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Isolation of Host-Specific Bacteriophages from Sewage Against Human Pathogens

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Abstract: Bacteriophages have been found to be effective against a wide variety of pathogenic bacteria as they are highly host specific. The present study describes the isolation of bacteriophages effective against few human pathogens such as *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*. A total of five isolates of bacteria were obtained from the sewage water, sampled from the sewage treatment plant located at Jinke Park, Bangalore, India. Based upon the colony morphology, biochemical characterization and growth on selective media, the isolates were identified as *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella* species and *Shigella* species. Out of the five different isolates three were sensitive to bacteriophages. The sensitive cultures belonged to the genera *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*. The phage filtrates, when spotted onto the lawn cultures of the respective host bacterium, resulted in the development of clear zones indicating the presence of lytic bacteriophages against the host bacteria. It was also found that each of the phages for *E. coli* and *Salmonella typhi* was only able to infect its original host bacterium, whereas, the phage for *Pseudomonas aeruginosa* was able to infect both *Pseudomonas* and *E. coli*. Studies of the morphology and characterization of these phages are currently being conducted. These isolated phages may hold a lot of promise as the first choice of prophylaxis (Phage Therapy) against nosocomial and secondary infections by deadly multi-drug resistant bacteria in the near future.

Key words: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, bacteriophages, multi-drug resistant bacteria, pathogens, phage therapy, sewage

INTRODUCTION

Viruses have been known as distinct biological entities for little more than a century. Humans have not only been subject to viral diseases throughout much of their history but have also manipulated these agents to suit their own needs (Flint *et al*., 2000). During the early decades of the twentieth century, the discovery of electron microscope revealed much information on viruses. The fundamental characteristic of viruses is their absolute dependence on specific host organisms for reproduction, therefore, they are rightly known as obligate intracellular parasites. In 1917, bacteriophages were recognized as epizootic infections of bacteria and were almost immediately deployed for antibacterial therapy and

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prophylaxis. Over the past few decades, indiscriminate use of antibiotics has led to resistance among various bacterial strains. This is a potentially dangerous situation that threatens to manifest itself in modern times.

Phages are thus being preferred because, unlike broad spectrum antibiotics, phages are highly specific and do not illicit resistance from untargeted bacterial strains (Sulakvelidze and Kutter, 2005). The use of bacteriophages for the treatment of diseases that are potentially life threatening is called Phage Therapy. Earlier study on phages suggested that the phages have cured 90% of the chronic suppurative bacterial infections (empyemas, peritonitis, osteomyelitis, etc.) in humans caused by antibiotic- resistant bacterial pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli* (Carlton, 1999).

Bacteriophages are widely distributed in the environment and can be isolated from sea water, soil, fresh water and sewage ecosystems (Jensen *et al.*, 1998). Attempts to isolate phages from sea water have been successful in some cases (Smith and Krueger, 1954; Hidaka, 1971). Wiebe and Liston (1968) isolated a bacteriophage active against a marine *Aeromonas* sp. from marine mud. Baross *et al.* (1978) reported the isolation of bacteriophages against *Vibrio* from marine water and sediments. Smith and Krueger (1954) isolated a bacteriophage against *Vibrio* from the mud of San Francisco Bay. The prevalence of large populations of pathogenic bacteria existing in close proximity in sewage water makes it a relevant source for the isolation of various bacteriophages. In this study, an attempt was made to isolate phages against specific human pathogens present in sewage water.

MATERIALS AND METHODS

The present study was conducted during the period from 26.11.2008 to 11.05.2009 at Jain Institute of Vocational and Advanced Studies, Chamarajpet, Bangalore, Karnataka, India. All the media used during the course of the study were obtained from Himedia Laboratories Pvt. Limited (A-406, Bhaveshwar Plaza, Mumbai-400086, India).

Isolation of Pathogens

Sewage water was sampled using sterile dark containers from the Sewage Treatment plant located at Jinke Park, Bangalore. The sample was first filtered through coarse filter paper to remove the debris. The filtrate was serially diluted using 0.85% sterile saline and plated onto different selective and differential media using the spread plate technique. All the plates were incubated at 37°C for 24 to 48 h. Following incubation, the isolated colonies were pure cultured and Gram stained. Biochemical characterization of the isolated colonies was carried out using standard protocols (Kannan, 2002). Identification was carried out according to Bergey's Manual (7th Edn.).

Demonstration of Bacteriophages

The isolation and demonstration of phages in the sewage sample were carried out as per the methods described by Spencer and Armon (Spencer, 1955; Armon and Kott, 1993). Five milliliter of Mueller Hinton Broth was prepared, autoclaved and inoculated with the respective organism. The tubes were incubated at 37°C overnight. Five milliliter of overnight culture was added to 150 mL Mueller Hinton Broth in a sterile shake flask together with each of the different bacterial strains in purified isolates and shaken for 2 to 3 h. Two hundred milliliter of raw sewage was filtered through normal filter paper to remove debris and was added to the contents of the flask (150 + 5 mL). This mixture was incubated at 25°C in a shaker incubator (Orbitek) for 2 to 3 h at 140 rpm and then further incubated overnight

without shaking. This mixture was centrifuged (Hettich U32R) at 5000 rpm for 30 min and filtered through Millipore Membrane Filter (0.22 μ). The filtrate was collected in sterile amber bottles. Ten microliter of the filtrate was spotted onto lawns of the bacterium prepared with Mueller Hinton Agar (Armon and Kott, 1993). The plates were incubated at 25°C overnight and examined for the appearance of clear zones of lysis (plaques).

Purification and Mass Multiplication of Bacteriophages

Materials from the centre of the plaques were scraped off using a sterile inoculation loop and were transferred to fresh sterile Mueller Hinton Broth containing the appropriate organism and incubated overnight for about 18 h at 25°C. This mixture was centrifuged at 5000 rpm for 30 min and filtered through Millipore Membrane Filter (0.22 μ). The filtrate was collected in sterile amber bottles. The phage assay was again carried out as mentioned earlier. The cycle was repeated for a minimum of three times to ensure the purity of the phages. All the phage lysates were stored at 4°C.

RESULTS

A total of five cultures of bacteria were isolated from the sewage water, sampled from the sewage treatment plant located at Jinke Park, Bangalore. Based upon the colony morphology, biochemical characterization and growth on selective media, the isolates were identified as *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella* sp. and *Shigella* sp. Upon Gram staining, all the sewage isolates were identified as Gram negative rods in scattered form. All the bacteria isolated in this experiment are aerobic mesophiles with the exception of *Pseudomonas* which is a psychrophile. *E. coli*, *Salmonella typhi*, *Klebsiella* and *Shigella* belong to Enterobacteriaceae family whereas *Pseudomonas* belongs to the Pseudomonadaceae family. Due to space constraint, we have included the biochemical characterization of only those bacteria that were found to be sensitive to the isolated bacteriophages. The result of partial biochemical characterization of the host bacteria is shown in Table 1.

Out of the five different isolates three were sensitive to bacteriophages. The sensitive cultures belonged to the genera *Salmonella typhi* (Fig. 1), *Escherichia coli* (Fig. 2) and *Pseudomonas aeruginosa* (Fig. 3). Results of the host specificity test indicated that the

Table 1: Partial biochemical characterization of the host bacteria

Biochemical tests	Isolate 1[a]	Isolate 2[b]	Isolate 3[c]
Gram's stain	-	-	-
Cellular morphology	Rods	Rods	Rods
Catalase activity	+	+	+
Indole production	+	-	-
Methyl red test	+	+	-
Voges proskauer's test	-	-	-
Citrate utilization	-	+	+
Gelatin liquefaction	-	-	+
Urease activity	-	-	-
Triple sugar iron test	A/A with gas	K/A with H_2S	K/A
Motility test	+	+	+
Sugar fermentation test			
Glucose	+/+	+/+	-/-
Sucrose	+/+	-/-	-/-
Lactose	+/+	-/-	-/-
Maltose	+/+	-/-	+/-
Mannitol	+/+	-/-	-/-
Xylose	+/+	-/-	-/-

[a]*Escherichia coli*; [b] *Salmonella typhi*; [c] *Pseudomonas aeruginosa*, For TSI Test: A; Acid Butt; K; Alkaline Slant, For Sugar Fermentation Test: Acid/Gas reactions; +; Positive; -; Negative

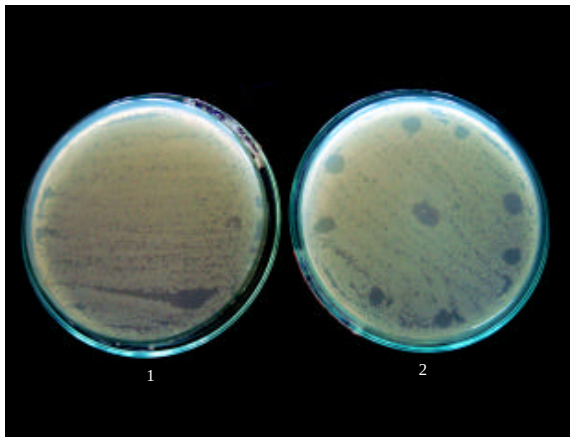

Fig. 1: Phage for *Salmonella typhi*; 1-Control and 2-Plaques on test plate

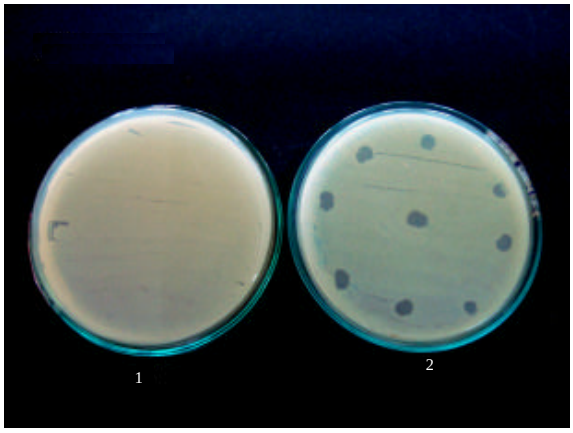

Fig. 2: Phage for *Escherichia coli;* 1-Control and 2-Plaques on test plate

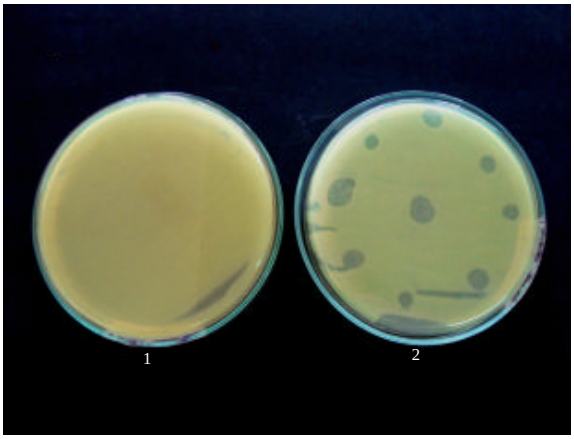

Fig. 3: Phage for *Pseudomonas aeruginosa*; 1-Control and 2-Plaques on test plate

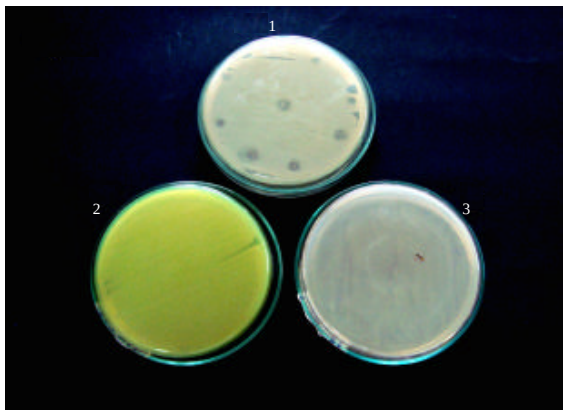

Fig. 4: Host Specificity test for *Salmonella typhi* phage; 1-*Salmonella typhi* and 2-*Pseudomonas aeruginosa*, 3-*E. coli*

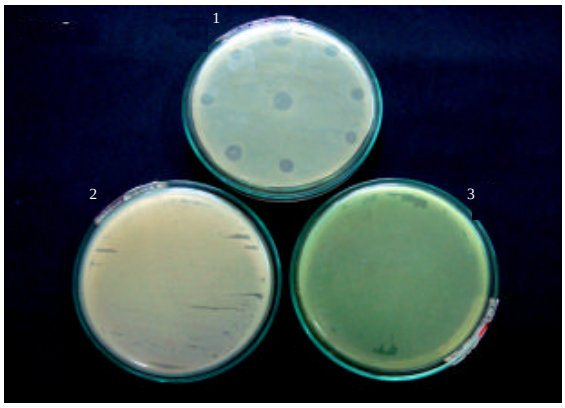

Fig. 5: Host Specificity test for *E. coli* phage; 1-*E. coli*, 2-*Salmonella typhi* and 3-*Pseudomonas aeruginosa*

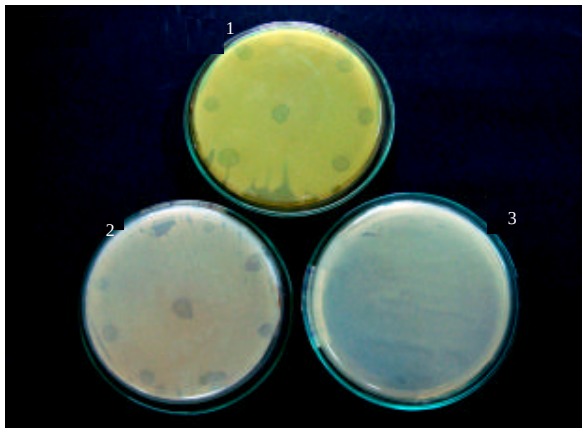

Fig. 6: Host Specificity Test for *Pseudomonas aeruginosa* phage; 1-*Pseudomonas aeruginosa*, 2-*E. coli* and 3-*Salmonella typhi*

phages for *Salmonella typhi* and *E. coli* were specific for the respective host pathogen (Fig. 4, 5), whereas, the phage for *Pseudomonas aeruginosa* was able to infect both *Pseudomonas* and *E. coli* indicating it to be a broad-host-range bacteriophage (Fig. 6).

DISCUSSION

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.

More and more people are dying every year because of the multi drug- resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and wild strains of *Salmonella typhi* and *E. coli*. 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 (Sulakvelidze and Kutter, 2005). Outside the host cell, the phages are almost non living and they always need a host cell for replication and other metabolic processes (Carlton, 1999). The lethal effect of bacteriophages on their bacterial hosts has been known since their discovery.

Phages can be isolated from a wide variety of sources such as sea water, sewage water/sludge, etc. These phages feed mainly on the locally available organisms and cells. They have an added advantage in that they are host specific and evolve along with the host.

Bacteriophages have been found to be effective against a wide variety of pathogenic bacteria. The present study describes the isolation of bacteriophages effective against few human pathogens such as *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*, the causative agents of typhoid, opportunistic and nosocomial infections, respectively. In this study we have isolated bacteriophages from sewage using the methods described by Spencer (1955) and Hidaka (1971).

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 (Fig. 1-3).

The host range of the bacteriophages was determined by Spot Test (Armon and Kott, 1993). It was found that each of the three virulent phages was able to independently lyse the original host bacterium. Bacteriophage for *Salmonella typhi* was able to infect *Salmonella typhi* only but not *Ps. aeruginosa* and *E. coli* (Fig. 4). Phage for *Escherichia coli* was only able to lyse the original host bacterium (Fig. 5). Studies of bacteriophage infection have revealed that the process is initiated when the virion interacts with host cell surface receptor molecules (Hayes, 1968). Many bacteriophages are known to be highly specific for their receptors and show little or no interaction with receptors with an even slightly different structure. This specificity forms the basis of numerous phage typing methods for the identification of bacterial species or subspecies. The results obtained thus clearly indicated that the bacteriophages for *E. coli* and *Salmonella typhi* were highly specific against their respective host organism.

The bacteriophage for *Pseudomonas aeruginosa* was able to completely lyse the host bacterium as well as *E. coli* (Fig. 6). This indicated the broad range of host specificity of the *Pseudomonas* bacteriophage. It is clear that some bacteriophages do productively infect a range of bacterial species. Of these broad-host range phages, P1 and Mu are the best studied. Bacteriophage P1 is a generalized transducing virus capable of plaque formation on several enteric species in addition to *Escherichia coli* (Yarmolinsky, 2004).

The prevalence of broad host- range phages relates to the origin of virus particles which compose such a large percentage of the dissolved organic carbon in marine ecosystems (Bergh *et al*., 1989; Fuhrman and Suttle, 1993; Torrella and Morita, 1979) and which are present in very large numbers in other ecosystems as well (Hennes and Simon, 1995). Earlier studies have reported the isolation of a bacteriophage from a freshwater pond and sewage with *Pseudomonas aeruginosa* and *E. coli* as hosts.

The experiments demonstrated that broad-host-range bacteriophages are readily isolated from aquatic environments and that some of these bacteriophages are capable of generalized transduction. The experiments supported the hypothesis that bacteriophages with a broad host range, as judged by plaque-forming ability, are frequently and readily isolated from complex natural microbial communities.

It is certain that many natural microbial communities will contain complex and rich assemblages of many bacterial species existing in close proximity (Jensen *et al*., 1998). Numerous studies have revealed the presence of large numbers of virus particles in aquatic and other ecosystems (Bergh *et al*., 1989; Fuhrman and Suttle, 1993; Hennes and Simon, 1995; Torrella and Morita, 1979). The existence of broad-host-range bacteriophages may partially explain these observations. The results of our study suggest that host specific bacteriophages against human pathogens are prevalent and can be readily isolated from sewage ecosystems. Present findings are in agreement with the works done by Jensen *et al.* (1998). They had isolated broad- host- range lytic bacteriophages from sewage. In addition, these phages have been found to remain viable even after 3 to 4 weeks of isolation, when stored at 4°C in amber or opaque brown containers. This provides an excellent opportunity for the long lasting phages to be used for the prophylaxis of bacterial infections as well as for the treatment of biofilms. Detailed studies on the morphological characterization of these phages are in progress.

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REFERENCES

Armon, R. and Y. Kott, 1993. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. J Applied Bacteriol., 74: 490- 496.

Baross, J.A., L. John and R.Y. Morita, 1978. Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples. Applied Environ. Microbiol., 36: 492-499.

Bergh, Q., K.Y. Børsheim, G. Bratbak and M. Heldal, 1989. High abundance of viruses found in aquatic environments. Nature, 340: 467-468.

Carlton, R.M., 1999. Phage therapy: Past history and future prospects. Archivum Immunol. Therapiae Exp., 47: 267-274.

Flint, S.J., L.W. Enquist, R.M. Krug, V.R. Racaniello and A.M. Skalka, 2000. Principles of Virology. ASM Press, Washington, ISBN: 1-55581-127-2, pp: 3–15.

Fuhrman, J.A. and C.A. Suttle, 1993. Viruses in marine planktonic systems. Oceanography, 6: 51-63.

Hayes, W., 1968. The Genetics of Bacteria and their Viruses. 2nd Edn., Blackwell Scientific Publications Ltd., London, UK., ISBN: 0632052007.

Hennes, K.P. and M. Simon, 1995. Significance of bacteriophages for controlling bacterioplankton growth in a mesotrophic lake. Applied Environ. Microbiol., 61: 333-340.

Hidaka, T., 1971. Isolation of marine bacteriophages from sea water. Bull. Jap. Soc. Scientific Fish. Kagoshima Univ., 37: 1199-1206.

Jensen, E.C., S.H. Schrader, R. Brenda, T.L. Thomas and K.W. Lee *et al*., 1998. Prevalence of broad-host-range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli* and *Pseudomonas aeruginosa*. Applied Environ. Microbiol., 64: 575-580.

Kannan, N., 2002. Laboratory Manual in General Microbiology. Panima Publishing Corporation, New Delhi, ISBN: 81-86535-40-5, pp: 117-119.

Smith, L.S. and A.P. Krueger, 1954. Characteristics of a new *Vibrio*-bacteriophage system. J. Genet. Physiol., 38: 161-168.

Spencer, R., 1955. A marine bacteriophage. Nature, 175: 690-691.

Sulakvelidze, A. and E. Kutter, 2005. Bacteriophage Therapy in Humans. Bacteriophages: Biology and Applications. CRC Press, Boca Rutan FL., ISBN: 0-8493-1336-8, pp: 381-436.

Torrella, F. and R.Y. Morita, 1979. Evidence by electron micrographs for a high incidence of bacteriophage particles in the waters of Yaquina Bay, Oregon: Ecological and taxonomical implications. Applied Environ. Microbiol., 37: 774-778.

Wiebe, W.J. and J. Liston, 1968. Isolation and characterization of a marine bacteriophage. Marine Biol., 1: 244-249.

Yarmolinsky, M.B., 2004. Bacteriophage P1 in retrospect and in prospect. J. Bacteriol., 186: 7025-7028.