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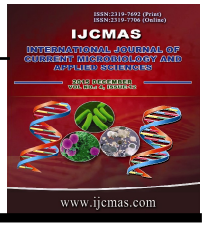

Original Research Article

Isolation and Characterization of Virulent Coliphages from Sewage Sample

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K e y w o r d s

Virulent Coliphages, Sewage Sample, *E. coli* host cells, Phage titer

A B S T R A C T

Enteric bacteria are normal inhabitants of the intestines of humans and other animals. Sewage contains high numbers of potentially very pathogenic enteric bacteria known as fecal coliforms In their natural habitat enteric bacteria are typically harmless but they can produce severe disease symptoms when ingested by susceptible individuals, Bacteriophages are viruses that infect bacterial cells. Phages are nonliving agents and thus require the use of the host's metabolic processes to replicate itself. In this study, the phages of interest are those that infect and lyses *E. coli* host cells. When phages are released from the ruptured host, distinct zones of clearing (plaques) form. The original *E. coli* host cells for this experiment came from a sample of raw sewage. In order to obtain the bacteriophage, a procedure of enrichment, isolation, dilution and seeding was followed, the presence of distinct plaques indicated that lytic bacteriophage had been successfully amplified, separated and grown. This study included determination of phage titre. Phage titer is determined by serial dilution of phage filterate (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}) the dilution factor gave the best countable number of plaques is10^{-6}. This dilution factor was then used for further characterization. The objectives of this study were to isolate phage from sewage sludge, identify its enteric bacterial host, and to determine the Phage titer.

Introduction

Bacteriophages are obligate intracellular parasites. They multiply inside a bacterium by making use of some or all of the host biosynthetic machinery. They enter the bacterial cell by 'landing' on the cell wall and injecting their DNA into the bacterial cytoplasm. After entry, the phage DNA acts as a template for production of phage proteins. These proteins replicate the phage and subjugate the cell, eventually causing lysis and death of the host cell.

A bacteriophage particle is even harder to see than a bacterium. Viruses are beyond the limits of resolution of the light microscope and can be seen only with electron microscopes. Fortunately, we can use a technique very similar to the colony-counting technique used to measure the number of bacteria to count phage particles, known as the plaque assay. Lytic phages are enumerated by this method. The plaque assay is originally a virological assay

employed to count and measure the infectivity level of the Bacteriophages (vlab.amrita.edu, 2011).

Enteric bacteria are normal inhabitants of the intestines of humans and other animals (Davis, 2005) but are often isolated from aquatic ecosystems after sewage has been introduced into the environment. Sewage contains high numbers of potentially very pathogenic enteric bacteria known as fecal coliforms. Coliforms are characterized as gram-negative, facultative anaerobic bacteria that ferment lactose within 48 h at 35^{o}C. Examples of fecal coliforms include *Escherichia coli* and *Enterobacter aerogenes*. In their natural habitat enteric bacteria are typically harmless but they can produce severe disease symptoms when ingested by susceptible individuals, particularly young individuals and individuals with weakened immune systems (Davis, 2005).

Aquatic environments contaminated with enteric bacteria posses a potentially serious threat to wildlife and human health (Beaudoin et al., 2007). Bacteriophage, which exist in many varieties, do not attack bacteria indiscriminately, they each usually attack only one specific kind (Roonest et al., 2008). Most phages are 24-200 nm in length. Each phage has a head (or *capsid*) and tail (many, but not all) structure, which can vary in size and shape. The head is composed of many copies of one or more type of protein, and it contains genetic material (i.e. nucleic acid). The genetic material can be ssRNA, dsRNA, ssDNA, or dsDNA between 5 and 500 kilo base pairs (kbp) long in either a circular or linear arrangement. Tails attached to the phage head, and is a hollow tube through which the nucleic acid passes during infection, and its size can vary considerably (Al-Mola et al., 2010).

Materials and Methods

The present study was conducted at Intermediate Reference Laboratory (IRL), Raipur. C.G. All the chemicals and media used during the course of the study were obtained from HiMedia.

Isolation of Pathogens

Sewage water was sampled using sterile containers. The sample was first filtered through coarse filter paper to remove the debris. The filtrate was serially diluted using phosphate buffer (pH- 6.8) and plated onto differential media using the pour plate technique. All the plates were incubated at 37°C for 24 hrs. Pure cultures were isolated. Gram staining and colony morphology were performed.

Biochemical Characterization

Biochemical characterization of the isolated colonies was carried out using standard protocols. To support strain identification; fermentation, methyl red, and Vogues-Proskauer tests were performed. Three-loopfuls of pure broth (24 h) cultures were inoculated into fermentation broth containing lactose, dextrose, sucrose, inositol, or trehalose as sole carbon source. Durham tubes were placed into the test tubes in the inverted position to trap gas (CO_2 and H_2), potential end products of fermentation. All fermentation tubes contained the pH indicator phenol red. Acid production from the fermentation of the sole carbon source was reported when the media changed color from red (pH- 7.4) to yellow (pH- 6.0). After 48 h incubation at 35^{o}C, inoculated fermentation tubes were scored on the basis of acid production (as indicated by a color change from red to yellow) and/or gas production (as indicated by gas bubbles trapped in the inverted Durham tube).

The Methyl Red test (Cappuccino and Sherman, 2001) was used to determine the host cell's ability to oxidize glucose with production of a high concentration of acid end products. Methyl red broth was inoculated with 24 h broth test cultures. Tubes were incubated for 24 h at 35^{o}C. After incubation 3-4 drops of methyl red indicator (red pH 7.0) were applied to the methyl red tubes. A change in the colour of the medium from amber to red (pH- 4.0) was scored positive, and a colour change from amber to yellow (pH- 6.0) was scored as negative. Vogues-Proskauer test was used to determine the test organism's ability to produce non-acidic or neutral end products from the fermentation of glucose (Cappuccino and Sherman, 2001). Vogues-Proskauer media tubes were inoculated with 24 h broth test cultures and incubated at 35^{o}C for 24 h. After incubation, 10 drops of Barritt's A (α-naphthol, 5.0 g; ethanol, 95.0 ml) solution followed by 10 drops of Barritt's B solution (KOH, 40.0 g; creatine, 0.30 g; dH_2O, 100.0 ml) were added to the test tubes. The tubes were shaken every 3-4 minutes for 15 minutes. A positive test was indicated by a colour change of the media from amber to rose. *E. Coli* (ATCC-35746) was used as a control for biochemical tests (Khairnar et al., 2014).

Isolation of a Bacteriophage from Sewage Sludge

Bacteriophages were isolated from sewage samples by using enrichment cultures. A total of five sewage samples were analysed which were collected from sewage near Kharun river, Dagania pond, Budha pond, sewage near Ramkrishna Hospital of Raipur C.G.

Approximately 35.0 ml of a filtered (Prefiltered to remove debris) sample was mixed with 35ml of 10× Nutrient broth and with 5.0 ml of pure cultures of isolated *E.coli*. After proper mixing the enrichment cultures were incubated for 24 h at 37°C to allow amplification of lytic Coliphages.10.0 ml of sewage bacteriophage culture was transferred into a centrifuged tube and the sample was centrifuged at 2000 rpm for 5 minutes.

Most of the remaining cells were pelleted. The supernatant was transferred to a 10.0 ml syringe barrel fitted with a 0.45 micron filter. The supernatant was filtered to remove bacteria from the phage sample. The filtrate (lysate) was stored at 4^{o}C (Lobocka and Szybacki, 2012).

Determination of Phage Titres in Lysates

Soft agar overlay method (Double layer agar method) was followed to determine the number of phage particles in the lysates. For this the lysates were serially diluted l0-fold.100 µl from each dilution was mixed with 500 µl of overnight culture of host strain in 3.0 ml molten soft agar (50°C, Agar- 0.8%) and dispensed onto nutrient agar plates. The plates were incubated overnight at 37^{o}C and dilutions showing countable plaques (30-300 plaques per plate) were counted for determining the phage titres in the lysates. (Al-Mola, 2010).

Results and Discussion

Isolation of Pathogens

A total of five cultures were isolated and purified from the sewage water, sampled from the sewage near Kharun river, Dagania pond, Budha pond, Sewage near Ramkrishna Hospital. Based upon the colony morphology, biochemical characterization and growth on differential media, the isolates were identified as *Escherichia coli*.

Table.1 Partial Biochemical Characterization of the Host Bacteria

Biochemical tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Gram's stain	Gram Negative	Gram Negative	Gram Negative	Gram Negative	Gram Negative
Cellular Morphology	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Catalase	Negative	Negative	Negative	Negative	Negative
Triple Sugar Iron Agar	Yellow/Yellow with Gas	Yellow/Yellow with Gas	Yellow/Yellow with Gas	Yellow/Yellow with Gas	Yellow/Yellow with Gas
Motility Test	Positive	Positive	Positive	Positive	Positive
Urease Activity	Negative	Negative	Negative	Negative	Negative
Methyl Red Test	Positive	Positive	Positive	Positive	Positive
Voges Proskauer's test	Negative	Negative	Negative	Negative	Negative

Table.2 Determination of Phage Titer

Plate no.	**Volume of Phage plated (ml)**	**Dilution**	**Dilution Factor (DF)**	**Plaque per Plate**	**Titer Calculation=Plaque no.x DF/Volume of Phage plated (ml)**	**Titer PFU**
1	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-1}}$	0	0	0
2	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-2}}$	0	0	0
3	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-3}}$	60	$60X10^{-3}/0.1$	$600X10^{-3}$
4	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-4}}$	72	$72X10^{-4}/0.1$	$720X10^{-4}$
5	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-5}}$	81	$81X10^{-5}/0.1$	$810X10^{-5}$
6	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-6}}$	90	$90X10^{-6}/0.1$	$900X10^{-6}$
7	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-7}}$	46	$46X10^{-7}/0.1$	$460X10^{-7}$
8	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-8}}$	23	$23X10^{-8}/0.1$	$230X10^{-8}$
9	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-9}}$	9	$9X10^{-9}/0.1$	$90X10^{-9}$
10	0.1	$\mathbf{10^{-1}}$	$\mathbf{10^{-10}}$	2	$2X10^{-10}/0.1$	$20X10^{-10}$

Figure.1 Plaques on Nutrient Agar Plates

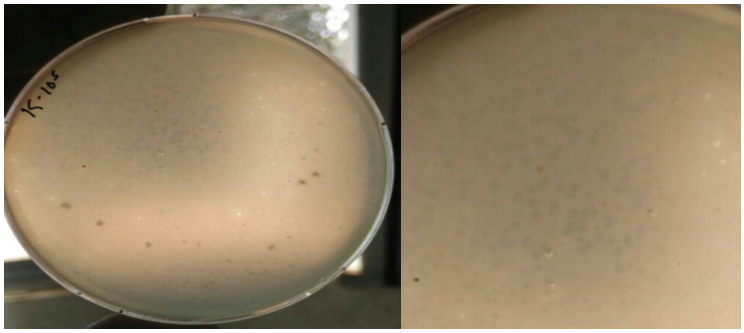
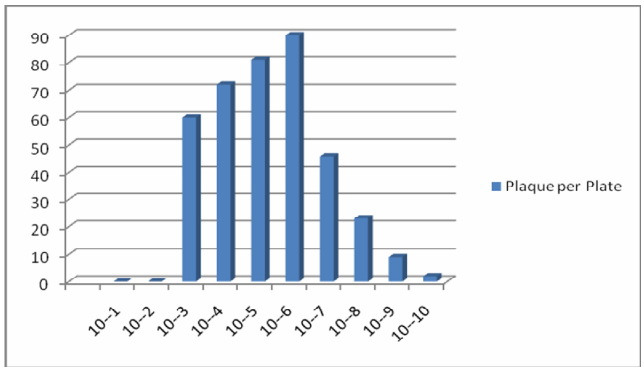


Figure.2 Plaques Per Plate against Dilution Factor



Bacteriophage Isolation

Enrichment double layer method was followed for the isolation of bacteriophage, Single plaques were observed after 24h incubation at 37^{o}C. A representative culture plate of plaques assay shown below;

Bacteriophages have been used to treat systemic and enteric diseases since the turn of the century in countries, such as Russia, Poland and China to some extent. They were found to have bactericidal properties as they largely feed on specific bacteria. The concept of these being used in medicine came up by the end of the 19th century. Bacteriophage Therapy Center located in the district of *Tbilisi* in Georgia has conducted a wide variety of research and has also used phages as a first choice of prophylaxis of various bacterial diseases. The emergence of drug resistance shows the ability of microbes to evolve with each generation. Phages are thus being preferred because, unlike broad- spectrum antibiotics, they are highly specific and do not illicit resistance from untargeted bacterial strains. Phage therapy was highlighted as one of seven approaches to "achieving a coordinated and nimble approach to addressing antibacterial resistance threats" in a 2014 status report from the National Institute of Allergy and Infectious Diseases (NIAID) (Madhusudan, 2014).

Sewage, in general, contains a large diversity of coliforms due to fecal contamination. Therefore, sewage water is a reservoir of enteric pathogens. The phages we obtained in this experiment were lytic. The development of clear zones of lysis against different host bacterium using

specific phage lysate indicated that all the phages isolated were lytic phages (Mahadevan et al., 2009).

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