Isolation of bacteriophage with antimicrobial activity against *Shigella Dysenteriae* from sewage sample

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. We have acknowledged all primary sources of help.

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Ethics Statement:

In this study, we have collected the sewage samples from the drain of the National Institute of Cancer Research & Hospital (NICRH), TB gate, Mohakhali, Dhaka. Therefore, no consent was necessary. All experiments were performed using the ethical guidelines of the BRAC University Microbiology and Biotechnology laboratory.

Abstract:

Shigellosis infection, caused by a genus of medically important bacteria called Shigella, results in diarrhea and other painful manifestations in humans. Annually, an estimated 74,000 to 600,000 people die of *Shigella* infection worldwide. Among the most lethal species of Shigella is *Shigella dysenteriae*, which produces the Shiga toxin upon infection. As a treatment, several antibiotics are prescribed to the patients suffering from shigellosis. However, recent studies have shown *Shigella dysenteriae*, along with several other species of bacteria, to have developed resistance against the common antibiotics; and, many of them were multidrug-resistant. In order to counter this problem, widespread research is ongoing in attempts to develop treatments of Shigellosis alternative to antibiotics intake, including phage therapy. Phage therapy is the therapeutic use of bacteriophages in treating bacterial infections. The initial step in the long process of developing successful phage therapy against a particular pathogenic infection is the isolation of phage(s) from the environment. In this study, we have isolated bacteriophages from hospital sewage and confirmed the phages' antimicrobial activity against ATCC strains of *Shigella dysenteriae.*

Keywords: phage isolation; *Shigella dysenteriae*; phage therapy

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List of acronyms:

NGS Next Generation Sequencing
DAL Double Agar Layer
S.dysenteriae Shigella Dysenteriae

Chapter 1
Introduction

1.1 *Shigella dysenteriae*

Shigella dysenteriae is a species of bacteria of the genus *Shigella.* Naturally, they are gram negative, facultatively anaerobic and morphologically rod-shaped. Moreover, they are nonmotile and non-spore-forming. The taxonomic classification of *S.dysenteriae* is given below (Castellani & Chalmers, 1919)

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Shigella*

Species: *Shigella dysenteriae*

S.dysenteriae have been classified under *Shigella* serogroup A and 15 common serotypes have been identified (Ansaruzzaman et al. 1995).

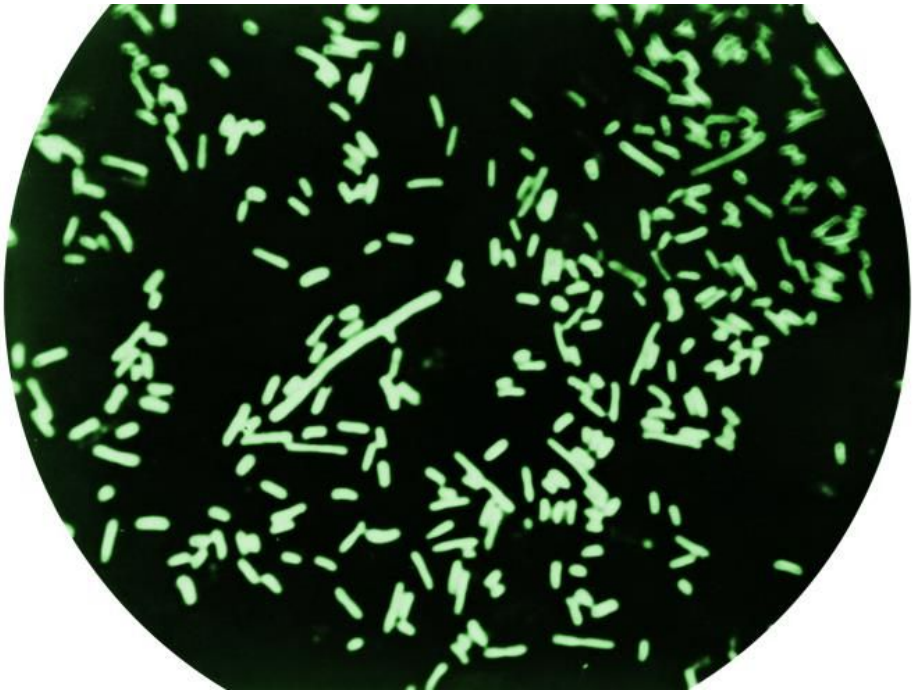

Figure 1: Dark field microscopy showing *S.dysenteriae*(AFIP,1964)

1.2 Shigellosis

Apart from humans, shigellosis may occur in other mammalian species including primates (Banish et al., 1990). Shigellosis usually manifests in the form of dysentery, vomiting, cramps, and in extreme cases leading to sepsis and seizures. It is one of the major bacterial causes of diarrhea globally, with the number of cases ranging between 80 and 165 million. Shigellosis occurs when *Shigella* is transmitted through the fecal-oral route as a consequence of poor hygiene and lack of proper sanitation facilities (CDC, 2016). Once the bacteria reach the small intestine, they release the enterotoxin called shiga toxin. The toxin then infects epithelial cells of the small intestine and then is absorbed into the circulation (Schüller, 2011). Treatment of Shigellosis includes oral or intravenous administration of fluids in order to replenish the lost water and salts from the body. Antibiotics are also prescribed to treat the infection (CDC, 2019). Unfortunately, with the astonishingly easy access and rampant use of antibiotics, multi-drug resistant species of *Shigella* have evolved over time. .

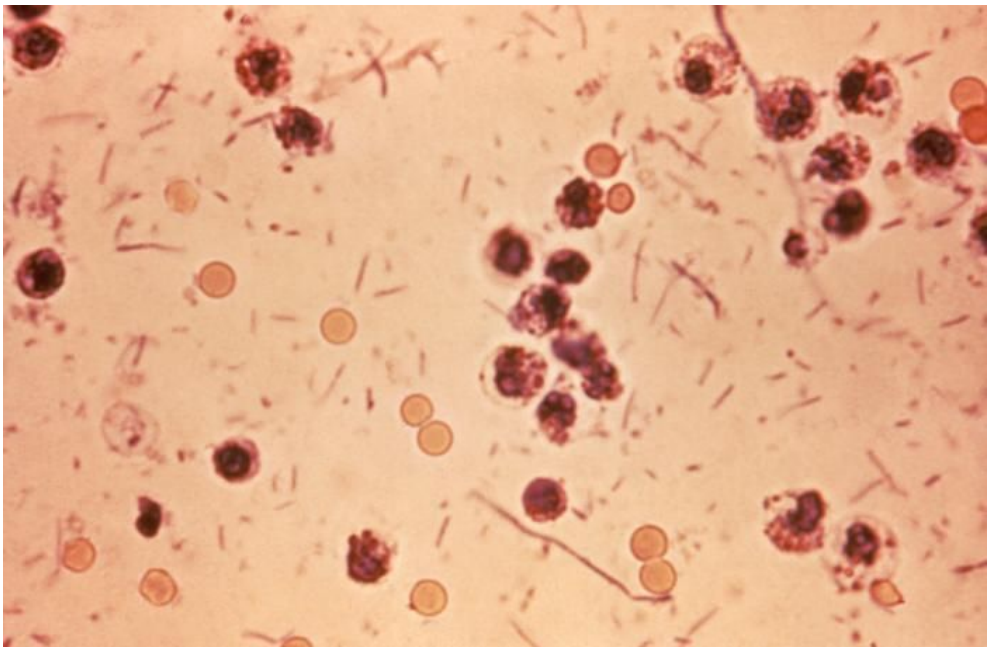

Figure 2: Photomicrograph of stool sample from a patient, showing presence of *S.dysenteriae* and blood cells (CDC, 2006)

1.3 Antibiotic Resistance

Generally, antibiotic resistance results from one of the following mechanisms: (1) innate resistance in specific strains of bacteria (2) gene mutation which gives the bacteria an ability to combat the antimicrobial mechanism (3) Horizontal gene transfer of resistance genes from one bacterial species to another (TUFTS., 2015). While mutations may occur completely spontaneously and randomly, uncontrolled use of antibiotics seem to speed up the rate of selection of bacteria that have the resistant mutations and thus are resistant (Holmes et al., 2016). In order to slow down this rapid development of antibiotic resistance by several species of bacteria numerous preventive measures are being suggested, such as using antibiotics only when required and using only narrow spectrum antibiotics instead of broad-spectrum antibiotics (Gerber JS et al., 2017). Besides these preventive initiatives against spread of antibiotic resistance, several alternative methods and treatments are being developed to combat bacterial infections.

Studies done across multiple countries show resistance of *S.dysenteriae* to traditional antibiotics, including ampicillin, tetracycline, cotrimoxazole, amoxicillin and fluoroquinolones such as ciprofloxacin and norfloxacin (Dutta et al., 2003).

1.4 Phage Therapy and Bacteriophages

One of these treatments is known as phage therapy. Phage therapy refers to the use of naturally-occurring viruses called bacteriophages to lyse and destroy infectious bacteria (Lin DM et al., 2017) . Bacteriophages are viruses that infect bacteria. Most phages are categorized into two types based on their life cycle. They are lytic and lysogenic phages. Lytic phages are the ones that infect bacterial hosts and quickly kill them by lysis of the cells. On the other hand, lysogenic phages integrate their genetic material into the host genome, and remain as plasmids within the bacterial cells (Clokie et al., 2011).

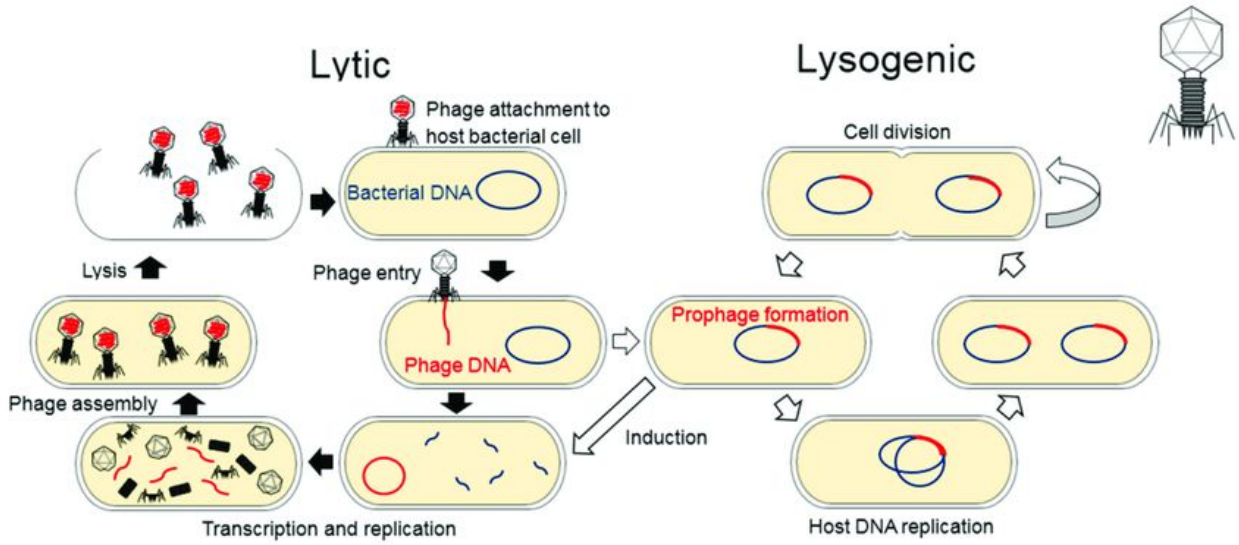

Figure 3: Lytic and Lysogenic life cycle of phages (Batinovic et al., 2019)

The lytic phages are the promising effectors in phage therapy. These phages have, in several in-vitro and in-vivo studies, shown antibacterial activity, thereby showing great potential to replace antibiotics in the fight against multi-drug resistant bacteria (Sulakvelidze et al., 2001).

Since the 1990s, phage therapy research has led to the discovery of numerous bacteriophages that show sustained lytic activity against common pathogenic and multidrug resistant bacteria. Researchers worldwide are continuously working in not only isolation of novel bacteriophages from the environment, but also in characterizing them and analysing the optimum conditions for their activity. Modern scientific literature often lists the various advantages (e.g low toxicity, low disruption of normal flora, stronger bactericidal activity compared to antibiotics etc) and disadvantages (e.g possible narrow host range, lack of expected activity in vivo etc.). Nonetheless, it seems that the advantages far outweigh the limitations of phage therapy. Through better phage selection, proper formulation as well as better clinical knowledge of phage application, the disadvantages can be dealt with.(Loc-Carrillo & Abedon, 2011) Currently, this is the goal of researchers- taking phage therapy promising prospects from the labs and making them available as a safe and effective treatment against infectious bacteria.

In this study, we collected sewage samples from the drain of the National Institute of Cancer Research & Hospital (NICRH), TB gate, Mohakhali, Dhaka, isolated bacteriophage that showed lytic activity against *S.dysenteriae,* and confirmed the presence of the lytic activity using a control.

Chapter 2

Materials and Methods

2.1 Standard Laboratory Practice

Glassware such as test-tube, conical flask, beakers was washed once with tap water
followed by a second time wash with distilled water. All culture media used in this study, pipette tips, centrifuge tubes, empty test-tubes were autoclaved at 121°C at 15 psi for 15 minutes before use and stored culture media at 4°C and autoclaved equipment in aseptic condition. During the experiment, a clean lab coat was worn and hand gloves were used and the experiments were performed inside a laminar flow cabinet which in prior was cleaned with 0.5 percent hypochlorite solution and 70 percent ethanol to avoid contamination.

2.2 Preparation of *Shigella* host

ATCC culture of *Shigella dysenteriae*, from the BRAC University Microbiology and Biotechnology laboratory, was used as the host for the bacteriophage isolation. Initially, *Salmonella-Shigella(*SS) agar was prepared to allow for differential growth of the host cells(in case the stock was contaminated). According to the manufacturer's instructions, 63g of SS agar media was added to 1.0L of distilled water, and heated in bunsen flame until all the powder was completely dissolved.The molten SS agar was then poured into multiple medium-sized sterile petri dishes, under the laminar flow cabinet, before allowing them to cool down and solidify. Once solidified, a sterile needle was used to streak S.dysenteriae onto the SS agar plates, in order to stimulate formation of single,pure colonies. The plates were then put in the incubator at 37°C

for 48 hours. Pink colored single colonies are recognized as S.dysenteriae. These plates were stored at 4°C.(Figure 4).

2.3 Sample Collection

A 500ml, autoclaved sample collection bottle was used to collect sewage samples. The samples were collected from the drain of the National Institute of Cancer Research & Hospital (NICRH), TB gate, Mohakhali, Dhaka. The bottle was tightly sealed and safely carried to the BRAC University Microbiology and Biotechnology laboratory for processing.

Figure 4: Single colonies of *S.dysenteriae* on SS agar

2.4 Sample processing

The bottle containing the samples was allowed to sit for a few hours so that solid particles would settle down. Later, 50ml of the suspension was gently transferred to a sterile conical tube. The suspension was then centrifuged at 3000rpm for 20 minutes. The supernatant was then filtered using 0.22 µm millipore syringe filters to filter out endogenous bacteria. The filtrate was stored in multiple 15ml conical tubes and later tested for the presence of bacteriophage with activity against S.dysenteriae (Jothikumar et al., 2000)

2.5 Plaque Assay

First, young bacterial host culture suspension was prepared in sterile Nutrient Broth. 13g of Nutrient Broth (NB) per 1.0L of water was prepared and allowed to autoclave at 121°C and 15psi for 30-45 minutes, before being poured into 15ml conical tubes. Then, using a sterile loop, single colonies were taken from the previously prepared SS agar plates and inoculated with the NB. The inocula were then placed in a shaker incubator at 37°C for 2-3 hours, to give rise to the young *S.dysenteriae* suspension.

Double Agar Layer method (Adams, 1959), followed by spot testing was used to check for presence of phage activity against *S.dysenteriae.* Two layers of Nutrient Agar were prepared for each plate. The bottom, more stable layer was the basal agar layer, while the one on top was the soft agar layer.

For the preparation of the basal agar, 13g of Nutrient Broth along with 1.5% bacteriological grade agar powder was required per 1.0L of distilled water. This was done for about 90ml of distilled water and poured into multiple conical flasks. The flasks were then sealed and allowed to autoclave at 121°C and 15psi for 30-45 minutes, for decontamination. Then, the basal agar was poured into multiple medium-sized petri dishes, under a laminar flow cabinet and allowed to cool down.

For the preparation of the soft agar, 13g of Nutrient Broth along with 0.7% bacteriological grade agar powder was required per 1.0L of distilled water. This was done for about 50ml of distilled water and poured into multiple glass test tubes (about 5ml of soft agar per tube). The test tubes were then sealed and allowed to undergo autoclave at the same conditions mentioned above. The tubes were then taken out and kept in a waterbath of 45°C.

After that, the bacterial suspensions were taken out of the shaker incubator and brought under the same laminar flow cabinet as the plates containing the basal agar. Test tubes of soft agar are brought into the cabinet from the water bath, one at a time. About 1ml of bacterial suspension is added to one test tube of soft agar, homogenized using vortexing, before pouring the mixture onto a plate of basal agar. In a few minutes, the soft agar solidifies and the double agar layer is formed. This step is repeated for all the test test tubes of soft agar.

Once all the double agar layers have cooled down and solidified, 7μm of sample was added to 4 designated spots on each DAL plate to test for bactericidal activity against *S.dysenteriae*. The plates were then placed in the incubator at 37°C for overnight. The next day, the plates were removed from the incubator and analysed for phage activity. Later, a further evaluation of the antibacterial activity was performed.

2.6 Storage

After analysis of the results, the conical tube containing the chunks of clear zones from DAL plates as well as the original filtered sample were both stored at 4°C. Later these phage solutions were enriched with the *S.dysenteriae* host, before undergoing filtration through 0.22 μm millipore syringe filters and storage at 4°C (Van Twest et al., 2009).

Chapter 3

Results

Isolation of bacteriophage : After filtering the sample, we added drops of the filtrate on three DAL plates as an initial check of antibacterial activity. After incubating the plates overnight at 37°C we observed that out of the three DAL plates containing drops of our samples only, two were discarded because of bacterial contamination, lack of stability of the agar and other similar issues. ONE of the remaining plates showed clear zones in ALL areas spotted with our sample, thereby confirming isolation of phage with activity against *S.dysenteriae* (Figure 5).

After analysis of the presence of clear zones on the plates, a micropipette was used to extract chunks of the clear zones on the DAL plates. The chunks were then stored in a conical tube containing chloroform solution (to eliminate any residual bacteria in the chunks) and stored at 4°C overnight, for further analysis of antibacterial activity using saline control.

Further Evaluation of bacteriophage activity: As further evaluation of antibacterial activity of our samples, we took drops of the solution from the stored conical tubes and added them on three DAL plates. We added 2 drops of our stored sample and 2 drops of saline on each plate. . After incubating the plates overnight at 37°C we observed that out of the three DAL plates that were tested with our samples and saline drops (control), two were discarded due to bacterial contamination, lack of stability of the agar and other similar issues. However, the remaining one plate showed positive result, i.e. clear zones in regions spotted with our solution containing chunks of clear zones from the previous experiment but no clear zones in regions spotted with saline (Figure 6)

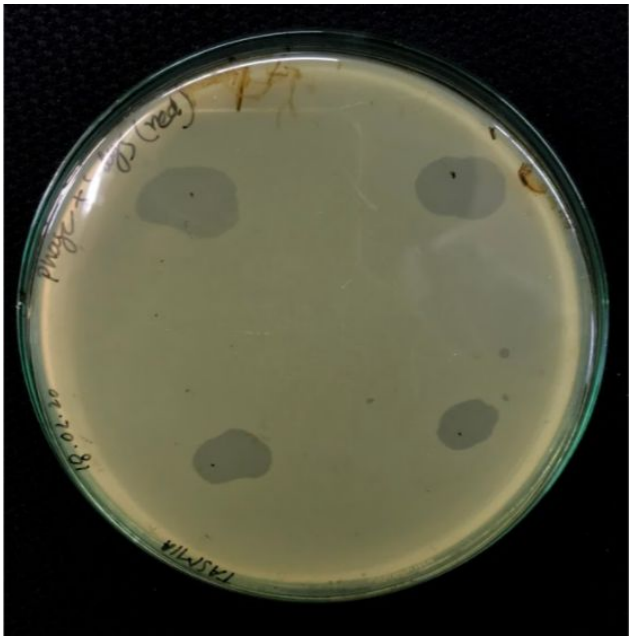

Figure 5: One of the three DAL plates shows clear zones in all four areas spotted with the filtered sample, thereby showing a potential bacteriophage activity against *S.dysenteriae.*

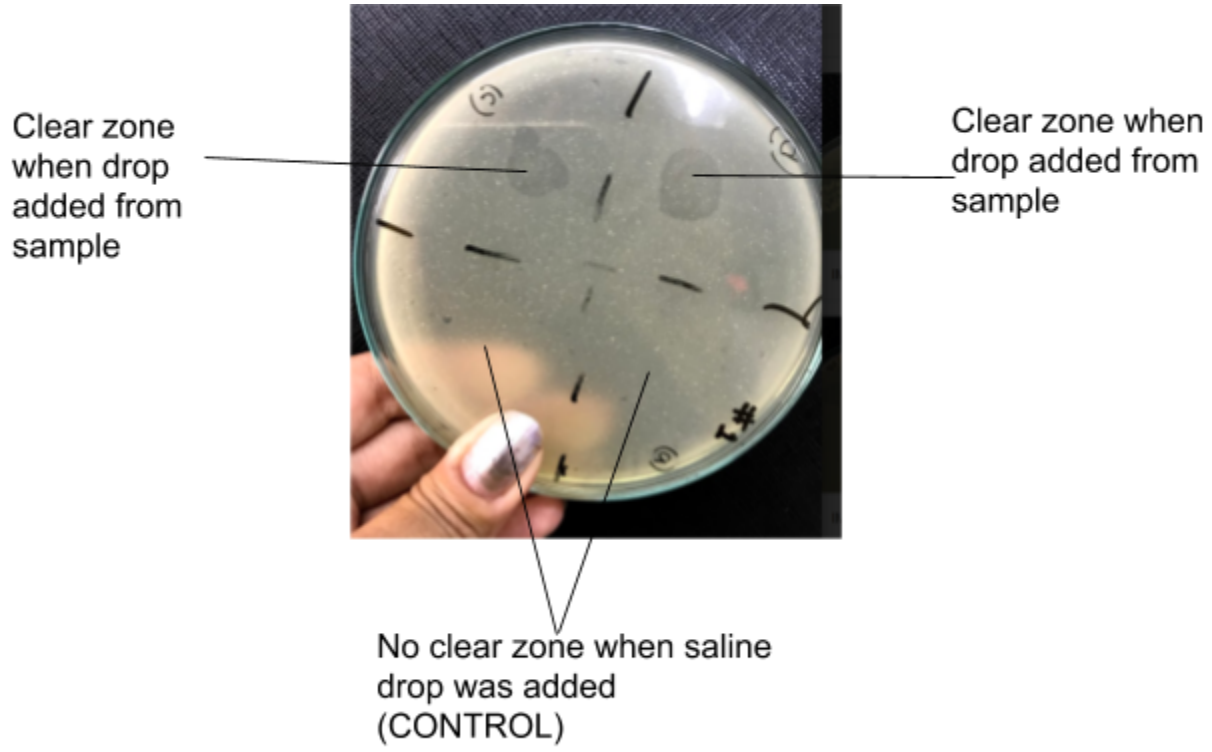

Figure 6: The two areas spotted with our solution shows clear zones, while areas spotted with the control show no clear zones.

Chapter 4

Discussion

Our results show clear zones(plaques) on the lawn of *Shigella Dysenteriae* , in places spotted by the processed sewage samples(Figure 5 and 6). Moreover, by comparing results with saline spots on the same petri dish, we were able to confirm that the clear zones were due to drops of samples added. From the results we obtained, we can conclude the presence of bacteriophage with activity against *S.dysenteriae*, in our sewage sample. Moreover, the sample was enriched with the host bacteria and stored in chloroform at 4°C for further investigations in the future.

While the testing with saline does confirm that the clear zones were not a false positive, our methods only identified the presence of bacteriophage against our host. Several more plates should be used for further investigation i.e replication must be done. Moreover the spot testing

method does not confirm whether the phages that were isolated were of obligately lytic or temperate nature. In order to be best suited for phage therapy, lytic phages are preferred. If we are to confirm that the phages we isolated have a lytic life cycle, further investigation, specifically plaques testing should be performed. The appearance and number of plaque forming units (PFU) may give us an idea of the nature of the phages. (Hyman, 2019)

Indeed, isolation and storage of bacteriophage is the first of the several necessary investigations before the phages may be deemed as suitable candidates for phage therapy. One of these steps is the characterization of the phage isolated. For use in phage therapy a broad host range within species, lack of toxin genes, efficiency in killing host cells etc. are all properties that are commonly screened for in newly isolated phages.Other ways to characterize phages include thermal and pH stability tests, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis(SDS-PAGE) in order to create structural protein profiles of the phage.Phage morphology and electron microscopy methods may help to classify the phages. Since NGS methods are relatively affordable now, DNA extraction followed by Whole Genome Sequencing of the phage genome may help detect presence of toxin genes or identifying phages that have similar genes. Pulse-field gel electrophoresis (PFGE) may be used to measure the size of the bacteriophage genome(Hyman, 2019). These investigations may be carried out in the future on the isolated and stored bacteriophages in order to check for utility in phage therapy.

Reference

Adams M.H. Discovery of bacteriophages and methods of study of bacterial viruses. Bacteriophages. Vol. 42. New York: Interscience Publication; 1959. pp. 447–448.

Ansaruzzaman M, Kibriya AK, Rahman A, et al. Detection of provisional serovars of Shigella dysenteriae and designation as S. dysenteriae serotypes 14 and 15. J Clin Microbiol. 1995;33(5):1423-1425.

Batinovic, Steven & Wassef, & Knowler, Sarah & Rice, & Stanton, & Rose, & Tucci, & Nittami, Tadashi & Vinh, & Drummond, & Sobey, & Chan, & Seviour, Robert & Petrovski, Steve & Franks, Ashley. (2019). Bacteriophages in Natural and Artificial Environments. Pathogens. 8. 100. 10.3390/pathogens8030100.

By Armed Forces Institute of Pathology, AFIP - https://phil.cdc.gov/Details.aspx?pid=22229, Public Domain,

By Centers for Disease Control and Prevention Public Health Image Library - Centers for Disease Control and Prevention Public Health Image Library, Public Domain

Clokie, M. R., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. Bacteriophage, 1(1), 31–45. https://doi.org/10.4161/bact.1.1.14942

Gerber JS, Ross RK, Bryan M, et al. Association of Broad- vs Narrow-Spectrum Antibiotics With Treatment Failure, Adverse Events, and Quality of Life in Children With Acute Respiratory Tract Infections. JAMA. 2017;318(23):2325–2336. doi:10.1001/jama.2017.18715

Holmes AH, Moore LS, Sundsfjord A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387(10014):176-187. doi:10.1016/S0140-6736(15)00473-0

Loc-Carrillo, C., & Abedon, S. T. (2011). Pros and cons of phage therapy. Bacteriophage, 1(2), 111–114. https://doi.org/10.4161/bact.1.2.14590

Isolation of coliphages specific to enterotoxigenic E. coli (ETEC)
Jothikumar N, Reddy CG, Sundari RB, Saigopal DV
J Environ Monit. 2000 Aug; 2(4):372-4.

Michael L. Bennish, Wasif A. Khan, Monira Begum, Emily A. Bridges, Sabeena Ahmed, Debasish Saha, Mohammad A. Salam, David Acheson, Edward T. Ryan, Low Risk of Hemolytic Uremic Syndrome after Early Effective Antimicrobial Therapy for Shigella dysenteriae Type 1 Infection in Bangladesh, Clinical Infectious Diseases, Volume 42, Issue 3, 1 February 2006, Pages 356–362, https://doi.org/10.1086/499236

Niyogi SK. Shigellosis. J Microbiol. 2005;43(2):133-143.

Schüller, S. (2011). Shiga Toxin Interaction with Human Intestinal Epithelium. Toxins, 3(6), 626–639. doi:10.3390/toxins3060626

Sulakvelidze, A., Alavidze, Z., & Morris, J. G., Jr (2001). Bacteriophage therapy. Antimicrobial agents and chemotherapy, 45(3), 649–659. https://doi.org/10.1128/AAC.45.3.649-659.2001

Sur D, Niyogi SK, Sur S, et al. Multidrug-resistant Shigella dysenteriae type 1: forerunners of a new epidemic strain in eastern India?. Emerg Infect Dis. 2003;9(3):404-405. doi:10.3201/eid0903.020352

Van Twest R., Kropinski A.M. (2009) Bacteriophage Enrichment from Water and Soil. In: Clokie M.R., Kropinski A.M. (eds) Bacteriophages. Methods in Molecular Biology™, vol 501. Humana Press. https://doi.org/10.1007/978-1-60327-164-6_2