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ONE STEP ISOLATION OF *E. COLI* HOST SPECIFIC BACTERIOPHAGES FROM SEWAGE SAMPLE

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ABSTRACT

Purpose: This study assessed the bacteriophages isolation from sewage water and evaluated the activity against *E. coli* organism. The application of lytic phages could be used as a control agent against pathogenic organisms.**Methodology**: In the current study, the bacteriophages are isolated from sewage water sample and evaluated for its antibiofilm activity against biofilm producing *E. coli* bacteria. The *E. coli* strain was isolated from sewage water and used for bacteriophage isolation. Morphological and biochemical characterization was done using specific tests. The biofilm activity was evaluated by tube assay method using crystal violet. **Findings:** The isolate was identified based on morphological and biochemical tests. Further, it was used for isolation of bacteriophages. The bacteriophage clearance activity was observed on MHA agar plates. In 10^{-4} dilution distinct plaques were observed and further dilutions the distinct plaques were reduced. **Originality**: This study suggests that the purified bacteriophages are potential to be used as therapeutic agents for *E. coli*.

Keywords: Phage therapy, Biofilm, Bacteriophages, Multi drug resistance, antibiotic resistance.

Introduction

Antibiotic resistant infections are wide spread across the globe and become as a major threat (Murray et al., 2022). The infectious agents belong to both gram positive and gram-negative bacteria (Peterson and Kaur, 2018). Among gram positive pathogens, *Staphylococcus aureus*, *Streptococcus species*, *Enterococcus faecium* and *Enterococcus faecalis* become as a pandemic infection and victimizes a greater number of people than the HIV/AIDS, cancer, diabetes, Parkinson's and other infections (Ventola, 2015). The gram-negative pathogens are particularly worrisome due to resistance to all antibiotics available for treatment (Karaiskos et al., 2019). The gram-negative pathogens are *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium, Acinetobacter baumannii, Shigella,* and *Campylobacter* (Tian et al., 2018). The misuse of antibiotics for treating various diseases results in the disturbance of normal flora of the body and the normal bacteria also gains resistance towards antibiotics (Langdon et al., 2016). Further, it results in multidrug resistant strains due to the production of biofilm serves as a control strategy for bacteria against antibiotics (Wang et al., 2017). World health organization (WHO) estimated that the growing global threat of antimicrobial resistance will kill at least 10 million per year by 2050 (Murray et al., 2022). Therefore, novel solutions like bacteriophages, probiotics, natural products (honey and aloe vera) and phytotherapy had attracted the attention of researchers for targeting the biofilm producing resistant strains (Moghadam et al., 2020). Among all, bacteriophages gained more attention in recent years due to its phage particles and their products for the treatment of bacterial infectious diseases (Lin et al., 2017). The current research on phages and its lytic proteins against multidrug resistant bacteria suggests the phage therapy (Furfaro et al., 2018). The phage therapy has potential to be used as an alternative or supplement to the antibiotic treatments (Lin et al., 2017). This phage therapy can be used in various applications like dentistry, medical and health care, agriculture and veterinary science (Keen and Adhya, 2015). Bacteriophages are omnipresent in nature, depends on the bacteria for its survival and both lies in the prey and predator relationship (Naureen et al., 2020). They are present in the environments like sewage water, ponds, soil, lakes, drainage system, river and ocean (Sala-Comorera et al., 2021). Bacteriophages are more effective than antibiotics due to its specificity to bacterial strains and they do not interfere with the host organism (Domingo and Delgado-Martinez, 2018). With increase of bacterial population, the concentration of bacteriophages also increases and effectively control the bacterial population by lysing the cells (Nabergoi et al., 2018). In the current study, the bacteriophages are isolated from sewage sample and evaluated against biofilm producing *E. coli* organism. Sewage water is the major source for spread of antibiotic resistance in the environment and acts as a potential

reservoir for multidrug resistant bacteria. Hence, In the present work the *E. coli* organism was isolated and checked for its biofilm formation. The isolated bacteriophages were evaluated for antibiofilm activity against biofilm producing *E. coli* organism.

Materials and Methods

The chemicals, reagents, media and solvents used in the current study were of A R grade (Merck, Loba, Qualigens, SLR and Hi-media purchased from local supplier.

Collection of sewage water to isolate *E. coli* bacterial strain

Sewage water was collected from the pond present near Vignan's Foundation for science and technology, Vadlamudi, Guntur (Dt.), Andhra Pradesh, India. The isolation and enrichment of specific *E. coli* organism was done using MacConkey agar media and M-Lauryl Sulphate agar media respectively. Pure culture was developed and stored for further phage activity.

Morphological and Biochemical characterization

The morphological features were identified using gram staining and biochemical characterization was done using selective medias and reagents for specific tests. Sugars like glucose, lactose, sucrose and mannitol were used for identification of bacterial strain for fermentation activity for respective sugars. Further, the indole, methyl red, voges proskauer, citrate utilization, triple sugar iron agar, urease, catalase and sulphate indole motility were used for evaluating the bacterial strain for aerobic or anaerobic nature and also motility of the bacterial strain (Lupindu, 2017).

Biofilm formation

Nutrient Broth Medium with 1% glucose was prepared and inoculated with pure culture of isolated *E. coli* bacterial strain and incubated at 37°C for 24 - 48 hrs. Uninoculated tube kept as a control. The tubes were decanted and washed with phosphate saline buffer to remove the planktonic bacteria and were allowed to dry. The dried tubes were stained with 0.1% crystal violet solution and excess stain was removed using distilled water and were dried in an inverted position and observed for biofilm production (Sujana et al., 2013).

Isolation of bacteriophages from Sewage water

Sewage water sample was collected from pond water and transferred into the centrifuge tubes. For every 5 ml of the sewage water sample 1ml chloroform was added and mixed for 10 minutes. The tubes are subjected to centrifugation for 20 minutes at 3,000 rpm at 4°C. After centrifugation the supernatant was collected and again subjected to centrifugation at 3,000 rpm for 10 minutes at 4°C. The step was repeated for 3 times and the supernatant was collected for phage activity (Shende et al., 2017).

Phage spot testing

For phage spot testing, bacterial lawn culture was prepared. The pure bacterial inoculum was added to Luria Bertani (LB) broth medium and incubated at 37°C till the optical density (600 nm) reaches 0.5 – 0.7. For bacterial lawn preparation sterile swab was taken, dipped into the bacterial

suspension and swabbed on the surface of Muller Hinton agar plate (MHA). The plates are then incubated at 37°C for 2 hours. After 2hrs of incubation the plates were taken out from the incubator. The bacteriophage supernatant (400-600µl) was dropped on the bacterial lawn culture and incubated at 37°C for overnight. After 24 hours, the plates were observed for clearance and phage activity (Hyman, 2019).

Bacteriophage concentration and purification

The Plates with phage activity or clearance were washed with TMG (Tris magnesium gelatin) buffer or mixed with TMG buffer using sterile swab stick. The mixture was collected in the centrifuge tubes and mixed with 1% (V/V) chloroform and kept for 10 minutes on rotary shaker. After shaking, the tubes are subjected for centrifugation at 10,000 rpm for 10 minutes at 4°C. The phage lysate supernatant was collected after repeating the centrifugation for 3 or 4 times (Bhargava et al., 2022).

Phage titration assay

The phage titration assay was done by taking seven collection tubes and label them as 1, 2, 3, 4, 5, 6 and 7. 1 ml of SM buffer (NaCl-5.8g, $MgSO_4.7H_2O$ – 2G, 1M Tris HCl – 50ml, $D.H_2O$ – 1000ml) was taken in tube no.1 and 900µl of SM buffer in remaining tubes. The phage lysate (10µl) obtained in previous step was added to tube no. 1. The dilution in this tube is 10^{-2}. Mix it by vertexing and add 100µl to tube no.2. The dilution is 10^{-3} and repeat this step up to 10^{-8}. In addition to this, seven more tubes are taken and labeled from 1 to 7. Add 300µl of bacterial suspension in all tubes and 100µl of respective phage dilution from 1 to 7 tubes. Mix it and kept at room temperature for 20min. LB agar plates are prepared and labelled as 1 to 7. The bacteriophage and bacterial suspension mixture from tube 1 was mixed with 4 ml of soft agar and poured on to the surface of LB agar plate marked with 1. The step was repeated for other dilutions and the plates were incubated at 37°C for overnight. The phage titre value was calculated by taking the formula:

Phage titre value = Number of plaques forming units/dilution factor × ml of lysate sample (Dulbecco and Vogt, 1953).

Results and discussion

Isolation and Identification of *E. coli* bacterial strain

The sewage water sample was collected from the pond present near Vignan's Foundation for science and technology, Vadlamudi, Guntur (Dt.), Andhra Pradesh, India. The bacterium was isolated from sewage water sample using MacConkey agar medium and further the enrichment of *E. coli* bacterial culture was done using M-Lauryl sulphate agar medium. The pure culture was developed using LB agar media and stored for further identification. The *Escherichia coli* was identified based on gram staining and biochemical tests. The morphological features were done by gram staining method and found that the bacteria are gram negative rod shaped. Further the biochemical features such as indole positive and lactose positive (Table

1) confirmed that the isolated bacterial strain was *E. coli*.

Table 1. Morphological and biochemical characterization

S. No	Biochemical test	Result
1	Indole	Positive
2	Methyl Red	Positive
3	Voges Proskauer test	Negative
4	Citrate utilization	Negative
5	Glucose	Positive
6	Lactose	Positive
7	Sucrose	Positive
8	Mannitol	Positive
9	Urease	Negative
10	Triple sugar Iron	Positive
11	Sulphate indole motility	Positive
12	Catalase	Positive

Biofilm formation

The biofilm formation was observed in the tube inoculated with *E. coli* in nutrient broth with 1% glucose solution and evaluated using crystal violet stain (Figure 1). Absence of biofilm was observed in the uninoculated tube consists of nutrient broth with 1% glucose solution.

Figure 1. *E. coli* biofilm formation

Bacteriophage isolation and phage spot testing

For isolation of bacteriophages chloroform was used and phage spot testing was done using MHA agar plate. After 24 hours of incubation the MHA agar plate was observed with phage clearance activity and the bacteriophage was purified using TMG buffer.

Bacteriophage purification

The bacteriophage sample after phage spot testing were collected and centrifuged for purifying the bacteriophage sample. The purified samples were stored in stock media (Peptone water) (Figure 7). The bacteriophages along with bacteria was inoculated into the tube containing the stock media which makes the bacteriophages inactive and helps in the storage of bacteriophages for further studies.

Phage Titration assay

The phage titration determines the number of plaque particles in the phage lysate solution. The phage titer value was calculated using the formula for the dilutions 10^{-2} to 10^{-8} and represented in the Table 2. In 10^{-2} and 10^{-3} dilutions distinct plaques were not observed due to complete lysis of bacterial cells. In 10^{-4} dilution distinct plaques were observed (Figure 2). From 10^{-5} to 10^{-8} dilutions the number of plaques were reduced due to higher dilutions of phage lysate.

Table 2. Phage titer assay values

Tube No.	Dilution	Number of plaques	Phage titre value
1	10^{-2}	0	0
2	10^{-3}	0	0
3	10^{-4}	220	2.2×10^{7}ml
4	10^{-5}	124	1.24×10^{8} ml
5	10^{-6}	82	0.82×10^{9} ml
6	10^{-7}	64	0.64×10^{10} ml
7	10^{-8}	45	0.45×10^{11} ml

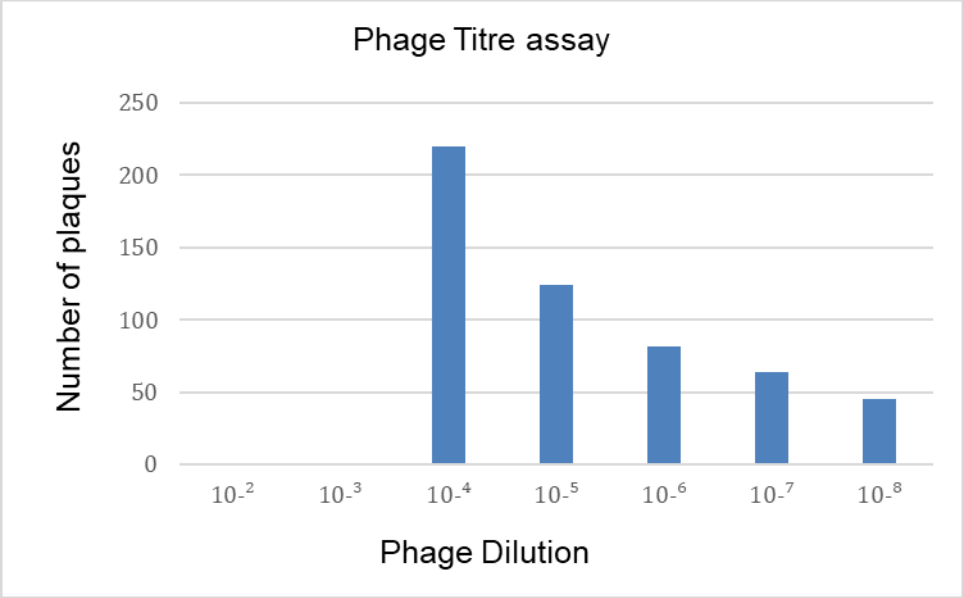

Figure 2. Phage titer assay

Conclusions

This study isolated the bacteriophages using specific *E. coli* as the host system. The purified bacteriophages are potential to be used as therapeutic agents for *E. coli*. However, there is a need to characterize the bacteriophages and understand the efficacy of bacteriophages in *in-vitro* and *in-vivo* studies.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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