

**Effects of *Clostridium difficile* on the human
immune response**

The Honors Program
Senior Capstone Project
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ABSTRACT

Clostridium difficile is a bacterium that is rapidly becoming a large issue in the medical community due to its tendency to infect hospital patients and its resistance to antibiotics. By studying the way in which the pathogen interacts with the human immune system, it is possible to better understand how the body naturally fights off the disease. This knowledge can allow medical professionals to develop treatments that can help curtail the infection before serious symptoms occur. Working under a grant program alongside Professors Kirsten Hokeness and Chris Reid, I was able to research the effects that exposure to the *C. difficile* bacteria has on healthy human immune cells. Our findings show that there is a heightened level of chemokine production in these cells, which is indicative of an immune response to combat the *C. difficile* infection.

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INTRODUCTION

Pathogenic bacteria cause millions of deaths worldwide each year. Pneumonia, anthrax, plague, and tuberculosis are just a sampling of the immense number of disease-causing bacteria found in our world. These imperceptible killers can enter your body through a number of ways and can remain undetected until the symptoms of their infection begin to appear. By this point, the pathogen may have caused enormous amounts of damage to the body. Luckily, modern science has developed ways of fighting back against these formidable opponents through the use of antibiotics. Alexander Fleming, who discovered penicillin, talks about his crucial discovery, “When I woke up just after dawn on September 28, 1928, I certainly didn’t plan to revolutionize all medicine by discovering the world’s first antibiotic, or bacteria killer. But I suppose that was exactly what I did”¹. Drugs such as penicillin and tetracycline are capable of targeting bacteria and destroying them by attacking key metabolic pathways or vital components of the cell wall. These antibiotics can either be highly specialized (narrow spectrum) to seek out a single type of microbe or nonspecific (broad spectrum) and capable of taking out a broad spectrum of bacterial species.

Despite how incredible these drugs have been in our continued fight against disease, they are also creating a new and persistent challenge and cause for alarm. Recent observations have shown that there is a current rise in antibiotic resistance amongst the very pathogens that these drugs target. Bacteria are developing the ability to survive antibiotic treatments and continue to cause disease within the patient. This evolution of antibacterial resistance can occur as a result of a number of different factors. Antibiotics are overused and overprescribed particularly in the Western world. Often times, the full course of the antibiotics are not taken which can lead to the development of favorable environmental pressures for the emergence of resistant strains of bacteria. These resistant strains multiply and continue to pass on their mutations acquired as a result of being in the presence of antibiotic, resulting in an accumulation of particularly pathogenic organisms that is of great concern, especially in hospitals where immunocompromised individuals are in high numbers.

The mutations rendering bacteria resistant to antibiotics can work in a number of different ways². They can prevent the drug from entering them by altering the antibiotic target site. Additionally, they can find a new metabolic pathway to function outside of the

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pathway targeted by the drug, which circumvents the drug's effectiveness. Lastly, the bacteria itself can secrete compounds that directly deactivate the antibiotic, rendering it useless. For example, some bacteria can produce penicillinase, an enzyme that is capable of hydrolyzing penicillin, rendering it completely ineffective by robbing it of its antibacterial properties. This ability of the bacteria to mutate so quickly is a direct result of the ability of the bacteria to multiply so rapidly.

These antibiotic-resistant organisms are the cause of close to 100,000 deaths per year in the United States alone according to the CDC¹. The resistance is occurring at a rate faster than we are able to come up with new therapies. Of greater concern is the fact that drug companies have halted a lot of their efforts to make antibiotics as they are targeting more chronic disease states due to financial reasons. In the past year, the FDA has only approved one new antibiotic for treatment while twenty new drugs were approved for conditions like cancer and heart disease. This is a public health problem that requires a lot of attention. Not only because it is a potentially deadly phenomenon but it also costly. Antibiotic resistance costs the United States more than \$20 billion each year¹. This has forced medical researchers to come up with alternative solutions and treatment targets for battling these pathogens.

Of specific concern is *Clostridium difficile*, a motile, Gram-positive bacterium which thrives in environments with little-to-no oxygen due to its anaerobic metabolic system³. *Clostridium difficile* infections typically occur after a patient has received a heavy dose of antibiotics to treat other infections the patient may have been suffering from. If a patient is exposed to the pathogen while the body is recovering from antibiotics, infection can be sudden and very harmful. When a patient is treated with broad spectrum antibiotics, the medicine will target a wide range of bacteria inside the body in addition to the pathogenic strains. Some of these bacteria are not harmful and may actually be beneficial to the body. The normal gut flora, which consists of many commensalistic or mutualistic bacteria that live in the human gut are important for normal body processes. Research has shown that these bacteria are able to assist the body in a number of ways. Some of these functions include training the immune system to respond to bacterial infection, absorbing remaining nutrients and energy that the body did not absorb, and shielding the body against infection from

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pathogenic bacteria and other disease-causing organisms by creating limited space and nutrients required for colonization by pathogens such as *C. difficile*⁴.

Pathogenesis of *C. difficile*

Following substantial antibiotic regimens, typical microflora levels are reduced so drastically, the gut becomes a prime target for opportunistic microbes to infiltrate, infect, and proliferate. Among these is *C. difficile*, which has developed a number of ways to ensure successful infection and colonization of the host body. The bacteria are capable of forming endospores, which are forms of the bacteria capable of survival in environments that would typically cause the bacteria to die. In a patient currently infected with the pathogen, mature cells will undergo the process of sporulation, in which they enter this dormant, non-reproductive spore form. They are then expelled from the rectum and can attempt to find a new host body to infect. These spores are transmitted via the fecal-to-oral pathway, meaning that after expulsion, they must be ingested orally. This is typically accomplished by the spores attaching to food products, which are then consumed by a healthy individual⁵.

Once ingested, the spores are carried into along the esophageal tract into the stomach. The resilient spore form of the bacteria protects it from the high acidity of the stomach's gastric juices, allowing them to pass into the intestines intact⁵. Once in the small intestine, the spores will then undergo germination, reverting back to the original mature bacteria cells. Some studies have suggested that bile salts found in the intestinal tract are be capable of promoting this germination process⁶. By the time they reach the large intestine and colon, the spores have fully reverted back into mature cells capable of reproducing and causing disease.

Not much information is available on the colonization process employed by *C. difficile* once it is fully germinated. However, we do know that it must create a way to adhere to the bowel walls. This is accomplished by the production of a surface layer of proteins found outside the cell wall. One protein in particular, called SlpA, has been found to be crucial to the adhesion process⁷. Once fully adhered to the bowel walls, the bacteria can begin to reproduce and propagate the colony throughout the bowel.

Following low level exposure, *C. difficile* can be asymptomatic, causing no pathogenic issues within the host body while living amongst the other microbes found in the gut.

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However, patients who experienced reduced gut flora levels and were exposed to *C. difficile* can have symptoms that can potentially be life-threatening. These devastating infections are most commonly seen in elderly patients and those that are immunocompromised due to chronic conditions that existed previously such as patients suffering from Inflammatory Bowel Disease (IBS) or Chron's disease.

The bacteria wreak havoc in patients primarily by the production of two well-described toxins Toxin A (TcdA) and Toxin B (TcdB). These two toxins act jointly to produce symptoms in patients, and studies have shown evidence that the two toxins may have been genetically linked by a duplication of one of their genes. This is evidenced by their very close proximity to one another, as they are both found within one 19.6 thousand base pair-long stretch of DNA on the *C. difficile* genome⁸. Further indication of this duplication is seen when looking at the 74% homology displayed between the N termini of these toxins⁹. Not unlike the *C. difficile* genome in general, both the tcdA and tcdB genes have low amounts of guanine and cytosine pairings in their genetic makeup⁸.

TcdA is an enterotoxin while TcdB is a cytotoxin; when combined, these two toxins are extremely detrimental to the intestinal cells. TcdA has a molecular mass of 308 kilo Daltons while TcdB is 270 kilo Daltons, which makes both these toxins rank among the most massive of toxins produced by bacteria⁸. These toxins function by first entering the cell through receptor-mediated endocytosis. The receptor is thought to be associated with Gal β 1-4GlcNac, a disaccharide that is a part of the I, X, and Y antigens for blood, as the cells expressing the molecule seem open to uptake of TcdA and, presumably, TcdB¹⁰.

After endocytosis occurs, the toxins now have access to the cytosol of the host cell, allowing them to go about altering cellular processes. There is evidence to suggest that the main target of these toxins is the actin cytoskeleton of the cells, which is critical in maintaining cell structure. The toxins appear to use enzymatic activity to interfere with the Rho family of GTPases, which has key roles in the regulation of cellular structure, replication, and motion¹¹. The toxic effects on these proteins are enough to sharply weaken the structure of the cell, eventually causing the cell to undergo apoptosis due to lack of functionality¹¹.

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These two toxins cause many of the symptoms commonly associated with *Clostridium difficile* infection, most notably diarrhea. The toxins infiltrate and kill of the cells lining the epithelial wall of the intestine, which causes extensive damage to the villi found there. Too much damage will allow fluid to seep into the intestine, which is then expelled from the bowels as it is not digestible. This causes the *C. difficile* associated diarrhea (CDAD) that is the mainstay of the bacterial infection¹². The toxicity can also cause pseudomembranous colitis, or *C. difficile* colitis. Excessive damage to the epithelium will invoke an inflammatory response from the body, as dead epithelial tissue will build up and cause ulcers to form in the gut and colon. Diarrhea and abdominal pain are very common symptoms when this occurs¹³. Death may also occur in extreme cases, as unchecked constant damage to the intestinal wall can allow some bacteria to infiltrate the blood stream, causing multiple organ failure as the immune system is overpowered and unable to effectively treat the infection.

Treatment and Growing Concerns

Treatment for *Clostridium difficile* infection (CDI) has been very difficult in recent cases, as the resistance of the bacteria to antibiotics limits the usual options available to the medical professionals. This is further complicated by the characteristics of the bacteria, especially the ability to form spores which are capable of survival under extreme stresses. Despite these characteristics, there have been some successes found with the use of antibiotics as a treatment option for all types of CDI.

There are two well-known antibiotics which have shown high success rates in treating CDI. Vancomycin has been used effectively to eradicate the *C. difficile* within the gut and allow the healthy gut flora to have a chance to proliferate within. Vancomycin specifically targets Gram positive bacteria by inhibiting with the synthesis of the peptidoglycan cell wall¹⁴. Specifically, it binds to the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides, which are the building blocks of the peptidoglycan backbone¹⁴. In fact, a recent study found that vancomycin was “superior” and involved far less drastic side effects when compared to metronidazole, which has been used a cost effective measure against CDI¹⁵. The comparison showed that severe cases of CDI are much better treated by vancomycin than other antibiotics.

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However, this information does not mean that vancomycin is the great eradicator of *clostridium difficile* that medical scientists have been searching for. Vancomycin is a very costly drug, about four times the price of metronidazole¹⁵. This may keep the drug from being a truly viable option for many patients who cannot afford such steep medical bills. Further hindering vancomycin's potential is antibiotic resistance. For decades, vancomycin has been used as a "last resort" drug when all other antibiotics have failed to work. It has seen significant success as a solution against *Staphylococcus aureus* strains that have become resistant to methicillin or other antibiotics. However, recent years have seen a rise in vancomycin-resistant *Staphylococcus aureus* (VRSA) strains¹⁶, which show an ability to adapt to the drug and resist its toxic effects. In strains that become resistant to the bacteria, the terminal D-alanyl-D-alanine residues are replaced with D-lactate which gets rid of the molecular target for the antibiotic and the drug is rendered ineffective as the bacterial cell wall is constructed normally¹⁶. Particularly disconcerting is the comparison between *S. aureus* and *C. difficile*, which have been shown to exhibit similar behaviors and have similar structures. This comparison establishes a historical precedent for the potential emergence of a vancomycin-resistant form of *C. difficile*.

While the vancomycin antibiotic treatments have been effective at treating nonrecurring CDI, this is not a truly viable option in many cases. *Clostridium difficile* infection has been shown to have a large chance of recurring within weeks or months of an antibacterial treatment regimen. Patients with this recurrent form of infection would need to take a powerful medication like vancomycin on a regular basis for the foreseeable future. These subsequent treatments can exact considerable stresses on the bodies and minds of the patients undergoing them. Even more noteworthy is the ability for *Clostridium difficile* to develop a resistance to antibiotics. This resistance potential has been observed repeatedly by a number of studies^{3, 17}. Routine exposure to a drug like vancomycin increases the chances that a resistant or even immune strain of *Clostridium difficile* could emerge. This would then cause even more treatment issues than were there to begin with. Other antibiotic treatments are being used as alternatives to vancomycin¹⁸, but these are still not ideal methods due to the threat of resistance building up.

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Luckily, there is another treatment possibility that have been shown to remove CDI recurrence with high levels of success and effectiveness. It has been called by many names, such as Fecal Bacteriotherapy or Stool Transplant, but it is most commonly referred to as Fecal Microbiota Transplantation. In this procedure, gut microflora is in essence taken out of a healthy donor via samples of fecal matter and used to facilitate healthy gut microflora colonization within the CDI patient. This can be done through a nasogastric tube or even repeated enemas, but the most common method of FMT treatment has been through a colonoscopy.

Studies have shown this treatment method to be a highly effective method for handling Multiply Recurrent *Clostridium Difficile* Infection (MR-CDI) and preventing recurrence. One study followed a 61 year old female who had a recurring form of CDI. Before her treatment, her gut did not contain any Firmicutes or Bacteroidetes bacteria, both of which are typically the most dominant found in the healthy gut. However, just one month after undergoing an FMT procedure, these categories of bacteria were found to be dominant at a normal level in her gut¹⁹. Another study was conducted in 2012 at Brown University's Alpert School of Medicine. The study looked at 26 MR-CDI patients with an average of 13 months of infection between them. They then performed the FMT on them over a 2 year period and tracked the signs of recurrence. They found that among the twenty six patients, twenty four of them showed no signs of either diarrhea or CDI after an average of 11 months tracking their symptoms. Their research and others show that FMT can be a highly effective and cost efficient way of dealing with CDI recurrence¹⁹. Fecal Microbiota Transplantation would also curtail the chances of antibiotic resistance developing in *Clostridium difficile*.

Despite these benefits, there are some drawbacks to treating *Clostridium difficile* with FMT. First and foremost is the use of human fecal matter to perform the transplant, which has a whole range of other health problems associated with it. The treatment can also be fairly expensive, although not as expensive as a vancomycin regimen would be. There is also a large issue of patients being unwilling to accept the treatment, as it is not the type of treatment that most patients would like to partake in.

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Immunogenicity

Our bodies naturally work tirelessly to prevent infection. The human immune system can be divided into two categories of response: the innate immune system and the adaptive immune system. These two subsystems work together to protect the body against pathogens. The innate immune system is the first to spring into action when a pathogen is first encountered. It is a broad, unspecific response that will function the same way no matter what the pathogen is. The adaptive system kicks in after the innate system, but it is highly specific towards the pathogen currently being targeted. It employs the process of antigen presentation to create antibodies, which are proteins used to target and eradicate the specific type of pathogen that the antigen was produced from. Altogether, the immune system is a complex and efficient process for healing and defending the body.

Even though not much is known about how the immune system works to combat *C. difficile* specifically, there has nonetheless been some research conducted on this relationship. For example, studies have shown that the surface layer proteins of the bacteria are used as antigens by the immune system, which is then able to make antibodies against the proteins²⁰. Another study showed that these SLPs provoke an immune response in the form of certain cytokines including IL-1 β , IL-6 and IL-10²¹. Other studies have shown that toxin A of *C. difficile*, incites a response in the form of dendritic cells and certain chemokines²². Despite these findings, our understanding of the response to an infection by this pathogen remains very vague and riddled with gaps.

A majority of what is known about *C. difficile* has revolved around understanding the role of the toxins in both establishing infection and in eliciting an immune response. Our goal was to further this understanding of the response to *C. difficile* by observing the interaction between the bacteria and human immune cells, in a strain that is lacking both TcdA and TcdB. This would give us a good look at what other parts of the bacterial cell wall may be doing to initiate the immune response if any, and provide a clearer understanding of the pathogenesis of this potentially deadly organism.

Chemokines are proteins that play a vital role in establishing immunity in that they are migratory proteins that elicit immune cell migration to a site of active infection²³. This is an

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absolutely critical step in initiating and maintaining an appropriate immune response to a pathogen such as *C. difficile*. Monocyte chemoattractant protein-1, or MCP-1, also called CCL2, which stands for chemokine (C-C motif) ligand 2, is a key innate chemokine²⁴. Specifically, this chemokine directs monocytes, memory T cells, and dendritic cells to areas of the body currently trying to stave off infection by outside pathogens²⁵. This makes MCP-1 vital in producing the immune effects that these cells have on the infected area, which includes the monocytes differentiating into macrophages and other immune cells that can battle the infection in a variety of ways.

A second well-described innate chemokine is macrophage inflammatory protein-1 α , or MIP-1 α . This protein is also referred to as CCL3, which stands for chemokine (C-C motif) ligand 3²⁶. MIP-1 α has been shown to be capable of recruiting leukocytes, especially macrophages, into the infected area of the body. These macrophages are then able to perform phagocytosis, which involves the engulfing and breaking down of infected cells and foreign cells, on the pathogen infecting the body. This response is made possible by the MIP-1 α chemokine, making it another vital component of the immune system²⁷.

It is easy to see how necessary cytokines like the MCP-1 and MIP-1 α proteins are to immune response, as without them, the body would be far less capable of fighting infections effectively. This is why they are so important to be studied if we are to understand the immune response to *C. difficile* infection. There has not been much prior research done to relate this section of the immune response to *C. difficile* infection, but research has been conducted on bacteria that have similar structural and functional characteristics. *Staphylococcus aureus* is a bacterium which has been well characterized, making it a very useful comparison model to base other studies on²⁸. *S. aureus* has been known to cause skin infections, pneumonia, meningitis, and food poisoning, among many other things. Like *C. difficile*, *S. aureus* is a Gram-positive, anaerobic bacterium that has shown drug-resistant tendencies in recent years²⁸. These similarities are useful in determining that *S. aureus* can be used as a starting point to base *Clostridium difficile* research upon.

Staphylococcus aureus has been studied relentlessly in order to determine how the immune system responds to the bacterial infection, including the role of MCP-1 and MIP-1 α

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in fighting off the infection²⁹. One study showed that both MCP-1 and MIP-1 α were observed to have been generated when exposed to the peptidoglycan found on the *S. aureus* cell wall³⁰. Peptidoglycan is the main determining factor in labeling bacteria as either Gram-positive or Gram-negative. Based on this study, it is logical to reason that a similar chemokine response would be seen in other Gram-positive bacteria. Because *Clostridium difficile* is also classified as Gram-positive, the MCP-1 and MIP-1 α proteins should be present in high amounts when exposed to this bacterium as well.

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METHODOLOGY

We chose to study the effects of *C. difficile* on macrophages which are key cells in the innate response. They are responsible for phagocytosis of infected cells, bacteria and cellular debris. In addition they can present antigen to help establish an effective adaptive response. THP-1 macrophages were cultured in flasks and suspended in a media broth. This media consisted of RPMI with 10% fetal bovine serum and 1% penicillin/streptomycin in order to provide the THP-1 cells with the nutrients necessary for cell survival and growth. The cells were incubated for extended periods of time at 37C with a 10% CO2 level at all times. The cells were routinely harvested from their flasks and counted using a hemocytometer. Once enough cells had grown in one flask, the cells were split between two new flasks with fresh media to maximize the amount of cells we could culture. This culturing process was repeated ad nauseum until there was a large enough stockpile of live, healthy THP-1 cells to conduct our research on.

In addition to THP-1 cells, bacteria cells also needed to be cultured. *C. difficile* (strain 70057 from ATCC which is toxin A,B, and C negative) and *S. aureus* were both grown in a 5 mL culture tube in a shaking incubator overnight at a temperature of 37C. Prior to use in the experiments, they were then diluted 1:1000 in Phosphate Buffered Saline (PBS). Optical density readings at OD600nm were used to observe and control for overall bacteria cell numbers.

The use of *S. aureus* in this research was to provide us with a control organism to compare *C. difficile* to. *S. aureus* has been extensively researched and its morphology, pathogenesis, and cellular function have been well documented over the years. More specifically, it was chosen as an optimal control for *C. difficile* due to observed similarities between the two organisms in terms of their function and behavior. In addition to these similarities, *Staphylococcus aureus* has shown tendencies towards antibiotic resistance, which serves to heighten the comparisons to *C. difficile*.

Once we had high reserves of both pathogens and the THP-1 cells, we could then begin to conduct our research trials. THP-1 cells were co-cultured with either *C. difficile* or *S. aureus* in order to simulate the infection process. These co-cultures varied in time of

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exposure, ranging from zero to twenty four hours of exposure. Zero exposure times were used to get a baseline reading on cellular activity without any reason for a heightened immune response. In addition, a control was also cultured with THP-1 cells in only media, with no pathogens present in the sample. This allowed us to further observe normal immune levels which could then be used to compare to the change in immune response once the pathogens were introduced.

Two kinds of analysis were conducted on these trials. The first of these was a western blot technique using gel electrophoresis. Western blots are used to detect the presence of a specific type of protein in a given sample. The samples are placed in wells atop a specialized agarose gel. Two electrodes are used to create an electric field that flows through the gel. This makes the proteins in the samples move down through the gel at different speeds. The smaller sized proteins will be able to move farther down the gel due to their smaller molecular mass, and larger proteins will move slower, getting caught up towards the top of the gel. This process effectively separates the components of the sample into different layers depending on their molecular size. The results of the gel electrophoresis are then transferred over to a membrane and stained to create the western blot. The results of this analysis can be observed in Figure 1 below.

The second analysis technique used in this research was the enzyme-linked immunosorbent assay, or ELISA. This technique is used to monitor the levels of soluble proteins in a sample. ELISAs are done by coating the bottom of a 96 well plate with a capture antibody. This antibody is coded to detect and attach to a specific type of antigen, or part of a cell. The samples are then added to the wells; if the antigen is present in the sample, it will bind to the capture antibody and remain in the well. A detecting antibody is then put in to bind the antigen once again, which enables it to become detectable when run through a specialized machine. This machine produces the charts that can be observed in Figure 2 and Figure 3. Figure 2 was detecting MCP-1 as the antigen, and Figure 3 was looking for MIP-1 α as its antigen.

RESULTS

Western Blot

The western blot analysis conducted for the transcription factors showed a definite sign of immune activity. The results of this analysis can be seen in Figure 1. In total, six samples were analyzed for their protein content. The first sample (A) was a negative control which contained only media without any THP-1 cells, and as expected, it did not contain any of the proteins we were looking for. Sample B was the positive control, which had THP-1 cells and media, but no exposure time to the *Clostridium difficile* strand. Only normal protein levels were detected for this sample. Co-culture samples of THP-1 cells and *Clostridium difficile* constituted the remaining trials (samples C through G). Among these, samples C and D were exposed to *Clostridium difficile* for only three hours, and samples E and G were exposed to the bacteria for a full 24 hours.

As a control protein to ensure cellular presence in the samples, we measured the levels of actin in the trials. Actin is a protein that plays a major role in the cytoskeleton of eukaryotic cells and is therefore found in all human cells, regardless of shape or function. Figure 1 shows that there was actin present in all samples that were supposed to contain the THP-1 cells, which shows that there was a high level of cellular presence in samples B through G. The level of actin in each trial can be seen as the bright yellow band in the first picture in Figure 1.

I κ B and NF κ B were the transcription factors observed in this western blot. I κ B can be seen as a thin orange band just below the actin on the first picture on Figure 1. The band gradually increases in intensity as the exposure time moves on from zero hours to three hours and then 24 hours. The NF κ B transcription factor was also seen in increasing amounts as the time frames moved forward. This can be seen in the second picture of Figure 1. The bright yellow band found here represents the level of NF κ B protein found in each sample. There is a very clear peak at about three hours for the NF κ B levels, which is very much in line with what we were looking for.

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MCP-1 ELISA

The levels of MCP-1 were analyzed in our cocultures using ELISA. The level of MCP-1 proteins found in the trial is measured on the vertical axis of the chart. It is measured in picograms per milliliter (pg/ml). Time of exposure was measured in hours and can be found on the horizontal axis.

In total, three trials were conducted for each of the three exposure scenarios. The media sample (blue line) refer to THP-1 cells incubated in media alone, with no pathogenic bacteria added to the culture. These samples were used as a control in order to ensure that no contamination was present in the samples. The lines labeled *S. aureus* and *C. difficile* refer to the particular pathogens that the THP-1 cells in those trials were exposed to.

The Media data (blue line) shows very low levels of MCP-1 production. These levels are in line with normal cellular amounts and do not represent any peak in immune chemokine production. We can conclude from the low levels of MCP-1 here that the controls did not contain any bacteria and there was no background that would serve to skew the ELISA results. On the other hand, the other two data lines are indicative of an immune response to their respective bacteria. The *S. aureus* trials (represented by the red data line) depict a steady increase in the MCP-1 production. By three hours, there was a large jump in the chemokine production from the THP-1 cells; by the 24 hour time point, the levels had continued to increase gradually. This is the expected result from these trials and is in line with previous knowledge of *Staphylococcus aureus* and its immune response.

The most important part of this graph is the activity seen in the *C. difficile* trials (displayed by the green line). There is a clear increase in MCP-1 production among these trials as exposure time increases. The three hour time point shows chemokine levels of approximately 175 pg/ml, and that measure increases to about 200 pg/ml at the 24 hour time point. This rapid upwards trend is in line with an increase in innate immune activity. Although the MCP-1 levels are lower than those observed in cells that were cultured with *Staphylococcus aureus*, there is still a noticeable jump in chemokine production when compared to the media trials.

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MIP-1 α ELISA

Figure 3 looks at the MIP-1 α chemokine activity observed. Like the MCP-1 chart detailed in Figure 2, this figure looks at three different exposure scenarios over three different time points. The levels of MIP-1 α found in each sample are measured on the vertical axis and measured in pictograms per milliliter (pg/ml). The time in hours is measured on the horizontal axis.

Once again, the trials are divided into three exposure scenarios. The first is the Media data, which consisted of THP-1 cells cultured in only RPMI media with no pathogenic bacteria exposure. These trials are represented by the flat blue line at the bottom of the graph. As was expected, the chemokine levels are very low for each of the three trials, as there is no bacterial presence to invoke an immune response. These low levels are in line with normal cellular function and show us that the control is uncontaminated and therefore a useable foundation to base our comparisons on with the other trials.

The other two data lines show the results observed when THP-1 cells were exposed to bacteria. The second dataset, shown as a red line, gives the MIP-1 α levels for the cells exposed to *Staphylococcus aureus*. This bacterium was once again used as a comparison organism to judge the *Clostridium difficile* trials against. The data clearly shows a spike in MIP-1 α production at the three hour time point, with a drop in the chemokine levels by the 24 hour mark. However, the levels never approach the Media trials once exposed, showing a continuous presence of MIP-1 α in the time during which the innate immune response typically operates.

The *Clostridium difficile* trials (represented in green) show a very similar MIP-1 α production to the *Staphylococcus aureus* trials. After three hours of exposure, there is a spike in production, as the chemokine level exceeds 120 pg/ml. This is lower than the *Staphylococcus aureus* levels, which is about 150 pg/ml, but there is still a clear increase in immune activity in comparison to the control trial. The 24 hour time point actually showed *C. difficile* to induce slightly higher chemokine levels than *S. aureus* (105 pg/ml and 95 pg/ml, respectively). The drop off in production is much less steep than the *S. aureus* trials, which

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may mean that the MIP-1 α would be produced for longer when these cells are exposed to *Clostridium difficile*.

LIMITATIONS

A potential limitation seen in this study is the use of in vitro research techniques. In order to study the pathogenic effects of *C. difficile*, we needed to use THP-1 cells that had been removed from their natural state of being (inside the body). This allows us to control the necessary variables while we conduct research. However, these techniques mean that we are not observing the exposure in a realistic environment, as actual *C. difficile* infection would be influenced by a number of variables related to the human body and its natural functions. We cannot ethically research a pathogen in vivo, or in the body, as this would mean exposing healthy patients to a disease. In vitro techniques are the closest we can ethically get to actually observing the infection of *C. difficile*, and is a well-established and accepted method of analysis.

CONCLUSIONS AND IMPLICATIONS

The results of this research show that there is a definitive immune response observed when THP-1 cells are exposed to *C. difficile*. This immune activity comes in the form of increased production of the transcription factors IFK and NFkB. There is also a notable spike in chemokine production, as both MCP-1 and MIP-1a levels increased as exposure time went on. These increases in immune activity indicate that the THP-1 cells recognize that there is a disease-causing agent that it must eradicate.

Also noteworthy were the comparisons to the trials that had been exposed to *Staphylococcus aureus*. The chemokine production displayed after exposure to *Clostridium difficile* was very similar to the production when *Staphylococcus aureus* was the pathogen added. This suggests that the two pathogens invoke a comparable response from the body. This is a potentially valuable comparison, as there is an extensive breadth of information available regarding the pathogenesis and functions of *Staphylococcus aureus*.

These studies provide us with a baseline of knowledge about how the bacteria that lack the potent and immunostimulatory toxins, can still evoke an effective immune response. This gives us great insight into the roles that other integral parts of the bacteria such as the cell surface sugars, may play in initiating infection and the corresponding immune response. This is of vital importance in the quest to discover new potential targets for the treatment of *C. difficile* infection.

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APPENDICES

Appendix A – Figure 1 – IKK and NFkB Western Blot

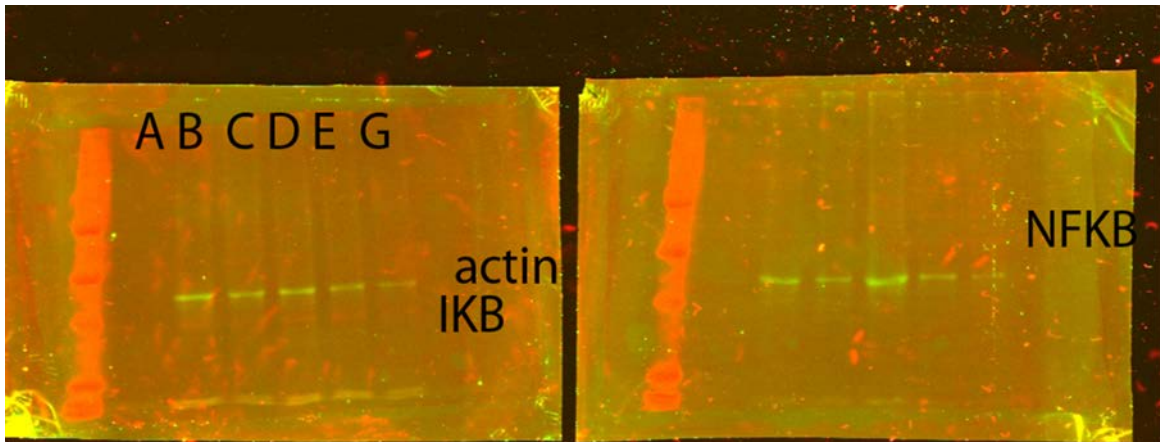


Figure 1 – Western Blot of Transcription Factors

Measure of the levels of IKB and NFkB found in THP-1 cells exposed to *C. difficile*. Actin control shown in yellow (first picture), IKB levels shown in orange (first picture), and NFkB levels shown in yellow (second picture).

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Appendix B – Figure 2 – MCP-1 Production Levels

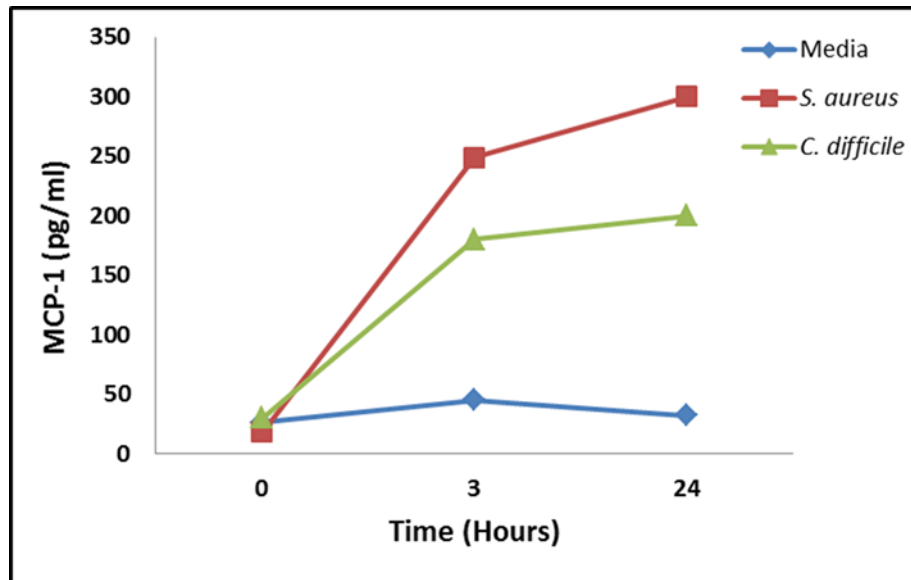


Figure 2 – MCP-1 ELISA results

Results of ELISA used to detect MCP-1 α levels in THP-1 cells exposed to *C. difficile* (shown in green) and *S. aureus* (shown in red).

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Appendix C – Figure 3 – MIP-1 α Production Levels

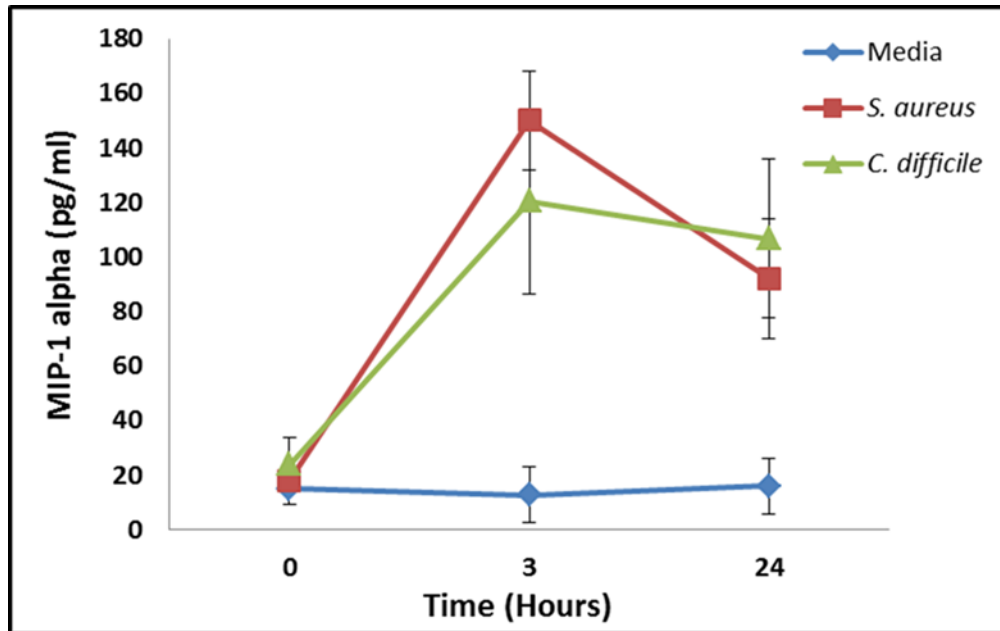


Figure 3 – MIP-1a ELISA results

Results of ELISA used to detect MIP-1 α levels in THP-1 cells exposed to *C. difficile* (shown in green) and *S. aureus* (shown in red).

REFERENCES

1. Tyson, Gregory. "Tipping Point: The Threat of Antibiotic Resistance." Northwestern.edu. 17 August 2012. Northwestern University. <<http://scienceinsociety.northwestern.edu/content/articles/2012/tipping-point-threat-antibiotic-resistance>>.
2. Martins, Ana, Attila Hunyadi, and Leonard Amaral. "Mechanisms of Resistance in Bacteria: An Evolutionary Approach." *Open Microbiology Journal*. 7. (2013): 53-58.
3. Lessa, Fernanda C, Carolyn V. Gould, and L. Clifford McDonald. "Current Status of *Clostridium difficile* Infection Epidemiology." *Clinical Infectious Disease*. 55. (2012): 65-70.
4. Guarner, Francisco and Juan-R Malagelada. "Gut Flora in Health and Disease." *The Lancet*. 360. (2003): 512-519.
5. Vedantam, Gayatri, Andrew Clark, Michele Chu, Rebecca McQuade, Michael Mallozzi, and V. K. Viswanathan. "*Clostridium difficile* infection." *Gut Microbes*. (2012): 121-134.
6. Heinlen, Latisha and Jimmy D. Ballard. "*Clostridium difficile* Infection." *The American Journal of the Medical Sciences*. 340. (2010): 247-252.
7. Calabi E, et al. "Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues." *Infection and Immunology*. 70. (2002): 5770-5778.
8. Voth, Daniel E. and Jimmy D. Ballard. "*Clostridium difficile* Toxins: Mechanism of Action and Role in Disease." *Clinical Microbiology Reviews*. 18. (2005): 247-263.
9. von Eichel-Streiber, C., R. Laufenberg-Feldmann, S. Saringen, J. Schulze, and M. Sauerborn. "Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B." *Medical Microbiology and Immunology (Berlin)*. 179. (1990): 271-279.

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10. Tucker, K. D., and T. D. Wilkins. 1991. "Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y." *Infection and Immunology*. 59 (1991): 73-78.
11. Just, I., J. Selzer, C. von Eichel-Streiber, and K. Aktories. 1995. "The low molecular mass GTP-binding protein Rho is affected by toxin A from *Clostridium difficile*." *Journal of Clinical Investigation*. 95. (1995): 1026-1031.
12. Moudgal, V and JD Sobel. "*Clostridium difficile* colitis: a review." *Hospital Practice*. 40. (2012): 139-148.
13. Viswanathan, VK, MJ Mallozzi, and Gayatri Vedantum. "*Clostridium difficile* infection." *Gut Microbes*. 1. (2010): 234-242.
14. Patti, Gary J., et al. "Vancomycin and Oritavancin Have Different Modes of Action in *Enterococcus faecium*." *Journal of Molecular Biology*. 392. (2009): 1178-1191.
15. Talk by Samir A. Shah. "*Clostridium difficile* in Inflammatory Bowel Disease: a dangerous mix." Given at Women's Medical Collaborative: 14 September 2012.
16. Smith, Theresa L. et al. "Emergence of Vancomycin Resistance in *Staphylococcus aureus*." *The New England Journal of Medicine*. 340. (1999): 493-501.
17. Silva Junior, Moacyr. "Recent changes in *Clostridium difficile* infection." *Einstein (São Paulo)*. 10. (2012).
18. Cornely OA, et al. "Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial." *The Lancet Infectious Diseases*. 12. (2012): 281-289.
19. Talk by Colleen R. Kelly. "Fecal Microbiota Transplantation: Treatment Option for *C. Difficile*." Given at Women's Medical Collaborative: 14 September 2012
20. Drudy, D, et al. "Human antibody response to surface layer proteins in *Clostridium difficile* infection." *FEMS Immunology and Medical Microbiology*. 41. (2004): 237-242.

Effects of *Clostridium difficile* on the human immune response
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21. Bianco M, et al. "Immunomodulatory activities of surface-layer proteins obtained from epidemic and hypervirulent *Clostridium difficile* strains." *Journal of Medical Microbiology*. 60. (2011): 1162-1167.
22. Lee, JY et al. "*Clostridium difficile* toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF-kappaB signaling pathway." *Journal of Molecular Medicine*. 87. (2009): 169-180.
23. Murdoch, C., and A Finn. "Chemokine receptors and their role in inflammation and infectious diseases." 95. (2000): 3032-3043.
24. Carr, MW, SJ Roth, SS Rose and TA Springer. "Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant." *Proceedings of the National Academy of Sciences*. 91.
25. Conti, P, et al. "Monocyte chemotactic protein-1 provokes mast cell aggregation and [3H]5HT release." *Immunology*. 86 (1995): 430-440.
26. Maurer, M. and E. von Stebut. "Macrophage inflammatory protein-1." *The International Journal of Biochemistry and Cell Biology*. 36. (2004): 1881-1886.
27. Menten, P, A Wuyts and J Van Damme. "Macrophage inflammatory protein-1." *Cytokine & Growth Factor Reviews*. 13. (2002): 455-481
28. Fournier, Benedicte and Dana J Philpott. "Recognition of *Staphylococcus aureus* by the Innate Immune System." *Clinical Microbiology Reviews*. 18. (2005): 521-540.
29. Verhoef, J., and E. Mattsson. 1995. "The role of cytokines in gram-positive bacterial shock." *Trends in Microbiology*. 3. (1995): 136-140
30. Wright, KM and JS Friedland. "Regulation of chemokine expression and secretion in *Staphylococcus aureus*-infected osteoblasts." *Microbes and Infection*. 6. (2004): 844-852.