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Research Article

Isolation of Bacteriophage Against the Drug-Resistant Clinical Pathogens and Host Range Analysis

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Abstract

Antibiotic crisis is a major challenge being encountered by the global population as a result of development of antibiotic-resistant bacteria. Thus, shifting the focus to explore alternative methods to treat bacterial infection. Bacteriophage, the natural predator of the bacteria is one of those alternatives. This study is aimed to isolate bacteriophages from different types of water samples which are capable of infecting drug-resistant clinical isolates and understand its host range capacity. The isolation of bacteriophage was done by Double Layer Agar Assay (DLAA) from 3 different water samples i.e sewage sample from Bagmati and ALKA hospital and from Lele river water. For the isolation of phage, host organisms used were Multi Drug Resistant (MDR) *E. coli* and *K. oxytoca* resistant to Amoxyclav, Cefotaxime, Ciproflaxcin, Erythromycin, and Nalidixic Acid. Phage against *E. coli* II was isolated from both sewage samples while *K. oxytoca* phage was isolated from Bagamti sewage only. Phage could not be isolated against *E. coli* I and *K. oxytoca* phage could not be sub cultured as no plaques were formed on sub culturing and it showed temperate properties. The host range analysis of *E. coli* II phage showed that the phage has narrow host range as it did not formed plaques against bacteria of other genera. This indicates that sewage is the best source for discovery of phage and the isolated bacteriophage are capable of lytic activity on MDR bacterial isolates. Even though the isolated bacteriophage has potential to be used in phage therapy, still further research should be done for its application.

Keywords: Antibiotic resistant; Double layer agar assay; sewage; lytic; phage therapy

Introduction

Antibiotics are the preferred method for the treatment of bacterial infections or diseases (Hanlon, 2011). However, the problem of antibiotic resistance is increasing everyday making it a major threat to human, animals and environmental health. This issue of antibiotic resistance is due to the emergence, spread and persistence of multidrug resistant (MDR) bacteria (Aslam *et al*., 2018). The development of antibiotic resistance is mainly due to the unregulated and overuse of antibiotics. Sir Alexander Fleming had warned about overuse of antibiotics as early as in 1945 (Ventola, 2015). Therefore, there is an enormous demand to develop novel antimicrobial alternatives. Antimicrobial peptides, bacterial cell wall hydrolases, and bacteriophages, are among some of the most optimistic alternatives (Parsien *et al.*, 2008).

Bacteriophages are the bacterial viruses that behave as the natural predators of bacteria making them the specially

designed weapon for bacteria (Lojewska and Sakowicz, 2021). They are known to infect and replicate only in host bacterial cells. Bacteriophages are ubiquitously and abundantly present in the environment (Kasman and Porter, 2022). They are usually classified according to their biological cycle, lytic or virulent and lysogenic or temperate bacteriophages. In lytic cycle, there is active production of viral particles while in lysogenic cycle, phage genome integrates with bacterial genome and remains in dormant form (Principi *et al*., 2019).

The isolation of phage is usually considered as the simple straightforward procedure of mixing sample containing phage with its host bacteria, followed by removal of bacterial cells through centrifugation or filtration process. The fundamental method for bacteriophage isolation has remained unaltered since it's development by Felix d'Herelle (Hyman, 2019). When bacterial cells are mixed with bacteriophage in soft agar, the bacterial cell will first grow to produce confluent lawn of cells. Then, the phage will attack the cells causing lysis and forming clear areas in the confluent lawn called as plaques. Each plaque is considered to be formed by the progeny of single virion, replicating and lysing the bacterial cells (Brown and Smith, 2017).

The use of bacteriophages to kill bacterial pathogens i.e. phage therapy has been around for nearly the century (Lin *et al*., 2017). Bacteriophage has been used in the treatment of various infectious diseases. Since the isolation of bacteriophage and it's use to treat bacterial dysentery by d'Herelle, phage therapy has been developed in many places around the world especially in the former Soviet Union countries (Kutateladze and Adamia, 2008). Phages can be employed as mixed phages or as a personalized phage therapy (Nikolich and Fillippov, 2020).

Bacteriophages are host specific as they are extremely precise and attacks only a particular strain of bacteria. Therefore, phage therapy can eradicate specific infection in patients without causing harm to normal flora, acting as a biomedical smart bomb (Keen, 2012). Phage therapy possess certain advantages to traditionalchemotherapy like bactericidal activity, auto dosing, low inherent toxicity, biofilm clearance etc. However, it is challenging to screen the suitable phage for phage therapy. (Loc-Carrillo and Abedon, 2011). Complex pharmacokinetics of phagotherapy and few interactions regarding interaction between phage and human are among other major challenges (Caflisch *et al*., 2019).

At present, whole world is struggling to solve the problem of antibiotic resistance. So, this study aims to explore the feasibility of isolating bacteriophages against multi drug resistant clinical isolates from sewage and water samples. Furthermore, we study the ability of those isolated phage to show lytic activity against other different bacteria.

Materials and Methods

Bacterial Test Organisms

Three bacterial test organisms (2 strains of *E. coli* and 1 strain of *K. oxytoca*) were obtained from ALKA hospital. Eight different bacterial sample were obtained from Department of Microbiology, D.A.V. college (*Pseudomonas, Salmonella, Shigella, Cholera, Bacillus, S. aureus and C. furendii and E. coli (MDR)*) were used for host range analysis**.**

Sample Collection

Samples were collected from sewage of Bagmati and ALKA hospital and river water of Lele. The samples were collected in sterile bottles by submerging the bottles about 5 cm inside the water body. Then these containers were properly labelled and transported to laboratory in an ice box maintaining temperature of 4^{o}C.

Antibiotic Susceptibility Test of Host Organisms

A bacterial suspension was prepared in nutrient broth and its turbidity was matched with that of 0.5 McFarland standard. Using sterile technique, all the agar plates was inoculated with respective test organisms with the sterile cotton swab covering the entire surface of the plates and allowed to dry for few minutes**.** With the sterile forceps antibiotic discs was placed in the plates and pressed gently. Incubated the plates at 37 ^{o}C for 24 hours. After incubation, zone of inhibition was measured and compared with the standard chart (Manandhar and Sharma, 2018).

Isolation of Bacteriophage

The isolation of bacteriophage was done by Double Layer Agar Assay (DLAA) method. For that, water/sewage sample was centrifuges at 4000 rpm for 30 minutes to remove unwanted contaminants and other cell debris. Furthermore, Syringe filter was done to remove bacterial contaminations and other unwanted materials with the help to membrane filter of size 0.22 µm. Thus, obtained filtrate was collected in sterile falcon and stored at 4^{o}C. Then, 1 ml of filtrate and 100µl log phase of bacterial growth was added to sterile tube and allowed to stand for attachment. Three ml of TrypticSoya Agar (TSA) (0.7% agar) was added to the tube, mixed and poured into the already prepared TSA agar plate (1.5% agar). After solidification, plates were incubated at 37^{o}C for 24 hours and observed for visible plaques (Kropinski *et al*., 2009).

Phage Lysate/Stock Preparation

For amplification of phage, different TSA plates were streaked with single plaque by continuous streaking method. Then,100µl of bacterial culture in LB was mixed with 3ml of soft agar and overlaid on streaked plates and incubated overnight after solidification. After that, 3 ml of SM buffer was poured ineach plates containing plaques and mixed properly and allowed agitating in shaker for 5-hours at 100rpm. SM buffer helps to absorb and detach the phage particles. The upper layer of soft agar was scrapped and

transferred to falcon tube and the mixture was centrifuged at 4000 rpm for 30 minutes to settle down debris. Again, by transferring the supernatant to another falcon centrifugation was repeated for 15 minutes. Then, the filtrate was filtered through a 0.22µm pore size syringe filter in sterile falcon tube. This filtrate is the phage stock. The stock was stored at 4°C (Fortier and Moinaeu, 2009).

Determination of phage titer

phage stock was diluted to different dilution up to 10^{-13} with SM buffer as diluent. Stock culture is 10X dilutions. Spot assay was done for titer determination. Grids were drawn on the bottom of TSA plates for spot test of each dilution. Bacterial lawn was prepared by pouring the mixture of 100 µl of log phase bacteria in 3 ml of soft agar in TSA plates. After drying, 5 µl of each dilution was spotted in respective grid as labelled. Only SM buffer was used as negative control. The droplets were allowed to soak and incubated at 37°C for 24 hours in inverted position. The plates were observed for clear zone of lysis. The last three dilutions which showed clear zone of lysis was used to perform DLAA. The plates were observed for plaques formation and distinguishable plaques were counted. The plaque forming unit per ml was calculated by using the formula (Anderson *et al*., 2011).

$$\text{Pfu/ml} = \frac{number\ of\ plaques\ formed}{dilution \times volume\ of\ sample}$$

Multi Host Range Analysis

DLAA was done for multi host range analysis. Different bacterial cultures (100 µl each) in log phase were mixed with 3 ml of soft agar in sterile falcon and poured on properly labelled TSA plates. Then, 5 µl of phage filtrate was added to all plates and incubated at 37°C for 24 hours and checked for the presence of or absence of bacterial lysis and clear zone (Verma *et al*., 2009).

Results

E. coli and *K. oxytoca* were the used as host organisms in this study for the isolation of bacteriophages. These organisms were identified by microscopic observation and biochemical tests. *E. coli* was seen as Gram negative short rods on microscopic observation and was Indole positive, Methyl red positive, Voges Proskauer negative, citrate negative and showed fermentative metabolism. While, *K. oxytoca* was seen as Gram negative rods on microscopic investigation. It was Indole positive, Methyl red negative, Voges Proskauer positive, citrate positive and showed fermentative metabolism(in OF test).

Both of the *E. coli* isolates were found to be Multi Drug Resistant but were sensitive to Gentamicin. *Klebsiella oxytoca* was also Multi Drug Resistant but was sensitive to Gentamycin and showed intermediate activity towards tetracycline (Table 1).

Among the 3 samples, bacteriophage against *E. coli* II and *K. oxytoca* were isolated from Bagmati ghat sample and bacteriophage against *E. coli* II was isolated from Alka hospital sample while none bacteriophage was isolated from Lele sample (Table 2).

Bacteriophage of *E. coli* II from Bagmati ghat showed activity lytic against *E. coli* (MDR) strain unlike against other bacteria of different genus. Host range analysis of phage isolated against *K. oxytoca* and *E. coli* II from ALKA sewage could not be conducted as those phages could not be amplified (Table 3).

Table 1: Antimicrobial Activity of bacterial isolates towards different antibiotics

S.N.	Antibiotics	*E. coli* I	*E. coli* II	*Klebsiella oxytoca*
1	Amoxyclav	Resistant	Resistant	Resistant
2	Cefotaxime	Resistant	Resistant	Resistant
3	Ciprofloxacin	Resistant	Resistant	Resistant
4	Erythromycin	Resistant	Resistant	Resistant
5	Gentamicin	Sensitive	Sensitive	Sensitive
6	Nalidixic Acid	Resistant	Resistant	Resistant
7	Tetracycline	Resistant	Resistant	Intermediate

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

S.N.	Water sample	Bacteriophage isolated against		
		E. coli I	*E. coli* II	*Klebsiella oxytoca*
1	Lele sample	-ve	-ve	-ve
2	Bagmati ghat	-ve	+ve	+ve
3	ALKA sewage	-ve	+ve	-ve

Table 3: Multi Host Range Analysis of Bacteriophage

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

Discussion

In this study host organisms (*E. coli* and *K. oxytoca*) were obtained from hospital. In hospitals and health care environments, sinks and other wastewater sites has been known to be a reservoir of antimicrobial-resistant *E. coli* and *Klebsiella* spp., particularly in the context of outbreaks of resistant strains amongst patients. It has been demonstrated that many hospital sinks are abundantly and persistently colonized with diverse populations of anti-microbial resistant as well as susceptible strains of *E. coli, Klebsiella pneumonie and Klebsiella oxytoca* and may be a part of transmission chains of outbreak of these pathogens leading to colonization and clinical diseases in patients (Constantinides *et al*.,2020). These organisms also have the highest incidence of being associated with healthcare associated infections (Weiner *et al*., 2016).

Whenever the bacteria are exposed to non-lethal level of antibiotics, they can use it as a signaling with regulatory functions in order to developed resistance. Alexander Fleming also had warned about this issue during the acceptance speech of Nobel price (Terrani *et al*., 2020). Therefore, we can observe bacteria being resistant to different antibiotics making them multi drug resistant. We will also observe different pattern of drug resistance in the same species depending on the exposure of different organism to different antibiotics.

Three different types of samples were used for the isolation of bacteriophages to determine the best source for phage isolation. Among the samples of Lele, Bagmati ghat and ALKA sewage, highest probability of discovering phage was from Bagmati ghat (66%) followed by ALKA sewage (33%) and Lele (0%). This result can be attributed to the natures of water samples. Water of Bagmati comes in heavy contact with humans as incineration of human dead body is performed in Bagmati ghat and garbage and sewage is also dumped in the waterway. All these activity increases the presence of different human pathogenic bacteria in Bagmati. 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 (Gill and Hyman, 2010). This vindicates that Bagmati ghat has highest probability for discovering phage among all the samples. The presence of phage in ALKA sewage can be due the presence of bacteria in sewage as the bacterial isolates was received from ALKA hospital. Similarly, the absence of bacteriophage in Lele can be due to the absence of our test organism in Lele sample. Phage was isolated against *E. coli* II from both the sewage sample of Bagmati and ALKA while phage of *K. oxytoca* was isolated from sewage of Bagmati only, while phage was not discovered against *E. coli* I. According to the study conducted by Mattila, *et al*. (2015) the probability for finding an infectious bacteriophage from sewage for different host bacterium varies substantially. In this study by Mattila, *et al*. (2015) the probability of discovering bacteriophage from sewage sample against *E. coli* was 90.6% while against *Staphylococcus aureus* the success rate was only 6.1%. This indicates that the discovery of phage may be feasible for some but not all bacteria. The difficulty in isolation of bacteriophages against some bacteria may be due to the host specificity of phages. This could be due to the absence of bacteriophage against test organism in the sample and may be due to the presence of bacteriophage in very low number in the sample as direct plating was done without enrichment of phage. The phage isolated against *K. oxytoca* could not be sub-cultured and amplified further. This may be due to the phage being a temperate phage, hence following lysogenic life cycle. Turbid plaques were formed by the *K. oxytoca* phage, which is traditionally used as presumptive indicator of temperate nature (Gill and Hyman, 2010). Another potential factor might be that the initial phage titer of the filtrate was very low due to which phage could not successfully lyse the bacteria.

The phage titer of phage stock was determined by spot assay followed by double layer agar assay. The phage titer was determined to be 4.5×10^{10} pfu/ml which indicates that 4.5×10^{10} plaques forming units of phage is present in the stock sample. The effectiveness of phage in affecting the bacteria depends on the quantity of phage. In a study by Lukman, et al. (2020) showed that $2.30\pm0.50\times 10^{19}$ of CI EPEC bacteriophage caused bacteria reduction of 57.15% in meat sample while $1.43\pm0.39\times 10^{10}$ pfu/ml of BL EHEC bacteriophage caused bacteria reduction of 99.04%. This

suggests that effective amount of phages is present in phage stock.

The phage isolated against *E. coli* II was able to lyse another MDR strain of *E. coli* but could not lyse *E. coli* I. Moreover, this phage was unable to infect *K. oxytoca, Pseudomonas, Salmonella, Shigella, Cholera, Bacillus, S. aureus and C. furendii.* This shows the narrow host range of the phage. The host range of a phage is influenced by numerous phage and host factors, like phage's receptors ability to recognize and bind to its specific receptors on bacterial surface, phage's capacity to properly discharge its genetic contents into the host cell, and intracellular elements like abortive infection mechanisms, bacterial restriction-modification systems, and recently identified CRISPR mechanism (Gill and Hyman, 2010).

This study aims at finding the better source for the isolation of bacteriophage and studying its lytic activity on different bacteria. Through this study we found that sewage to be the best source for the isolation of many bacteriophages and there are high chances of presence of phages where there is presence of host bacteria. Furthermore, the isolated phage showed narrow host range showing its lytic activity against only *E. coli* only and not against bacteria of other genera. This phage can potentially be used for phage treatment but further characterization and genomic study should be done to determine its effectivity.

Conclusion

Even though, bacteriophages are found widely in nature and are present for almost all types of bacteria, the highest probability of isolating bacteriophage is from the environment where there is possible presence of host bacteria. The isolated phage from sewage against *E. coli* II was able to lyse different MDR strains of *E. coli*, also indicating its lytic activity. Thus, showing its ability to infect *E. coli* strains. However, it was unable to lyse bacteria of other genus (*K. oxytoca, Pseudomonas, Salmonella, Shigella, Cholera, Bacillus, S. aureus* and *C. furendii*) indicating the phage to be of narrow host range. Therefore, the isolated bacteriophage can potentially be used for phage therapy in treating drug resistant *E. coli* strains after further characterization and genomic study.

Author's Contribution

All authors contributed equally at all stages of research and preparation of the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare no conflict of interest with the present publication.

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