ANALYSIS OF PLASMA IMMUNE RESPONSE TO *CLOSTRIDIUM DIFFICILE* PROTEINS IN HOSPITALIZED PATIENTS AT HEALTH SCIENCES NORTH, SUDBURY, ONTARIO, CANADA

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Biology

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis
Titre de la thèse: ANALYSIS OF PLASMA IMMUNE RESPONSE TO CLOSTRIDIUM DIFFICILE PROTEINS IN HOSPITALIZED PATIENTS AT HEALTH SCIENCES NORTH, SUDBURY, ONTARIO, CANADA

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Degree
Diplôme: Master of Science

Department/Program
Département/Programme: Biology

Date of Defence
Date de la soutenance: March 03, 2017

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ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

Clostridium difficile infection (CDI) is the leading cause of nosocomial diarrhea worldwide. Prevention and treatment of CDI requires an intimate understanding of the immune response to the bacterium. Thus, this study investigated the plasma immune response of 79 patients at Health Sciences North in Sudbury, Ontario, Canada to identify immunodominant *C. difficile* proteins. Plasma samples were obtained from patients diagnosed with *C. difficile* infection, patients positive for *C. difficile* without symptoms, and patients who were negative for *C. difficile*, with and without symptoms. The plasma samples were tested for the presence of antibody reacting to *C. difficile* protein extracts using Western Blot analysis, Wes immunoblotting, and subsequent characterization by 2-D immunoblot analysis and mass spectrometry. Candidate immunodominant *C. difficile* proteins were found to be enolase, acetyl-coA acetyltransferase, and the 50s ribosomal protein, L7/L12. However, the presence and/or levels of antibodies that recognized these proteins in patient plasma were not statistically different between patient cohorts. Further analysis of the potential immunogenicity of these proteins could be useful to CDI treatment and prevention.

Keywords

Clostridium difficile infection (CDI), immune response, immunodominant, asymptomatic carrier, plasma IgG, Wes immunoblotting.

Acknowledgments

I would like to begin by thanking Dr. Francisco Diaz-Mitoma for his valuable mentorship in my journey through academia. Not only have you provided guidance in the progression of my research, you have helped me become a better student and professional. You allowed me to take a leap of faith toward my dream, teaching me that anything is possible.

I would like to thank Dr. Robert Lafrenie for all his support in the final stages of my project. You've assured that my degree continued to move forward and I am grateful for your support, your kind words, and your sense of humor. Without you, I would not be at the stage I am today.

Thank you to the members of my committee – Dr. Stacey Ritz, Dr. Eric Gauthier, and Dr. Reza Nohkbeh. Together, you've challenged my way of thinking and taught me to truly value the unique input of others. I am grateful for your investment in this project and my academic journey. Thank you, as well, to all those involved in the revision of this document.

To my classmates, thank you for your support and encouragement both in times of stress and times of joy. Not only have I learned about the world of science, writing, and laboratory protocols, I have gained a handful of life-long friends.

Thank you.

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Abbreviations

APC – antigen presenting cells

CDI – *Clostridium difficile* infection

MHC – major histocompatibility complex

sIg – secretory immunoglobulin

EMR – electronic medical record

PPE – personal protective equipment

T_H – T-helper cell

1 Introduction

1.1 Introduction

Clostridium difficile infection (CDI) is the leading cause of nosocomial, or hospital-acquired diarrhea worldwide and has recently been classified as an urgent threat to public health [1]. CDI is a communicable bacterial disease that can be acquired in hospital settings, especially in long-term care facilities. The bacterium poses a threat to public health due to its antibiotic resistance [2]. *C. difficile* is a pathogen that is transmitted via the fecal-oral route, that is to say due to contamination of the hands by excreted bacteria, which is facilitated by its bacterial spores that are not easily decontaminated [3].

In 2012, CDI cost the Canadian health care system an estimated 271 million dollars for the treatment and hospitalization of nearly 38,000 cases of CDI [4]. Moreover, CDI is directly attributable to an estimated 14,000 deaths per year in the United States [1]. Not only does *C. difficile* threaten the health and well-being of the population, it imposes a burden on the healthcare system. Furthermore, CDI is frequent in the most vulnerable cohorts of the population, such as the elderly [5]. Prevention of CDI is not only of interest for the protection of the population but also for the prevention of monetary loss in a presently strained health care system. Thus, prevention of CDI could have profound effects. In order to prevent CDI, it is important that clinicians and public health authorities be aware of *C. difficile* presence through better diagnostic testing methods [5].

This study investigated the immune response of patients to *C. difficile* proteins in an attempt to identify any immunodominant bacterial proteins that could act as epitope for detection of the bacterium through immunoassays.

1.2 Characteristics of *Clostridium difficile*

Clostridium difficile is an anaerobic bacillus that acts as an opportunistic human gut pathogen [6]. *C. difficile* produces highly resilient spores when facing nutrient and water shortage, such as in the distal environment of the colon, providing a survival mechanism that allows the organism to persist under harsh environmental conditions like extreme desiccation or in the presence of alcohol [7]. The formation of spores aids in the transmissibility of the pathogen in health-care and community settings [7]. *C. difficile* spores enter the body through the fecal-oral route. The transmission begins with the excretion of fecal matter, containing germinated, live cells and spores [7]. The excretion is usually in the form of symptomatic diarrhea, frequent liquid stool, increasing the probability of contaminating surrounding surfaces such as toilet seats, sinks, doorknobs, etc. [8]. The contaminated surfaces, as previously mentioned, are dry environments in which spores are able to survive and in which germinated cells will perish [9]. If not properly decontaminated, the spores are easily transmitted to another person if they touch the surface with their hands [8]. With no or improper handwashing, the spores can be transmitted to the mouth through eating or any other hand-to-mouth contact [10]. Once in the oral cavity, spores are swallowed where they survive through the stomach and into the duodenum [10]. Here, spores come into contact with appropriate germination factors before becoming actively growing vegetative cells in an ideal anaerobic environment [11]. Once germinated, the vegetative cells reproduce, and proliferate in the low-oxygen environment of the intestinal lumen. Here, the bacteria secrete exotoxins, which enhance its virulence by causing widespread damage and symptoms, provided the presence of

certain predisposing factors that are discussed in Section 1.3 [3]. Both the toxin-A and toxin-B compromise the cytoskeleton of the human gut cells, allowing *C. difficile* to penetrate the gut epithelium, enter the underlying intestinal mucosa and result in symptomatic infection by inducing inflammatory processes [7].

Asymptomatic colonization with *C. difficile* is thought to be an important factor in the transmission of the bacterium from person to person [8]. The proportion of the population that is colonized with *C. difficile* at any given time without symptoms varies dependent on the patient population. In healthy adults, up to 15% of the population is colonized, while in long-term care facilities, rates can exceed 50% [12]. The presence of the bacterium within asymptomatic carriers increases the risk of *C. difficile* transmission to other persons, especially to those who are immunocompromised and in long-term care facilities where the incidence is higher [13]. It is important to emphasize that *C. difficile* isolates are resistant to various broad-spectrum antibiotics, including cephalosporin and fluoroquinolones, which are commonly prescribed for pneumonia and urinary tract infections, respectively [14,15,16]. Thus, when an individual carrying *C. difficile* receives a course of these broad-spectrum antibiotics, the commensal microflora of the body is disrupted, which is especially important in protecting the colon [17]. Due to their susceptibility to antibiotics, the protective gut bacteria are unintentionally killed during the treatment, while *C. difficile* and other resistant pathogens remain alive [17]. The composition and the quantity of the gut microflora changes, with a decrease in proportions of protective phyla and an increase in proportions of opportunistic species, such as *C. difficile* [17]. Clearly, this imbalance results in risk of infection by opportunistic and harmful species. With ample opportunity to grow, resistant *C. difficile* remain unchallenged

and begin to proliferate in an ideal environment [18]. Growth of *C. difficile* within the gut results in an array of symptoms that can be very dangerous, especially in immunocompromised individuals [18].

1.3 *Clostridium difficile* Infection Risk Factors

Clostridium difficile infection (CDI) is characterized by the presence of *C. difficile* in stool, as detected by a polymerase chain reaction (PCR) to detect *C. difficile* DNA [19]. CDI is also characterized by the presence of biological signs related to infection, such as fever and frequent diarrhea [19]. *C. difficile*-associated diarrhea can vary from mild to severe, and may eventually result in complications that include pseudomembranous colitis, toxic megacolon, and death [14]. The reason for the variation in the severity of disease is not well understood but is thought to be related to the overall health of the infected individual [20]. This is to say that individuals with underlying health condition are at risk of infection [19]. Nevertheless, the population most at risk for developing CDI are those who are immunocompromised, that is those who have a lower than normal functioning immune system [20]. It appears as though an effective immune response is an important factor in protection from disease, being that susceptibility to *C. difficile* infections is much higher in immunocompromised patients [21]. Populations of patients at risk of being immunocompromised include those receiving radiation treatment, those who are diabetic or arthritic, patients undergoing surgery and any other treatments that diminish antibody production, require immunosuppression, or destroy mature immune cells [20]. As evidence

shows that immune integrity is protective against infection, understanding the immune response to *C. difficile* could aid in immunotherapeutic intervention of CDI.

Another major risk factor in mounting an effective immune response is old age. Infants less than six months old have little protection from infection, aside from the antibodies passively received by their mother through lactation or those able to cross the placenta in utero [21]. In addition, the protective gut microflora is not fully developed and requires widespread environmental exposure to become diverse [22]. These factors put infants of less than six months of age at risk for developing CDI [20]. On the other hand, as an individual becomes elderly, the time it takes to mount an immune response increases, while the intensity of that mounted response diminishes, which can result in an insufficient protection against the pathogen [21]. Thus, individuals greater than 70 years of age are at a higher risk for developing an infection, as shown by the higher than normal incidence of CDI in the elderly [4,20]

Another important yet overlooked factor in CDI are sex and gender. It has been shown that sex hormones have a significant influence on immune function [23]. Estrogen and progesterone appear to be related to greater cellular and humoral immune responses, with a higher average level in women, resulting in less incidence of infection in women as compared with men [24]. Despite differences like these, sex and gender have been neglected in recent immunological studies [23]. In *C. difficile* immunological studies, the same is true. Many studies use cohorts of people or mice that are biased towards one sex/gender. One study looked at the interaction between gut microbiota and immunological function with no mention of the sex or gender of the subjects, despite ample evidence suggesting major differences in the microbiome between sexes and genders

[25,26]. Another study investigated intravenous immunoglobulin treatment for CDI with a majority female cohort [27]. This trend continues both within specific *C. difficile* studies as in general immunological research. This disregard for the significance of sex and gender has generally been acceptable in publications, which can have an important impact on the application of treatment for both sexes and genders. For these reasons, sex and gender are considered in this study to ensure that appropriate attention is paid to sex/gender factors that may be relevant in clinical decision making intervention.

Although general immune integrity, age, and sex/gender are important for the risk of CDI, they are not exclusive. Recently, younger adults have fallen ill with CDI having no previous sign of immune impairment [28] [13]. Often but not always, a patient has received a course of wide-spectrum antibiotics for treatment of other infections [28]. For those who have not received antibiotics, the cause of their susceptibility is less understood. For those who have received antibiotics, prevention of CDI requires the proper monitoring of *C. difficile* presence and its role in this controllable and preventable risk factor [29]. Thus, a better understanding of the immune response in conjunction with antibiotic stewardship could prove useful in CDI control.

1.4 CDI Prevention

C. difficile infection (CDI) symptoms vary from mild to severe. Approximately 61% of those infected with *C. difficile* suffer from mild to moderate symptoms, with the remaining proportion have severe and fulminant infection [4]. Of these severe cases, about 6% of patients die from causes attributable to their CDI symptoms [30]. Among patients who

improve after treatment with CDI-specific antibiotics, there is a 20% change of these patients experiencing a recurrent episode of diarrhea after cessation of antibiotics [4]. This can become psychologically, emotionally, and physically taxing on patients [31]. CDI patients can be hospitalized and isolated for months at a time as a result of their uncontrollable diarrhea and potential for transmission to others [32]. This experience can have a negative emotional effect on the patient, as many patients in isolation report loneliness or other similar feeling, which can result in a decreased quality of life and poor health outcomes [31].

Currently, prevention methods for CDI appear to be suboptimal. Prevention and control strategies are currently focused around prevention of the acquisition of *C. difficile* spores, prevention of the development of CDI induced by antibiotics, or protection of individuals through immunotherapies [33]. A few specific strategies include hand-washing, antibiotic stewardship, and vaccination. Hand-washing and sanitization are effective methods for preventing the transmission of the spores and the live *C. difficile* cells, if properly done [34]. Transmission of *C. difficile* occurs via the fecal-oral route, which illustrates the important role of the hands in the spreading of this disease, thus hand-washing in an important barrier to acquiring the disease [8,35]. However, in order to be effective, hands must be washed with sufficient soap, water, and mechanical friction or "scrubbing" [34]. Furthermore, this does not ensure removal of all spores [34]. Studies suggest the best decontamination method for *C. difficile* spores is to use highly abrasive material, comparable to sand, in order to ensure sufficient removal of spores, which is impractical when applied to the delicate human epidermis [34]. Thus, hand-washing as a

CDI prevention mechanism is a problematic approach when considering the level of compliance to scrubbing protocols needed to fully decontaminate the hands.

In terms of environmental cleaning to decontaminate against *C. difficile*, bleach-based disinfectants must be used to ensure the destruction of all spores and cells due to *C. difficile*'s resistance to most sanitization solutions [36]. Applying this to hand washing, these are no such disinfectants that can be applied to the skin, further demonstrating the barriers of hand-washing as a prevention mechanism. These products are effective in decontaminating many surfaces, however their effectiveness is reliant on careful cleaning by hospital staff [37]. Furthermore, research on hazard exposure in workplace settings shows that personal protective equipment (PPE) strategies are the least effective methods of harm prevention [38]. Personal protective strategies focus on putting a barrier between the person and the harm [39]. In healthcare, PPE includes surgical gloves, masks, gowns, glasses, handwashing, etc. They are the least effective method of harm prevention as they are highly reliant on the compliance of each individual person [40]. This is not to say that they are unnecessary, nor futile – PPE strategies must be enforced to the greatest extent. However, even in health care settings, PPE compliance is low among health care providers who often go without gloves, glasses, or gowns even in known infective environments [41]. Thus, if *C. difficile* prevention is reliant only on the compliance of health care professionals, visitors, and patients to engage in hand-washing and other sanitization, it becomes problematic and introduces a level of risk when compliance is low. It is more effective to supplement PPE with controls at a higher level that do not require significant individual compliance by the public, such as vaccination or antibiotic stewardship [42].

Antibiotic stewardship is a program that involves the regulation of antimicrobial prescriptions by physicians [43]. It ensures that antibiotics are used only when needed and are used for the complete course of treatment to reduce the chance of creating antimicrobial resistant organisms [43]. Health Sciences North in Sudbury, Ontario, the hospital at which this study was conducted, is part of a partnership in an antimicrobial stewardship program between several hospitals in Ontario, with the aim to reduce antimicrobial-associated infections [43]. Although the combination of these methods could yield promising results, they are not specific to *C. difficile* colonization.

More specific prevention strategies for *C. difficile* can help to ensure more effective infection control [44]. Vaccination is one such method that would elicit a specific immune response and could provide individuals with immunity against *C. difficile* [21]. However, there are no current commercial vaccines available. There are several vaccines in clinical trials, but all target *C. difficile* toxins [45] [35]. This is a promising strategy to reduce symptomatic infection but the vaccines remain years away from production and their effectiveness is still unknown. Additionally, these vaccines target the product of live cells, and do not elicit an immune response against colonization, or against the bacterium itself. This is an area in which this study seeks to fill a gap in knowledge and aid in the progression of research toward a surface protein based immunotherapy.

The prevention strategy that is of most interest to this study is the screening of patients for the presence of *C. difficile* colonization then implementing specific treatments to prevent symptomatic disease. There are currently no screening programs in place and testing for *C. difficile* does not usually occur unless there is suspicion of infection [19]. Normally, by the time patients show signs and symptoms of infection, the bacteria have

already proliferated to the point where they have caused tissue destruction [3]. Thus, screening for *C. difficile* before a wide-spectrum antibiotic is prescribed could increase the chance of preventing a *C. difficile* infection that is induced because of its resistant to the given antibiotics [46]. This screening could allow for physicians to be aware of the risk in patients and prevent severe, even fatal complication. If the prescribing doctor was aware of the presence of *C. difficile* in the patient's gut, the choice of a wide-spectrum antibiotic could be avoided, to prevent inducing CDI, and a more appropriate antibiotic to which *C. difficile* is susceptible could be prescribed (e.g. vancomycin) [46]. This could likely prevent complication.

However, the current method of *C. difficile* testing poses a barrier to implementing this prevention strategy. Current gold standard testing for colonization by *C. difficile* is a PCR-based DNA amplification of the toxin gene (GeneXpert) and toxigenic culture of organisms present in the patient stool samples [47]. When considering screening and prevention methods, the testing of patient stool becomes problematic. It relies on a bowel movement by the patient, which may not happen during the time they spend at the hospital. The patient may not pass a movement because they are constipated due to side effects from their medication, because they are nervous, or because they are reluctant to provide a stool sample due to embarrassment [48]. Conversely, blood samples can be more easily withdrawn at the time of admission and are frequently being withdrawn for other purposes [49]. This provides an easily accessible avenue for widespread screening of *C. difficile* before the administration of antibiotic. Patient blood could be tested using the remainder of the blood from other tests, as was done in this study. Accordingly, a blood test capable of detecting *C. difficile* would be an ideal tool for detecting and preventing the

infection, especially if designed to be sensitive enough to work on minute quantities of blood that could be administered immediately upon admission. It could be used before the administration of antibiotics to avoid any induced infection by allowing the physician to identify this risk in the patient. This type of test could also be used as a widespread screening strategy to identify and further investigate the characteristics of those asymptomatically carrying the bacteria. However, in designing such a test, the immune response to *C. difficile* must be well understood.

1.5 Immune Response to *C. difficile*

In order to design a blood test capable of detecting *C. difficile* antibodies, it is important to understand the interaction between the immune system and the bacterium. The first interaction between *C. difficile* and the gut mucosal immune system occurs in the stomach, where stomach acids efficiently destroy live bacterial cells [50]. Spores, however, can survive and move into the small intestine, where they come into contact with bile salts, initiating germination [20,51]. *C. difficile* germination factors include inorganic phosphate and potassium chloride [11]. Following this interaction, spores germinate to become vegetative cells [11]. This becomes the first opportunity for host antigen presenting cells (APCs) to encounter the bacterial surface layer proteins involved in germination [21]. As the vegetative cells move along the intestine, they adhere to the surface of the colon through bacterial cell surface proteins specialized for adhesion [52]. Again, this is an opportunity for APCs in the colon to encounter surface proteins and initiate an immune response [21]. Once *C. difficile* cells adhere to the colon's mucosa, they reproduce and begin

to secrete toxins [21]. As mentioned, the surface proteins that are involved in adhesion can be recognized by the immune system, providing an interesting immunotherapeutic target [53]. Although these proteins have been widely identified and characterized, their function in the immune system is less clear. The characterization of the immune response to these antigens is the aim of this study.

C. difficile toxins and other antigens interact with the innate immune response through APCs and micro-fold cells (M cells), which endocytose antigens from the gastrointestinal lumen and present them to naïve T-cells in the intestinal mucosa through MHC Class II [21]. This interaction activates T-cells, which will initiate a mostly T_H17 response. This response initiates the synthesis and secretion of pro-inflammatory cytokines, including IL-8. IL-8 induces neutrophil chemotaxis, which has been associated with an increased severity of symptoms in CDI by increasing the amount of inflammation and damage in the mucosa [54].

C. difficile toxins and other antigens activate an adaptive immune response in several ways. Due to the protein nature of the *C. difficile* toxins and most of the other antigens on the surface of the cell, antibody production is T-cell dependent [21]. This means that a B-cell activated by exposure to *C. difficile* antigens will be co-stimulated by T_H cells and will result in a strong humoral adaptive response to be mounted, including the production of affinity matured and class switched antibodies [21]. The first class of antibody produced is a secretory IgA (sIgA), which is secreted into the lumen of the intestine [55]. This is an important class of antibodies as it is the main antibody associated with toxin neutralization at the mucosal level [56]. Insufficiency in sIgA production has been associated with the development of more severe CDI symptoms [57]. Secretory IgA

production is the first stage in adaptive immunity and attempts to eliminate the pathogen before it breaches the mucosal lining [56]. The production of anti-*C. difficile* IgG occurs both in the mucosa and systematically to combat the pathogen during the later stages of infection [21]. Theoretically, secretory IgA would be a useful tool for measuring current CDI through the stool, while systemic IgG would provide a better indication of past or chronic exposure to *C. difficile* as it is produced during the later stages of infection [21]. The greater affinity of IgG to antigens also makes this class of antibody a more promising candidate for blood testing [21]. Thus, this study focuses on the plasma IgG immune response to *C. difficile* proteins, as they apply to both current and past CDI, in a large cohort of patients [21].

Using the patient plasma, antibodies specific to *C. difficile* proteins could help identify any antigens that were able to generate high affinity antibodies by the patient as they were theoretically able to mount a specific immune response and prevent CDI. Because the antigenic proteins on the surface of the bacterium are not well understood, an array of patient blood samples could help to further characterize these antigens. Screening a large population of *C. difficile* positive patients could allow us to identify the antigens most frequently encountered by their immune systems. Furthermore, using patients with different symptoms – such as asymptomatic carriers and severely symptomatic patients – it should be possible to indicate which antibodies were protective to the patient and indicate potential virulent targets for immunotherapy on the bacterium.

Previous evidence shows that asymptomatic carriers of *C. difficile* possess higher levels of serum anti-*C. difficile* IgG antibodies than their symptomatic counterparts [53]. Therefore, it is hypothesized that asymptomatic patients in this study will have a

measurable difference in their immune response as compared with controls. It is also hypothesized that these patients will have a measurable difference in their response to specific *C. difficile* proteins that may serve to indicate any proteins to which immunogenicity might be protective against CDI. This could also specify proteins to which an immune response is significant enough to be detected through serum and serve as a screening tool for the detection of *C. difficile* in the gut.

1.6 Project Objectives

In this study, the plasma IgG immune response of hospital patients to an array of *C. difficile* proteins was investigated. The goal was to characterize the pattern, if any, of immune response to *C. difficile* proteins and identify any immunodominant proteins that could be used in the design of a blood test for the detection and potential treatment of *C. difficile* infection. The objectives of the study were to:

1. *Construct a patient cohort consisting of equal proportions of male and female patients, meeting all inclusion criteria (see Material and Methods).*
    I. *Classify patients according to presence/absence of C. difficile in stool and presence/absence of symptomatic diarrhea*
2. *Investigate general IgG immune response patterns to C. difficile proteins through testing of 8 patient blood samples (2 from each of the four cohorts) by Western blot.*
    I. *Identify potential regions (in molecular weight) of immunodominance among C. difficile proteins.*

3. *Further investigate and quantify IgG immune response to C. difficile proteins through testing of all patient samples by Wes capillary immunoblotting*
    I. *Identify and quantify immunodominant regions of C. difficile proteins.*
4. *Characterize the identity of any immunodominant proteins through 2-dimensional Western blot and mass spectrometry.*
    I. *Identify potential targets for further analysis in C. difficile immune response.*

2 Materials and Methods

2.1 Patient Blood Sample Collection & Classification

Patient samples were obtained following informed consent from patients admitted to Health Sciences North in Sudbury, Ontario, Canada between March 2014 to August 2015. All patients admitted to one of two wards within the hospital were given the opportunity to participate in the study. The two wards surveyed were the oncology (MED-4) and respiratory (MED-6) wards, which are populations consistent with those most at risk for developing CDI. Patients were informed that any blood remaining after their standard blood tests would be used for the study. Patients were also asked to provide stool samples for confirmation of *C. difficile* presence within their stool. Patients were included in the study if both blood and stool were received. Patients were excluded from the study if one of the two were not received. There were no other inclusion or exclusion criteria.

Patient cohorts consisted of equal proportions of men and women. The mean age for all of the patients was 67.6 years old, with the youngest patient being 21 and the oldest being 92. The mode age for the cohort was 80 years old. Patients were classified as positive or negative for CDI based on PCR testing of their stool for *C. difficile* toxin DNA sequences. Additionally, toxigenic stool culture was performed to confirm *C. difficile* presence. Patients were classified according to their signs of disease based on physician documentation in the patient's electronic medical records. In summary, the four patient cohorts were as follows and as shown in **Table 1**:

- CDI positive, Symptomatic (n=30): CD-positive, with diarrhea
- CDI positive, Asymptomatic (n=17): CD-positive, no diarrhea

- CDI negative, Symptomatic (n=16): CD-negative, with diarrhea
- CDI negative, Asymptomatic (n=16): CD-negative, no diarrhea

Positive, symptomatic patients were those diagnosed by a physician to have *Clostridium difficile* infection (CDI) with confirmed presence of diarrhea. Conversely, positive, asymptomatic patients were those with a *C. difficile*-positive stool test that exhibit no diarrhea. These patients were not suspected of having disease; they consented to have their stool tested and were admitted for unrelated reasons, but tested positive for *C. difficile* without symptoms of the bacterium's presence. These patients are considered carriers and pose a risk of silent transmission.

Negative symptomatic patients were individuals suffering from diarrhea without the presence of *C. difficile*. The causes of their diarrhea range from celiac disease, stress, unrelated bacterial infection, etc. This population provides an observation of immune responses unrelated to *C. difficile* in a group of cases where there may have been a suspicion of CDI. This group could serve as an important cohort in distinguishing between specific CDI versus non-specific immune responses.

Lastly, negative asymptomatic patients serve as negative controls. These individuals are neither exhibiting diarrhea nor colonized with the bacteria. It is important to note that absolute positive and negative controls are not possible in this study as many of the important variables are currently unknown. It is possible that patients who are negative for *C. difficile* in their stool could have encountered the bacterium at a previous time in their life and could have elicited an immune response resulting in antibody production. Thus, perfect negative controls might be difficult to obtain.

Patient plasma was received less than 24 hours after being drawn from the patient in an EDTA-coated draw tube to inhibit blood clotting. Blood samples were centrifuged at 2000g (Eppendorf 5810 Centrifuge) for 10 minutes to separate cellular blood components from plasma. Patient plasma was removed and stored in 15μL aliquots at -80°C. Plasma was not thawed more than three times to preserve antibody integrity.

2.2 *C. difficile* Protein Sample

The *C. difficile* strain used to produce protein extracts for analysis of patient antibody responses was a hypervirulent, toxigenic, ribotype 027 strain, given the arbitrary name CD186 by the Health Sciences North Research Institute. This specific isolate was locally isolated from a clinical patient stool sample. Among all isolated strains, CD186 was chosen for its NAP-1 characterization, which is a subtype of *C. difficile* that is associated with the most severe cases. Further, patients exhibited robust responses to this strain in preliminary testing. The CD186 protein extracts were provided by Dr. Justo Perez and Dr. Hoang-Thanh Le, prepared using a proprietary formation of bile salts. Extraction was performed with 2% bile salts at 37°C for 60 minutes using 50mg wet weight of bacteria and 20mL bile salt preparation. Protein samples were stored at 4°C and handled under Biohazard Safety Level 2 conditions.

2.3 Western Blotting

Western blot analysis was used to assess the general immune response of patients to *C. difficile* proteins. Protein extracts of *C. difficile* CD186 were loaded on a 12%

polyacrylamide gel containing sodium dodecyl sulphate (SDS-PAGE) and subjected to electrophoresis on a BioRad Tetracell for 60 minutes at 110V (**Figure 1**) [58]. The amount of protein was determined using a BCA protein assay (Fisher Scientific) with bovine serum albumin, as the standard, and 20μg of protein extract was resuspended in loading buffer (0.125 M Tris-HCl, pH 6.8, 5% (v/v) beta-mercaptoethanol, 4% SDS, 20% glycerol, and 10 ug/ml bromophenol blue), heated at 95°C for 5 minutes, and loaded into each lane of the gel. Following electrophoresis in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3), the proteins in the gels were transferred to a nitrocellulose membrane using a BioRad semi-dry apparatus for 45 minutes at 25V with Towbin transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol, pH 8.3). The transferred nitrocellulose membrane was blocked overnight at 4°C by incubation in 2% skim milk dissolved in phosphate buffered saline, pH 7.5, 0.05% Tween (PBST) solution. Next, the membrane was washed three times for 10 minutes each in PBST before being incubated overnight at 4°C with a patient plasma sample diluted 1:10 000 in a 2% skim milk:PBST solution. The blots were washed three times with PBST and then incubated with an anti-human rabbit IgG-horseradish peroxidase secondary antibody (Biorad) diluted 1:20 000 in 2% skim milk:PBST solution for 1 hour at 22°C. The blots were washed three times with PBST and then incubated for 1 minute in enhanced chemiluminescent reagent (ECL, Fisher Scientific) before being exposed to autoradiography film.

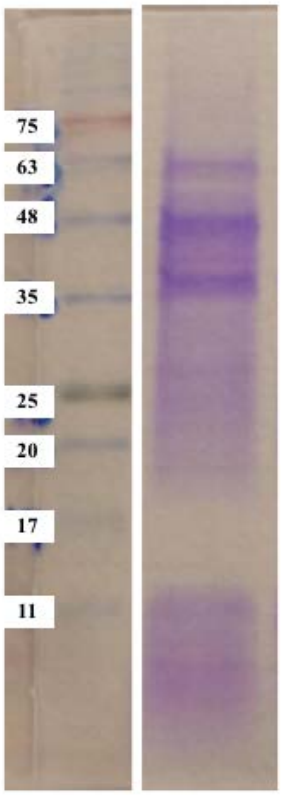

Figure 1 - SDS-Page Electrophoresis of C. difficile Proteins Stained with Coomassie Blue

Protein samples (20ug) were resolved on 12% SDS-Page gel at 110V for 45min and stained with 0.5% Coomassie Blue in 40% methanol, 10% acetic acid for 1 hour, and destained in 40% methanol and 10% acetic acid overnight. Froggobio's BLUeye Prestained Protein Ladder used to discern molecular weights.

2.4 Wes Immunoblotting

Once the general presence and pattern of the immune reactive proteins was observed, the reaction of the *C. difficile* proteins with the patient plasma was tested using the automated immunoblotting system, Wes, manufactured by ProteinSimple (**Figure 2**). This system uses the Western blot principle in a micro-capillary medium, where all reagents are loaded into the plate at one time and run in an automated fashion. This system increases the number of samples that can be analyzed and reduces the run time [59]. This reduces the opportunity for human error, as is the case with the Western blot, which involves much handling over many days.

The amount of protein loaded into the Wes plate was 0.2μg, as suggested by the manufacturer. A sample of patient plasma was diluted to 1:100 in the provided antibody diluent II. Lastly, the same secondary anti-human IgG antibody as that used in the Western blot was used at a concentration of 1:1000 in the same diluent. These concentrations were increased due to the drastically decreased Wes sample volume and were consistent with manufacturer recommendations. The Wes protocol designed for the 25-well Whole Protein Size was followed according to provided manuals and analyzed using ProteinSimple's Compass software.

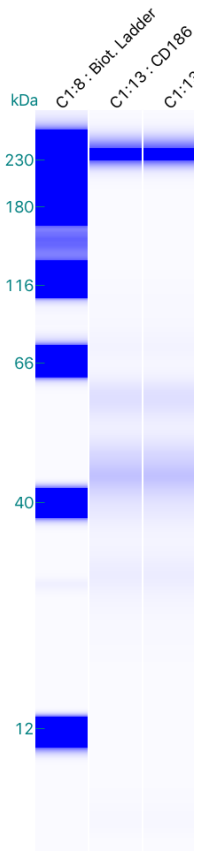

Figure 2 - Wes Capillary System Electrophoresis of C. difficile Proteins
Protein samples (0.2ug) loaded into Wes 25-capillary plate and run according to Wes Total Protein protocol and stained with Total Protein Biotin Labelling Reagent (analogous to Coomassie Blue) and visualized with ProteinSimple's Compass Software.

2.5 2-D Western Blot

A 2-D western blot was performed to resolve the bacterial proteins by isoelectric point and molecular weight. The same *C. difficile CD186* extracts used in the Western blot analysis and in the Wes experiments were prepared for 2D electrophoresis. The amount of protein subjected to isoelectric point analysis using ReadyStrip IPG, pH 3-10 electrofocusing gels (BioRad) was 50 μg. The protein extracts were diluted 1:100 with rehydration buffer and a 7 cm immobilized pH gradient (IPG) strip was rehydrated with 100 μl of sample overlaid with 400 μl Dry Strip Cover fluid overnight at 20ºC. Isoelectric focusing (IEF) was performed at 50μA per strip at 500V for 12000 volt-hours using a BioRad Protean IEF system. The IEF strip was then equilibrated by incubation in Equilibration buffer containing iodoacetamide. The 2nd dimension, or SDS-PAGE electrophoresis, was performed using a 12% acrylamide resolving gel and 4% stacking gel as described for Western blots. The equilibrated strip was laid on top of the stacking gel and sealed using the agarose sealing mixture and subjected to electrophoresis at 100V for approximately 2 h. The resulting gels were transferred to nitrocellulose membranes using a semi-dry BioRad transfer machine (as indicated for Western blots). One of the duplicate membranes was stained using 0.5% Ponceau S in 1% acetic acid and an image taken to identify the relative position of the major staining protein spots (alternately, SDS-PAGE gels were stained by incubation in 0.5% Coomassie Blue in 40% methanol, 10% acetic acid). The second membrane was prepared for western blot analysis. The membrane was incubated with patient plasma in 2% skim milk:PBST at a 1:100 dilution overnight and then washed three times with PBST. The membrane was then incubated with an anti-

human rabbit IgG-horseradish peroxidase secondary antibody, as described for Western blot analysis. The blots were washed three times with PBST and then incubated in ECL chemiluminescence reagent for 5 min and exposed to X-ray film. Comparison of the duplicate blots was done and protein spots corresponding to the position of the immunoreactive proteins of interest were excised and characterized using mass spectrometry.

2.6 Protein characterization by Mass Spectroscopy

Protein characterization of immunodominant protein bands at 15 and 45kDa was performed using western blot and 1-D SDS-PAGE excision technique. Bands (1 corresponding to the 45 kDa protein and 3 corresponding to the 15 kDa protein) were excised, submerged in 10% glacial acetic acid and characterized by mass spectrometry by Ottawa Hospital Research Institute (OHRI). The OHRI Proteomics staff analyzed the sample following digestion using trypsin. Mass spectrometry was performed using an Orbitrap LUMOS mass spectrometer and the size of the produced peptide fragments obtained. Subsequent analysis of the generated fragment size data was performed using Mascot and Scaffold programs.

2.7 Statistical Analysis

One-way ANOVA, T-test, and Kruskal-Wallis non-parametric tests were performed on all Wes immunoblotting values. These tests were chosen to measure any statistically

significant immunofluorescent values within the study, indicating a greater immune response to a given protein. Variance tests were performed in the following ways:

- Between each protein, inclusive of all cohorts.
- Between each cohort, individually for each protein.
- Between *C. difficile* positive and negative patients, inclusive of all proteins and individually for each protein.
- Between sexes/genders, inclusive of all proteins and individually for each protein.

An alpha value of $p = 0.05$ was chosen as a confidence interval. Any p values obtained by the variance testing was considered to be statistically significant if below 0.05.

3 Results

3.1 Patient characteristics

Samples were obtained from 79 patients, divided into 4 separate cohorts based on presence of CDI and symptom of diarrhea (**Table 1)**. Refer to **Appendix Table 7** for gender and age distributions.

Table 1 - Representation of Patient Cohort Characterized by positive or negative PCR result and presence or absence of diarrhea

Positive, With Diarrhea		Positive, No Diarrhea		Negative, With Diarrhea		Negative, No Diarrhea	
Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
B028	B088	B040	B045	B018	B053	B025	B032
CD001	CD003	B135	B051	B063	B069	B066	B059
CD004	CD012	B170	B119	B109	B150	B163	B100
CD006	CD013	B182	B131	B156	B097	B071	B267
CD026	CD027	B278	B142	B193	B077	B215	B070
CD025	CD014	B274	B080	B319	B346	B285	B300
CD028	CD029	B338	B179	B341	B289	B301	B345
CD030	CD008	B277	B159	B339	B178	B329	B326
CD005	CD019		B347	**n = 16**		**n = 16**	
CD021	CD018	**n = 17**					
CD010	CD020						
CD022	CD017						
CD002	CD047						
CD007	CD046						
CD040	CD042						
n = 30							

3.2 Western Blotting

Western blot analysis using the plasma isolated from 8 patients – 2 from each cohort – against the *C. difficile* protein extract served as a preliminary investigation into possible patterns of immune response between patients of various cohorts (**Figure 3**). The samples chosen were from patients that exhibited the same symptoms as the others within the cohort, thus were considered to be representative of the group as a whole. Western blot analysis showed unique patterns of protein recognition for each patient sample, but some trends in blotting pattern were observed. One of the samples from the CD positive patients with no symptoms, (B045) reacted only with one *C. difficile* protein band at 15kDa while the second patient sample (B051) recognized *C. difficile* proteins of around 15kDa, 25kDa, and 35-50kDa. In the CD positive patients, with diarrhea, both samples of patient sera (CD003 and B080) identified similar sized protein bands at around 15kDa, 25kDa, and 35-50kDa, with high intensity. The control samples, from the CD negative patients without diarrhea (B032 and B086), did not identify any strongly reactive protein bands, as expected from negative patients. One of the plasma samples from the CD negative patients with diarrhea (B053) identified a relatively weak band at 15 kDa, while the second (B267) showed no reaction at all.

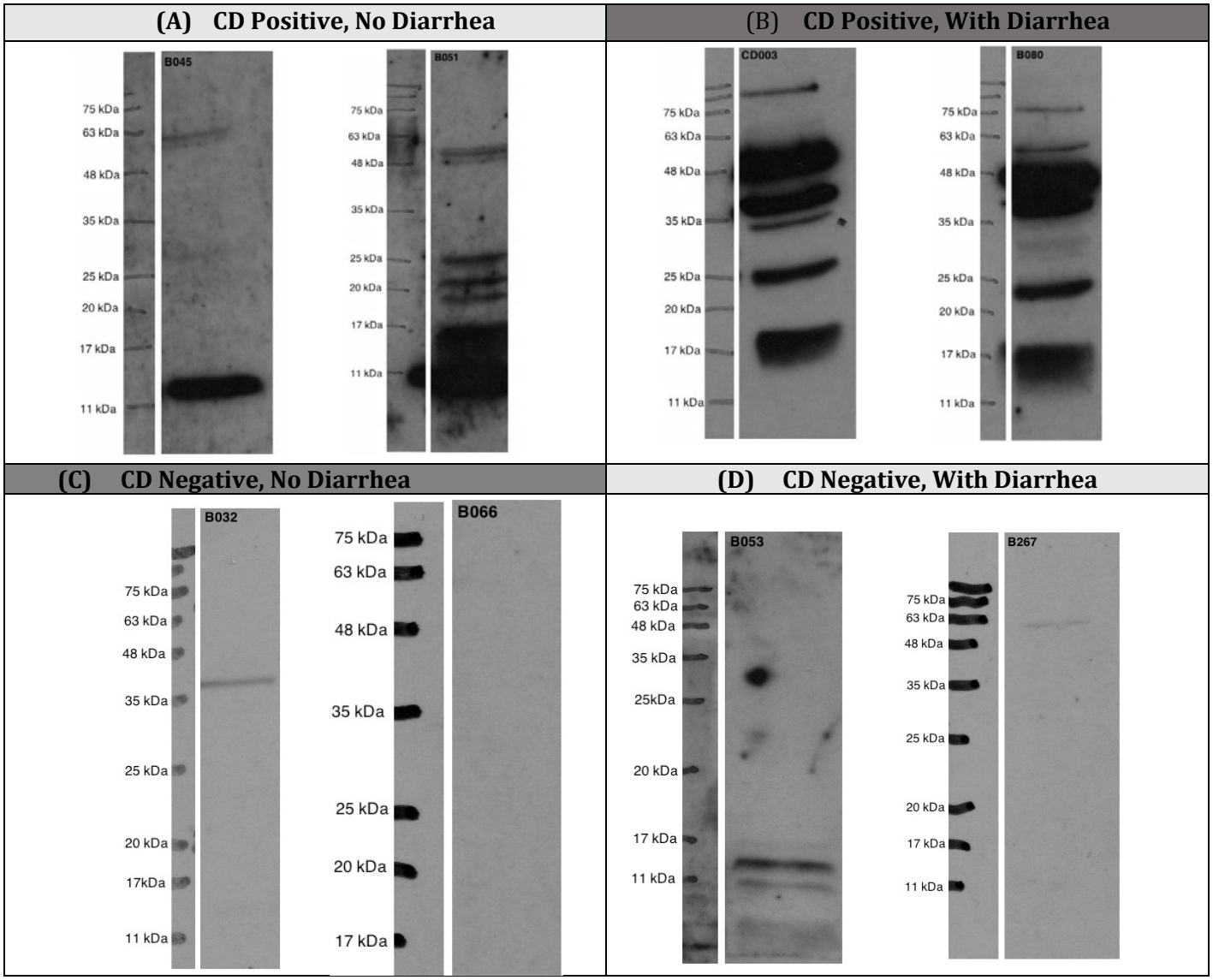

Figure 3 - Western blot results ; Protein samples (25ug) were resolved using 12% SDS-PAGE, transferred by semi-dry method to nitrocellulose membrane. Membranes blocked using 2% PBST-SM overnight and washed 3 times. Primary antibody (patient plasma) incubated in PBST-SM overnight, washed 3 times, and probed with secondary antibody (anti-human rabbit IgG) for 1 hour at RT. Membranes incubated in ECL and exposed via autoradiography. (A) Sera from *C. difficile* positive patients with no symptoms; (B) Sera from *C. difficile* positive patients with symptomatic diarrhea; (C) Sera from *C. difficile* negative patients with no symptoms; (D) Sera from *C. difficile* negative patients with symptomatic diarrhea.

3.3 Wes Immunoblotting

All of the samples from the patients within this study (n= 79) were tested for reaction against the *C. difficile* protein extracts using the Wes immunoblotting system. The Wes immunoblotting analysis results differed from the Western blot analysis results but the most prominent bands, particularly the band at 15 kDa, were conserved between methods (**Figure 4**). Most patient samples also tested with Western blot analysis had significant differences in their Wes immunoblot protein recognition. Differences were less frequent in patients with a less robust response in the Western blot. The most robust responses in the Western blot resulted in prominent differences in Wes recognition.

Refer to **Tables 3 to 6** for cohort specific Wes result summary. Refer to **Tables 8 to 10** for a numerical summary of cohort responses.

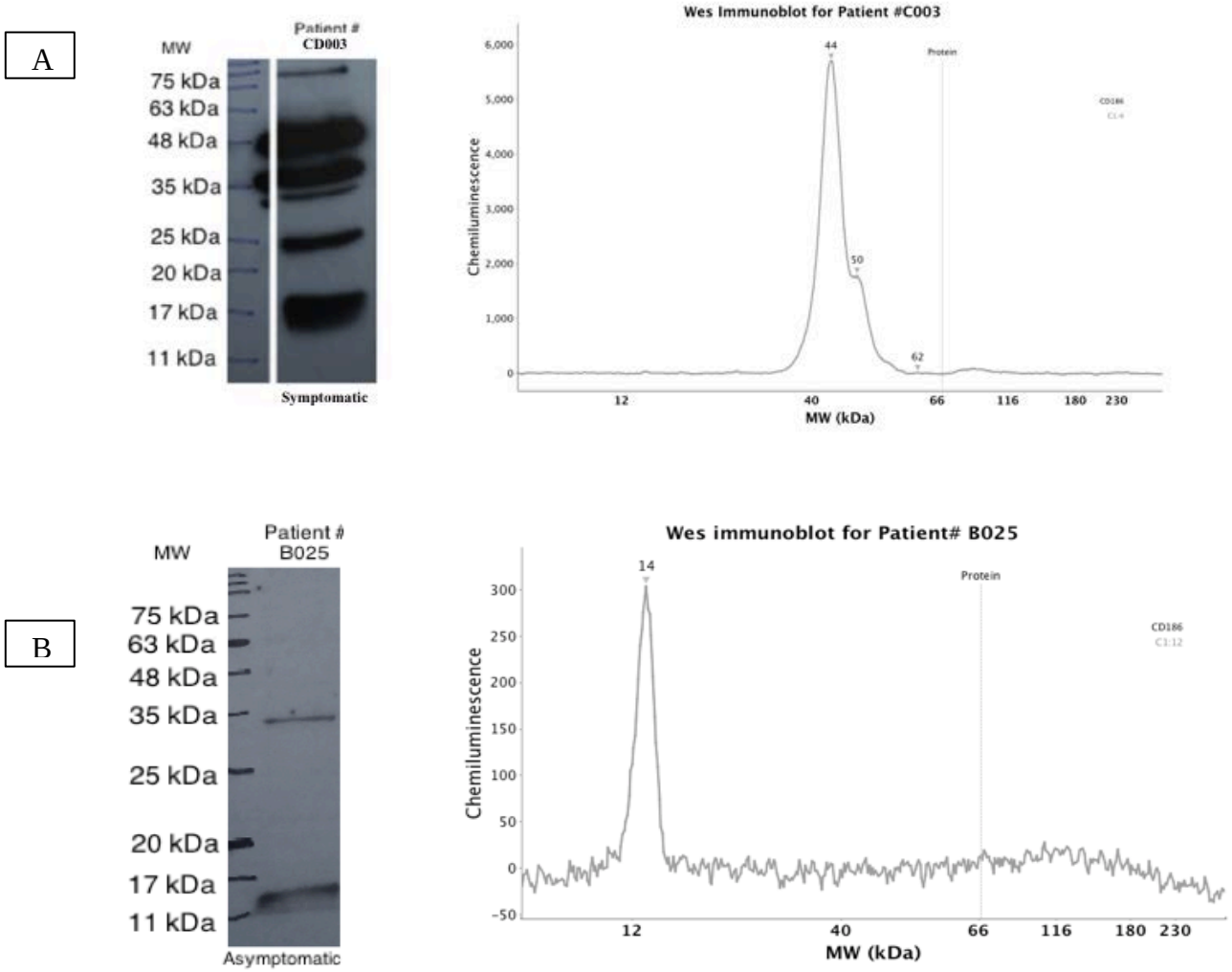

Figure 4 – Comparison of Western blot to Wes automated immunoblot using Patient Plasma.

Western blot performed on *C. difficile* proteins (20 μg) using SDS-PAGE, semi-dry transfer, incubation with primary antibody (patient plasma 1:10000) overnight and secondary antibody (anti-human rabbit IgG 1:20000) for one hour at room temperature. Wes immunoblotting was performed using ProteinSimple's Wes capillary system. The same C. *difficile* proteins (0.2ug versus 20ug), primary antibody (1:100), and secondary antibody (1:1000) were used in both the Western blot and Wes experiments. Blocking solution and wash solution was provided with the Wes system. The entire Wes immunoblotting run was conducted over a 5-hour period. (A) Patient CD003, a positive symptomatic patient, showed significant difference in immunofluorescent signal between the Western blot and Wes methods, with the only conserved band found at 44-50kDa. (B) Serum samples from Patient B025, a negative asymptomatic patient, showed less significant differences in protein recognition between methods, with conservation of the 15kDa band and elimination of ~30kDa band.

Figure 5 illustrates the results of the Wes immunoblot analysis for all of the tested patient samples overlaid over one another. Protein bands detected within the 15 kDa and 45-50kDa regions appear to be immunodominant for the entire cohort, based on the quantity and height of the absorbance peaks within these areas. Additional reacting protein bands were present at 66, 116, 180 and 230 kDa but these bands appeared for only a single or a small number of patient plasma samples. There were 7 patients where the plasma did not contain antibodies that recognized any of the *C. difficile* proteins – 1 positive asymptomatic, 2 negative symptomatic, 4 negative asymptomatic. Repetitions were performed on approximately 20% of serum samples, to illustrate the conservation of bands. **Figure 6** shows a representative group of 3 patient samples that were retested by the Wes system. The repeated spectra of these 3 patients shows that the size of the recognized protein bands was conserved but that the strength of the immunochemiluminescent signal was diminished between trials.

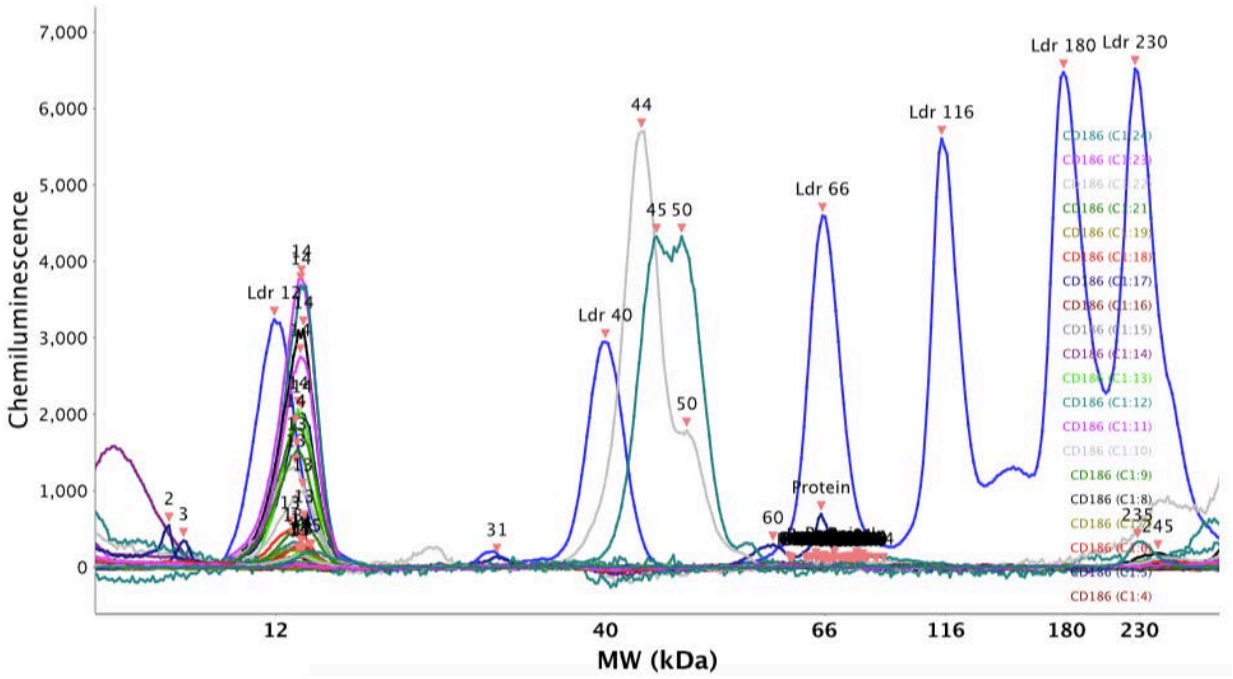

Figure 5 - Overlay of Entire Patient Cohort Wes Immunoblot, Each Line Representing One Patient Shows Abundant Recognition at ~15kDa

Wes immunoblotting was performed using the Whole Protein Size protocol provided by ProteinSimple utilizing *C. difficile* proteins, patient plasma (1:100), and consistent anti-human rabbit IgG-HRP (1:1000). The graph shows the chemiluminescent results for all of the patient graphs overlaid to observe relative frequencies between patients and general patterns of recognition. The blue Ldr line indicates the standard protein size ladder that serves as a point of reference. Patterns of recognition can be seen around the 15, 45, 50, and 60 – 85 kDa regions, with the most significantly frequent recognition at 15kDa.

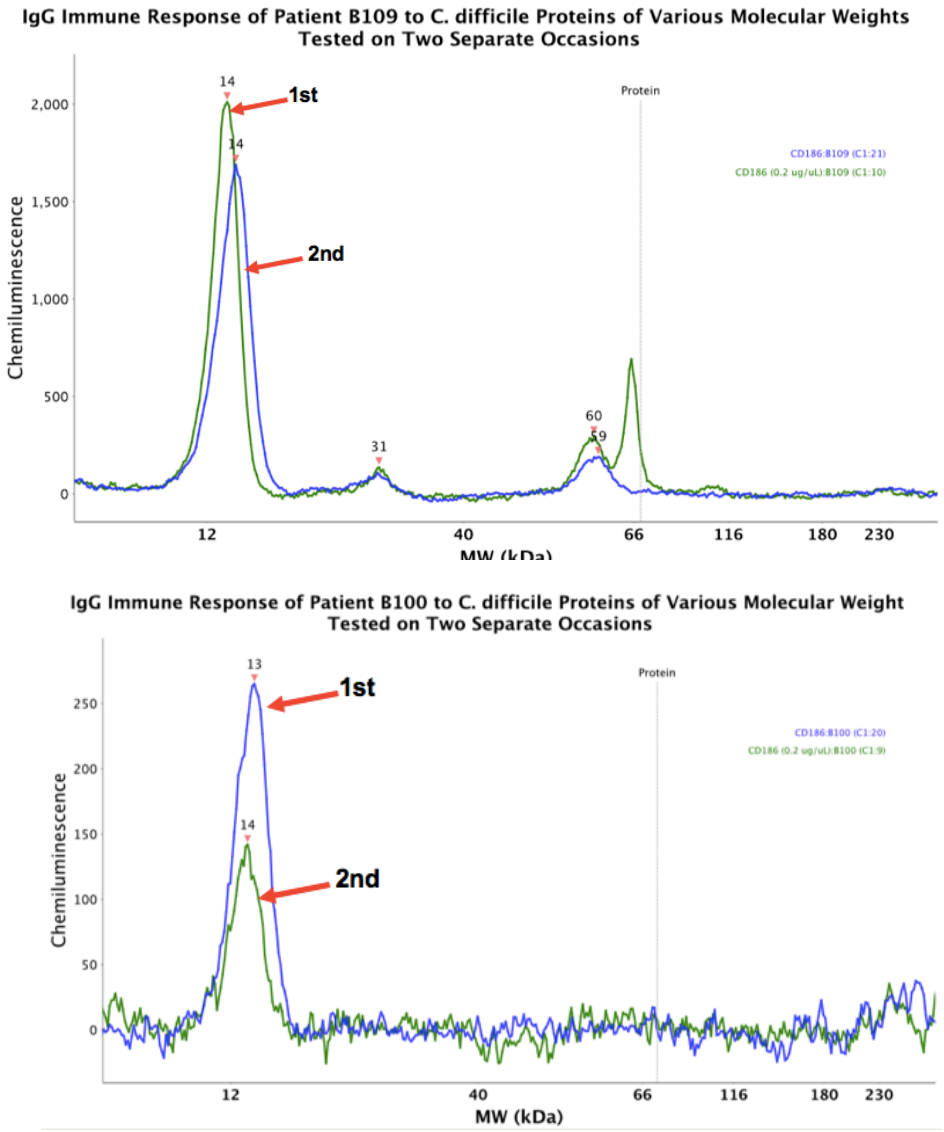

Figure 6 - Repetition of Wes Immunoblot for 2 Patients Shows Consistency in Recognition. Wes immunoblot performed according to Wes Protein Size 25-well protocol using primary antibody (patient serum 1:100), secondary antibody (anti-human IgG 1:1000), and *C. difficile* protein extraction. Repetitions performed two months apart. Each figure below represents one individual patient and their repeated blot. Recognized proteins are conserved between trials, with the strength of recognition diminishing likely due to freeze-thaw antibody stress.

The frequencies of protein recognition were analyzed for each patient cohort (**Figure 7, and 8, Table 2**). The 15kDa protein region was stained by 35% (28/79) of all of the patient samples tested and between 30 - 47% of the tested patient samples depending on the cohort: 30% of the patients who were positive for *C. difficile* but negative for symptoms identified the 15kDa band while 47% of patients positive for *C. difficile* and positive for symptoms showed a reaction. However, the differences in incidence between the different cohorts was not statistically significant. The 65 and 70kDa bands similarly were recognized by antibodies present in 19-34% of patients tested and these incidences were not significantly different between the different cohorts. The relative intensity of staining for each of the bands detected by the patient plasma showed that the average intensity of the 15kDa band was very similar among patient samples in all cohorts (Figure 5). The intensity of the 45 and 50kDa bands showed a much higher staining intensity in the two *C. difficile* cohorts although this result was driven by very high values obtained from only two of the patients and was not statistically significant.

The detection of only one of the proteins was found to be significant – the strength of association between recognition of the 15kDa protein with the entire patient cohort was statistically significant ($p < 0.05$) as compared with all other detected proteins (**Figure 7, Figure 8)**. This protein was concluded to be the most immunodominant among all types of patients, with no significant difference in incidence between genders or patient cohorts. All remaining values corresponding to positive responses were analyzed statistically and found not to be significantly associated between all cohorts, genders, and between positive/negative patients.

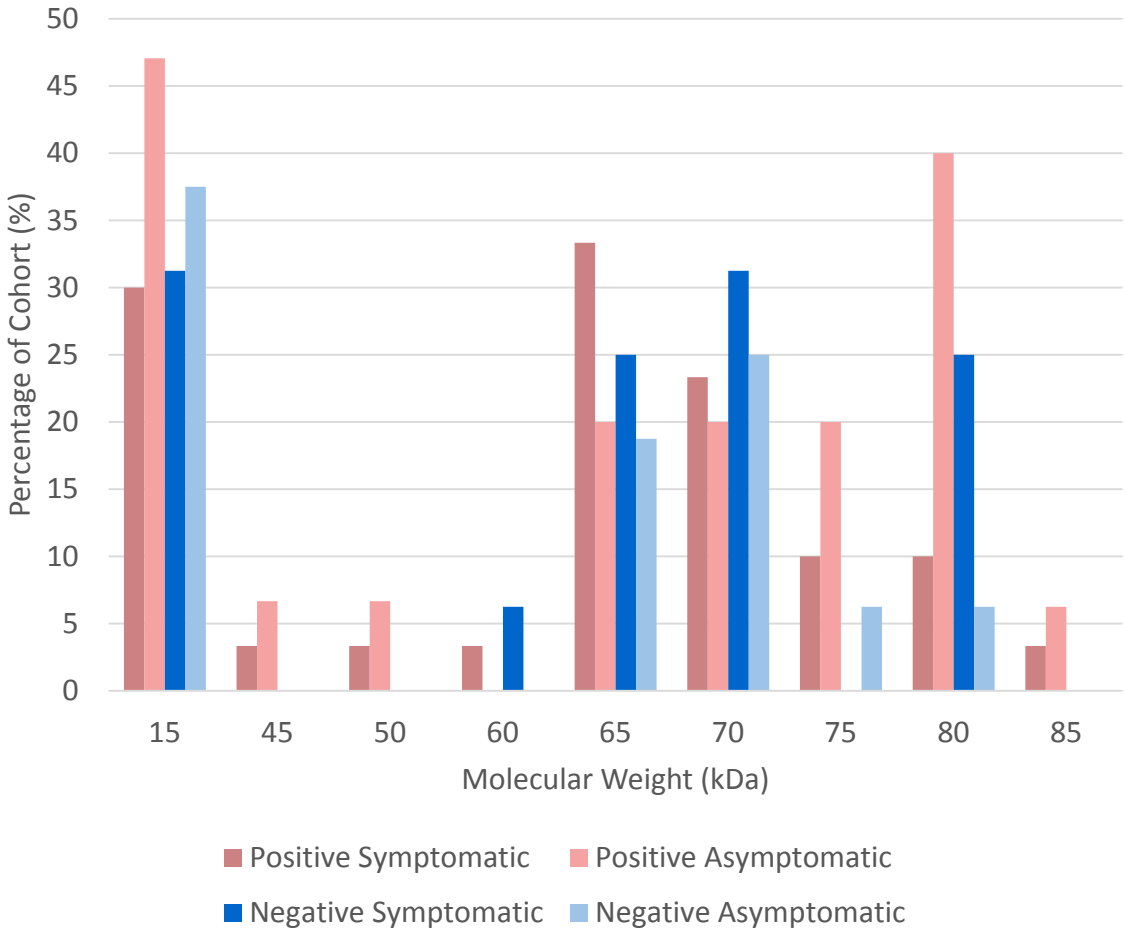

Figure 7 - Frequency of Immune Response Among Patient Cohorts to C. difficile Proteins of Various Molecular Weight (n=79)
Frequency of recognition is expressed as percentage on the y-axis and corresponds to a positive result in the Wes immunoblot assay utilizing patient plasma against unknown *C. difficile* proteins of various molecular weights. Intensity of recognition is omitted from analysis. Patient cohorts are expressed by color, as indicated below the graph in the color legend.

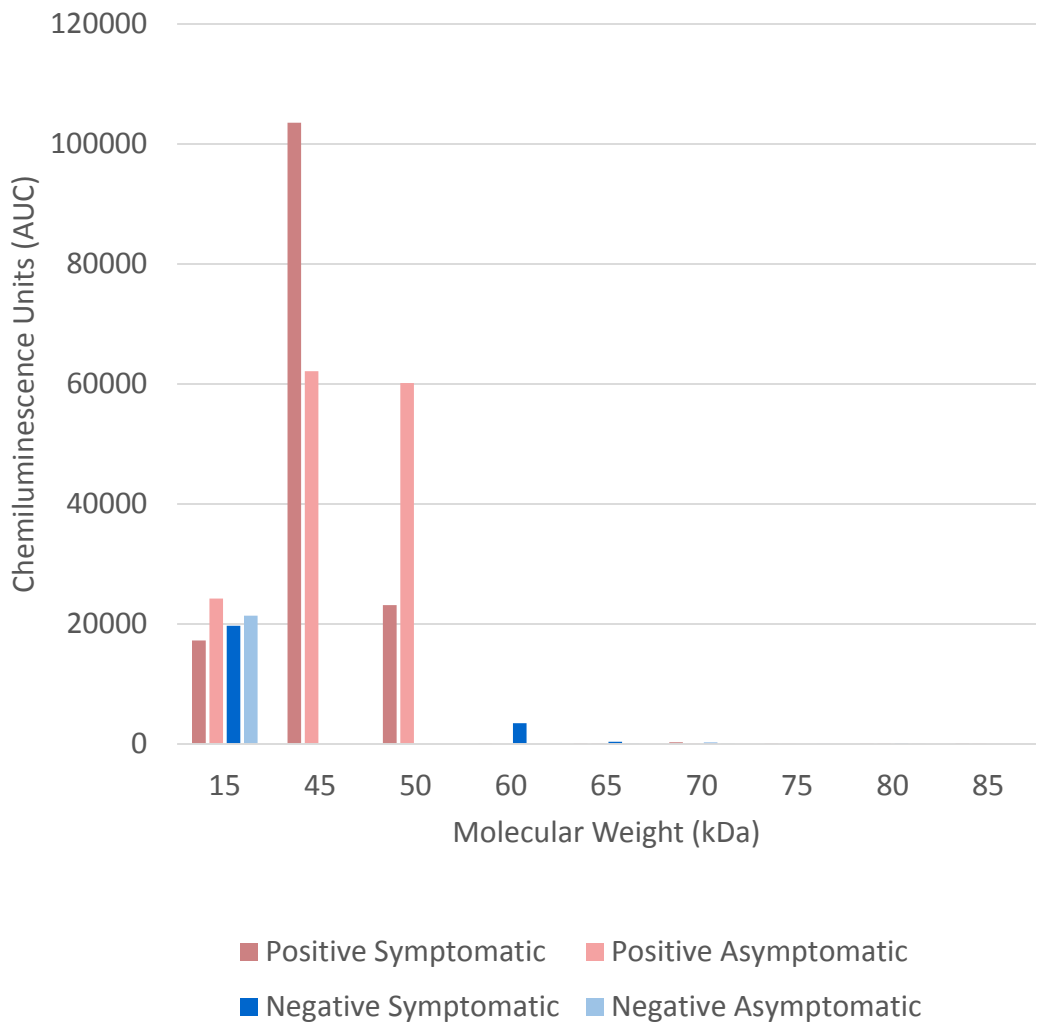

Figure 8 - Average Intensity of Patient Immune Response to C. difficile Proteins of Various Molecular Weights Classified by Presence or Absence of C. difficile in Stool and Presence or Absence of Diarrhea (n=79)

Intensity of patient recognition by Wes immunoblot is expressed using chemiluminescent units, as measured and reported by ProteinSimple's Compass Software. Patients characterized by symptom and presence/absence of *C. difficile* according to color legend below graph. Values expressed as an average of the patient cohort's response to the protein. Amount of patients exhibiting this response is omitted from analysis, thus intensity of response must be compared to Figure 6. The most intense response was measured in the 45 and 50kDa regions, in positive symptomatic and positive asymptomatic patients. The second most intense responses were measured in all cohorts against 15kDa protein, with a notable response exhibited at 60kDa by negative symptomatic patients.

3.4 Protein Identification

The preliminary characterization by 1-D Western blot of the 45kDa protein (**Figure 9**) was excised and subjected to mass spectroscopy and was identified as three different proteins, Acetyl-coA acetyl transferase, Enolase, and/or NAD glutamate dehydrogenase as the potential immunogenic proteins. The Western blot was performed 3 times with identical results and characterized by mass spectroscopy for one of the experiments.

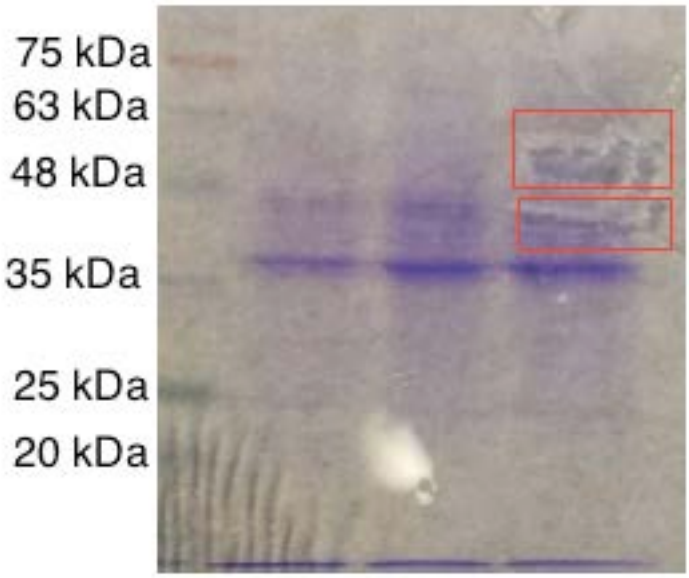

Figure 9 - Excision of immunodominant band within 1-dimension SDS-page
Protein sample (20ug) was resolved using 12% SDS-PAGE at 110V for 45 minutes. Extreme caution used as to not contaminate gel with human or other proteins. Gel stained with Coomassie Blue and destained with water. Protein weights of interest excised using scalpel and placed within 0.5mL Eppendorf tube containing 10% acetic acid. Protein samples were shipped with dry ice to Ottawa Hospital Research Institute (OHRI).

The immunodominant 15kDa protein was also characterized by 2-D SDS-PAGE and Western blots and identified by mass spectrometry (**Figure 10**) to correspond to three separate candidate proteins specific to *C. difficile*: Enolase, Acetyl-coA acetyl transferase, and the 50S ribosomal protein L7/L12 (**Figure 11**). Mass spectrometry was only performed once, however, the 2-D immunoblot was replicated 3 times with similar results.

Figure 10 - Immunoblot of 2-Dimensionally separated C. difficile proteins
(Above) Coomassie stained 12% SDS-PAGE gel of 2-D separation of *C. difficile* proteins. Isoelectric focusing performed on a 7cm IPG strip immobilized pH gradient (IPG) strip was rehydrated overnight at 20ºC. IEF was performed at 50mA per strip at 500V for 12000 volt-hours using a BioRad Protean IEF system. The IEF strip was then equilibrated by incubation in Equilibration buffer containing iodoacetamide SDS-PAGE electrophoresis, was performed using a 12% acrylamide resolving gel and 4% stacking gel. The equilibrated strip was laid on top of the stacking gel and sealed using the agarose sealing mixture and subjected to electrophoresis at 100V for approximately 2 h. The gel was stained using Coomassie Blue.
(Below) 2-D Western blot was performed on a duplicate transferred gel, run in accordance with the protocol above. Gel was transferred to nitrocellulose by semi-dry method and blotted with patient serum (1:10000) overnight at 4C and detected with anti-human IgG over 1 hour at RT. Proteins exhibiting responses corresponding to Ponceau stained triplicate membrane and Coomassie stained gel are identified in Figure 9 as Protein 1 and Protein 2. These proteins were excised and characterized by mass spectrometry.

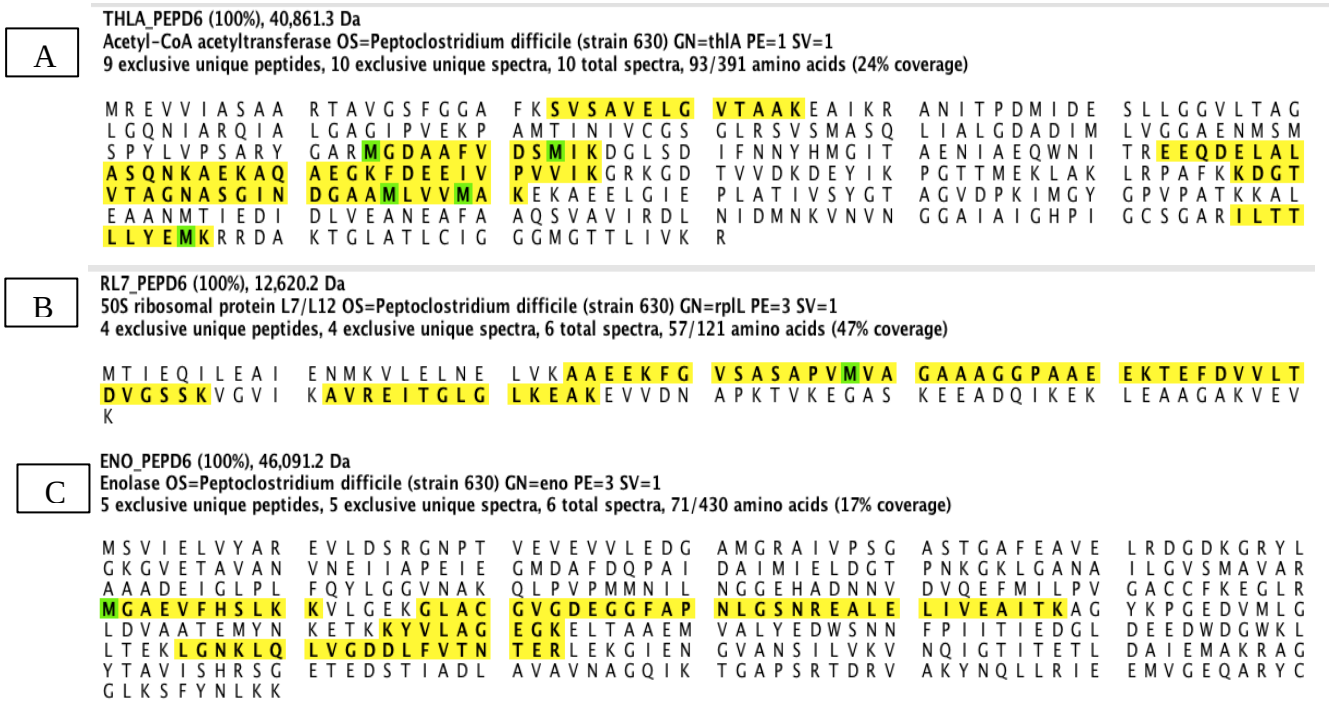

Figure 11 - Coverage of Mass Spectrometry Identified Protein Fragments

Mass spectrometry was performed on the immunodominant protein spots, as identified by 2-D Western blot. Spots were excised from the 12% SDS-PAGE gel and submerged in 10% acetic acid before being shipped on ice to OHRI. OHRI performed digestion with trypsin and subsequent mass spectrometry was carried out with an Orbitrap LUMOS mass spectrometer. Peaks were analysed using Mascot and Scaffold programs.

A. Fragment identification and coverage of Acetyl-coA acetyltransferase, B. Fragment identification and coverage of 50S Ribosomal Protein L7/L12, C. Fragment identification and coverage of Enolase.

3.5 Statistical Analysis

The only statistically significant finding was the recognition of the 15kDa protein by the entire patient cohort, as compared individually against all other proteins (all $p < 0.05$). All other data resulted in p values much greater than 0.05 thus were not statistically significant.

4 Discussion

Clostridium difficile is an emergent public health concern [1]. The prevention and detection of the bacterium in patients at risk could potentially help to prevent disease and death. This study looked to explore the patterns, if any, of serum immunoglobulin response to *C. difficile* proteins of various molecular weights. However, no statistically significant difference was observed in the immune response to *Clostridium difficile* proteins between sick patients and asymptomatic carriers in this study. This is an interesting finding that is contrary to other evidence showing an increase in protection through toxin neutralizing and other non-toxin anti-*C. difficile* IgG antibodies in asymptomatic *C. difficile* carriers [59] [53].

Western Blot and Wes Immunoblotting Methodology Limitations

In the analysis of the immune responses, there were differences in the Western blot pattern and the Wes capillary immunoblotting pattern (**Figure 4**) which can possibly be attributed to the significantly smaller aliquot utilization and surface area of antibody interaction in the Wes method. This introduces a possible source of error in the results that could be rectified in later studies by optimizing the concentration used in the Wes method to better match the Western blot results. The difference can be observed by the significant bands in the Western blot that were not observed in the Wes observed in **Figure 4(A)**. In the Western blot, bands were observed in the 30, 35, 48, and 55 kDa region, with some in the 17 and 25kDa region. However, in the Wes immunoblot, only one protein in the 15kDa region was observed with high intensity. It is important to note that not all samples had

differences in their Wes vs Western blot, as in the case of **Figure 4(B**). Here, the differences between methods are much subtler, indicating more consistent results. It should also be noted that the strongest immunoreactive bands detected by Western blot analysis were also detected by the Wes analysis, however some of the weaker reactive bands by Western blot analysis were not detected using the Wes. This could possibly have been a function of the concentration of the Wes sample, as previously mentioned. Regardless, the study was interested in identifying the most immunodominant bands thus the potential loss of lower intensity bands would not affect the intended analysis as the study was looking for the higher intensity bands that were more conserved between methods. Moreover, purpose of the study was not to investigate the differences between the two methods, but to determine the most systematic and controlled method of investigating a large number of patient sera. The best method for screening of multiple samples with high reproducibility was determined to be the Wes capillary system, which allowed controls in each well, ensuring that the same running time, temperature, and reagents were used during each assay [58]. We were satisfied with the quality of the Wes results as they exhibited internal consistency between trials and between patient samples. Repeated analysis of the same sample showed that the Wes system identified immunoreactive bands at the same size. However, there were some differences in relative intensities of the bands between the replications of the patient samples likely due to freeze-thaw protein integrity damage [60]. The Wes allowed for controlled conditions and inclusion of standards within each well to allow comparability, something that was not easily possible with Western blot analysis.

Due to limitations in funding and resource, an optimization of the Wes conditions was not performed. Given the appropriate resources, the study would be repeated by

including an optimization of the *C. difficile* protein concentration and patient plasma antibody concentration in order to best match Wes results with the results of the Western blot. This would likely eliminate or reduce the inconsistencies observed in between both methods. In addition, given the lack of positive and absolute negative controls in the Western blot, the study would be repeated with appropriate controls to ensure viability of the results in the absence of Wes analysis. One method of control could be the use proteins from a ubiquitous gram-positive organism within the Western blot in its own lane. Also, a Western blot was not performed for each patient which limits the comparability between methods. Thus, in a repeated study, each patient's Western blot result would have been performed. Lastly, an increase in patient cohort number would also be incorporated to further analyze the statistical difference between patient cohorts as it is believed that a larger cohort might increase the power of the study to detect differences in immune response.

Patient Cohort Characteristics and Their Limitations

As it relates to the patient cohort characteristics, the diagnostic criteria for the inclusion of patients within this study were consistent with recommended guidelines for the diagnosis of CDI [19]. The inclusion of patients within the CDI-positive, symptomatic cohort was dictated by a physician diagnosis. If the patient expressed symptomatic diarrhea and a liquid stool was collected, in addition to a positive *C. difficile* stool PCR, we are confident in the diagnosis of patients within this cohort. In the case of positive, asymptomatic patients, the inclusion criteria included the absence of liquid stool, and a positive test for *C. difficile* toxin by PCR. Again, this criteria for asymptomatic carriers is in

accord with recommendations and has been used in other similar studies [19]. However, when analyzing any trends in the presence of an immune response as it relates to the patient cohorts, it must be noted that the patient cohorts are not perfectly definitive. It is possible that a proportion of the *C. difficile* negative patients had previously encountered the bacterium. This would suggest that some negative cohort patients would have an immune response to the *C. difficile* proteins despite not being currently infected as determined by the absence of *C. difficile* DNA in stool. It was not possible to eliminate this factor within the study, as patients may not know that they encountered bacterium if it was asymptomatically or if they had mild symptoms for which they did not seek medical attention. On the same note, an absence of a measured immune response is not perfectly definitive of the absence of antibodies present towards *C. difficile* proteins. It is possible, even likely, that some plasma samples contained antibodies that simply did not react, which would indicate a negative or faint result in the study but might not be indicative of the actual biological reaction. Again, this factor was not controlled for within this study. A positive control within each sample could have been used, such as lipopolysaccharide or another ubiquitous antigen [61]. Further studies would benefit from the use of this positive control. In summation, the characterization of the patient cohorts could have affected the analysis of the results, by introducing the potential for memory of previous immune responses to *C. difficile,* which allow the detection of high-affinity *C. difficile*-specific antibodies, skewing the average absorbance values for that patient cohort.

Furthermore, it is uncommon for CDI studies to include negative, symptomatic patients, as was included in this study, which may have served to complicate the results and analysis. As previously discussed, negative controls are not easily obtained in this type

of analysis. A negative control could have been serum from a germ-free mouse or a similarly raised animal to absolutely ensure no *C. difficile* specific antibodies were present. Alternatively, the inclusion of an *E. coli* protein extract could have served well as a control, to distinguish CD-specific from non-specific protein binding.

Lastly, sex and gender have been associated with differences in immune response, especially with regard to mounting humoral responses. In this study, there were no statistically significant differences between the immune responses of males and females in of this patient population. Aside from two outlier patients, both female, who exhibited extreme responses to the 45-50kDa region (**Figure 5, Figure 8)**, all other patients showed statistically similar immune responses. This lack of difference between sexes/genders is reassuring with regard to *C. difficile* immunity. Had differences been found, it could have complicated the study and subsequent design of immunotherapies. As mentioned, there were 2 of the 79 patients that exhibited an extremely high response to the 45-50kDa protein region, however it was not statistically significant. It does merit further analysis in later studies. Thus, sex/gender was not a differing factor in this study, but the only way to confirm this was to ensure equal representation of female and male patients within a cohort. This still remains an important issue in scientific research and should be addressed at every opportunity, especially in studies that may result in immunotherapeutic interventions that must be universal between sexes and genders.

Protein Identification

Among proteins tested in this study, the only immunodominant protein region associated with the patient samples and confirmed by statistical analysis was found in the

15kDa region. Although there were no significant differences between patient cohorts, the 15kDa band was the most immunodominant protein identified for the entire patient population, compared with all other proteins examined. This suggests that proteins in this region might be immunodominant because a large proportion of patients had antibodies that were able to bind with high affinity to a protein or a group of proteins within this region. It is possible that a non-specific response occurred, as all cohorts of patients have these antibodies at frequencies that were too similar to show statistical significance. However, it should also be noted that there were differences in frequency, or proportion of the cohorts that had a response to the protein: 47% of CDI patients without diarrhea had antibodies that recognized the 15kDa protein while 30% of CDI patients with symptoms and 30% of all uninfected patients had antibodies that recognized the 15kDa protein. Although a small difference that does not meet statistical criteria, this trends in the direction of supporting the idea that the ability of *C. difficile* carriers to present with no symptoms might be associated with an immune response to this region of proteins. It is also possible, even likely, that there are several immunoreactive proteins in the 15kDa range and that only a subset of these are specific for C. *difficile,* while up to 30% of all patients recognize a non-specific, non-*C. difficile* 15kDa protein. Non-specific proteins could include general antigens present in Gram-negative bacteria or *C. difficile* proteins that may have similar protein sequences to more ubiquitous bacteria. More studies to distinguish among potential immunoreactive proteins within this region in the different cohorts might be informative.

The immunodominant proteins identified – acetyl-coA acetyltransferase, L7/L12, and enolase – have previously been show to play important roles in the metabolism and

pathogenicity of *C. difficile* [62,63]. This supports the finding of these proteins as immunodominant candidates identified by patient plasma.

First, acetyl-coA acetyltransferase is an enzyme that is involved in over 10 metabolic pathways [62]. It is an intracellular enzyme that can be found in the cytosol of prokaryotes as well as in the mitochondria and peroxisomes of eukaryotes [62]. This *C. difficile* protein was excised on two different occasions that used two different methods, both the 1-D and the 2-D mass spectrometry results, suggesting that it is likely a key protein targeted by the immune response. It was also identified by others investigating immunogenic *C. difficile* proteins [63]. However, the actual molecular weight of the protein is inconsistent with the molecular weight location at which it was excised the second time, in the 2-D gel mass spectrometry. Acetyl-coA acetyltransferase has an expected molecular weight of 41kDa, consistent with the 1D excision, despite being excised from the 15kDa region following 2D gel analysis [64]. One explanation for this inconsistency could be possible fragmentation of the *C. difficile* protein during preparation or while being stored at 4°C for the length of the project [65]. Additionally, no protease inhibitors were used in the preparation, which could have allowed protein destruction, further supporting this hypothesis [60]. Thus, it is possible that the acetyl-coA enzyme suffered fragmentation, but remained recognizable by the antibodies in both its full and fragmented forms. Regardless, the identification of acetyl-coA acetyltransferase as an immunogenic protein is supported in studies with other species. In *Clostridium chauvoei*, for example, acetyl-coA acetyltransferase was identified as an immunogenic surface protein [66]. Furthermore, in studies with *Staphylococcus epidermidis*-induced sepsis, acetyl-coA acetyltransferase conferred protection when administered, reducing in the systemic bacterial load [67]. Thus, this enzyme as an

immunogen in *C. difficile* is highly probable. However, the intracellular location of the enzyme poses a challenge for the accessibility by the immune response, as *C. difficile* is an extracellular pathogen [53]. Significant bacterial death would likely be required for the immune response to have access to high levels of acetyl-coA acetyltransferase. However, because other studies have identified this protein as a surface antigen, it is possible that it could be expressed on the surface of *C. difficile* but has yet to be characterized in this area.

Another identified protein, the 50S ribosomal L7/L12, is also an intra-cellular protein [68]. This protein is an important part of *C. difficile*'s 50S ribosome [68]. L7/L12 serves as an important antigen in infection with *Brucella abortus*, inducing a T_H1 immune response, which suggests a macrophage activation is possible using this antigen [69,70]. The importance of this protein to the bacterium and in the function of its ribosome was illustrated in a study using *Escherichia coli*, wherein removal of this protein from the bacteria significantly inhibited the translational output of the ribosome, in turn, reducing the amount of protein it could produce [68]. This could be an interesting area of intervention in CDI, as its main pathogenic factors are the protein toxins it produces, toxin A and toxin B [56]. Thus, targeting and disrupting L7/L12 could potentially decrease the protein production of the cell and, in turn, reduce symptomatic disease. *C. difficile* spores are heavily populated with ribosomes [71]. If they were to be phagocytized and successfully destroyed, APCs would come into contact with high levels of ribosomal L7/L12 and could present this antigen to T_H2/T_H17 cells, eliciting a humoral immune response. This could explain, to some degree, why there was detection of IgG immune response to *C. difficile* L7/L12 in this patient population. However, all patients recognized the 15kDa proteins with the same intensity, which might indicate a non-specific immune response to

L7/L12 that could have been elicited from another species of ubiquitous bacteria. After all, 50S ribosomes are a large part of all prokaryotes and show significant similarities among species. Overall, L7/L12 provides an interesting target in *C. difficile* treatment as is it specific to 50S ribosomes, which are not found in eukaryotic cells [72]. Many antibiotics utilize this difference between eukaryotic and prokaryotic ribosomes by targeting them [72]. Thus, successfully targeting this protein might give the host an advantage in mounting an immune response.

The last immunogenic protein identified was enolase. Enolase is an enzyme utilized in both eukaryotic and prokaryotic metabolism, catalyzing the 9th step in glycolysis [62]. Interestingly, this enzyme is expressed on the cell surface of bacteria in an inactive form, aiding in bacterial adherence and pathogenesis [73]. The immunogenicity of enolase has been illustrated in studies with other organisms, particularly as an immunogenic surface antigen in *Clostridium chauvoei* [66]. Moreover, immunization with enolase in *Candida albicans* has induced protective immunity, which strongly supports the relevance of the finding of immunogenicity in *C. difficile* within this study [74]. Much like acetyl-coA, enolase posed interesting molecular weight inconsistencies. Enolase is a 46kDa protein that was excised from this region in the 1-D gel but was then excised from the 15kDa region in the 2D blot, as with acetyl-coA [62]. Again, it is possible that fragmentation with long-term protein storage could account for this inconsistency. Furthermore, there is evidence that enolase is secreted to the surface of the cell in an inactive form, which does not require the entire enzyme to be present [75]. Thus, it is plausible that fragments can be excreted to the surface, providing an explanation for the identification of enolase at such a low molecular weight. Enolase is also a protein that had been shown to be involved in bacterial cell

adhesion [76]. Additionally, enolase was identified in both protein characterization methods, by 1-D and 2-D analysis, lending support to its potential as a target protein. Overall, enolase seems to be an interesting candidate for further investigation in *C. difficile* immunity due to its extracellular location, its function in adherence, and its immunogenic properties in other species. Unfortunately, enolase is not specific to prokaryotes and the *C. difficile* enolase protein sequence has similarities with the human enolase enzyme. This could potentially result in auto-reactive antibodies or serves as a convenient tool for *C. difficile* to hide from the immune system through the tolerance of self. Regardless, it is an interesting area of investigation for future studies.

Due to the single separation by molecular weight of the 15kDa proteins in the Wes capillary system, the immune response was measured to the molecular weight group as a whole. Thus, the measured immune response to each protein within the 15kDa is unknown. For example, the two-dimensional electrophoresis study showed that there were multiple protein species present at approximately 15kDa, with different isoelectric points, that were recognized by the different patient plasma samples used for these experiments. This supports the idea that the different immunoreactive bands recognized by the Wes immunoblotting could be comprised of multiple proteins and that there might even be differences in recognition of individual proteins by the patient antibodies. Consequently, there were no significant differences in immune response to the group of proteins between patient cohorts. However, this does not eliminate the possibility that the immune response to each individual protein within the 15kDa region – that is enolase versus acetyl-coA acetyltransferase versus L7/L12 – might have a significant difference in immune response on their own. Future studies could investigate each protein separately to further

characterize the specific immune response. This could be done by purifying each protein and performing an enzyme-linked immunosorbent assay on each protein separately, and measuring the immune response between patient cohorts. This could result in a different conclusion. Regardless, this information provides interesting opportunities for future studies of *C. difficile* infection.

Lastly, it is important to mention that, although immunodominant proteins were identified in this protein sample, it does not exclude other candidates that may have not been present in this extraction. It is well known that variations in methods of protein extraction result in different protein contents [77]. Thus, the immunodominant proteins identified might be unique to the bile salt extraction method and may not be repeatable with a glycine or other extraction. Nonetheless, the immunodominant proteins identified are theoretically sound according to evidence from the previous studies cited and appear to be interesting candidates for future studies.

5 Conclusions

In this study, the immune response to *C. difficile* proteins did not seem to differ significantly between *C. difficile* positive symptomatic or asymptomatic, and *C. difficile* negative patients. Immunodominant protein candidates within our sample were found to be enolase, acetyl-coA acetyltransferase, and 50S ribosomal protein L7/L12. Future studies are needed to confirm the role these proteins have in the immune response to *Clostridium difficile* and further characterize the immune response of *C. difficile* carriers.

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Appendix

Table 2 - Frequency of Immune Response Among Positive Asymptomatic Patients to C difficile Proteins of Various Molecular Weights

Positive recognition by each patient to proteins of varying molecular weights indicated by + symbol within the table. Patients described using their assigned number and sex/gender indicated by color of patient cell (blue corresponds to male, pink corresponds to female). Graph to the right of the table demonstrates all immunoblots for patients within this cohort overlaid over one another, with the standard Ldr ladder curve. No patients in this cohort recognized proteins in the 60kDa region. Patterns of recognition were especially clear in the 15, 65, 70, and 75kDa regions.

	Molecular Weight (kDa)								
Patients	15	45	50	60	65	70	75	80	85
B347	+						+		
B131								+	
B142							+		
B045	+					+			
B051	+							+	
B080		+	+		+				
B119									+
B159	+				+				
B179						+			
B277								+	
B040	+						+		
B135					+				
B170	+						+		
B182	+				+				
B278						+			
B338	+								
B274								+	

Figure 11 - IgG Wes Immunoblot of Positive Asymptomatic Patients Against C. difficile Proteins of Varying Molecular Weights

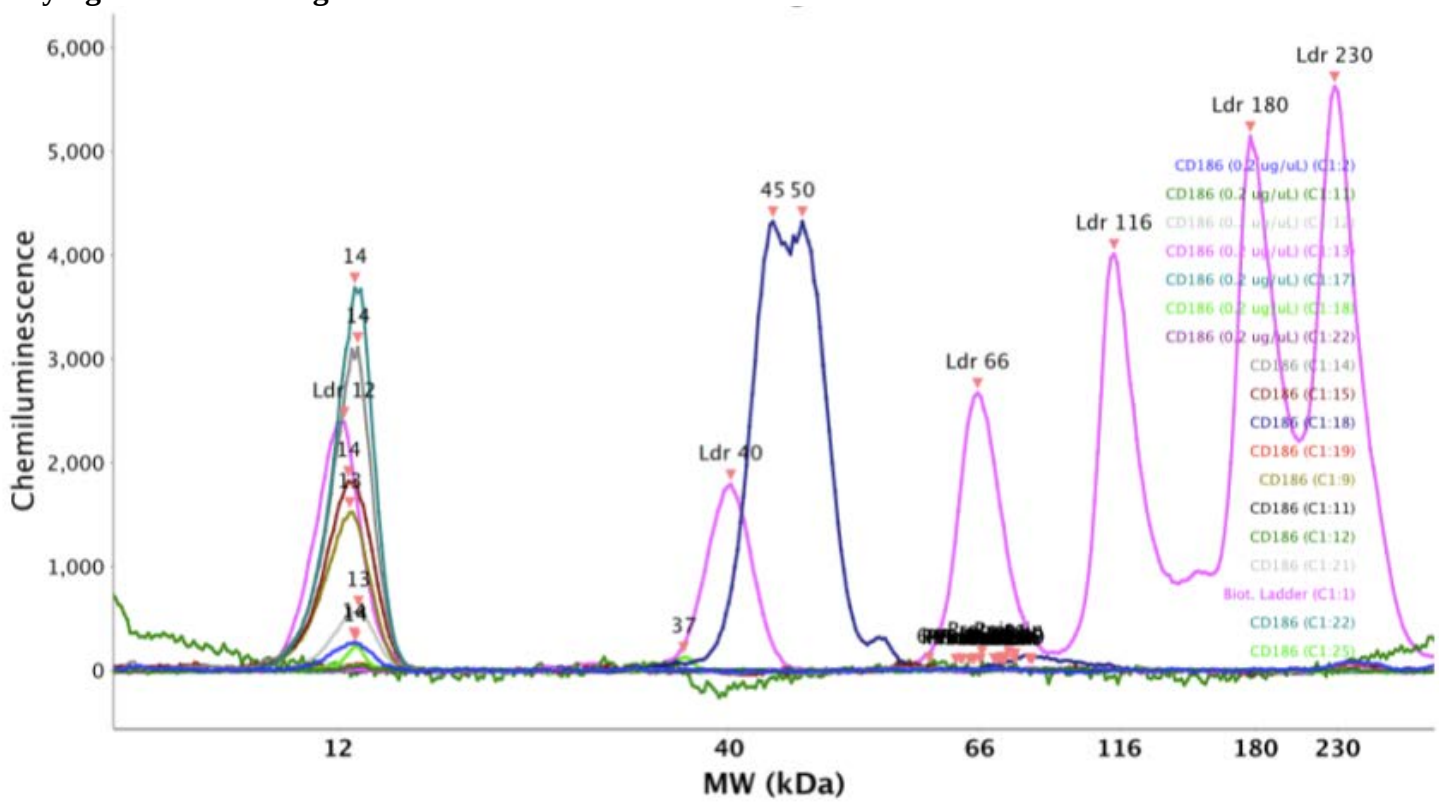


Table 3 - Frequency of Immune Response Among Positive Symptomatic Patients to C. difficile Proteins of Various Molecular Weights

Positive recognition by each patient to proteins of varying molecular weights indicated by + symbol within the table. Patients described using their assigned number and sex/gender indicated by color of patient cell (blue corresponds to male, pink corresponds to female). Graph to the right of the table demonstrates all immunoblots for patients within this cohort overlaid over one another, with the standard Ldr ladder curve. Patients in this cohort recognized all proteins. Patterns were especially clear in the 15, 65, and 70kDa regions.

	Molecular Weight (kDa)								
Patients	15	45	50	60	65	70	75	80	85
CD012	+				+				
CD013							+		
B088					+				
CD014						+			
CD003		+	+						
CD042					+				
CD046									
CD047	+				+				
CD018						+			
CD019									
CD020					+				
CD027					+				
CD017	+				+				
CD029	+							+	
CD008						+			
CD005									
CD007						+			
CD010	+						+		
CD021								+	
CD022	+			+					
CD025	+					+			
CD028									+
CD040							+		
CD030							+		
B028								+	
CD001						+			
CD002						+			
CD004					+				
CD006	+				+				
CD026	+				+				

Figure 12 -IgG Wes Immunoblot of Positive Symptomatic Patient Against C. difficile Proteins of Varying Molecular Weights

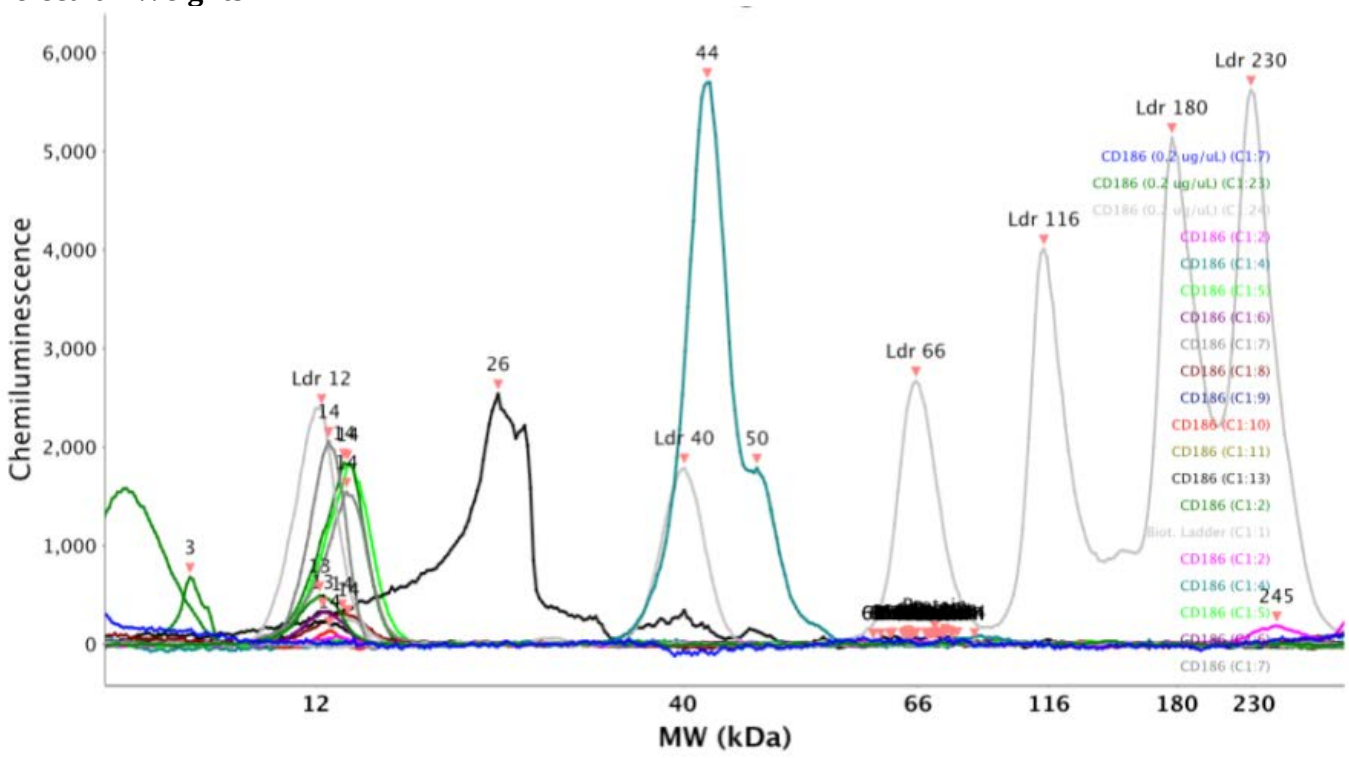


Table 4 - Frequency of Immune Response Among Negative Symptomatic Patients to C. difficile Proteins of Various Molecular Weights

Positive recognition by each patient to proteins of varying molecular weights indicated by + symbol within the table. Patients described using their assigned number and sex/gender indicated by color of patient cell (blue corresponds to male, pink corresponds to female). Graph to the right of the table demonstrates all immunoblots for patients within this cohort overlaid over one another, with the standard Ldr ladder curve. No patients in this cohort had responses to proteins in the 45, 50, 75, and 85 kDa. Patterns were observed in the recognition of proteins around the 15, 60, 65, 70, and 80 kDa.

	Molecular Weight (kDa)								
Patients	15	45	50	60	65	70	75	80	85
B077									
B097									
B150								+	
B053	+				+				
B069						+			
B178	+				+				
B346	+								
B289	+							+	
B109	+			+		+			
B063						+			
B156								+	
B193						+			
B215					+				
B018								+	
B339	+								
B319					+				

Figure 13 - IgG Wes Immunoblot for Negative Symptomatic Patients to C. difficile Proteins of Varying Molecular Weight

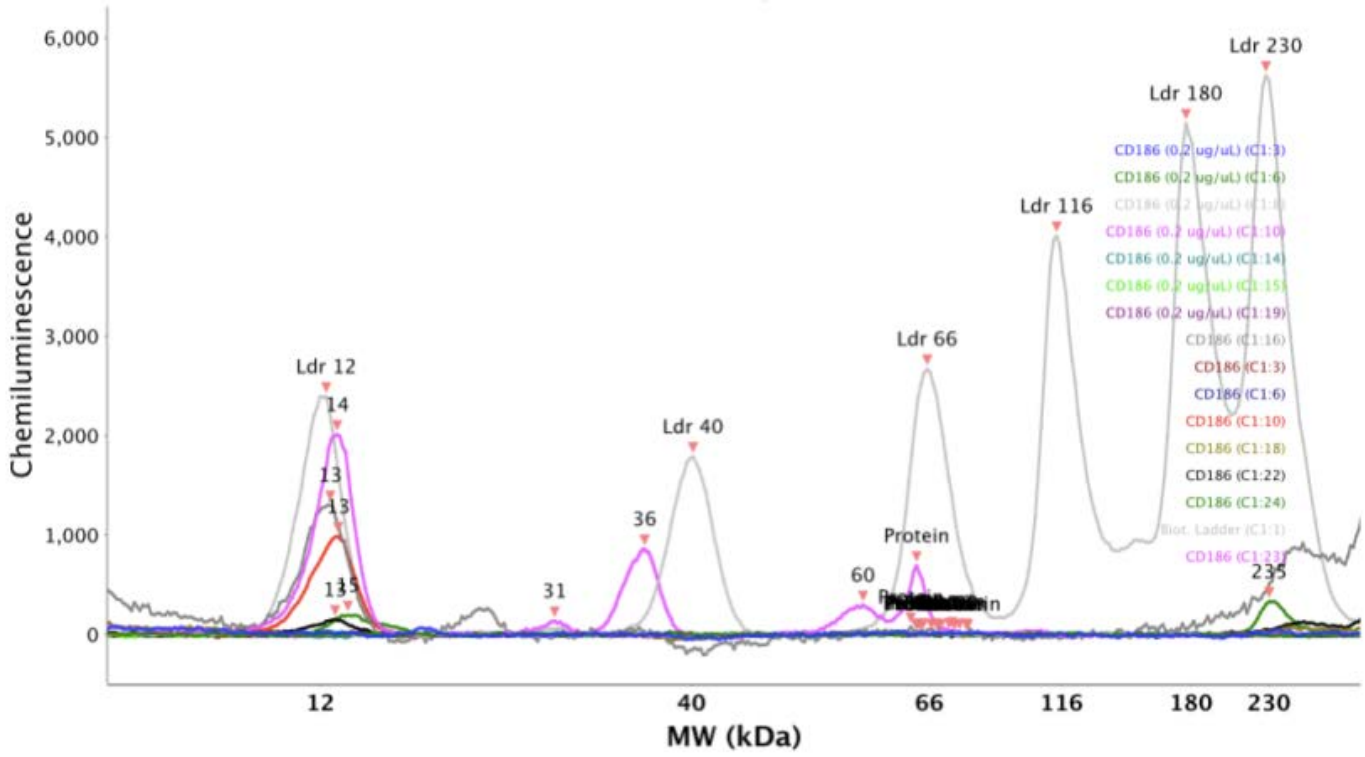
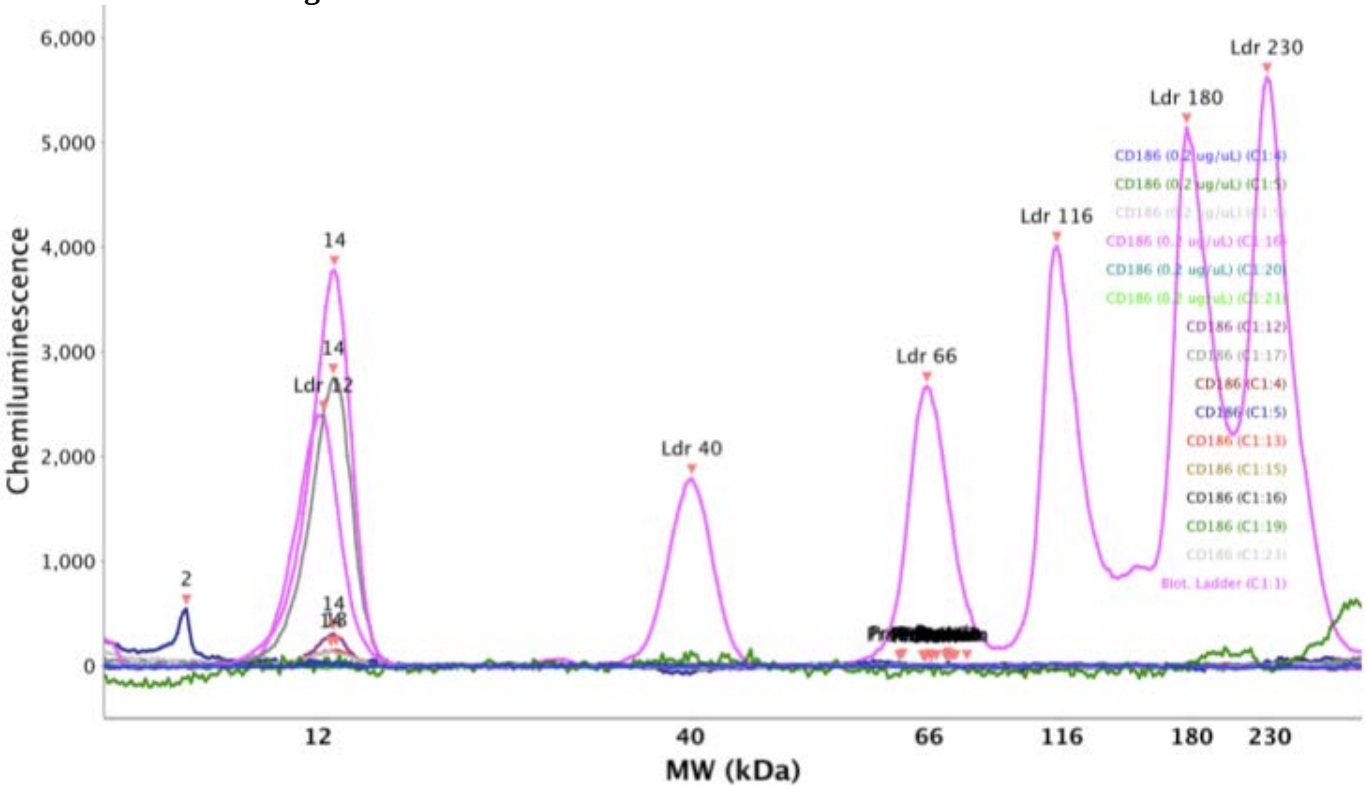


Table 5 - Frequency of Immune Response Among Negative Asymptomatic Patients to C. difficile Proteins of Various Molecular Weights

Positive recognition by each patient to proteins of varying molecular weights indicated by + symbol within the table. Patients described using their assigned number and sex/gender indicated by color of patient cell (blue corresponds to male, pink corresponds to female). Graph to the right of the table demonstrates all immunoblots for patients within this cohort overlaid over one another, with the standard Ldr ladder curve. No patients within this cohort recognized proteins in the 45, 50, 60, nor 85kDa. Trends in response were found in the 15, 65, 70, 75, and 80kDa.

	kDa								
Patients	15	45	50	60	65	70	75	80	85
B267									
B070					+				
B100	+						+		
B059	+								
B032								+	
B300						+			
B326									
B345					+				
B341					+				
B071									
B163	+					+			
B025	+				+				
B066							+		
B285	+						+		
B301					+				
B329									

Figure 14 - IgG Wes Immunoblot of Negative Asymptomatic Patients Against C. difficile Proteins of Various Molecular Weights



Summary

Table 6 - Summary of Patient Cohort Demographics Patient

Group	N	Median Age (years)	Females (n)	Age (years)	Median Age (years)	Males (n)	Age (years)	Median Age (years)
CD+, with diarrhea	30	74	15	66 – 80	80	15	55 – 86	68
CD+, no diarrhea	17	67	9	21 – 76	64	8	37 – 92	71
CD -, with diarrhea	16	71	8	38 – 75	56	8	60 – 90	72
CD -, no diarrhea	16	71	8	29 – 92	70	8	57 – 86	72

Table 7 - Summary of Immune Response of Patient Cohorts to Proteins of Various Molecular Weights

		Frequency of Patient Recognition of *C. difficile* Proteins								
Patient Group	N	**15kDa** n (%)	**45kDa** n (%)	**50kDa** n (%)	**60kDa** n (%)	**65kDa** n (%)	**70kDa** n (%)	**75kDa** n (%)	**80kDa** n (%)	**85kDa** n (%)
Total patient population	79	28 (35)	2 (2)	2 (2)	2 (2)	23 (29)	16 (20)	11 (14)	11 (14)	3 (3)
CD +, with diarrhea	30	9 (30)	1 (3)	1 (3)	1 (3)	10 (33)	7 (23)	4 (13)	3 (10)	1 (3)
CD +, no diarrhea	17	8 (47)	1 (6)	1 (6)		4 (23)	3 (17)	4 (23)	4 (23)	1 (6)
CD -, with diarrhea	16	6 (38)			1 (6)	4 (25)	4 (25)		4 (25)	
CD - ,no diarrhea	16	5 (31)				5 (31)	2 (12)	3 (19)		1 (6)

Table 8 - Summary of Immune Response of Male vs Female Patients of Specific Cohorts to Proteins of Various Molecular Weights

		Frequency of Patient Recognition of *C. difficile* Proteins																	
Patient Group	N (M:F)	**15kDa** n (%)		**45kDa** n (%)		**50kDa** n (%)		**60kDa** n (%)		**65kDa** n (%)		**70kDa** n (%)		**75kDa** n (%)		**80kDa** n (%)		**85kDa** n (%)	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
CD +, with diarrhea	30	4	5		1		1	1		3	7	4	3	3	1	1	2	1	
CD +, no diarrhea	17	4	4		1		1			2	2	1	2	2	2	2	2		1
CD -, with diarrhea	16	2	4					1		2	2	3	1			2	2		
CD - ,no diarrhea	16	3	2							3	2	1	1	2	1		1		

Table 9 - Summary of Immune Response of Male vs Female Patients Regardless of Cohort to Proteins of Various Molecular Weights

		Frequency of Patient Recognition of *C. difficile* Proteins								
Patient Group	N	**15kDa** n (%)	**45kDa** n (%)	**50kDa** n (%)	**60kDa** n (%)	**65kDa** n (%)	**70kDa** n (%)	**75kDa** n (%)	**80kDa** n (%)	**85kDa** n (%)
Female	40	15 (37)	2 (5)	2 (5)		13 (32)	7 (17)	4 (10)	7 (17)	1 (2)
Male	39	13 (33)			2 (5)	10 (25)	9 (23)	7 (18)	5 (12)	1 (2)