, is of concern in this situation (Wu et al. 2007). Additionally, C. difficile toxins are relatively unstable and degrade easily at room temperature (CDC 2012). As a method of detecting the 63 kDa protein of interest that may be present in concentrations undetectable to MALDI, we explored the idea of exploiting a toxinspecific substrate (Boyer et al. 2011). 4-Nitrophenyl β -D-glucopyranoside (PNPG) is a substrate of C. difficile toxins (Darkoh 2012). Toxins A and B cleave the O-glycosidic bond of PNPG, producing 4-nitrophenol as a product (Figure 4). Although this product is too small to be detected with MALDI, the cleavage event can be monitored with a spectrophotometer at 410 nm. This method of toxin detection also allows for toxin quantitation, as under optimal conditions (temperature 35-40°C, pH 8) and unlimited PNPG, the amount of 4-nitrophenol is directly proportional to the amount of toxin present. Therefore, this method of confirming the presence of the 63 kDa active domain can help us determine the limits of detection for C. *difficile* toxins in minimally processed stool samples using MALDI.



Figure 4. *C. difficile* toxins A and B cleaving the O-glycosidic bond of PNPG to produce 4nitrophenol. This autocleavage event can be monitored with a spectrophotometer at 410 nm (Taken from Darkoh 2012)

Objective

The goal of this research is to design a protocol which allows for the detection of *C*. *difficile* toxins A and B in stool samples with minimal sample preparation. The protocol relies on the detection of the 63 kDa active domain in clinical stool samples with MALDI. Such a protocol would allow for the possibility of MALDI to be used as a clinical diagnostic tool for the diagnosis of CDIs, as it would be a fast and direct method of confirming the presence of *C. difficile* toxins in stool. The use of toxin-specific substrates will also be explored as a possible alternative toxin detection method.

MATERIALS AND METHODS

Induction of autocleavage in commercially purchased toxin A

Toxin A 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 was 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 gave a resuspension buffer consisting of 50 mM Tris, pH 7.5, 50 mM NaCI, and 0.1 % trehalose.

Autocleavage of the toxin was attempted using varying concentrations of dithiothreitol (DTT) and inositol hexakisphosphate (InsP6). These concentrations were much higher than those attempted in previous studies (Koeck et al. 2013; McClean et al. 2014; Lam et al. 2014), ranging from 4 to 10 mM and 8 to 20 mM, respectively. Each reaction was adjusted to pH 4.5 with sodium acetate to mimic the environment of an endosome. Incubation times ranged from 0.5 to 72 hours both at room temperature and at 37°C (Table 1).

Table 1. Concentrations and volumes of DTT and InsP6 used to induce autocleavage in 1 μ g
of toxin A. Samples were incubated at room temperature or 37 °C for 0.5 to 72 hours.
Reactions adjusted to 4.5 and volumes adjusted to 26 μ l with deionized water.

Toxin A (µg)	DTT	InsP6	Total volume (µl)
1	4 mM	8 mM	26
1	6 mM	12 mM	26
1	8 mM	15 mM	26
1	10 mM	20 mM	26

Samples were taken before and after incubation and analyzed using MALDI in an attempt to view the 63 kDa active domain known to be released through autocleavage (Giesemann et al. 2008). Samples were taken after 0.5, 4, 8, 12, 24, 48, and 72 hours of incubation.

Clinical stool sample preparation

Known toxin and antigen positive and negative stool samples from Royal Inland Hospital were stored at -80°C and transported to Thompson Rivers University, where they were stored at 4°C. Samples were confirmed toxin and antigen positive or negative by Royal Inland through enzymatic immunoassay (EIA). Those testing negative for *C. difficile* toxins but positive for the antigen were subjected to PCR testing for PaLoc.

Approximately 10 µl of each sample was diluted in 100 µl deionized water, phosphate buffered saline (PBS), or PBS with 10 mM EDTA as a protease inhibitor. Diluted samples were then vortexed for up to 5 minutes until a relatively homogeneous mixture was achieved. Samples were then centrifuged at 1000 xg for 20 seconds and the pellet removed or filter sterilization with 0.22 micron syringe filters. An equal volume of acetonitrile was added and the solution incubated for 30 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 xg for 10 minutes at room temperature and the supernatant removed.

The pellet was then suspended in 10, 25, 50, 200, 300, 400 μ l of deionized water or TA30 (3:7 HPLC grade acetonitrile: 0.1% trifluoroacetic acid in deionized water). This solution was designated as the crude protein fraction.



Figure 5. Overview of methods for stool sample preparation. All samples were processed according to the protocol outlined in the arrows above. Listed below each step are variations to the protocol explored.

MALDI parameters and matrix selection

The crude protein fractions and samples from autocleavage experiments 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 2hydroxy-5-methoxybenzoic acid), or CHCA (α -Cyano-4-hydroxycinnamic acid) matrix using the dried droplet method. This consisted of adding 1 µl of the sample to 1 µl of matrix, briefly mixing by pipetting up and down, and spotting 1 µl of this mixture on the target plate. Ratios of 1:2 and 2:1 crude protein fraction to matrix was also spotted. 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%.

PNPG as a toxin-specific substrate

A PNPG solution consisting of 2 mM PNPG, 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 100 μ M MnCl₂ was prepared fresh daily and the protocol was carried out as outlined by Darkoh et al. (2011). Briefly, 100 μ l of crude protein fractions from three toxin positive samples (109, 111, and 112) and one toxin negative sample (113) was added to 200 μ l of the PNPG solution and incubated with an AnaeroPack for 3 hours. Samples were taken every half hour and 40 μ l of 3 M Na₂CO₃ was added to stop the reaction. Cleavage of PNPG was monitored by measuring the absorbance at 410 nm using a Novaspec II spectrophotometer.

RESULTS

Induction of autocleavage in commercially purchased toxin A

MALDI analysis was unable to detect the presence of proteins in the 63 kDa range, suggesting that the attempts to induce autocleavage had failed (data not shown). Furthermore, MALDI was also unable to detect the whole uncleavaged toxin at 300 kDa, since MALDI is not well suited for detecting proteins above 100 kDa (van Remoortere et al. 2010) (Figure 5).



Figure 6. Unintelligible MALDI spectra produced from attempts to view the whole 300 kDa *C. difficile* toxin A protein. Although some individual ion signals can be seen, they were not reproducible between trials. MALDI is not well suited for the detection of proteins above 100 kDa (van Remoortere et al. 2010).

Clinical stool samples

MALDI analysis of clinical stool samples showed no peaks at 63 kDa. A peak was seen at 56 kDa in three samples (Table 2 and Figure 6), although the presence of this peak was inconsistent between replications. All samples gave relatively similar spectra, with similar ion signals between 20-60 kDa and few inconsistent ion signals beyond 70 kDa (data not shown).

Table 2. Sample number, EIA results, and PCR results obtained by Royal Inland Hospital as well as MALDI results obtained in the present study. A=antigen, T=toxin. Only samples with inconclusive EIA results were subjected to PCR analysis. Peaks at 56 kDa were inconsistently seen in 3 samples, however no peaks in the 63 kDa range were seen in any sample.

Sample number	EIA	PCR	MALDI peak at 56 kDa
105	A+ T-	+	N
106	A+ T-	+	Ν
107	A+ T-	+	Y
108	A+ T-	+	Y
109	A+T+	n/a	Ν
110	A+ T-	+	Ν
111	A+T+	n/a	Y
112	A+T weak +	+	Ν
113	A- T-	n/a	Ν
114	A- T-	n/a	Ν



Figure 7. MALDI mass spectra of stool samples 107 (pink), 108 (green), and 111 (black). Samples 107 and 108 tested EIA negative for C. difficile toxins and PCR positive for PaLoc. Sample 111 tested EIA positive for C. difficile toxins. All spectra show a hump containing an unresolved complex mixture in the mass range 20- 60 kDa and individual ion signals at approximately 22, 24, 27, 28, 34, 39, 48, and 56 kDa. All samples co-crystallized in a 1:1 ratio with SA matrix.

No differences in MALDI spectrum quality were seen between samples diluted in deionized water and samples diluted in PBS or PBS with 10 mM EDTA. 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 intelligible spectra showing individual ion signals while protein precipitation with ammonium sulfate did not (Figure 7).

No difference in MALDI spectra was seen between pellets resuspended in deionized water compared to those resuspended in TA30. Sample pellets resuspended in 10, 25, 300, or 400 μ l of deionized water or TA30 provided lower quality spectra than pellets resuspended in 50 or 200 μ l of solvent. No difference in spectra quality was seen between sample pellets resuspended in 50 μ l of deionized water or TA30 compared to those resuspended in 200 μ l of deionized water or TA30.

Sample co-crystallization with a SA matrix produced intelligible spectra, while samples co-crystallized with a SDHB matrix or CHCA did not. Samples spotted in a 1:1 ratio with SA matrix showed the highest quality spectra.



Figure 8. MALDI spectra comparing a *C. difficile* toxin positive stool sample protein precipitated with (a) acetonitrile and (b) ammonium sulfate. Sample co-crystalized in 1:1 ratio with SA matrix. Acetonitrile protein precipitation produced intelligible MALDI spectra while ammonium sulfate did not.

PNPG as a toxin-specific substrate

Spectrophotometer readings taken every half hour showed a continuous increase in absorbance in only one sample testing EIA positive for *C. difficile* toxin (Figure 8).



Figure 9. Optical density (OD) readings at 410 nm verses time (hours) of stool samples testing EIA positive for *C. difficile* toxins (109, 111, and 112) and one sample testing EIA negative for toxins. Only sample 112 showed an increase in OD over time.

DISCUSSION

As previous studies have attempted to induce autocleavage in purchased toxins A and B with In6P and DTT in concentrations up to 15 mM and 8 mM, respectively (Koeck et al. 2013; McClean et al. 2014; Lam et al. 2014), the current research attempted autocleavage with concentrations of In6P and DTT ranging from 5-20 mM and 2-10 mM, respectively. The pH was adjusted to 4.5 and the reaction was incubated at 37 °C to mimic the environment of an endosome. However, MALDI analysis showed no evidence of successful autocleavage, as none of the samples showed peaks in the 63 kDa range. When reconstituted, the commercially purchased toxin A is suspended in a buffer which contains 0.1% trehalose. Trehalose is a sugar that is found in lower and higher life forms, although it is not found in mammals (Jain et al. 2009). The purpose of including trehalose in solutions which contain proteins is to ensure the stability of the protein and to prevent its degradation. It is speculated that the presence of this protein stabilizer interfered with the induction of autocleavage in the commercially purchased toxin A.

A peak was seen at 56 kDa in three samples (107, 108, 111), although this result could not be consistently replicated. 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 for CDIs, regardless of its identity.

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 heterogeneous samples such as stool. Depending on the protocol used, only $0.5 - 1 \mu l$ 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 homogeneous 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.

In general, stool is a complex combination of 75% water and 25% solid matter (Wu et al. 2007). 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. Surprisingly, all MALDI spectra acquired were relatively similar (Figure 3), barring a few individual ion signals such as the 56 kDa signal. All individual ion signals were seen between 20 and 60 kDa and above 70 kDa (data of the latter not shown). 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 stool 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 CDIs.

Unfortunately, further research into the relevant scientific literature proved the results of the PNPG tests to be insignificant, as the beta-glucuronidase activity of *Escerishia coli*

enzymes cleave PNPG in much the same way as the 63 kDa active domain of *C. difficile* toxins A and B (Aich et al. 2001). The notion of utilizing a toxin-specific substrate is still viable however, as it may help to overcome some of the disadvantages of working with the 63 kDa active domain of *C. difficile* toxins. Because *C. difficile* toxins are unstable proteins, degrading at room temperature within a few hours (CDC 2012), there is concern that stool samples may not contain high enough concentrations of intact toxin for MALDI detection. Although MALDI has been shown to be a very sensitive tool that is able to detect femtomoles (10-¹⁵) of protein, MALDI analysis of more complex samples will not allow for that level of sensitivity (Lin et al. 2007). As discussed in the Introduction, MALDI analysis of a complex sample such as the crude protein fraction analysed here allows for the possibility of the ion signal from the protein of interest to be supressed by the ion signals of other components in the sample. This ion suppression effect is of particular concern in the present research, given the unstable nature of *C. difficile* toxins. If a substrate truly specific to *C. difficile* toxins can be found, one which results in products easily detectable with MALDI, it may be coupled with MALDI analysis to indirectly verify the presence of the toxin.

Although this study was unsuccessful in using MALDI to detect the 63 kDa active domain, improvements can be made to the present protocol to better target the recovery and detection of the 63 kDa active domain from stool samples. Filters with a molecular-weight cutoff at 40 kDa would allow for the reduction of the size distribution of proteins present and reduce the ion suppression effect. Improvements can also be made to the matrix solution preparation, as certain matrix preparation procedures have been found to better target proteins of certain size ranges (Cohen et al. 1996). Immunoglobulins specific to the 63 kDa active domain may also be useful in amplifying the MALDI signal (Joanna Urban personal communication, 2015). In addition, protein purification techniques such as dialysis can be utilized to remove the trehalose protein stabilizer from the purchased toxin to allow for autocleavage to occur.

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APPENDIX A

The certificate of analysis for toxin A from C. difficile from List Biological Laboratories, Inc.



Product #152A

540 DIVISION STREET • CAMPBELL • CALIFORNIA 95008-6906 • USA 408-866-6363 • 800-726-3213 • FAX 408-866-6364 • EMAIL info@listlabs.com WEBSITE WWW.listlabs.com CERTIFICATE OF ANALYSIS

Toxin A from C. difficile Lot #15218A1A

Contents

Each vial, when reconstituted with 0.1 ml of water, contains 2 µg of *C. difficile* toxin A enterotoxin in 50 mM Tris, 50 mM NaCl, pH 7.5 and 0.1% trehalose. **Handle product gently; do not vortex.**

Concentration

Protein concentration is determined by the Bradford method¹ using bovine serum albumin as the standard.

Purity

This preparation migrates as a single major band with an apparent molecular weight of 300,000 daltons when run on a 3-8% gradient polyacrylamide-SDS gel.

This product has been tested for endotoxin levels and was found to be acceptable.

Activity

Each lot of *C. difficile* toxin A is tested to confirm binding activity to fresh rabbit red blood cells using a hemagglutination assay; the results were comparable to the previous lots. In addition, *C. difficile* toxin A has been tested to confirm activity in a cell rounding assay using human foreskin cells and was found to be comparable to previous lots. Since each cell type exhibits a different sensitivity, testing a range of toxin concentrations is recommended.

Packaging and Storage

This product is packaged aseptically, lyophilized, and sealed under vacuum. Store at 2-8°C prior to reconstitution. Following reconstitution, use immediately; do not store. <u>Do not freeze.</u>

Handling

Good laboratory technique should be employed in the safe handling of this product. Wear appropriate laboratory attire including a lab coat, gloves and safety glasses. Nitrile gloves are recommended for use when handling lyophilized material.

This product is intended for research purposes by qualified personnel. It is not intended for use in humans or as a diagnostic agent. List Biological Laboratories, Inc. is not liable for any damages resulting from the misuse or handling of this product.

FOR RESEARCH PURPOSES ONLY. NOT FOR HUMAN USE.

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1. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

Production: TP Date: 5/31/12 Management: NS Date: 5/31/12 QAVQC: Lat Date: 5/31/12