

The Use of the λ Bacteriophage to Neutralize the Pathogenic Effects of Shiga Toxins from *Escherichia Coli* to Combat Antimicrobial Resistance: A Research Protocol

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Abstract

Introduction: Resistance to antibiotics is becoming a global health crisis. Certain strains of *Escherichia coli* bacteria are resistant to antibiotic drugs and thrive without competition. Pathogenic effects of *E. coli* are caused by Shiga toxins they produce. Instead of attempting to kill the pathogens, we propose altering the bacterial genome of *E. coli*, causing the expression of globotriaosylceramide (Gb₃) receptors, effectively neutralizing produced toxins.

Methods: Phages are introduced to cultured *E. coli* cells and incorporate their DNA into the bacterial genome. Mutation vectors are transformed into *E. coli* cells using electroporation, causing expression of Gb₃ receptors and preventing the lysing of the cell. Next, exposure to UV light causes phages to enter the lytic cycle, and mutated phages can be collected. Then, *E. coli* cells will be administered to 28 Wistar rats, and phage treatments causing expression of Gb₃ receptors on *E. coli* cells will be administered to the treatment group. Rats in both groups will be monitored for symptomatology of *E. coli* poisoning, and stool samples will be collected and analyzed for quantities of Shiga toxins.

Anticipated Results: We anticipate that phage-treated *E. coli* cells will express Gb₃ receptors. We expect that, in the control group, symptoms of *E. coli* poisoning and quantities of Shiga toxins will increase over the duration of the study. Upon adequate expression of Gb₃ receptors, we expect symptoms of *E. coli* poisoning and quantities of Shiga toxins to be lower in the treatment group than in the control group throughout the study.

Discussion: Confirmation of our anticipated results through immunofluorescence spectra visualizing Gb₃ receptors, histogram plots of symptoms, and lateral flow assays detecting quantities of Shiga toxins will prove our methods to be a valuable asset for decreasing the effects of *E. coli* poisoning and related diseases.

Conclusion: Antibiotic resistance is becoming a serious threat. As antibiotic drugs are designed to kill bacteria, we propose an alternative method to neutralizing the pathogenic effects caused by *E. coli* through the incorporation of Gb₃ receptors, and allowing the proliferation of the bacteria. This suggests an approach to slowing the acceleration of antibiotic resistance.

Keywords: Shiga toxin; *Escherichia coli*; λ bacteriophage; globotriaosylceramide receptor; antibiotic resistance; recombineering

Introduction

A major challenge for modern medicine is the prevalence of antibiotic resistance to human pathogens [1]. There are currently 18 antibiotic-resistant bacteria and fungi that are classified as either urgent, serious, or concerning threats to human health, and many more are currently developing antibiotic resistance [2]. Antibiotics are used to target susceptible bacteria, however, bacteria with protective mutations and other factors have survival advantages that allow them to flourish on available nutrients, gaining a competitive fitness advantage [1]. Instead of killing the bacteria, we propose genetic

modification of the bacteria infecting a given organism to impair toxin effectiveness, thereby reducing bacterial virulence.

Shiga toxin-producing *Escherichia coli* is estimated to cause 2,801,000 global infections annually [3]. Shiga toxins can be absorbed and neutralized by bacteria possessing an extracellular Gb₃ receptor [1]. Gb₃ receptors function by recognizing the B₅ subunit of the Shiga toxin, triggering endocytosis. This will allow for the cell to internalize the receptor and its coupled toxin, where the cell will degrade the toxin into separate components. This Gb₃ receptor can be synthesized in *E. coli* strains possessing a *waaO*

mutation in the lipopolysaccharide (LPS) biosynthesis genome locus [1]. This mutation prevents LPS from being fully synthesized on the bacterium's outer membrane, therefore providing an origin site for the Gb₃ receptor to be synthesized. Without this mutation causing partial synthesis of LPS, Gb₃ receptors would lack a location to be synthesized. Additionally, when the *lgtE* and *lgtC* genes are introduced into mutant *E. coli*, Gb₃ receptors are synthesized in place of LPS [1]. This allows *E. coli* to neutralize produced toxins.

Bacteriophages can exist in either a lytic cycle that kills the host bacteria, or a lysogenic cycle that incorporates the phage DNA into the bacterial chromosome [4]. Switching from the lysogenic to lytic cycle (prophage induction) occurs when a lambda repressor protein (C1) stops repressing lytic genes. When bacteria are stressed, RecA proteins are synthesized, which cleave C1 repressors, thereby allowing lytic genes to be expressed. By introducing the phage mutation λ cl *ind*-, cleavage by RecA

proteins is greatly reduced, and the prophage is more likely to stay in the lysogenic cycle [5]. This mechanism can be harnessed to genetically modify bacteria without killing them.

We hypothesize that treating rats with a recombinant phage containing *E. coli* DNA encoding a Gb₃ receptor will alleviate the pathogenic symptoms caused by Shiga toxin-producing *E. coli* and decrease *E. coli* poisoning recovery time.

Methods

Subjects

Twenty-eight healthy, pathogen-free, male (n=14) and female (n=14) Wistar rats at 8-weeks old (p56) will be used in this study [6,7] (Figure 1). Rats will be acclimatized to a light-dark cycle in which lights turn on at 8:00 and turn off at 20:00, and at a temperature of 21°C. Rats will be kept at 90% of their free-feeding body weight, and all monitoring of symptoms will occur during their light cycle.

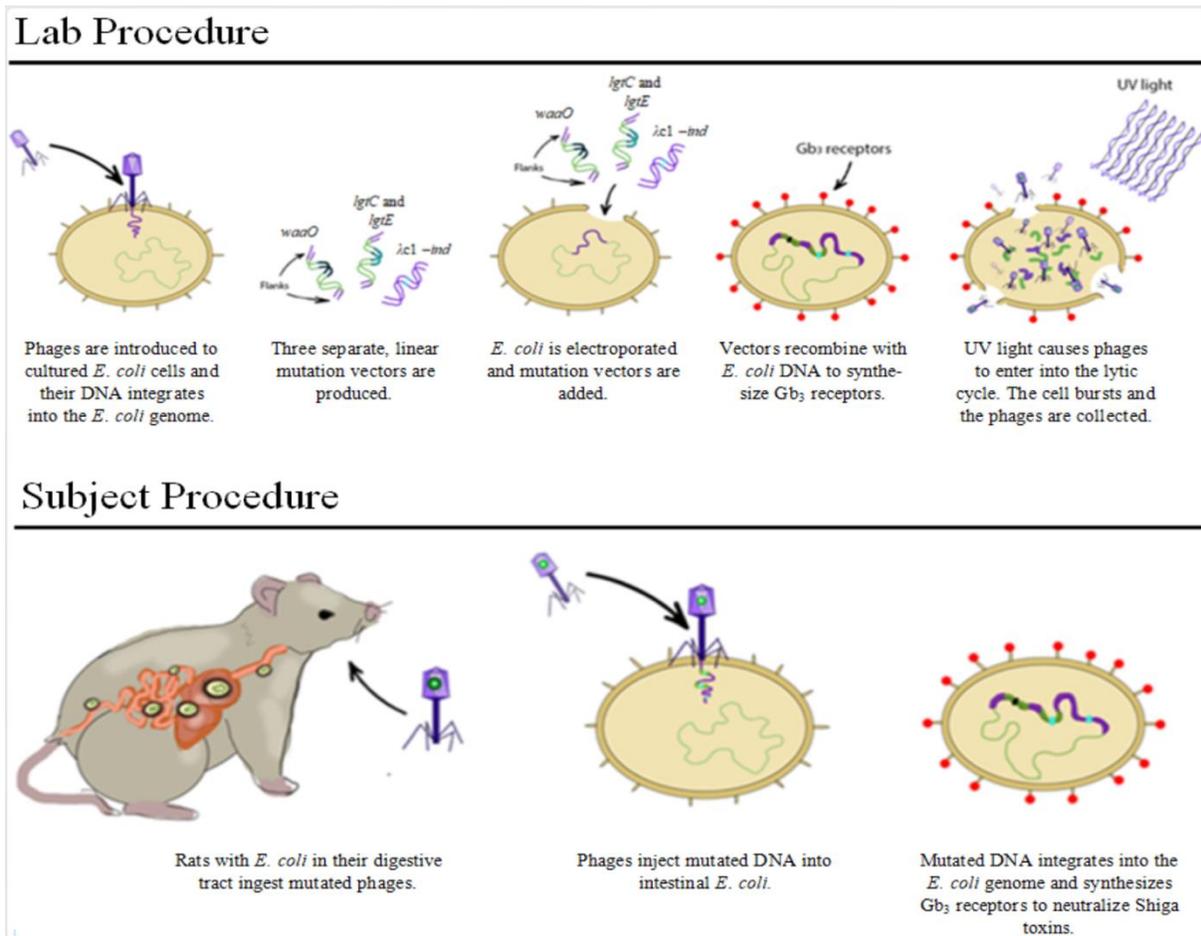


Figure 1. Mutant DNA recombineering process with λ phage DNA in the lab, then in rat subjects. This figure was created using Adobe Photoshop.

Development of Mutation Vectors

To induce *waaO* mutations in *E. coli*, the *waaO* mutant DNA of *waaO* will first be synthesized by substituting an in-frame deletion [8]. Through asymmetric polymerase chain reaction (PCR), primers A, B, C, and D will be used to create the deletion, then primers A and D will be

used for amplification (Table 1). For the desired mutations to recombine with λ phage DNA, the phage flanking sequences J and K (Table 1), will be added to the 5' and 3' ends of the *waaO* sequence, respectively, through recombination [9].

Table 1. Primers used for PCR and flanking [1,5,8]. The flanking sequences were chosen to incorporate mutations into regions of bacteriophage between coding regions and recombining sites [16].

	Sequence description	Function	Sequence
A	Upstream of <i>waaO</i> gene	PCR primer	5'-CGCGGATCCTCATTGCGGATGCCAGTAT-3'
B	Upstream of <i>waaO</i> gene	PCR primer	5'-CCCATCCACTAACTTAAACAGCCATAAGCAACATGGAATG-3'
C	Downstream of <i>waaO</i> gene	PCR primer	5'-TGTTTAAGTTTAGTGGATGGGGCTCGTTATTGCGCTAAGCA-3'
D	Downstream of <i>waaO</i> gene	PCR primer	5'-CGCGGATCCCCCTCGTAAAAGCGTGAGTAA-3'
E	Upstream of <i>lgtC/E</i> gene	PCR primer	5'-CAGACGGGATCCGACGTATCGGAAAAGGAGAAAC-3'
F	Upstream of <i>lgtC/E</i> gene	PCR primer	5'-GCGCGCAATATATTCACCGCCACCCGACTTTGCC-3'
G	Downstream of <i>lgtC/E</i> gene	PCR primer	5'-GGCAAAGTCGGGTGGCGGTGAATATATTGCGCGC-3'
H	Downstream of <i>lgtC/E</i> gene	PCR primer	5'-CATGATGGATCCTGTTTCGGTTTCAATAGC-3'
I	λ cl <i>ind</i> - single base pair mutated at underlined C.	Mutation sequence	5'-TGTTTTTTTCTCATGTTTCAGGCAGGGATGTTCTCACCCGAGCTTAG AACCTTTACCAAAGGTGATGCGGAG-3'
J	Binding site 1 of λ phage	Flanking	5'-TGTTACAGGTCACTAATACCATCTAAG-3'
K	Binding site 2 of λ phage	Flanking	5'-TAGTTAGTTCATAGTGACATAT-3'
L	Binding site 3 of λ phage	Flanking	5'-TTCTCGTTCAGCTTTTTTATACTAAGTTGGCATT-3'
M	Sequence after binding site 3 of λ phage	Flanking	5'-ATAAAAAGCATTGCTTATCAATTTGTTGCAAC-3'

The second mutation will be induced by inserting *lgtE* and *lgtC* genes into the *E. coli* [1]. *lgtE* and *lgtC* will be mutated by overlap extension PCR using primers E, F, G, and H, purified, then amplified using primers E and H (Table 1). Next, to allow for recombination with λ phage, the phage flanking sequences L and M (Table 1) will be added to the 5' and 3' ends of the *lgtE* and *lgtC* sequence, respectively [9].

The third mutation, λ cl *ind*-, will be synthesized through oligonucleotide synthesis by adding nucleotides in a cyclic 4 step process (blocking, coupling, capping, and oxidation) to a growing oligonucleotide chain, resulting in the sequence, I [5,10] (Table 1). This sequence is complementary to the λ cl gene except for a single base pair mutation [5]. This DNA segment will then be amplified by PCR [8]. Since the λ cl *ind*- gene contains entirely phage DNA, no flanking insertion elements will be needed.

The three mutations, *waaO*, *lgtE* and *lgtC*, and λ cl *ind*- will be amplified by PCR. Then, in 2 separate solutions, *waaO* and *lgtE/lgtC* will be digested with their respective flanking sequences by *Bam*HI, treated with phosphate, then ligated to their respective flanking sequences to form 2 separate, linear vectors [1,10]. Since both the *waaO* and *lgtE/lgtC* mutations and their respective flanking sequences contain restriction sites for *Bam*HI, it is important that we digest each mutation in separate solutions, to prevent the ligation of the 2 mutations to each other. This process is not required to create the λ cl *ind*- mutation vector, as the addition of flanking sequences onto this mutation vector is not necessary. After the vectors are produced, they will then be transformed into *E. coli* cells via the recombinering process discussed below. Incorporation of manufactured mutation vectors into the λ bacteriophage genome within the *E. coli* genome is possible due to either the presence of phage DNA within the vector (ie., λ cl *ind*- mutation vector) or the addition of flanking sequences via digestion with *Bam*HI (ie., *lgtE/lgtC* and *waaO* mutation vectors).

Recombineering Process

First, the *E. coli* cells will be infected with phages purchased from Sigma Aldrich to introduce the phage DNA into the bacterial cell [9]. After 125 minutes, the phage DNA should be fully incorporated into the bacterial chromosome as a prophage in the lysogenic cycle [11]. The *waaO*, *lgtE* and *lgtC*, and λ cl *ind*- vectors will be introduced into the cell through electroporation [9]. The prophage DNA then binds with the complementary flanks on the vectors, incorporating the mutations into its genome. Despite the λ cl *ind*- mutation preventing the prophage from entering the lytic cycle, stress from UV light (315-400nm) can overcome this, causing the phages to replicate and lyse the cell [12]. The phages can then be collected and tested through immunofluorescence as described below through phage testing [9].

Phage Testing

To test the ability of the phages to modify Gb₃ receptors *in vitro* before administering treatments *in vivo*, immunofluorescence will be used. There will be a control group (an *E. coli* cell culture that is not administered phage treatment) and phage treatment group of *E. coli* cell cultures that will both be treated first with primary antibodies specific to Gb₃ receptors, then with secondary antibodies specific to the primary antibodies that also contain a fluorophore. After washing away excess antibodies, the amount of fluorescence corresponding to the quantity of Gb₃ receptors present in the samples of *E. coli* cells can be quantified through spectroscopy. Both the control and treatment groups of *E. coli* cells will be tested to allow for comparison of the quantity of fluorescence between the groups. Adequate fluorescence quantified from the phage treatment group of *E. coli* cell cultures when compared to the control group will indicate that the Gb₃ receptors are being expressed on the *E. coli* cells through phage treatment effectively.

Treatment Protocol

E. coli (O157:H7 strain) and λ bacteriophages will be purchased from Sigma Aldrich chemical company to be used in this study. Rats will be separated into a control group (n=4 males and n=4 females) (infected with *E. coli*) and a treatment group (n=10 males and n=10 females) (infected with *E. coli* and given phage treatment). Phage treatments will consist of recombinered phages that were confirmed to contain the proper mutations necessary to cause expression of Gb₃ receptors in the phage testing process. Both groups will be fed 0.1 mL (5 x 10⁷ CFU) samples of *E. coli* O157:H7 using a micropipette on day 0 [6,14]. The phage treatment will be administered to the treatment group orally in 0.1 mL samples on days 0-3, twice per day, 2.5 hours apart to allow for full integration of treatment into the body before the next treatment is administered [6]. Control rats will be fed 0.1 mL of oral saline solution without phage treatment concurrently. To establish the effectiveness of phage therapy, stool samples collected daily will be analyzed for mutated *E. coli* using Lateral Flow Assay (LFA). LFA will be used to detect quantities of Shiga toxin in stool samples through the use of a pair of monoclonal antibodies. The LFA will produce a coloured line at the test line position that varies in saturation according to the presence and quantity of Shiga toxins within the stool samples. Symptoms such as bloody diarrhea, vomiting, and fever will also be monitored daily [15]. The experiment will continue for 10 days (Figures 2, 3, 4) [6]. Depending on susceptibility of the rats to *E. coli*, a possible limitation may arise if too many rats die before sufficient data is collected.

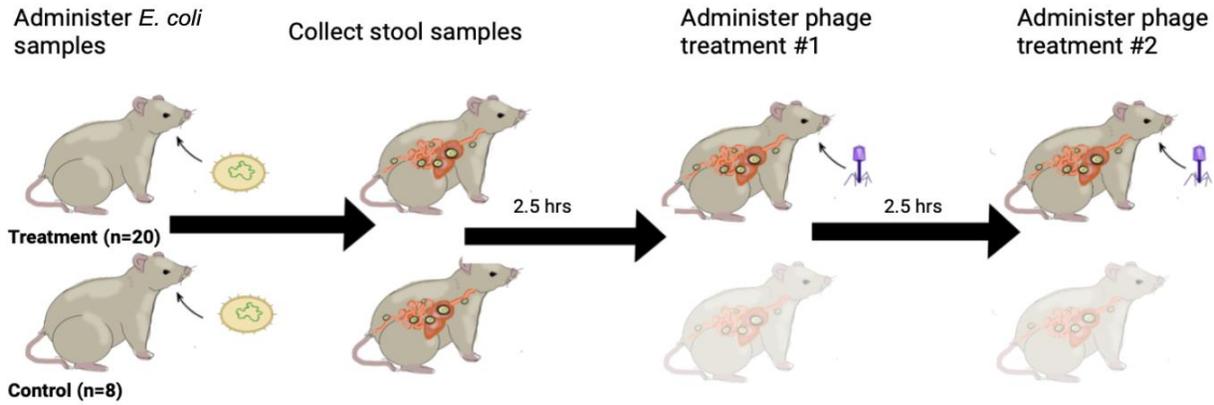


Figure 2. Day 0 treatment protocol: Administer *E. coli* to control and treatment groups, followed by stool sample collection from both groups, followed by two phage treatments to treatment group only [6]. This figure was created using Adobe Photoshop.

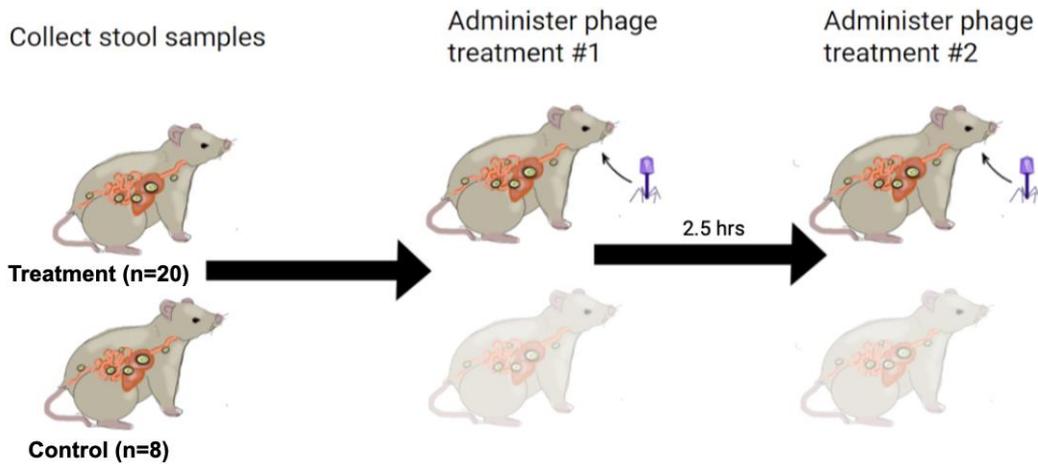


Figure 3. Days 1-3 treatment protocol: Stool sample collection from both groups followed by two phage treatments to treatment groups only [6]. This figure was created using Adobe Photoshop.

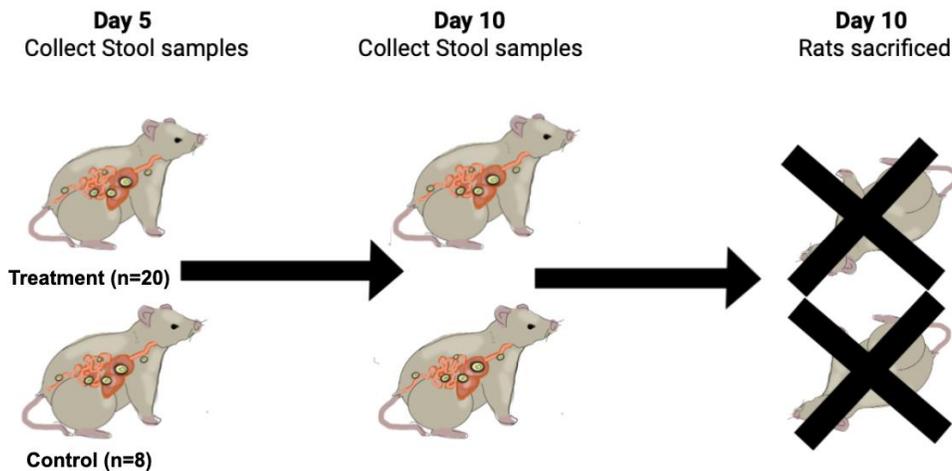


Figure 4. Days 5, 10 treatment protocol: Stool sample collection from both groups followed by sacrifice of the rats on day 10 [6]. This figure was created using Adobe Photoshop.

Results

As what we discussed here is proposed research, experimental results were not yet obtained, and the following discussion will be limited to expected results only.

We expect that the group of *E. coli* cells that received phage treatment prior to being administered to test subjects will fluoresce significantly when compared to the control group of cells, due to the insertion of Gb₃ receptors via recombination. Secondly, we expect that the quantity of symptoms related to *E. coli* poisoning will be significant in the control group, increasing as the study proceeds. We expect that the quantity of symptoms in the treatment group will be much smaller when compared to the control group, and it may be possible that the treatment group presents no visible disease throughout the course of the study following phage treatment on day 0. Lastly, we anticipate that the amount of Shiga toxins present in the stool samples of the treatment and control groups will follow the same relationship as the symptoms of *E. coli* poisoning. Upon adequate expression of Gb₃ receptors, we expect that the treatment group's stool samples will present smaller quantities of Shiga toxin when compared to the control group.

Discussion

The use of immunofluorescence allows for the confirmation of Gb₃ receptor presence in a given sample. The introduction of primary antibodies specific to Gb₃ receptors, followed by the introduction of fluorophore-conjugated secondary antibodies specific for the primary antibodies, allows for the visualization of Gb₃ receptors. Therefore, if our phage treatment was successful in the treatment group of *E. coli* cells, we should see a significant amount of fluorescence in the treatment sample when compared to the control sample. Once antibodies have been administered, the fluorescence present in the treatment and control group samples will be visualized using a fluorescence microscope and quantified using a spectrofluorometer. The spectrofluorometer will measure and quantitatively collect the fluorescence produced by the fluorophores through absorbance readings. When analyzing the spectra produced by the spectrofluorometer, we expect a significant increase in absorbance at a wavelength that is associated with our selected fluorophore for the treatment sample, and minimal to no absorbance at that given wavelength for the control sample. This will indicate that our treatment was successful in causing the expression of Gb₃ receptors in *E. coli* cells in the treatment group, and we will be able to proceed with the administration of *E. coli* cells and phage treatments to live subjects.

After administration of *E. coli* cells to both control and treatment groups and the introduction of phage treatment to the treatment group only, an unbiased observer who is unaware of the purpose of our research will monitor and quantify the symptomology of both the treatment and

control groups of subjects daily. The observer will monitor both groups of rats and will record the frequency of any symptoms of *E. coli* poisoning that appear in the subjects. The observer will record frequencies of symptoms such as vomiting, diarrhea, and fatigue. Internal temperature of the rats will be attained daily to determine the presence of fevers, which will also be recorded. Once the study has been completed after 10 days, frequencies of symptoms for the treatment and control groups will be plotted on respective histograms to allow for comparison of symptomatology between the groups. We expect that as the study proceeds, the quantity of symptoms in the control group will increase and plateau. For the treatment group, as the study progresses, we expect quantities of symptoms to be lower compared to the control group, dependent upon adequate expression of Gb₃ receptors, and therefore a decrease in Shiga toxin quantities. This will indicate that the phage treatment was successful in treating *E. coli* infection in the treatment group.

Stool samples will be collected daily from both the treatment and control group to be analyzed for quantities of Shiga toxin using LFA. We expect an increasing quantity of Shiga toxin in the control group as the study progresses. Therefore, we expect to see an increasing amount of saturation at the test line position of the LFA for the control group as the study progresses. For the treatment group, we expect to see a decrease in quantity of Shiga toxin upon adequate expression of Gb₃ receptors on *E. coli* cells. As a result, for the treatment group, we expect to see a smaller amount of saturation at the test line position of the LFA when compared to the control group, upon adequate Gb₃ receptor expression. This will indicate that the phage treatment was successful in causing expression of Gb₃ receptors in the treatment group *E. coli* cells, and that the Gb₃ receptors were successful in degrading the Shiga toxins produced by the *E. coli* cells in this group. With these results, we can conclude that our phage treatment methods may provide a possible alternative therapeutic capable of mitigating the effects of *E. coli* related diseases.

It is possible that the survival of rats in the phage treatment group may be attributed not to our proposed methods, but to phages with the λ c1 *ind*- mutation failing to maintain their lysogenic cycle, resulting in the lysing of *E. coli* cells. We may be able to tell if the λ c1 *ind*- mutation is successful within the phages during the production of recombiner phages. This is because during production of the phages, we need to use UV light to allow the phages to overcome the λ c1 *ind*- mutation and lyse the cell. However, if we see that cells are being lysed before the addition of UV light, we would know that the mutation was unsuccessful at keeping the phage in its lysogenic cycle, and we would have to modify our methods before administering this phage treatment to the subjects. To further confirm that our phage treatment methods are successful and *E. coli* cells are not being lysed by our recombiner phages, we could conduct further testing

using quantitative PCR (qPCR) assays to determine the quantities of *E. coli* cells in collected stool samples. If our methods are working as proposed, we would expect to see a consistent quantity of *E. coli* cells in the treatment group's stool samples throughout the experiment. However, if our phage treatments were in fact causing lysing of the *E. coli* cells, we would see a decrease in the quantity of *E. coli* cells in the treatment group's stool samples throughout the experiment and would again need to modify our methods.

Conclusions

Our proposed approach to mitigating the pathogenic effects of *E. coli* focuses on neutralizing Shiga toxins by recombining mutant DNA with *E. coli* through the use of phage infection, instead of killing the bacteria. By keeping mutated *E. coli* cells alive and allowing them to proliferate with Gb₃ receptors that mitigate the pathogenic effects of the Shiga toxins, we will effectively create a population of *E. coli* cells in the organism that may not be harmful. These methods are important as they bypass the recurring issue of antibiotic resistance, as keeping the mutated *E. coli* cells alive prevents any resistant *E. coli* from having a selective advantage by having access to their resources. With these proposed methods, we offer a potential therapeutic for *E. coli*-related diseases and suggest an alternative method to prevent further acceleration of antibiotic resistance. If the results of this study are as expected, future directions for this research include beginning clinical trials in humans to test the efficacy of this phage treatment against *E. coli* poisoning. As well, future research may be conducted to further understand the bacteriophage-pathogen relationship underlying our methods and to determine if they may be used to mediate the effects of other pathogens on human health.

List of Abbreviations Used

Gb₃: globotriaosylceramide
LPS: lipopolysaccharides
C1: lambda repressor protein
PCR: polymerase chain reaction
LFA: lateral flow assay
qPCR: quantitative polymerase chain reaction
UV: ultraviolet
E. coli: *Escherichia coli*

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study did not require ethics approval or participant consent, as it is a proposal for research, and no humans, animals, or tissues were used.

Author's Contributions

JMK: made substantial contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.

BDP: made substantial contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.

ELV: made substantial contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.

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