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Potential of Bacteriophage Therapy in Treating Hospital Wastewater

Alfredo V. Corpuz [1]

[1] *University of Northern Philippines*

Abstract: ***Hospital wastewater contains increased amounts of antibiotics, disinfectant, and by-products of hospital operations. The indiscriminate discharge of hospital wastewater is considered as a significant sanitation problem and a mode of transmission of diseases.***

The study evaluated the efficacy of phages as an alternative method for controlling the occurrence of pathogenic bacteria in hospital wastewater. Specifically, it determined (1) the identity of the bacteria isolated from the hospital wastewater; (2) the concentration of the phage lysate that can effectively destroy the pathogens isolated from the wastewater; and (3) if significant difference exists on the effect of the different phage lysate concentrations on the growth and development of the pathogens isolated from the hospital wastewater.

The findings of the study show that there were three bacterial pathogens isolated from the hospital wastewater. These were Escherichia coli (Extended Spectrum Beta Lactamase positive), Klebsiella pneumoniae, and Pseudomonas putida. These bacteria are all clinically significant as they cause different diseases and is one reason for increasing morbidity and mortality worldwide. The results of the mean plaque forming units per milliliter revealed that Escherichia coli exhibited the highest number of clearing (plaques) in the 100% phage lysate concentration, having a mean pfu/mL of 1.481 x 10^4. E. coli was followed by Klebsiella pneumoniae, with a mean pfu/mL of 6.307 x 10^3 and Pseudomonas putida, with a mean pfu/mL of 5.891 x 10^3.

There is a direct proportional relationship between the phage lysate concentration and the mean pfu/mL. It reveals a positive agreement between these two variables, meaning that as the phage lysate concentration increases, so does the mean pfu/mL, and it happens in all the bacteria isolated.

The result of the One Way Analysis of Variance reveals that even if the 100% phage lysate concentration exhibited the highest number of plaque forming units per milliliter, statistical analysis shows no significant difference in the pfu/mL between these bacteria.

In conclusion, the presence of bacterial isolates (like Escherichia coli, Klebsiella pneumoniae, and Pseudomonas putida) in hospital wastewater confirms that the current treatment process of some secondary hospitals is not effective in destroying the clinically infectious pathogens found in the wastewater these hospitals generate. Therefore, treatment of hospital's wastewater can be achieved by bacteriophages as bio-control agent.

For future direction of the study, the researcher recommends to include more wastewater samples to isolate more strains of bacteria. Furthermore, the investigator suggests to identify the bacteriophages present by performing genomic sequencing. Other recommendations include the determination of specific host range of bacteriophages among target bacterial cells, and the promotion of bacteriophage therapy as an alternative way to treat hospital wastewater especially to small health centers and hospitals that cannot afford the establishment of their own water treatment facility.

Keywords: Bacteriophage, Hospital Wastewater, sewage, bacteria, virus

1. INTRODUCTION:

Wastewater generated by hospitals is described as a significant source of environmental contamination loaded with pathogenic microorganisms, partially metabolized pharmaceutical drugs, radioactive elements, and toxic chemical substances. Hospital wastewater contains increased amounts of antibiotics, disinfectants, by-products of hospital operations, and patient treatment, which results in a higher incidence of multi-drug resistant or even chemical disinfectant-resistant bacteria. Hence, indiscriminate discharge of hospital wastewater is considered an essential sanitation problem and a mode of transmission of diseases.

Often, this wastewater is discharged to septic tanks or allowed to flow through canals without any pre-treatment. In urban areas, however, hospital wastewater is discharged to municipal wastewater treatment plants (WWTP). But the wastewater is not properly treated by these management facilities because they are not designed to remove persistent pharmaceuticals. Also, hazardous wastewater may spread during flooding and may contaminate bodies of water.

Conventional chemical-based methods for treatment of sewage and wastewater such as chlorination, ozonation, and ultraviolet (UV) irradiation have their negative effects on the treated water. Therefore, all hospital wastewater treatment plant effluents contain residues of pharmaceuticals due to their inefficient removal in the conventional treatment systems.

There are many challenges in disinfecting hospital wastewater. In the Philippines, the Department of Health (DOH) is encouraging hospitals to undergo pre-treatment of their water wastes before connecting existing sewage lines to the provincial/municipal agencies in charge of water supply and sewage.

In spite the fact that current research has developed some ways to treat wastewater generated by human consumption, there is an urgent need to develop practical, cost-effective and environment-friendly antimicrobial treatments for the inactivation of pathogenic microorganisms in water. This reality is justified by the recognition that indiscriminate disposal of hospital wastewater is a critical emerging public health problem.

In a study conducted by Periasamy and Sundaram (2013), they used phages isolated from sewage water to reduce the pathogen population in wastewater. It turned out that the inoculated bacteria were 100% lyzed by phages after 14 hours of incubation. Inspired by this research, the researchers conducted this study to determine the efficacy of bacteriophages in treating hospital wastewater of pathogenic bacteria.

The use of water in hospitals and health care facilities is particularly important since the operation of these enterprises is water-intensive. As such, one of the most complex compliance issues facing them involves meeting effluent standards. The increased enforcement of discharge regulations under the Philippine Clean Water Act (Republic Act 9275) is requiring many hospitals and healthcare facilities to initiate the reduction and recycling of their wastewater.

In today's climate, however, most primary and secondary hospitals do not have a proper wastewater treatment facility to meet the effluent standards set by regulatory agencies. As a consequence, they discharge untreated or partially treated wastewater into receiving water bodies. To their point of view, the installation of a wastewater treatment facility in an additional operating cost without a return of investment (ROI). The so-called business mentality of no ROI poses a gigantic challenge to the industrial world in keeping their business afloat. These hospitals need to demonstrate responsible environmental management to the consumers and stakeholders for them to be locally and globally competitive.

This is the driving force that propelled the researcher to investigate the use of viruses to eliminate pathogenic bacteria generated from hospitals and healthcare facilities' operation. The investigator hopes to develop a practical, cost-effective, and environment-friendly method of managing hospital wastewater through the use of bacteriophage therapy.

This study determined the potential of bacteriophage therapy in treating hospital wastewater. Specifically, it identified the bacteria isolated from the hospital wastewater, determined the concentration of the phage lysate that can effectively destroy the pathogens in the hospital wastewater, and determined if significant difference exists on the effect of the different phage lysate concentrations on the growth and development of the pathogens isolated from the hospital wastewater.

On Hospital Wastewater

Hospital use of water varies from around 400 to 1200 liters per bed per day depending on the type and location of the hospital (Laber et al., 1999). This fact leads to the production of a significantly large volumes of wastewater per day.

The saprophytic bacteria from the soil and atmosphere, the water employed in hospital and patient and hospital staff excreta influenced the composition of the microbial flora of hospital wastewater (Nunez and Moretton, 2007). Once these microbiotic flora reach hospital wastewater, they are exposed to many chemicals such as pharmaceuticals, disinfectants, radionuclides, solvents used in hospital operations and many non-metabolized drugs passed in patient's excreta, especially antibiotics (Meirelles-Pereira, et al., 2002; Baquero, et al., 2008). These chemicals act as a selective pressure for the development of antimicrobial resistance. Bacteria exposed to disinfectants routinely used in the hospitals such as, glutaraldehyde, may be less sensitive to that disinfectant compared to other type or strain.

Hospital practices of wastewater treatment and handling vary widely. Four possible options exist for hospital wastewater treatment: untreated discharge to the environment, discharge into a municipal water treatment plant, on-site wastewater treatment before release to the environment, and on-site waste water treatment before discharge into a municipal water treatment plant (Pauwels and Verstrate, 2006).

While there are no formal studies conducted investigating hospital wastewater treatment practices in the Philippines, we infer a few conclusions from studies made on municipal wastewater and health care waste disposal in general. Although there are international standards regulating hospital effluent contents and the Philippine Healthcare Waste Management Manual recommends that all hospitals should have their wastewater treatment plant, this is not strictly enforced since there are no legislative or executive regulations justifying its implementation (Molina, 2002). Only a few tertiary and specialized hospitals across the country have their wastewater treatment facilities (Cruz et al., 2014). Many hospitals are, likewise, not registered as hazardous waste generators required by Republic Act 9003. Overall, public policy has not been responsive to this potential problem, even if some studies have indicated the need to address this issue, up to requiring hospitals to have their treatment plants before releasing their wastewater to the municipal wastewater system (Pauwels and Verstrate, 2006).

On Pathogens Found in Wastewater

Wastewater generated by hospitals is described as an important source of environmental contamination by pathogenic microorganisms such as enteric viruses and antibiotic resistant bacteria, such as *Pseudomonas aeruginosa* (Chitnis et al., 2000; Fuentefria et al., 2011). Hence, discharge of hospital wastewater is considered a significant sanitation problem and a mode of transmission of pathogens. This condition is exacerbated if the pathogens in question are transmitted through fecal-oral route, such as *Vibrio cholerae* (Sozzi et al., 2015). There are many challenges in disinfecting hospital wastewater since it contains increased amounts of antibiotics, pharmaceuticals, disinfectants, by-products of hospital operations and patient treatment which results in a higher incidence of multidrug resistant and even chemical disinfectant-resistant bacteria (Chitnis et al., 2004).

The identification of bacteria is a careful and systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture. It produces benefits for many aspects of the research of microorganisms. Count of viable cells is important in measuring the number of bacteria in a sample. The bacteriological analysis like the number of bacterial colonies, number of total coliforms, and fecal coliforms are measured by standard plate count (SPC), most probable number (MPN) and fecal coliform count (FCC) respectively. The samples are also plated in specific media to isolate the potentially dreadful pathogens, gram staining, and subjected to further characterization to identify the organisms as per the standard procedures (Periasamy and Sundaram, 2013).

On Enterobacteriaceae

The Enterobacteriaceae is a huge family of bacteria consisting of gram-negative bacilli and coccobacilli. The organisms are frequently encountered in the clinical laboratory and are associated with infections of almost every area of the human body. These bacteria are found in soil, water, and on plants. Some are also considered to be resident flora of the gastrointestinal tract (GIT) of many animals and humans. Infections are associated with lapses in personal hygiene via the fecal-oral route, poor sanitation in impoverished countries, and colonization of the skin and respiratory tract of hospitalized patients. These bacteria are known to cause disease in poultry, livestock, fish, and vegetable crops in addition to being significant human pathogens.

Escherichia coli is a natural inhabitant of the lower gastro-intestinal tract of humans and animals and is normally found in the stool. It is the most common clinical isolate in the Family Enterobacteriaceae, which also makes part of the coliform bacteria. These coliform bacteria are used as a bacterial indicator of sanitary quality of foods and water. They are rod-shaped, gram-negative, non spore-forming bacteria which can ferment lactose with the production of acid and gas at 35^0C to 37^0C. The presence of Escherichia coli in water indicates fecal contamination and may indicate the possible presence of disease causing pathogens, such as bacteria, viruses, and parasites. Although most strains of Escherichia coli are harmless, certain strains such as E. coli 0157: H7 may cause illness such as severe bloody diarrhea, stomach cramps and even urinary tract infections (Colom et al., 2015). In general, an increased level of fecal coliforms provides a warning of failure in water treatment. Fecal coliform bacteria can enter rivers through direct discharge of waste from mammals, birds, and from human sewage.

Klebsiella pneumoniae is a Gram-negative, non-motile bacteria with a prominent polysaccharide capsule. When cultured, this species produces distinctive yeasty odor, and bacterial colonies have a viscous/mucoid appearance. It is commonly found in the human digestive tract and part of the natural microflora and is often the cause of hospital acquired or nosocomial infection involving the urinary and pulmonary systems. Because of the presence of capsule, this pathogen possesses many virulence factors that allow it to go undetected by the host's immune system and cause infection in a variety of ways. This bacterium produces an enzyme called carbapenemase, which makes it resistant to drug carbapenem. Its habitat is not limited to humans but is ubiquitous to the ecological environment, which includes surface water, sewage, and soil.

Pseudomonas putida is a rod-shaped, flagellated, gram-negative bacterium, found in most soil and water habitats where there is oxygen. It grows optimally at 25-30 degrees Celcius and can be easily isolated. *Pseudomonas putida* is a versatile bacterial species adapted to the soil and its fluctuations. Like many other species living in soil, *P. putida* often faces water limitation. Alginate, an exopolysaccharide (EPS) produced by *P. putida*, is known to create hydrated environments and alleviate the effect of water limitation (Gulez et al., 2014).

On Bacteriophage

Bacteriophages, also called phages, are viruses. These are the obligate intracellular organisms which infect bacteria, seize their replication machinery, replicate into thousands of new progenies, and lyse the cell for escape. Phages are the most abundant entities on earth and help in regulating microbial balance in the environment (Mulani et al., 2015).

Like all viruses, the genome of bacteriophages consists of nucleic acid surrounded by a protein coat, capsid. Phages range in size from 24-200 nm in length. T4 is among the largest phages; it is approximately 200 nm long and 80-100 nm wide. All phages contain a head structure, which can vary in size and shape. The head encloses the nucleic acid and acts as the protective covering. Some of the phages have tails attached to the head of the phage. The tail is hollow tube where the nucleic acid passes the infection. T4 tail is surrounded with contractile sheathe, which contracts during infection of the bacterium. At the end of the tail, T4 phages have a base plate and one or more fibers that are attached to it. The base plate and tail fibers bind the phage to the bacterial cell. Not all phages have base plates and tail fibers.

The T4 bacteriophage is a type of virus that commonly infects bacteria known as *E. coli*, which is one of the most studied bacteria in the field of molecular biology. The T4 bacteriophage belongs to a family of viruses known as T-phages, also referred to as lytic phages because they always lyse and kill the host bacterium. The bacteriophage uses the host cell to replicate and make more bacteriophages. It utilizes the bacterial cell's machinery to multiply and replicate, and lyzes the bacterial cell to release newly formed viruses. The new viruses will infect other *E. coli* and repeat the cycle over again. Phages are bacteria-dependent. Specific virus will infect a particular bacterium. They also evolve and mutate in order to kill bacteria. Phages attack only specific bacterial host cell thus reducing damage in environment of microbes.

On the Lifecycle of Bacteriophage

Phages are classified as temperate or virulent. Virulent phages involve replication of new phages inside the host, therefore, resulting in lysis, whereas temperate phages also do this, but they can incorporate their genomes into the host and changes the virulence. This process is called lysogenic conversion.

Phages usually follow one of two lifecycles: lytic (virulent) or lysogenic (temperate). Lytic phages take over the machinery of the bacterial cell to make phage components. They then destroy or lyse the cell releasing new phage particles. Lysogenic phages incorporate their nucleic acid into the chromosomes of the host cell and replicate with it as a unit without destroying the cell. Under certain conditions, lysogenic phages can be induced to follow the lytic cycle.

Lytic bacteriophages (as opposed to lysogenic bacteriophages) have remarkable bactericidal property. However, translating this potential into the actual application has had to hurdle numerous obstacles. The pharmacodynamics of any bacteriophage treatment is somewhat intricate, due to the ability of bacteriophages to multiply. Hosts have a significant impact as well; sometimes a mixture of bacteriophages is used to maximize the chances that the target bacteria will indeed be infected (Chan et al., 2013). Induction of resistance to bacteria phage is a major limiting factor in deploying bacteriophages. However, bacteriophages also constantly evolve to circumvent host defenses, and bacteria that have developed resistance to bacteriophages are often less viable or less virulent (Connerton & Connerton, 2005). The virus will either enter the lytic or the lysogenic cycle. When the virus enters its host bacterium, it is adsorbed to the cell wall surface. The virus will then inject its genetic material into the host.

On Phage Therapy

Many sewage waste treatment systems is aiming for complete pathogen removal, which necessitates the search for novel approaches that does not harm the environment. One such innovative approach is exploring the possibilities of bacteriophages for pathogen removal (Periasamy and Sundaram, 2013).

Due to the emergence of pathogens and increased antibiotic resistance among existing ones, meaningful research is essential in looking for new avenues of treatment and prevention of infections in humans, animals, and plants. One of these avenues includes bacteriophage therapy, the utilization of bacteria-targetting viruses in the treatment and prevention of bacterial infections. They can be isolated from all reservoirs where bacterial hosts are present, such as in the soil, sewage, sea water, or even in the intestines of animals. As compared to other viruses, phages are detected by simple, inexpensive, and rapid procedures.

Bacteriophage therapy is becoming more acceptable field of therapeutics. Since bacteriophages are highly specific to the bacteria they target, these are non-toxic to animals and plants alike. Moreover, due to the inherent characteristics of bacteriophages, they multiply faster in situations where there is a large number of bacteria present. These qualities make them highly ideal in combating bacterial infections. Phage therapy is also known as biocontrol, simply because phages are organisms that control other organisms which are the bacteria. Hence, they are called bacteriophages. Bacteriophages are considered ideal as antibacterial agents because they are highly specific, more effective, and does not illicit resistance.

Many studies have been made describing the application of bacteriophage therapy in ensuring food safety, especially in chickens (Atterbury et al., 2007, fresh-cut fruits and processed foods (Huff et al, 2006). Phage therapy has also been applied to cultured fish in aqua culture and corals (Efrony et al., 2007). Likewise, several studies have also been done on humans but regulatory approval has not been forthcoming due to several problems about its application (Sulakvelidze et al., 2001).

Although current research has developed some ways to treat wastewater generated by human consumption, there is a need for, and interest in, developing practical, cost-effective and environment-friendly antimicrobial treatments for the inactivation of pathogenic microorganisms in water. This is due to the recognition that disposal of hospital wastewater is an important emerging public health problem. Bacteriophage has proven to be beneficial to health and the environment in many ways. Hence it can be evaluated in this study.

2. Materials and Methods

Research Design

The study utilized the experimental research design. The different steps involved in the experiment were: (1) collection and characterization of the wastewater; (2) isolation, quantification, identification and culture of pathogens and bacteriophages; (3) electron microscopy; (4) preparation of culture media using different phage lysate concentrations; and (5) determination of the effects of the bacteriophage on the growth and development of pathogens in the wastewater.

Collection of Hospital Wastewater

The specimen was collected from a secondary hospital in Ilocos Sur, using three 1-liter sterile and leak-proof glass bottles. The samples were brought to the College of Health Sciences, University of Northern Philippines laboratory for processing after collection.

Isolation and Culture of Pathogens

Ten (10) sterile tubes were labelled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, to 10^{-10}, which indicated their dilution factor. To each test tube, nine (9) mL of Tryptic Soy Broth (TSB) was transferred. One (1) mL of the hospital wastewater was added to the first tube, mixed and an mL of the mixture was transferred to the second tube. The serial dilution continued until the 10^{th} tube. To maintain the same volume, one (1) mL from the last tube was discarded. All 10 tubes were mixed thoroughly to obtain a homogenous mixture.

Five hundred (500) microliters from each diluted test tubes was transferred to each properly labelled plate of the Nutrient Agar after the serial dilution. The plates were then covered with nutrient agar and incubated overnight at 37^0C inside an incubator. After the incubation period, the colonies were differentiated based on their color, morphology, and texture. Likewise, Gram's staining was performed to observe the morphology. The VITEK 2 ID machine confirmed the identity of the bacteria isolated.

Collection, Isolation and Purification of Phages

The phages utilized in the experiment were collected from the UNP lagoon. A 200 mL samples were collected from four (4) different sites using four (4) sterile Erlenmeyer flasks. The water samples were brought to the laboratory, where they were mixed. The homogenized sample was filtered, transferred to a test tube, and centrifuged at 3,500 rpm for 30 minutes. One (1) mL of the supernatant was aseptically transferred in an Erlenmeyer flask containing 48 mL of TSB together with 1 mL of the known isolated 5-hour bacterial suspension from the culture of the confirmed pathogen. The electronic shaker mixed the solution 24 hours. After the 24 hour mixing, representative of the mixture was taken up into a 5 mL syringe. From the 5 mL syringe, a 0.44 microns pore sized filter was attached and filtered in a sterile tube. Then it was filtered again using a 0.22 microns pore sized filter into another test tube. The filtrate set the final filtered TSB containing phage.

Phage Forming Assay Using Soft Agar Overlay

The researchers utilized the method described by Wilson & Artkinson (2000) for the isolation, purification, and determination of phage titer. One (1) mL of the ultrafiltrate was serially diluted from 10^{-1} to 10^{-10} in nine (9) mL TSB. To determine the phage lysate concentration, the researcher considered the one mL of the ultrafiltrate as 100% and come up with the following lysate concentrations: (1) 25% = 250 µL, (2) 50% = 500 µL, (3) 75% = 750 µL, (4) 100% = 1,000 µL or 1 mL.

Each phage lysate concentration was diluted in the corresponding amount of TSB to be able to get a total solution of 1 mL phage lysate in preparation for the 10-fold serial dilution and was incorporated with 1 mL of hospital wastewater in a 3 mL soft trypic soy agar (TSA) maintained at 45^0C. It was thoroughly mixed before adding on the surface of the pre-heated TSA. These plates were allowed to solidify and then inverted and incubated at 37^0C for 18-24 hours.

Phage Counting Technique

Four representative squares were counted and multiplied to a factor of 65 to get the phage count. Plaque formation was calculated using the following formula:
Plaque forming unit (pfu/mL) = number of plaques/d (v) x 100; where d is the dilution, v is the volume of the diluted virus added to the plates.

Transmission Electron Microscopy (TEM)

The morphological and internal characteristics of the bacteriophage were determined using the TEM. Collection and isolation of phage were done by getting a portion of agar with plaques and placed it in five mL of TSB then centrifuged for 10 minutes. The supernatant

was separated in a clear and sterile tube and sent to the Research Institute for Tropical Medicine for visualization.

Statistical Analysis

A One Way Analysis of Variance (ANOVA) was employed to determine the significant difference on the effect of the different phage lysate concentration data derived from the experiment.

3. RESULTS AND DISCUSSION

The Pathogenic Bacteria Isolated in the Hospital Wastewater

Three pathogenic bacteria were isolated from the hospital's wastewater. These were *Klebsiella pneumoniae, Pseudomonas putida* and *Escherichia coli* (Extended spectrum beta lactamase positive). These isolates were known clinically to be pathogenic. The presence of these pathogenic bacteria in the hospital's wastewater is an indication that the wastewater is not treated before discharged.

The isolation of *Pseudomonas putida* and *Klebsiella pneumoniae* in hospital wastewater was congruent to other the studies. The emergence of drug and carbepenen-resistant *Pseudomonas putida* causes the problematic treatment of nosocomial infection in severely ill patients. *Escherichia coli*, especially Extended spectrum beta lactamase-positive, is known to cause many diseases. According to Wenke (2001), diarrheal diseases remain to involve in increasing morbidity and mortality worldwide. Even *Escherichia coli*, a normal intestinal flora, was found to adopt and obtained genetic material which make them pathogenic and harmful. *Klebsiella pneumonia*, which is known to cause different respiratory infections, was found in hospital wastewater (Morris et al. 2016). These bacteria can be a secondary invader in bronchiectasis, influenza and worst, tuberculosis, even though it comprises a small percentage in pneumonia reported cases.

Optimum Concentration of the Phage Lysate

Table 1 presents the phage forming unit per milliliter (pfu/mL) of *Escherichia coli*. It shows that the higher the concentration of the lysate added into the culture medium, the higher is the plaque formed in the plates. The highest was exhibited at 100% lysate of *Escherichia coli*, producing 1.481 x 10^4 pfu/mL (14,810) and the lowest was the 25% lysate (6.325 x 10^3 pfu/mL or 6,325).

Table 1. Plaque Forming Assay for *Escherichia coli*

Concentration of Phage Lysate	Trials			Mean (pfu/mL)
	1	2	3	
100%	$1.497x10^4$	$1.4930x10^4$	$1.455x10^4$	$1.481x10^4$
75%	$1.118x10^4$	$1.064x10^4$	$1.093x10^4$	$1.092x10^4$
50%	$7.688x10^3$	$7.378x10^3$	$8.098x10^3$	$7.721x10^3$
25%	$5.920x10^3$	$7.161x10^3$	$5.894x10^3$	$6.325x10^3$

Table 1 further illustrates that the higher the lysate concentration, the greater is the plaque formation. There was a positive agreement between the pfu/mL and phage lysate concentration, indicating that their relationship is directly proportional. The graph below best illustrates the data presented in the table:

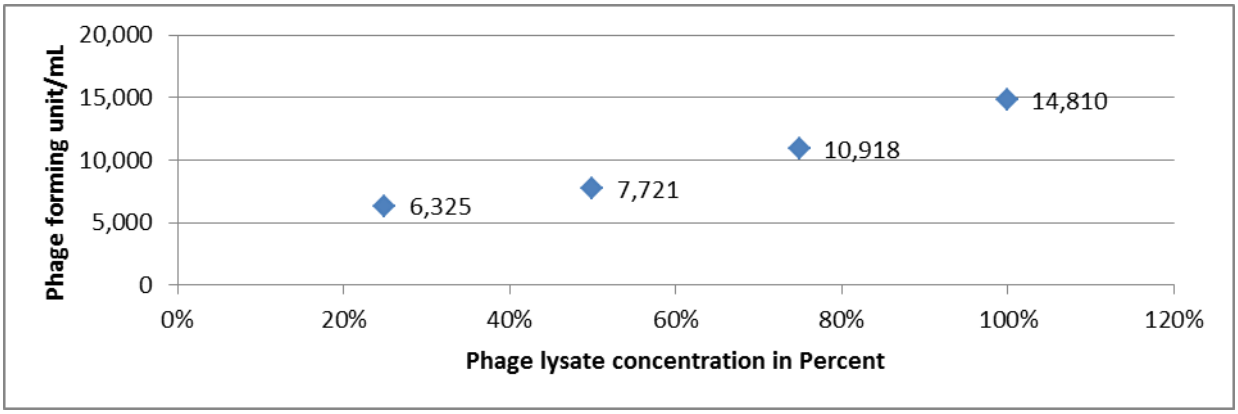

Figure 2. Graphical presentation of the Phage Forming Assay for *Escherichia coli*

This phage forming assay result of *Escherichia coli* is similar to the result of the study made by Periasamy and Sundaran (2013), where the isolated bacteriophage acts specifically on *Escherichia coli*. The study focused on the isolation and characterization of the bacterial species and phages to develop a new strategy for the control of bacterial pathogens in wastewater. Bacteriophages were tested for the biocontrol of *Escherichia coli*. The target pathogens were inoculated separately as well as with specific bacteriophages, and time course study was done to determine the survival rate of the pathogens (Periasamy and Sundaran, 2013).

Table 2 below shows the different amounts of pfu/mL of phage lysate at various concentrations on *Klebsiella pneumoniae*. The highest number of pfu/mL lysate was found at 100% phage lysate concentration ($6.307 \times 10^{3)}$ and the lowest was at 25% ($2.385 \times 10^{3)}$. It clearly shows that the higher the phage lysate concentration, the higher is the formation of phage in the agar plate.

Table 2. Plaque Forming Assay for *Klebsiella pneumoniae*

Concentration of Phage Lysate	Trials			Mean (pfu/mL)
	1	2	3	
100%	6.307×10^3	6.409×10^3	6.204×10^3	6.307×10^3
75%	4.262×10^3	4.740×10^3	4.170×10^3	4.391×10^3
50%	3.263×10^3	2.949×10^3	3.304×10^3	3.172×10^3
25%	2.602×10^3	2.5421×10^3	2.010×10^3	2.385×10^3

Figure 3 shows the graphical presentation of the phage forming unit for *Klebsiella pneumoniae*. The graph revealed that there was a positive agreement in the two variables. The pfu/mL is linear with the phage lysate concentration. The relationship between the two is directly proportional, which means that as the phage lysate concentration increases, the pfu/mL also increases. The destruction of *Klebsiella pneumoniae* by the virus or phage was manifested by the plaques formed on the surface of the culture medium.

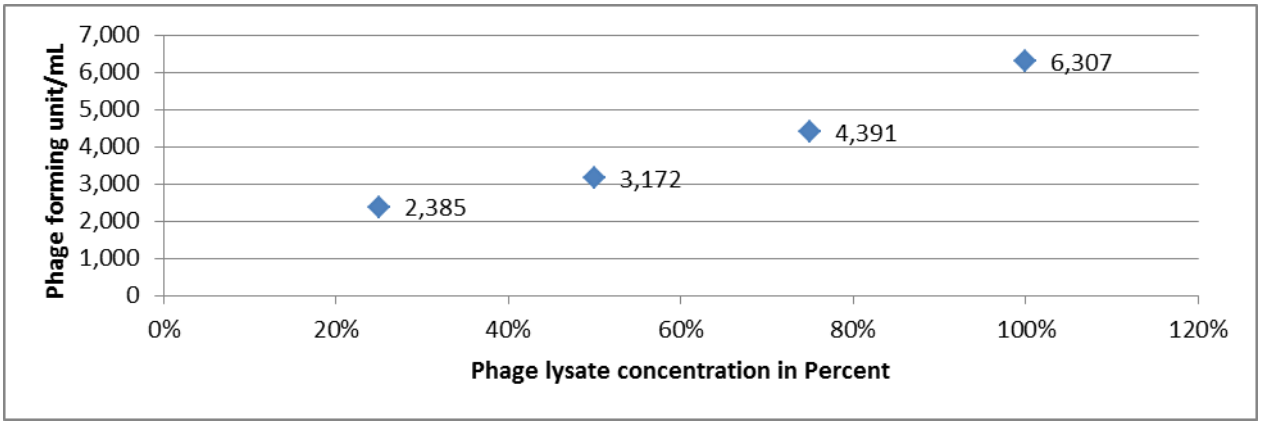

Figure 3. Graphical presentation of the Phage Forming Assay for *Klebsiella pneumoniae*

A similar study was conducted by Kumari et al (2010) using sewage samples from commercial establishments. Each of the bacteriophage isolated was found to be specific against *Klebsiella pneumoniae* strain. In another study, bacteria were isolated anaerobically from a wastewater sample, and the filtered effluent was screened to isolate bacteriophages. A lytic phage was then isolated and was found to belong to the *Klebsiella pneumonia* strain (Hoyles et al., 2015).

Table 3 shows the mean pfu/mL of *Pseudomonas putida* at various lysate concentrations. Similarly, the lysate concentration is directly proportional to the pfu/mL, i.e., the higher the lysate concentration, the greater is the number of plaque forming unit/mL. The 25% produced the least pfu/mL ($3.160x10^3$) while the 100% generated the highest number of pfu/mL ($5.891x10^3$).

Table 3. Plaque forming Assay for *Pseudomonas putida*

Concentration of Phage Lysate	Trials			Mean (pfu/mL)
	1	2	3	
100%	$6.065x10^3$	$5.095x10^3$	$6.514x10^3$	$5.891x10^3$
75%	$5.036x10^3$	$4.201x10^3$	$5.074x10^3$	$4.770x10^3$
50%	$4.915x10^3$	$3.477x10^3$	$4.186x10^3$	$4.193x10^3$
25%	$4.007x10^3$	$2.281x10^3$	$3.194x10^3$	$3.160x10^3$

Figure 4 exhibits a positive agreement between the concentration and pfu/mL variables. The relationship between the phages lysate concentration and mean pfu/mL is directly proportional.

The result is in agreement with the study of Soleiman-Delfan (2012), when *Pseudomonas putida*, a known plant antagonist against plant's bacterial pathogens, was employed for bacteriophage enrichment regime.

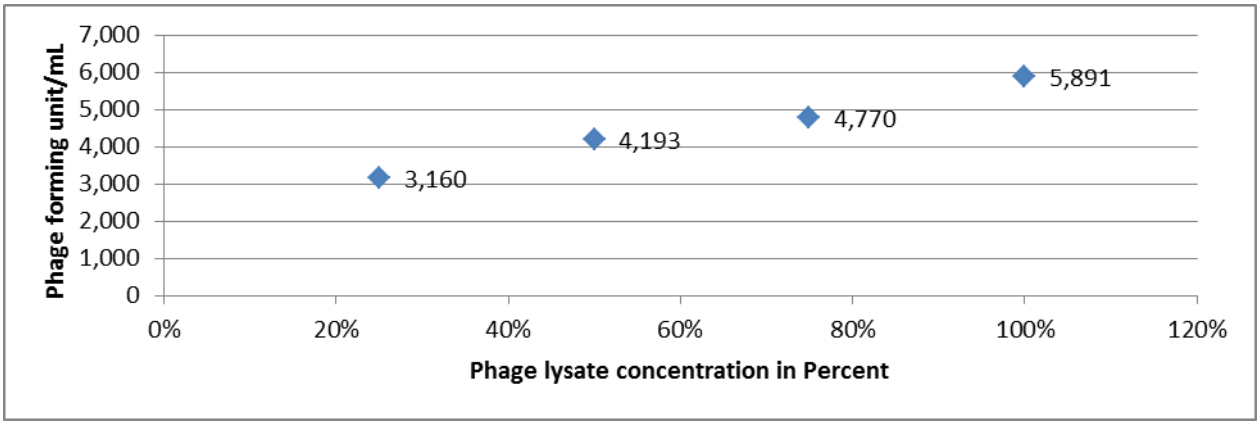

Figure 4. Graphical presentation of the Phage Forming Assay for *Pseudomonas putida*

The data above prove that the 100% phage lysate concentration yielded the most number of plaques. The same data indicate that the virus destroyed more pathogens during the lytic stage. The data also provide input on the aggressive action of bacteriophage against *E. coli* than that of *P. putida* and *K. pneumoniae*, as shown in a large number of pfu/mL on the culture plates.

In contrast with the control, the lawn of bacteria remained intact and did not have any clearing zone. The pfu is believed to be an in-vitro predation capacity of bacteriophage against *E. coli, P. putida,* and *K. pneumoniae*. The ESBL *E. coli* which could be multi-drug resistant have not escaped the lytic action of the bacteriophage, indicating a non-effect of its property against bacteriophage.

Moreover, these data could serve as bases for using bacteriophage as a disinfectant agent for hospital contaminated water against antibiotic resistant bacteria. *Klebsiella* spp., *Pseudomonas* spp., and *Escherichi coli* are the most frequently identified bacteria in the hospital wastewater according to Moges et al (2014). These findings are likewise consistent with the various studies made on the antibiotic-resistant bacteria such as beta lactamase-producing Enterobacteriaceae, tetracycline resistant *Salmonella enterica* (Guillarme et al., 2011) and multi-drug resistant *Pseudomonas aeruginosa* (Fuentefria et al., 2011) which are commonly isolated in hospital wastewater even after treatment. In another study, Nunez and Moretton (2007) isolated bacteria with reduced susceptibility to disinfectants such as chlorhexidine and povidone-iodine from hospital wastewater. These results are consistent with the well-documented finding that increased antibiotic consumption leads to the emergence and increase in bacterial population with antibiotic resistances, especially considering that due to the large volume present, the antibiotics in wastewater are present in sub-inhibitory concentrations (Davies, 2016; Auerbach et al., 1983).

Areas handling hospital wastewater such as municipal treatment plants are considered very good environments for the evolution of antibiotic resistance in bacteria due to the presence of large amounts of nutrients able to sustain a high bacterial growth rate coupled with repeated seeding with both antibiotics and antibiotic-resistant bacteria (Iverson et al., 1799). The findings of Jun et al. (2016) had shown similarity to this study when they used the bacteriophage which was isolated from a sewage system and discovered that it could infect and destroy a waterborne pathogen.

Morphological Characteristics of Isolated Phage

The phages that were isolated from the sewage water was an Enterobacteriophage T4-like. Enterobacteriophage T4 belongs to a family of Myoviridae. Bacteriophage T4 is 86 nanometer wide, 120 nanometers long and has an elongated icosahedral head. The T4 phage is a huge phage and has a tail that can inject a nucleic acid into the host cell that it is infecting

and targeting specific host. The tail consists of fibers that help the phage in recognizing the host cell and determine its phage host range. T4-like phages contain a contractile tail and intricate base plate that has six (6) long fibers that branch-out from it, and 90% grows in *Escherichia coli* and other members of the Enterobactericeae family (Comeau, 2009).

Three types of bacteriophages were isolated from the UNP hospital lagoon. Accordingly, these phages are members of Myoviridae family and could infect and lyze bacteria that belong to the Enterobacteriaceae family such as *E. coli, P. putida,* and *K. pneumoniae.*

The results of the research are congruent with the studies of Klausa et al. (2003), where they found out that wastewater and sewage water contain the highest population of coliphages. They also observed that 37^0C was the optimum plaque forming temperature for wastewater and 30^0C for sewage water.

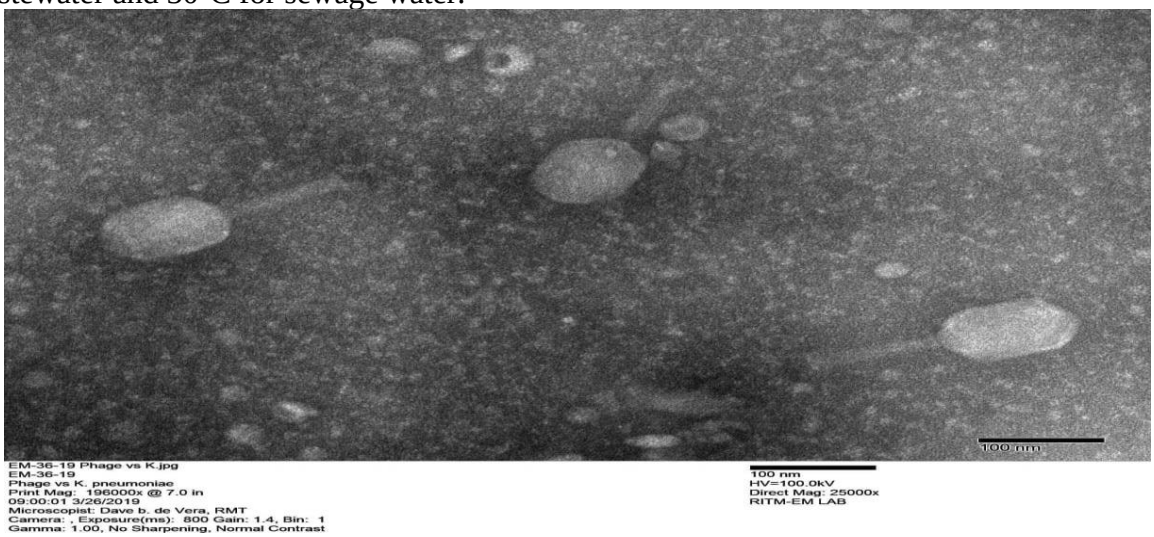

Figure 5. Bacteriophages isolated from the pond examined under the Electron Microscope.

4. LEVEL OF SIGNIFICANCE

One Way Analysis of Variance (ANOVA) shows that there is no significant difference on the different concentrations on phage lysate used against *E. coli, K. pneumonia*, and *P. putida* since the critical p value (2.87) is greater that 0.05 (p=2.87>0.05) (Table 4). The null hypothesis was, therefore accepted. This result means that the number of phages present in every concentration of phage lysate was enough to kill an enormous number of pathogenic bacteria in the wastewater. The different concentrations of the phage lysate can disinfect the hospital wastewater due to its high specificity and easy adaptation to its environment.

Table 4. Differences on the Effect of the Different Concentrations of the Phage Lysate on the Growth and Development of *Pseudomonas putida, Escherichia coli*, and *Klebsiella pneumoniae*

Groups	Df	Critical Value (α 0.05)	Statistics F	Analysis	Decision
Phage lysate in different concentrations on *P. putida*	3/36	2.87	0.20	Not Significant	Accept Ho
Phage lysate in different concentrations on *E. coli*			0.23	Not Significant	Accept Ho
Phage lysate in different concentrations on *K. pneumoniae*			0.31	Not Significant	Accept Ho

5. ANTIBACTERIAL PHAGE DISINFECTION AGENT

The result of the study reveals that the 100% phage lysate concentration produced the most number of plaque forming units per milliliter. However, statistically, there is no significant difference between the concentrations used in the experiment. Thus, it is suggested that any of the four concentrations (25%, 50%, 75%, and 100%) can be used as a hospital wastewater disinfectant agent formulation.

It is likewise recommended that the phage lysate shall be prepared based on the procedure stated in this study. Twenty five (25) millilitres of the 25% phage lysate is applied to one (1) liter of hospital wastewater. However, if higher phage lysate concentration is used, greater volume of the hospital wastewater is recommended.

6. CONCLUSION

The presence of bacterial isolates in hospital wastewater confirms that the current treatment process of hospital management is not effective in destroying these clinically infectious pathogens found in the wastewater generated by the hospital.

This study proved that the treatment of hospital wastewater could be achieved by a bacteriophage as a biocontrol agent. Twenty five percent (25%) of the phage lysate is as efficient as 50%, 75%, and 100%.

The bacteria that were isolated from the hospital wastewater were all belonging to the Enterobacteriaceae family while the phages that were isolated from the sewage water were believed to belong to the Myoviridae family. These Myoviridae phages are specific mostly to those bacteria under the Enterobacteriaceae family.

Therefore, this study proves that the bacteriophages isolated from the stagnant pond water can be efficient in controlling the bacterial pathogens that were present in the hospital wastewater. Regardless of the phage lysate concentration, it would be efficient in the treatment of the hospital wastewater.

7. RECOMMENDATIONS

For the further direction of the study, the researcher recommends that (a) further isolation and identification of other strains of bacteria present in the hospital wastewater be conducted; (b) further isolation, identification, and characterization of bacteriophage in sewage water be performed; (c) genomic sequencing of the bacteriophages isolated for identification purposes, promotion in an advanced research, and future use of phages in hospital wastewater management; and (d) determination of the specific host range of bacteriophages among target bacterial cells should be conducted.

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