

Isolation of Host Specific Bacteriophages against Salmonella and Methicillin Resistant Staphylococcus aureus (MRSA) From Hospital Waste Water

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Abstract: Worldwide emergence of new resistance mechanisms in constantly increasing infections of Multidrug Resistance (MDR) bacteria (such as Methicillin resistant Staphylococcus aureus (MRSA) and Salmonella) is generating a major problem in treatment or prevention of infection against these pathogens. Development of resistance reduces the effectiveness of antibiotics against them. Antibiotics are costly and also harm useful microbes of host body, thus there is a need to adopt drug-independent remedial strategies to defeat pathogens. Phage therapy has therefore been considered as one of the strongest alternatives to antibiotics. Host specific Bacteriophages naturally and specifically kill their host without disturbing the normal microbes of host organism also provides cost effective treatment. Present Study describes the isolation of MRSA and Salmonella specific Bacteriophages from hospital sewage water sampled from sewage treatment plant at local hospital in jaipur and in vitro analysis of their lytic efficiency by plaque assay. Bacterial host one MRSA strain and two Salmonella typhi strain for this experiment collected from Sawai Man Singh hospital jaipur. Successful isolation of host specific Bacteriophages and determination of their lytic efficiency support the evidence of phage therapy as promising approach against multidrug resistant pathogenic hosts bacterium in present also since it started and hope to take world advantage and application of bacteria and phage relation to treat MDR infections naturally as much as possible.

Keywords: Multidrug resistance, phage therapy, Methicillin resistance Staphylococcus aureus (MRSA), Salmonella typhi, Bacteriophages, sewage, pathogens, plaque assay.

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I. Introduction

The use and misuse of antimicrobials or antibiotics in both humans and animals has given rise to the emergence of infectious bacteria displaying resistance towards many effective antimicrobials (European Food Safety Authority. 2004; Velgeet *al.* 2005; Arletet *al.* 2006), which are mainly used in treatment of hospital patient. As a result high proportions of hospital acquired infections are being seen around the world. A high percentage of hospital acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug resistant Gram-negative bacteria such as *Salmonella typhi*. People with MRSA have about 64% more mortality than people with a non-resistant form of the infection according to WHO. Although the treatment of MDR bacterial infections are extremely important; during 1998-2003, only nine new antibiotics were approved by the Food and Drug Administration; out of which two antibiotics have a novel mode of action on antibiotic resistant infections [Service 2004;]. There are currently no new classes of effective antibacterial compounds, which can prevent or treat antibiotic resistant bacterial infections (Falconer *et al.* 2009). Therefore, there is an urgent need to develop novel therapies to control these pathogens (Cohen, 1994). So the potential of therapeutic application of isolated phages from hospital waste is of current interest to scientific community. Thus this study has been planned to isolate bacteriophages from hospital waste water and to test their potential as therapeutic agents against already collected bacterial pathogens.

II. Materials and methods

Tryptic soy broth (TSB), Tryptic Soy agar (TSA), SM buffer (composition: NaCl 5.8g, MgSO₄.7H₂O 2.0 gm, 1M Tris HCl pH 7.4 50 ml, 2% gelatin 5ml, Quantity total make up to 1 liter, sterilize by autoclaving), chloroform 1% v/v, Ethanol, Syringe filter (0.45µ), Top agar : 0.7% TSA soft agar, Bottom agar : 1.5% TSA, pour into 90 mm hi media autoclavable Petri plates.

Bacterial strain

This experiment conducted with 3 bacterial strains 1 MRSA and 2 Salmonella as host strains which were collected from local government Sawai Man Singh hospital (source clinical isolates of human patients) at jaipur. Strains further confirmed by growth on mannitol salt agar and cefoxitin resistance in MRSA, and Xylose lysine deoxycholate agar used for salmonella biochemical test Catalase test done for both the strains. Overnight Bacterial cultures were prepared in tryptic soy broth, incubated at 30°C for 18 hr in shaking conditions.

Isolation of Bacteriophages

Isolation of Bacteriophages was carried out from raw sewage water sample collected from waste water treatment plant at nearby local hospital at jaipur. 100ml of freshly collected sewage sample in sterile autoclavable bottle transferred to the lab and preserve at 4°C. 50 ml of whatman paper filtered sewage sample mixed with 5ml of respective host culture and 50 ml of freshly prepared TSB. The mixture was incubated at 37°C overnight with shaking. After incubation 100µl of chloroform added to 10 ml of mixture with gentle shaking, leave for 30 min to kill the bacteria. The mixture was centrifuged at 4000g for 15 min, supernatant carefully pour off in sterile flask and sterile filtered through 0.45µm nylon membrane filter (Randisc). To detect the presence of host specific bacteriophage in filtered phage lysate and their lytic efficiency. Serial 10 fold dilutions 10^{-1} - 10^{-10} of filtrate prepared with SM buffer. 100 µl of diluted phage and 200 µl of target bacteria were mixed with 2ml of 0.7% TSA soft agar (prewarmed at 45°C in waterbath) in sterile test tube followed by spreading on Tryptic soy agar plates (prewarmed at 37°C in incubator.(Sambrook ,j Russell 2001) Leave plates for 15 min in laminar until soft agar solidified .After this plates inverted and incubated overnight at 37°C. Thus for each strain 10 dilutions are prepared and result in 10 TSA plates for analysis. Total of 30 plates (1 MRSA and 2 *Salmonella typhi*) were prepared for bacteriophage plaque analysis.

Purification of Bacteriophages

A sterile inoculation loop used for the purification of phages. Materials from the centre of the plaque scrapped using loop and transferred aseptically to the TSB broth containing respective organism, incubated o/n for about 18 hr at 25°C. after incubation mixture was centrifuged at 5000 rpm for 30min and filtered through 0.45µm membrane filter. The filtrate stored in sterile amber bottles. Plaque assay again carried out ,thus cycle repeats 3 times to ensure the purity of phages. All the phage lysate stored at 4°C.

III. Results and Discussions

Three known reference Strain one of MRSA and two of *Salmonella typhi* strains collected from SMS hospital jaipur gives positive growth of MRSA on mannitol salt agar incorporated with cefoxitin disc and positive for catalase test. On the other hand *Salmonella typhi* gives yellow colonies on XLD agar and are positive for catalase test. Results confirmed their identity as strain 1 was MRSA, Strain 2 and strain 3 were *Salmonella typhi*.

Table 1: Biochemical tests to confirm the characteristics of host bacteria.

Biochemical test	Strain 1	Strain 2	Strain3
Growth on mannitol salt agar incorporated with cefoxitin	Yellow colonies	Test not applied	Test not applied
Growth on XLD agar	Test not applied	Yellow colonies	Yellow colonies
Catalase test	Positive	Positive	Positive

Strain 1: Mannitol salt agar contains a high concentration (about 7.5%-10%) of salt (NaCl) which makes it selective for Gram-positive bacteria *Staphylococcus* since this level of salt is inhibitory to most other bacteria. Yellow color of colonies indicates fermentation of mannitol produces acid which turns phenol red in the agar to yellow, thus confirms its identity as mannitol fermented *Staphylococcus aureus* . Growth of colony in presence of cefoxitin disc indicates the resistance against this antibiotic which is found in Methicillin resistant *Staphylococcus aureus* (MRSA). Catalase test is always positive for MRSA.

Strain 2: XLD agar have suagar Xylose and phenol red indicator. Fermentation of xylose produce acid which turns into yellow colour .*Salmonella*, fermented the sugar xylose to produce acid thus gives yellow color colonies.*Typhi* strain of *Salmonella* had confirmed by positive results of methyl red test. Catalase test is always positive for *Salmonella*.

Strain 3: XLD agar have suagar Xylose and phenol red indicator. Fermentation of xylose produce acid which turns into yellow colour .*Salmonella*, fermented the sugar xylose to produce acid thus gives yellow color

colonies. Typhi strain of *Salmonella* had confirmed by positive results of methyl red test. Catalase test is always positive for *Salmonella*.

Bacteriophages observed in the form of plaques in their respective host plates. Below are the results shown the presence of Bacteriophages against respective host in sewage sample.

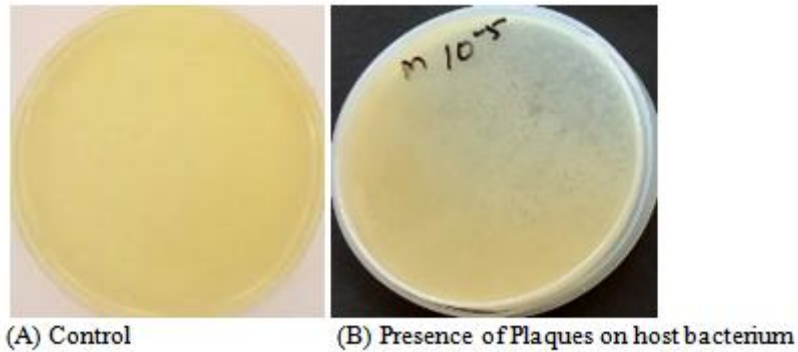


Fig 1: Methicillin resistance *Staphylococcus aureus* showing plaques formation at dilution 10^{-5}

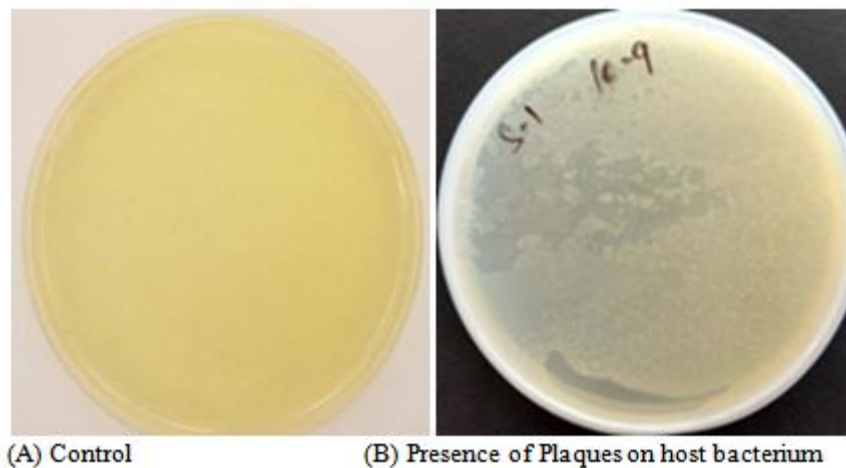


Fig 2: *Salmonella typhi* strain 1 showing clear plaques formation at dilution 10^{-9}

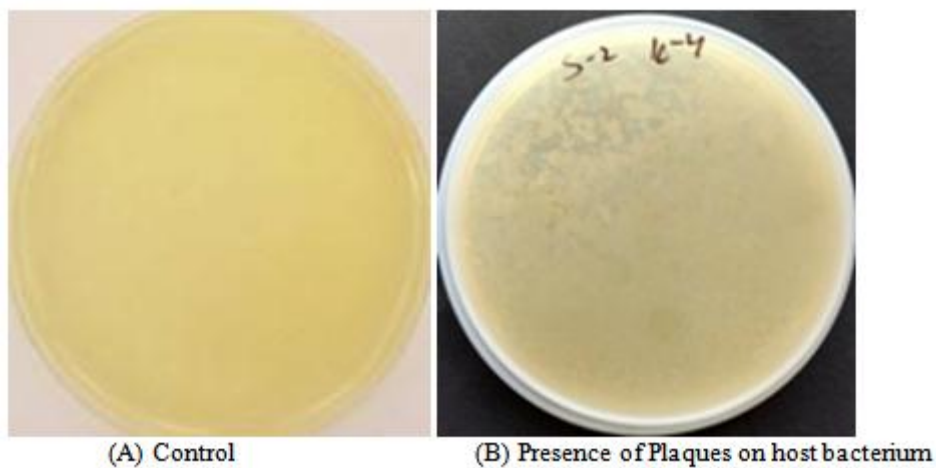


Fig 3: *Salmonella typhi* strain 1 showing clear plaques formation at dilution 10^{-4}

Plaques were analysed at different dilutions for all the three host strain :
 MRSA as host: Clear zone of lysis in small area of plates were visible in 10^{-5} dilution highest, 10^{-7} , 10^{-8} and 10^{-9} while lower dilution 10^{-1} - 10^{-4} , 10^{-6} and also 10^{-10} shows no zone of clearing.
S. typhi strain 1: Clear zone of lysis in large area of plates were visible at 10^{-8} and 10^{-9} dilution highest, from 10^{-7} - 10^{-1} zone of lysis decreases continuously. 10^{-10} has similar results with 10^{-7}
S. typhi strain 2 : Clear zone of lysis in large area of plates were visible at 10^{-9} and 10^{-10} dilution maximum followed by 10^{-8} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-7} had produce very less zone of lysis 10^{-1} , 10^{-2} and 10^{-6} dilutions unable to produce visible plaques or zone of lysis on plates.

Table 2: Showing lytic efficiency of isolated Bacteriophages in SM buffer dilutions.

S.NO	Tenfold serial dilution	Zone of lysis (plaque) visibility / area of plate under observation		
		MRSA	<i>S.typhi</i> 1	<i>S.typhi</i> 2
1	10^{-1}	-*	+ ¼	-
2	10^{-2}	-	+ ¼	-
3	10^{-3}	-	+ 2/4	+ 2/4
4	10^{-4}	-	+ 2/4	+ 2/4
5	10^{-5}	Max. Zone of lysis	+ 2/4	+ 2/4
6	10^{-6}	-	+2/4	-
7	10^{-7}	+ 3/4 area	+ ¾	+ 2/4
8	10^{-8}	+ 3/4 area	Max. Zone of lysis	+3/4
9	10^{-9}	+ 3/4 area	Max. Zone of lysis	Max. Zone of lysis
10	10^{-10}	-	+3/4	Max. Zone of lysis

-*Means no zone of clearing

Results shows that visibility of plaques increases with increasing dilution .visibility of plaque clear and higher between 10^{-5} - 10^{-10} dilutions.

IV. Conclusion

Global existence of large number of Bacteriophages in air, water and soil makes them susceptible for isolation and treatment of pathogenic bacterial infections. In our study we successfully isolated the Bacteriophages against MRSA and *Salmonella typhi* strain. Further characterization and morphology were also done. Variation in lysis zone at different phage dilutions shows that optimum concentration of phages is required to see visible plaques on agar plates.

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