One Health Grant Proposal

Exploring Novel Nanoparticle Treatments for *C. difficile*

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Abstract

When presented with a bacterial infection, medical professionals often prescribe antibiotics as a conventional method of treatment. However, overuse and misuse of antibiotics can lead to the development of antibiotic resistance, causing adverse effects to human health and making future infections increasingly difficult to treat. This is particularly evident in the bacterium *Clostridium difficile*, an opportunistic pathogen within the human GI tract that leads to antibiotic-resistant infection after antibiotics are used to treat another condition. This in vitro experiment seeks to answer how the treatment trajectory of *C. difficile* infection will be affected when utilizing inorganic nanoparticle therapy, in comparison to conventional methods of treatment using further antibiotics. Current methods of treatment for antibiotic resistance are insufficient and continue to exacerbate the cycle of antibiotic use. Because of the complexity of this issue, antibiotic resistance requires an interdisciplinary, One Health solution. The One Health Initiative connects the health of people with the health of animals and the environment we coexist in, drawing in professionals from these fields. By utilizing inorganic nanoparticles to target antibiotic strains of bacteria, we circumvent the use of further antibiotic treatment and implement a technology that sits at the intersection of many disciplines within science. This experiment uses the sol-gel method to expose *C. difficile* to nanoparticles. Based on existing research, inorganic metal oxide nanoparticles are expected to be a more effective treatment for *C. difficile* than antibiotic alternatives.

Section 1 - What is your idea?

Bacterial infections that are resistant to antibiotics have become a serious health problem that emphasizes the need to develop new treatments. The infection that arises from the bacteria *Clostridium difficile* actually stems from the use of antibiotics to treat a different infection and altering the balance of the gut microbiota. *C. difficile* is listed by the Center for Disease Control (CDC) as “the leading cause of antibiotic-associated diarrhea and one of the most common healthcare-associated infections in the United States” and is enlisted in the CDC’s Emerging Infectious Disease surveillance program. The need for novel treatments to combat antibiotic resistance is increasingly apparent and research on nanoparticles as an alternative to antibiotics may provide a solution to these persistent, detrimental infections. Current antibiotic treatments fail to eliminate *C. difficile* spores in an effective and timely manner, causing chronic dysbiosis and intestinal inflammation.

Nanoparticles are a more effective and faster treatment than traditional antibiotics. In fact, nanoparticles are less likely to promote antibiotic resistance compared to most antibiotics because they act directly on bacterial cell walls without the need to penetrate the cell. In this experiment, *C. difficile* is used to reaffirm that hypothesis and test the efficacy and biocompatibility of the following inorganic metal oxide nanoparticles: silver, titanium dioxide, magnesium oxide, and superparamagnetic iron oxide.

This approach is novel for its selection of inorganic nanoparticles and *C. difficile*. Silver and titanium dioxide are well-studied whereas superparamagnetic iron oxide is relatively new, but all are known to be effective (Beyth et al., 2015). The approach is novel also for its focus on the speed at which treatment takes place. Time is an important factor when treating a patient and speed poses a significant advantage for nanoparticles over antibiotics. Additionally, nanoparticle development brings together a unique group of researchers such as microbiologists, engineers, and environmental scientists.

It is hypothesized that the use of inorganic nanoparticles as a treatment for persistent *C. difficile* infections will decrease the overall treatment time of the infection, as compared to conventional treatment with regular antibiotics. Inorganic nanoparticles, such as silver and titanium dioxide, offer a more efficient mechanism for treating antibiotic resistant bacteria, where the nanoparticle exhibits bactericidal qualities that effectively lyse bacterial cells.

Figure: Vancomycin

As compared to the more common treatment using broad-spectrum Vancomycin, the use of nanoparticles in combating antibiotic resistant *C. difficile* infections allows for a decrease in overall drug exposure. This would close the loop of antibiotic use that may exacerbate antibiotic resistance, and can be designed to penetrate cellular barriers that antibiotics may not be able to do in order to kill the bacteria intracellularly. Overall, the efficacy of inorganic nanoparticles silver, titanium dioxide, magnesium oxide, and superparamagnetic iron oxide in the treatment of antibiotic resistant *C. difficile* is greater than that of the conventional treatments using the antibiotic Vancomycin due to the specificity and potency of nanoparticle treatment. For these reasons, it is hypothesized that inorganic nanoparticle treatment for *C. difficile* infections will affect the treatment trajectory by decreasing the overall time of treatment for the infection, when compared to traditional antibiotics.

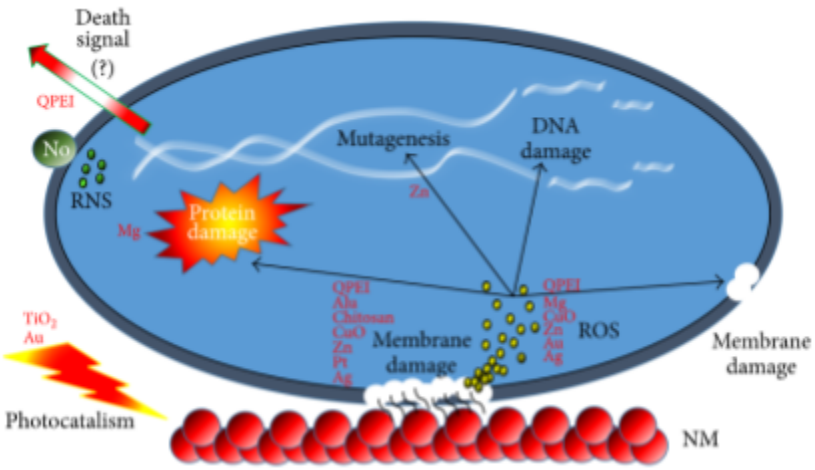

Figure 2. A diagram depicting how a nanoparticle invades a cell. A nanoparticle reacts electrostatically with a membrane, damaging it and producing free radicals, atoms with an unpaired valence electron, which damage DNA and proteins within the cell.

Section 2 - What informs this project?

To understand this proposal, it is important to have a foundational understanding of *C. difficile* infections and its connection to antibiotic resistance, as well as nanomaterials and the mechanisms behind nanoparticle therapy. As this is a fairly recent development in antibiotic resistant bacteria treatment, the key studies that informed this article have all been written in the past decade-- primarily literature reviews of progress that has been made in nanoparticle technology from peer-reviewed journals.

C. difficile is one of the most common nosocomial infections, a hospital acquired infection. It is a type of life-threatening bacteria that causes inflammation of the colon (Centers for Disease Control, n.d.). More commonly affecting patients 65+ years and healthcare workers, it often starts from the use of antibiotics that are used to treat other infections or from transmission of the infection at hospitals. *C. difficile* produces spores when attacked by antibiotics (Banawas, 2018), a potent factor that creates antibiotic resistance. The spores will still be present in the body regardless if the *C. difficile* bacteria is killed. *C. difficile* alters the balance of the gut microbiota, meaning that the toxins attack the intestinal wall that will eventually lead to an ulcer or sore. These symptoms are similar to food poisoning, where you might experience symptoms including diarrhea or cramping. If patients affected by *C.difficile* are left untreated for longer, their symptoms include weakness, dehydration fever, nauseous, and potentially blood in your

stool. As more malignant strains of *C. difficile* begin to emerge and become more resistant to standard therapies, it is important for upcoming research of nanomaterials to be an alternative to antibiotics that kill *C. difficile* bacteria.

Because antimicrobial resistance is driven by many different aspects in medicine, the environment, and other human activity, an interdisciplinary solution is required to address the complexity of this growing issue. The One Health perspective is imperative to finding solutions for antimicrobial resistance to ensure that the issue isn't further compounded and tackles the root of the issue. Nanoparticle therapy is an emerging treatment in antibiotic resistant bacteria that combines different scientific disciplines as a potent treatment for antibiotic resistance.

It is important to note that although nanoparticles may be a viable alternative to antibiotics, additional research is still required. Studies are currently being carried out concerning the toxicity of nanoparticles, especially long-term effects. The study of nanoparticle adverse effects and toxicity is commonly referred to as nanotoxicology (Yang et al., 2021, 280). Nanoparticles are described to have similar dimensions of biological molecules, which is the key reason why they travel easily both in the body and in the environment. Upon exposure, these nanoparticles may interact with organs, tissues, cells, and biomolecules (Ramakrishna et. al, 2011). Due to the variety of nanoparticles a case-by-case study of each nanoparticle's toxicity would be required since each may react differently depending on their physicochemical properties (Beyth et al., 2015). For example, even if the core of the nanoparticle is the same, slight variations within the nanoparticle can result in significantly different toxicity and biodistribution. In short, before we can fully evaluate the toxicity of each nanoparticle, the structure and corresponding physicochemical properties need to be completely characterized and understood.

To this respect, studies are still preliminary and require further research in order to apply this technology to clinical practice. Researchers must better understand the biocompatibility of the metal oxides used in nanomaterials, especially in their long term effects, as there is risk of toxicity and cell damage from the nanomaterials and the mechanisms used to eradicate bacterial strains. There is a possibility that the reactive oxygen species that is created by the nanoparticle can damage surrounding tissues and cause permanent damage to the genome of human cells. Maximizing the efficiency of the bactericidal effects of the nanoparticles while preventing any damage to non-target cells will require further studies on nanoparticle design that utilizes the mechanisms of antibiotic resistant treatment. By finding an optimal

design and pattern for these nanomaterial structures, nanoparticle therapy will be able to better target areas of infection and engage the mechanisms simultaneously under human cell conditions.

Literature concerning nanoparticles as an alternative to antibiotics is comprehensive, but not thorough. A subsection of this research is the study of individual nanoparticles, some of which are well-documented with other pathogens and others not. This paper looks at four different nanoparticles, each with different properties and backgrounds: silver, titanium dioxide, magnesium oxide, and superparamagnetic iron oxide (Hemeg, 2017). Thus far no research has studied the effects of these nanoparticles on *C. difficile*.

Silver (Ag) is one of the most common metal nanoparticles, its medicinal qualities known for at least two thousand years. The metal fell out of favor in the last century around the advent of antibiotics like penicillin, but now has regained interest as a nanoparticle. Silver is known to be effective against bacteria, fungi, and viruses, and is used for disinfecting surfaces and minor injuries. It is less likely for bacteria to form resistance to silver than traditional antibiotics. Despite its effectiveness, the antibacterial method of silver is unknown. However, observations show that silver can cause pitting in cell walls and can inhibit protein synthesis.

Similarly, the antibacterial properties of titanium dioxide (TiO_2) have also been well-studied and has a longer history of use than other nanoparticles. The oxide is best known for its effectiveness against gram-positive and gram-negative bacteria and more recently viruses. It is photocatalytic, meaning it accelerates a photosensitive reaction. Titanium dioxide responds to visible and ultraviolet light and causes a burst of reactive oxygen species, damaging cell membranes and DNA. When combined with silver, titanium dioxide can be even more effective.

Magnesium oxide (MgO) are known to be efficient against bacteria and are already used in economically viable products. It is effective against viruses, spores, and gram positive and negative bacteria. Magnesium oxide is not as well-studied as other nanoparticles. However, other magnesium-containing nanoparticles such as MgF_2 are known to prevent biofilm formation and enzyme transportation. This method may translate to MgO.

Unlike the last three, superparamagnetic iron oxide (SPION) is a relatively new nanoparticle and has not been researched significantly. It could present a new approach to nanoparticle treatment not yet exhibited in any other. Researchers used SPION and magnetic fields to cause localized hypothermia and damage cells. Others paired SPION with silver to penetrate biofilms (Beyth et al., 2015).

For our experiment, we plan on conducting an in vitro study by collecting samples of C. diff from infected patients and incubating the samples in either plates or tubes that mimic conditions of a human cell. In vitro refers to work or research that is conducted outside a living organism, while in vivo refers to research that is done with a living organism, like humans or animals. This can be done by looking at cell cultures. We chose in vitro because we are testing a new method to kill C. diff bacteria. In vitro testing is helpful for determining where or not the inorganic particles we picked have any toxic or carcinogenic effects.

Given this information, our proposal seeks to replicate human cell conditions within the gastrointestinal tract throughout the incubation process to enrich a culture of *C. difficile* and test these different nanomaterials through nanoparticle therapy. In doing so, we will be able to explore the biocompatibility and effectiveness of these different metal oxides and bridge the gap in pre-existing research. The results of our proposed research could potentially serve as a novel treatment that ends the cycle of antibiotic use in *C. difficile* infections and could be applied to several different antibiotic resistant infections in medicine.

Section 3 - How will we test it?

Our procedure utilizes *in vitro* techniques of bacterial plating in order to measure the rate of zones of clearance formation of the different nanoparticles, as compared to the vancomycin treatment, in *C. difficile.* In order to undergo this, the following preparation methods will be used prior to observation and analysis.

Preparation

1. Preparation of Anaerobic Chamber

C. difficile requires an anaerobic condition, meaning it is necessary to keep the C.difficile culture in an oxygen-free environment for a successful controlled experiment. An airlock must be used to avoid oxygen contamination before introducing the C.difficile plates into the chamber. It is also important to never leave the anaerobic chamber open for longer than the amount of time required to move items in and out of the interchange to avoid oxygen contamination. Keeping that in mind, we planned our approach to have minimal frequency of moving items in and out of the chamber. According to other studies that have used C.difficile, they recommend using two purge cycles of nitrogen gas to remove the oxygen inside the

chamber. Then proceed to fill the anaerobic chamber with the recommended gas mix: 5% CO_2, 10% H_2, 85% N_2 (Edwards et al., 2013). This ensures that there is no oxygen in the anaerobic chamber.

2. Preparation of *C. difficile* Plates in vitro

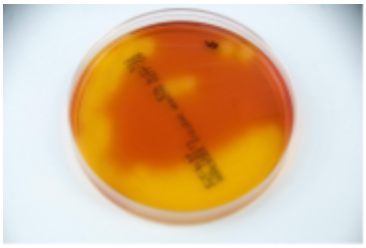

Figure: CCF Agar with *C.difficile*

Bacterial Strains and Media. The type of *C Difficile* strain used would be 027/NAP1/BI since it is the most common strain type and also the most harmful as it appears to show an increased virulence. Because *C. difficile* forms spores, alcohol or heat treatment of samples may be used to reduce or eliminate the growth of other bacterial organisms within the stool. As mentioned above, it is critical to pre-reduce all plates for at least 1-2 hours in the anaerobic chamber prior to use to ensure the removal of oxygen contamination. CCFA, an agar medium containing cycloserine, cefoxitin, fructose, and egg yolk would be used to culture the C.difficile strain. This agar is extremely sensitive and preferred to maintain a reliable culture of *C.difficile*. We plan to use two plates of each nanoparticle (Mg,Ti, Ag and SPION) one control plate mixed in with vancomycin totaling 9 plates.

The limitations of growing C difficile on plates are as follows:

1. Other organisms may grow on the medium but do not typically affect the characteristics of C difficile
2. Yellow fluorescence of *C. difficile* is detectable after 24 hours and last for 5-6 days

3. Preparation of Nanoparticles

A common way of preparing nanoparticles for exposure to bacteria is the sol-gel method, which involves the heating of monomers into a liquid colloidal solution (sol) such as a metal alkoxide and the transformation of said solution into a solid, uniformly structured polymer (gel). Research shows that the sol-gel method is a viable method for all four nanoparticles in this proposed study, however variations in the sol-gel method are expected for each nanoparticle.

For silver, scientists use a non-aqueous version of the sol-gel method. A gel is prepared by heating the colloidal solution at 250 degrees C for one hour (Petit et al., 2013). Sol-gel preparation is not always an exact science. Researchers stirred a liquid TiO2 solution before heating it in a microwave oven to create a gel (Kang et al., 2016). Magnesium oxide nanoparticles are prepared typically with

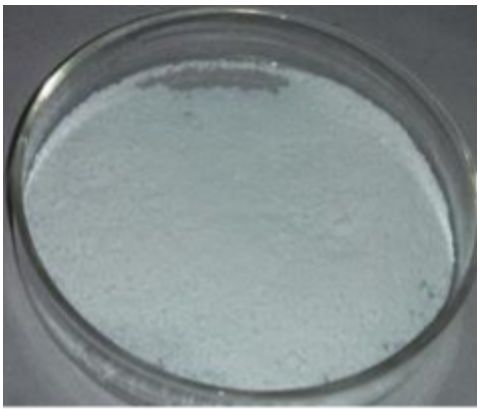

Figure: MgO Powder Precipitate

magnesium nitrate (MgNO3.6H2O). NaOH and water are added to magnesium nitrate, stirred together, then filtered and methanol washed before being dried and heated. The final product is magnesium oxide powder (Sharma et al., 2018). Finally, scientists use ferric nitrate to prepare superparamagnetic iron oxide nanoparticles. Ferric nitrate is dissolved in ethylene glycol, stirred, and evaporated with heat (Ansari et al., 2019). Finally, for superparamagnetic iron oxide, scientists dissolve ferric nitrate in ethylene glycol before heating into a gel.

Methods

After preparation of the incubation chamber, the CCFA plates, and the nanoparticles to be used, the plates can then be inoculated with *C. difficile* and the variable nanoparticles.

Inoculation of Nanoparticle Plates: On each plate, the *C. difficile* strain will be inoculated on top of the CCFA medium using sterile technique to limit contamination as much as possible. The bacteria will be spread across the entirety of the CCFA plate, so as to obtain a lawn of growth and not isolated colonies. After being inoculated with the *C. difficile,* different nanoparticles will then be introduced to the inoculated plates. Using the sol gel method specific to each nanoparticle, outlined in the preparation section, as the nanoparticle is still suspended in the liquid solution, it will be inoculated using sterile technique onto the center of the CCFA plate containing *C. difficile.* The point of inoculation will be kept to the size of the antibiotic disc, around 5 mm wide. It will then be allowed to incubate in the chamber for the duration of the experiment.

The incubation chamber will replicate the conditions of the GI tract as close as possible, with temperatures of the chamber kept steady at 39 °C, which is about the temperature of the human gastrointestinal tract. In addition, the inoculated plates will be placed in an anaerobic chamber with a clear lid, and a gaspak sachet will be placed within the chamber. This will allow for anaerobic conditions to be maintained while still allowing for the camera to record the formation of zones of clearing. The plates will be incubated until there is a complete zone of clearing or until a 48 hour time period passes.

Inoculation of Vancomycin Plate (control): In the control plate of *C. difficile* growth, a disk diffusion test will be conducted to compare against the plates inoculated with nanoparticles. After the *C. difficile* plate has been inoculated, a paper disc loaded with vancomycin will be placed in the center of the plate and allowed to incubate in the same conditions as the other plates, again using sterile technique to limit contamination. Commercially available Vancomycin VA30, AST 5X50, will be used in the control plate.

Observations and Analysis

In order to observe our procedure, cell death of *C. difficile* will be measured as the time that it takes for a complete zone of clearing to form in the inoculated plate. A timelapse camera will be placed above the plates, with a ruler in frame for each of the plates so as to measure the size of clearing. The plates will be allowed to incubate once inoculated with *C. difficile* and the metal oxide nanoparticle, with 1 control plate of *C. difficile* with the vancomycin disc, in order to compare the use of nanoparticles against conventional treatment methods of antibiotics.

To analyze the treatment trajectory of the inorganic nanoparticles on *C. difficile,* we looked at the zone of clearing after a period of incubation. The zone of clearing is the area of a treated surface that is checked for antibacterial efficiency. We want our zone of clearing to be large spread and inhibit *C. difficile* growth on the entirety of the CCFA plate, indicated by a lack of growth surrounding the point of inoculation. The larger the zone, the more sensitive the *C. difficile* bacteria is to the inorganic nanoparticles introduced and the quicker the clearing forms, the shorter the treatment time for *C. difficile*

The time it takes for a complete zone of clearing to form will be measured through the camera's timestamp. If the nanoparticle or the control plate fails to produce a complete zone of clearing, having the ruler in the camera frame will allow for the rate at which clearing formation occurs, by measuring the diameter of the clearing and dividing by the time it takes for the clearing to form. Based on our observations, our expected results should indicate that the rate of clearing formation is faster in the experimental nanoparticle plates, as compared to the control vancomycin plate, indicating that the time of treatment has decreased overall for *C. difficile* infection when using nanoparticle therapy.

Conclusion

Next Steps. With successful results we can continue further studies on the best performing nanoparticle and start performing in *vivo* studies, using mice models as a precursor to human clinical studies. We plan to study how the specific nanoparticle will perform within the GI tract of an organism that has *C. difficile* infection. By introducing this method to *in vivo* studies, we will be able to further explore the biocompatibility of the nanoparticles and analyse the toxicity concern of the nanoparticle. Ultimately, this will help determine proper dosage for in-human treatments and inform other studies looking to use nanoparticle therapy for different antibiotic resistant bacterial infections.

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