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Isolation and Host Range Analysis of Bacteriophages Against Multidrug-Resistant *Escherichia* coli

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Abstract

Due to the emergence of pathogens that are resistant to antibiotics, antibiotic crisis is one of the biggest problems facing the world's population. Therefore, attention is now being directed towards investigating different approaches of treating bacterial infections. The bacterium's natural predator, the bacteriophage, is one of those options. This study aimed to identify the host range capacity of bacteriophages and separate them from various sewage samples that can lyse drug-resistant clinical bacterial isolates. The isolation of bacteriophage was done by Double Layer Agar Assay (DLAA) from 4 different sewage samples i.e. sewage sample from tertiary care hospital balkhu, tertiary care hospital nakhu, Chovar sewage outlet and Nayabazar sewage. For the isolation of phage, host organisms used were Multi Drug Resistant (MDR) *Acinetobacter* spp., *Pseudomonas* spp. and *Escherichia coli* resistant to Cefotaxime, Ciprofloxacin, Gentamicin, Imipenem, Amoxyclav, Ceftazidime. The plasmid extraction, 16S RNA analysis with PCR and Gel Agarose Electrophoresis, was done. Phage was isolated against *Escherichia coli* from both the hospitals sewage samples. Phage was also isolated against *Pseudomonas* spp. from Chovar sewage outlet but could not be sub-cultured because of its temperate properties. Phage could not be isolated against *Acinetobacer* spp. The host range analysis of *Escherichia coli* phages showed that the phages has narrow host range as they formed plaques against only one bacterium i.e. *Klebsiella pneumoniae*. Therefor, hospital sewage is probable place to find bacteriophages that can lyse MDR bacterial strains. Despite the isolated bacteriophage's potential in bactericidal activity, more research is necessary to fully understand it's potential towards phage therapy.

Keywords: Antibiotic resistant, double layer agar assay, sewage, lytic, phage therapy

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Introduction

The recommended treatment for bacterial infections or illnesses is the use of antibiotics [1]. However, the issue of antibiotic resistance is getting worse every day, posing a serious risk to the health of people, animals, and the environment. As early as 1945, Sir Alexander Fleming issued a warning against the overuse of antibiotics [2]. Consequently, there is a huge need to create innovative substitutes. Among the most promising substitutes are bacteriophages.

Bacteriophages are bacterial viruses that operate as the bacteria's natural predators, making them a bacteria-specific weapon [3]. It is well known that they can only infect and multiply in bacterial host cells. Their biological cycle is typically used to categorizes them as lytic or virulent, lysogenic or temperate bacteriophages. While the phage genome merges with the bacterial genome and remains in a dormant state throughout the lysogenic cycle, the lytic cycle involves the active generation of viral particles [4].

It is generally accepted that phage isolation is a straightforward technique that involves combining a phage-containing sample with its host bacterium and then removing the bacterial cells using a centrifugation or filtration method. Since its creation by Felix d'Herelle, the basic technique for bacteriophage separation has not changed [5]. First, the bacterial cell will expand to form a confluent lawn of cells when combined with the bacteriophage in soft agar. Plaques are clear patches in the confluent grass that are created when the phage attacks the cells, producing lysis. It is believed that each plaque is created by a single virion that replicates and lyses the bacterial cells [6].

Because they are host specific, bacteriophages are incredibly accurate and exclusively target a certain type of bacteria. Thus, phage therapy functions as a biomedical smart bomb, eliminating a particular illness

in patients without harming healthy flora [7]. Compared to conventional chemotherapy, phage therapy has a number of benefits, including bactericidal action, auto-dosing, but finding the right phage for phage therapy is difficult [8].

The world is currently grappling with the issue of antibiotic resistance. Therefore, the purpose of this study was to investigate whether it is feasible to separate bacteriophages from sewage and water samples in order to combat clinical isolates that are resistant to several drugs. We also investigated whether those isolated phages have lytic activity against other types of bacteria.

Materials and methods

Bacterial Test Organisms: Three bacterial test organisms (1 strain of *Escherichia coli,* 1 strain of *Psesudomonas* spp., 1 strain of *Acinetobacter* spp.) were obtained as bacteriophage hosts and 10 different Multi-Drug Resistant (MDR) bacterial samples were obtained for host-range analysis from the Phage Bank of Central Department of Biotechnology, Tribhuvan University.

Sample Collection: Sewage **s**amples were collected from tertiary care hospital balkhu, tertiary care hospital nakhhu, Nayabazar sewage, Chovar outlet sewage in sterile bottles by submerging about 5 cm inside the water body. Then containers were labelled and transported to laboratory in an ice box maintaining temperature at 4°C.

Antibiotic Susceptibility Test of Host Organisms: The antibiotics susceptibility test was carried out by using standard protocol of Kirby bauerer disc diffusion method and the zone of inhibition was evaluated following incubation at 37°C and compared to the standard chart [9].

Table 1: Antimicrobial activity of bacterial isolates towards different antibiotics

S. N	Antibiotics	*E. coli*
1	Amoxicillin	Resistant
2	Cefotaxime	Resistant
	Ciprofloxacin	Resistant
4	Gentamicin	Resistant
5	Meropenem	Sensitive
6	Imipenem	Resistant
7	Amoxicillin/Clavulanic acid	Resistant
8	Ceftazidime	Resistant
9	Norfloxacin	Sensitive
10	Nitrofurantoin	Sensitive

Isolation of Bacteriophage: The Double Layer Agar Assay (DLAA) method was used to isolate the bacteriophage. For that, sewage samples were centrifuged for 30 minutes at 4000 rpm in order to exclude additional cell debris and undesirable pollutants. In addition, a syringe filter was used to eliminate undesired materials and bacterial contaminations using a 0.22 µm membrane filter. The filtrate was collected in sterile falcon and kept at 4°C. After that, a sterile tube was filled with 1 ml of filtrate and 100 µl of the bacterial growth log phase, and the tube was left to stand for attachment. The tube was filled with three milliliters of Tryptic Soya Agar (TSA) (0.7% agar), combined, and then transferred into the TSA agar plate (1.5% agar). Plates were solidified and incubated for 24 hours at 37°C to check for any visible plaques [10].

Phage Lysate/Stock Preparation: Using the continuous streaking method, a single plaque was streaked across many TSA plates to amplify the phage. Following solidification, streaked plates were incubated for night. The plates with plaques were then filled with SM buffer, mixed and agitated. To remove any remaining debris, the mixture was centrifuged and soft agar was scraped off and moved to a falcon tube. Again, centrifugation was carried out by transferring the supernatant to another falcon tube using syringe filter. This filtrate serves as the phage stock [11].

Multi Host Range Analysis: DLAA was done for Multi-Host Range Analysis. Different bacterial cultures (100µl each) in log phase was mixed with 3 ml of soft agar in sterile falcon and poured on properly labeled TSA plates. Then, 5 µl of phage filtrate was added to each plate and incubated and checked for the presence of or absence of bacterial lysis and clear zone [12].

Bacterial Plasmid DNA Extraction: Alkaline Lysis Method was utilized in order to extract the plasmid DNA of bacterial isolates.

16S rRNA gene amplification and analysis: The appropriate primers were used for 16S rRNA amplification. By following the standard PCR techniques and Gel Agarose Electrophoresis, amplification of 16S rRNA was done.

Results

E. coli, Pseudomonas spp. and *Acinetobacter* spp. were used as host organisms in this study for the isolation of bacteriophages. These organisms were identified by microscopic observation and different biochemical tests by following standard microbiological protocol. The *E. coli* isolates were found to be Multi Drug Resistant. The details are in Table 1.

Table 2 : Isolation of Bacteriophage from different water sample against bacterial isolates

S. N	Water sample	Bacteriophage isolated against		
		MDR *E. coli*	MDR *Pseudomonas spp.*	MDR *Acinetobacter spp.*
1	Tertiary care hospital balkhu sewage	+ve	-ve	-ve
2	Tertiary care hospital nakhu sewage	+ve	-ve	-ve
3	Chovar sewage outlet	-ve	+ve	-ve
4	Nayabazaar sewage	-ve	-ve	-ve

Table 3: Multi-Host Range Analysis of Bacteriophage

S. N	Test organisms	Activity of *E. coli* phage
1	*E. coli* strains	-ve
2	*E. coli* (MDR)	+ve
3	*Klebsiella pneumonae*	+ve
4	*Pseudomonas*	-ve
5	*Salmonella*	-ve
6	*Shigella*	-ve
7	*Cholera*	-ve
8	*Bacillus*	-ve
9	*S. aureus*	-ve
10	*C. furendii*	-ve

Among the 4 different sewage samples, bacteriophage against *E. coli* were isolated from both hospitals' sewage samples i.e. tertiary care hospitals. Whereas bacteriophage against *Pseudomonas* spp. was isolated from Chovar sewage outlet which could not be further sub-cultured. Bacteriophage could not be isolated against *Acinetobacter* spp. from any sewage samples. The details are given in **Table 2**.

Bacteriophage of *E. coli* from tertiary care hospital balkhu sewage sample showed lytic activity against MDR *E. coli* and *Klebsiella pneumonae* obtained from Phage lab of Central Department of Biotechnology, Tribhuvan University. Host range analysis of phage isolated against *Pseudomonas* spp. from Chovar sewage outlet could not be conducted as those phages could not be amplified. The details of results are given in **Table 3.**

The result of bacterial plasmid extraction (MDR *E. coli*) and Gel Agarose Electrophoresis has been shown in the figure 1, Whereas the result 16S rRNA gene amplification and analysis showed 16s RNA band (MDR *E.* coli) against ladder on Gel Agarose Electrophoresis after PCR, has been presented in **figure 2**.

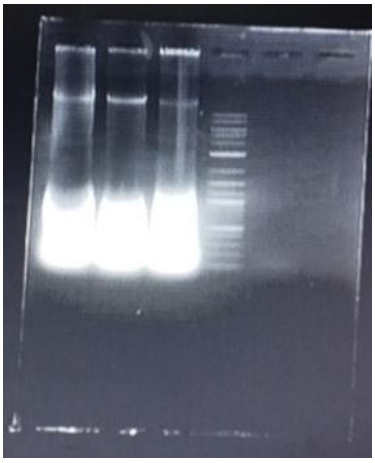

Figure 1: Band after bacterial plasmid extraction (MDR *E. coli*) and Gel Agarose Electrophoresis

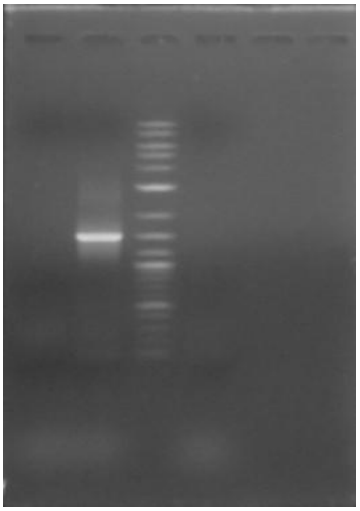

Figure 2: 16s RNA band (MDR *E. coli*) against ladder on Gel Agarose Electrophoresis after PCR

Discussion

In this study, host organisms (*E. coli, Pseudomonas* spp. and *Acinetobacter* spp.) were obtained from the Phage lab of Central Department of Biotechnology, Tribhuvan University. Sinks and other wastewater systems in hospitals and healthcare settings have been found to harbor antimicrobial-resistant *E. coli* and *Klebsiella* spp., especially when patient outbreaks of resistant strains have occurred. Numerous hospital sinks have been

shown to be heavily and persistently colonized with a variety of susceptible and antimicrobial-resistant strains of *E. coli*, *Klebsiella pneumoniae*. These strains may be a part of the transmission chains of these pathogen outbreaks, which can result in colonization and clinical diseases in patients [13]. Additionally, these organisms are most frequently linked to diseases related to healthcare [14].

The bacteria can employ antibiotics as a signaling mechanism with regulatory functions to evolve resistance whenever they are exposed to non-lethal levels of the drugs.

In his Nobel Prize acceptance address, Alexander Fleming also cautioned about this problem [2]. As a result, we can see that bacteria are multi-drug resistant. Depending on how different organisms are exposed to various medicines, we may potentially see distinct patterns of drug resistance within the same species.

Four different types of sewage samples were used for the isolation of bacteriophages to determine the best source for phage isolation. Among the sewage samples of tertiary care hospital balkhu, tertiary care hospital nakhu, Chovar sewage outlet and Nayabazar sewage, higher probability of discovering phage was from tertiary care hospital balkhu sewage (33%), tertiary care hospital nakhu sewage (33%) and Chovar sewage outlet (33%) and Nayabazar sewage (0%). This result can be attributed to the nature of the sewage samples which is medical sewage and other sewage which has been mixed with hospital sewage has hardly 1/3 of probability of being detected with bacteriophage. Sewage water of tertiary care hsopital balkhu and tertiary care hospital nakhu, comes in heavy contact with medical waste and is especially prone to medically significant bacterial samples. All these activity increases the presence of different human pathogenic bacteria in tertiary care hospitals. As it is assumed that, any environmental surrounding which contains the pathogen of interest is expected to contain phages which are able to infect that particular organism [15]. In this preliminary study involving four sampling sites, hospital sewage yielded bacteriophages more frequently than the other sources investigated. Further studies involving larger numbers of environmental samples are required before general conclusions can be drawn.

Phage was isolated against MDR *E. coli* from both the hospital sewage sample that is tertiary care hospital balkhu and tertiary care hospitals nakhu. While phage against *Pseudomonas* spp. was isolated from sewage of Chovar outlet only, while phage was not discovered against *Acinetobacter* spp. The host specificity of phages may be the reason why it was challenging to isolate bacteriophages against certain bacteria. This might have been because the sample did not include any bacteriophage against the test organism.

The phage isolated against *Pseudomonas* spp. could not be sub-cultured and amplified further. This could be because lysogeny assays and phage titer determination were not performed, these explanations remain speculative and require further investigation. The phage isolated against MDR *E. coli* was able to lyse another MDR strain of *E. coli* and MDR *K. pneumonae*. Moreover, this phage was unable to infect *Pseudomonas, Salmonella, Shigella, Cholera, Bacillus, S. aureus and C. furendii.* This shows the narrow host range of the phage. Numerous phage and host factors affect a phage's host range, including the phage's receptors' capacity to identify and bind to specific bacterial surface receptors, the phage's ability to appropriately discharge its genetic contents into the host cell, and intracellular components like bacterial restriction-modification systems, abortive infection mechanisms, and the recently discovered CRISPR mechanism [15].

The purpose of our study was to identify the most reliable source for bacteriophage isolation and investigate its lytic efficacy against various bacteria. According to our investigation, hospital sewage yielded bacteriophages more frequently than the other sources investigated., and the existence of phages is likely in environments where host bacteria are present. Moreover, the isolated phage's limited host range demonstrated that it only exhibited lytic activity against MDR *Klebsiella pneumoniae* and MDR *E. coli*, not other genera of bacteria. Therefore, additional characterization and genetic research are necessary to determine the efficacy of this phage.

Conclusion

In this preliminary study involving four sewage sampling sites, hospital sewage yielded bacteriophages more frequently than the other sources investigated. However, these observations require validation through studies involving a large number of environmental samples before broader conclusions can be drawn.

Author's contribution

Final form of manuscript was approved by all authors. Shashi Bhushan Chaturwedi was the project in-charge, whereas the project conceptualization was done by David Pun. The original draft preparation was also done by David Pun and the review & editing was performed by Rajani Malla, Preety Chaudhary and Pradeep Kumar Shah.

Conflict of interest

The authors declare no conflict of interest.

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