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IN VITRO EVALUATION OF BACTERIOPHAGES AGAINST BACTERIAL PATHOGENS FROM WOUND INFECTIONS

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ABSTRACT

The insurgence of antimicrobial resistance is the most common attrition in the management of infectious diseases. In modern medicine, the antibiotics are considered as bedrock in treating drug resistant bacteria. By thinking the alternative, the authors proposed a research by determining *in vitro* analysis of antimicrobial activity of isolated specific bacteriophages. The main objective of this study is to construct bacterial specific phages from the environmental sources and evaluate *in vitro* effectiveness against wound infection causing bacterial pathogens. The isolation procedures were optimized and bacteriophages were isolated from raw sewage. After confirming the bacterial pathogens from clinical samples, the phage activity was performed. As a result, plague forming units were identified and counted to confirm the bacterial phage particles. Further, it was identified that the isolated phages were infected the bacterial host cells and no significant observation of the bacterial growth. In conclusion, phage therapy may be an important alternative to antibiotics for treating multidrug resistant pathogens causing superficial infections.

KEYWORDS: Bacteriophages, sewage, plague forming units, antibacterial activity, *in vitro.*

INTRODUCTION

Increased with multidrug resistant (MDR) pathogens is responsible for the increased duration of hospitalization, cost of management, morbidity and mortality of many patients.[1] Failure in antibiotic therapy and the emergence of MDR pathogens have further hampered against infectious diseases. Antibiotics are the foundation of modern medicine, but due to misuse, abuse and overuse, it has been functionless. But in the very near future, we're going to learn to live without antibiotics once again. And it's going to get wicked, unless other modalities of treating these drug resistant bacteria are assessed.[2]

Escherichia coli, Proteus spp., *Pseudomonas* spp., *Staphylococcus aureus* and *Enterococcus* spp. are the most frequent pathogens contributing to progressive and widespread tissue destruction.[3] Wound infections are often poly-microbial[4] and isolation of Methicillin-resistant *Staphylococcus aureus* (MRSA) has been commonly observed.[5] The increasing association of MDR pathogens with wound infection further compounds the challenge faced by the physician or the surgeon in treating wounds without resorting to amputation. Infection with MDR pathogens is also responsible for the increased duration of hospitalization, cost of management, morbidity and mortality of the patients.[1]

The use of herbal medicine in wound care has been very encouraging and several researchers around the globe have started to practice. Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain an active ingredient, which contains parts of plants, or other plant materials or combinations. Before the advent of modern medicine, people of all continents have used medicines from plant origins since pre-historic times. Other complementary medicines, including phage therapy are also being practiced throughout the universe to get cured of infections. Thus, treating the drug resistant bacteria with bacteriophages is quite interesting nowadays. Bacteriophage therapy is a method of antibacterial treatment that harnesses the bacteria-killing properties of harmless viruses.[2,6,7]

Screening procedures for isolation of the bacteriophages were designed and used for the isolation of stable integrated phages construct specific to the bacterial genus by filtration and centrifugation.[8] Most of the bacteriophages are lytic by infecting the host cell leads to production of virions and death of the host cell by lysis.

As an antibiotic therapy, phage therapy is not yet fully established alternative to antibiotics due to the requirement of repeated treatment with phages, rapid absorption by the tissues or superficial, deep layers and mucous membrane.[9,10]

There are additional attribute for phages that are used for therapy

1. Host specificity
2. Potential values with the trait
3. Ideal molecule for co-therapy with antibiotics

The emergence and spread of resistance in several microorganisms have rendered the management of wound infections in hectic manner. Failure to discover newer antimicrobial agents has further hampered the war against infectious agents. Hence the present study is planned to isolate bacterial specific phages from the environmental sources and evaluate the *in vitro* effectiveness against various wound infections causing bacterial pathogens.

MATERIALS AND METHODS

Ethical clearance

The study was conducted in accordance with the permission from institutional research board (IRB) approval of the institutional ethical committee (IEC) of the institute (Reference: CMCH&RC/ IEC-25 dated 28.04.2014).

Collection of wound swabs

A total of 50 wound swabs was collected from wound infection cases admitted in the inpatient departments of Surgery and Orthopaedics to screen for possible bacterial pathogens by standard culture method.[11]

Characterization of bacterial isolates

Wound samples were collected using sterile cotton swabs (fresh pus). The pus specimen was inoculated with blood and MacConkey agar plates. The streaked plates were incubated at 37°C for 24 hours. Identification of isolates were done based on colony morphology, Gram staining, motility, catalase test, oxidase test, coagulase test and biochemical tests.[12]

Formulation and Isolation of specific bacteriophages from raw sewage

Aseptically 5ml of culture broth and 45ml of raw sewage water were added, mixed thoroughly and incubated for 24hours at 37^{o}C. Following incubation, the culture-sewage mixture was centrifuged at 2500rpm for 20 minutes. Without disruption, the supernatant was transferred to the sterile flasks. Through 0.45µ membrane filter unit, the supernatant was filtered aseptically. The filtrate was stored for further analysis. This method was followed separately for all the three bacterial cultures. A set of 5 test tubes with 5ml tryptone broth each were taken. In the first broth tube, one drop of phage filtrate was added and 2, 3 and 4 drops were added appropriately.

To this phage filtrate - tryptone broth, 0.1ml of specific bacterial cultures was added. This mixture was mixed thoroughly. Tryptone agar plates were prepared. On the surface of the agar, 0.1ml of the phage filtrate bacterial cultures was inoculated by a standard spread plate method using L-rod. These plates were incubated for 24hours at 37^{o}C. Further the plates were dispensed for the detection of plaque formation by counting the Plaque forming units (PFUs).

Detection of specific phage lysis

Luria Bertanii (LB) agar plates were prepared. The specific bacterial pathogens were spread on the agar surface using L-rod. The wells were cut with standard distances aseptically. The mixture of plague containing tryptone broth and 0.1M calcium chloride solution was added in all wells appropriately. Then the plates were incubated for 24hours at 37^{o}C. Following incubation, the plates were observed for the plaque forming units around the wells.

Determination of phage interaction with bacteria

Plaque assay by double layer method

Hundred (100) µl of early exponential phase bacterial culture and 50µl of respective lysates were mixed with $CaCl_2$ and $MgSO_4$ (0.1 M final concentrations) into 3ml of melted LB soft agar tube. It was then poured on LB agar plate and incubated at 37°C overnight. Negative control contained no lysates.[13,14]

Spot assay by double agar layer method

Hundred (100) µl of the early exponential phase culture of bacterial culture was mixed into 3ml of melted LB soft agar and plated on a LB agar plate. After solidification, 10µl of phage lysate were applied on the bacterial lawn and incubated at 37°C overnight.

RESULTS

The isolates from wound specimens were confirmed as *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The microscopy, colony morphology and biochemical reactions also supported the same. The bacteriophage isolates showed positivity to all isolates and primarily confirmed as specific phages included coliphage, pseudophage and staphylophage. The plaque forming units also supported the same. The detailed results related to the PFUs in each isolate are depicted in table 1.

Table 1: PFUs of selective phages

S.No.	Bacterial culture	Concentrations	PFUs/ml
1	*Escherichia coli*	1 drop	54
		2 drops	98
		3 drops	172
		4 drops	TNTC*
2	*Pseudomonas aeruginosa*	1 drop	20
		2 drops	28
		3 drops	32
		4 drops	45
3	*Staphylococcus aureus*	1 drop	12
		2 drops	19
		3 drops	24
		4 drops	38

*Too numerous to count - Count more than 300 PFUs

The highest PFUs found in each bacterial specific phages are denoted in figure 1. This highlighted the importance of centrifugation and membrane filtration that specifically required for the isolation, separation and purification of the phages from the environmental samples. The selection criteria denoted that the phages may adapt to the environment with selection pressure and other stresses.

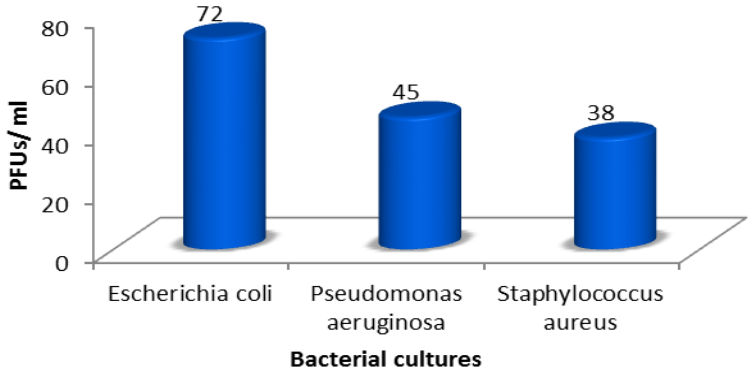

Figure 1: Observation of PFUs verses bacterial specific phages

The determination of the antibacterial activity using the specific bacteriophages was highly observed with the formation of plaque forming units around the wells done. This showed that the *in vitro* confirmation of the phages against the bacterial pathogens may largely be useful in the fields of orthopaedics, Burns and other related infection control practices. This *in vitro* analysis provided a platform to the modern medicine to overcome the antimicrobial resistance. The table 2 highlights the data related to the detection of phage that lyse the bacterial pathogens and the observation related to the lysis phenomena also depicted in figure 2. Further the plaque and spot assay of phage on the lawn of multi drug resistant *P. aeruginosa* were also analyzed and impregnated in figure 3a and b respectively.

Table 2: Detection of phage that lyse the bacterial pathogens

S.No.	Bacterial culture	Concentrations	Observation
1	*Escherichia coli*	1 drop	No lysis
		2 drops	Lysis
		3 drops	Lysis
		4 drops	Lysis
2	*Pseudomonas aeruginosa*	1 drop	Lysis
		2 drops	Lysis
		3 drops	Lysis
		4 drops	Lysis
3	*Staphylococcus aureus*	1 drop	Lysis
		2 drops	Lysis
		3 drops	Lysis
		4 drops	Lysis

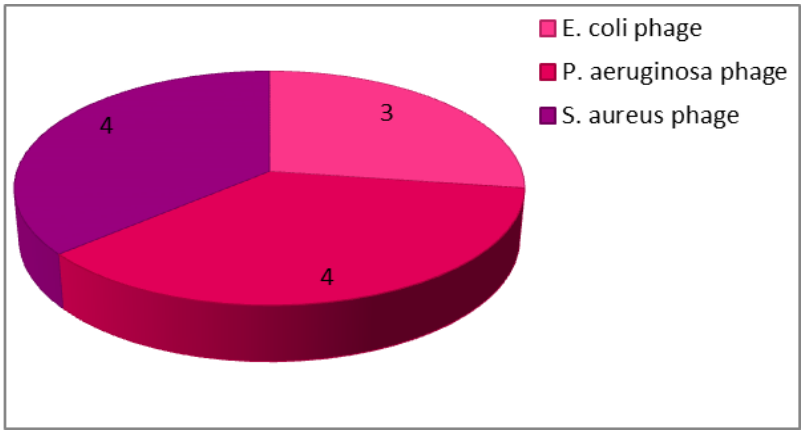

Figure 2: Observation of lysis phenomena

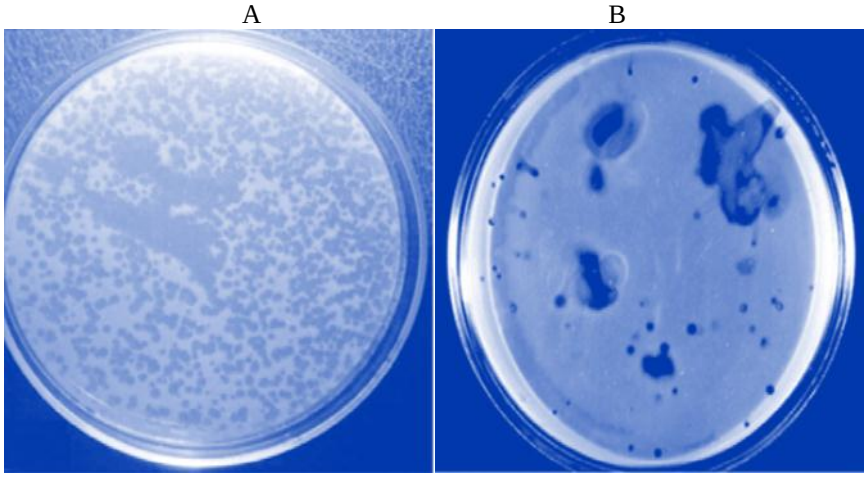

Figure 3: Plaque assay of lytic phage

A. Plaque assay of phage on the lawn of multi drug resistant *P. aeruginosa.*
B. Spot assay of phage on the lawn of multi drug resistant *P. aeruginosa.*

DISCUSSION

Bacteriophages have very effective bactericidal activity and several advantages over other antimicrobial agents. Most notably, phages replicate at the expense of bacteria, are available in abundance where they are most required and so far, no serious or irreversible side effects of phage therapy have been described.[5] Although there are no phage therapy products in the Western country market at the moment numerous companies have developed or are in the process of developing phage-based products against *Escherichia coli*, *Pseudomonas* and *Staphylococcus aureus* infections.[15]

Bacteriophages have also been used to reduce the catheter- associated biofilms of bacterial strains.[1,16] Plaque-forming ability of the phage depends directly on the composition of the medium used and the temperature of incubation. A rich medium and at a temperature optimal for the growth of the host cells, fails to produce discernible plaques, probably owing to the rapid growth of the host cells followed by a decline in their metabolic activities, which interferes with phage adsorption. However, in a medium which is barely sufficient for growth of cells, visible plaques are formed.[3,17] The clear plaque mutant observed in mass cultures essentially has the same characteristics as does the parent phage.

The authors believed some reasons that phage therapy has not been globally recognized and applied may be due to following major concerns which are already discussed.[18]

1. The rapid lyses of a large numbers of microbes, especially Gram negative that may release endotoxin (i.e. LPS) leads to pyrexia and Jarisch-Herxheimer reaction.
2. The concern is the "by-standard effect", where phages may destroy other non-target microbes and disturb the normal flora.
3. Phages are highly receptor-specific.
4. Retrospective history of using phage administration by different routes in several countries has reported.
5. There have been almost no report of serious complication related to their use as phages are common entities in the environment and regularly consumed in foods, the development of neutralizing antibodies should not be a significant obstacle during the initial treatment of acute infection, because the kinetics of phage action or lytic

enzymes is much faster than immune recognition and antigen processing system by the adaptive immunity.
6. If antibodies are generated by a host against a particular host's immune system, it is unlikely that the same host will be receiving the exact same phage therapy twice.
7. There is a possibility that phage preparations may contain residual bacterial antigens or endotoxins.

To address this, bacteriophage production for clinical trials have to follow specific Good Manufacturing Practice (GMP) guidelines with appropriate quality controls and to meet specific standards for purity and sterility.

CONCLUSION

Individual components of phages (e.g. lysins) can also be used as antibiotic substances. So far, resistance has not occurred despite comprehensive testing. Due to their specificity, phages do not cause a selection of resistance in the bacteria that live in and on the body. Phages are found throughout nature. This means that it is easy to find new phages when bacteria become resistant to them. The data obtained in this report provide the newer ideas to the biomolecular formulation to overcome the antimicrobial resistance. This phage product may be highly useful in the treatment of burns, deep wounds (acute or chronic), catheter oriented infections, dental implants and other orthopedic issues.

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