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THERAPEUTIC EFFECT OF MANGROVE-DERIVED BACTERIOPHAGES ON BIOFILM FORMING BACTERIA ISOLATED FROM MICE BEARING LUNG-CANCER

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ABSTRACT

Therapeutic potential of phages was examined against biofilm forming bacteria (BFB) *Pseudomonas stutzeri* (PS) and *Vibrio parahaemolyticus* (VPH) in lung cancer animals. Phages were isolated from mangrove soil. The latent period of the infectious cycle was determined as 20 and 55 min for PS and VPH phages respectively. The mean burst size was found to be 102 and 156 pfu per bacterial cell respectively. The disruption of bacterial cell membrane with respect to incubation time was visualized by Epi-Fluorescence microscopy (EFM). *In vitro* biofilms clearance study showed the biofilm removal due to phage activity varied between 23 and 92%. Toxicity study proved that the injected phages showed no toxicity in mice. Survival and stability of PS and VPH phages were measured in animals after i.p. and i.v. injection. And the phage count was measured in blood and lungs tissues and the maximum phage counts in lungs and blood were obtained 6 h of post injection. Route of i.v. injection provided the most significant (87%) protection. In phage-treated animal groups, as compared with untreated control groups, there was a signification reduction in biofilm forming bacteria (BFB) in mice bearing lung cancer. Mice intravenously injected with PS and VPH phages in addition to BFB had a much lower death rate than mice administered with BFB alone. Treatment of mice with PS and VPH phages proved to be effective as measured by reduction in cfu and body temperature and histopathologic analysis. These results demonstrated that bacteriophages had therapeutic potential for both localized and systemic infections caused by bacterial pathogens in animals.

KEYWORDS: Bacteriophages, Biofilm forming bacteria, Mangroves, Phage therapy.

INTRODUCTION

Bacteriophages can be useful as phage therapy in many fields from medicine to agriculture. They may be used as a tool to treat bacterial infections because of their remarkable antibacterial activity that too because of their characteristic host-specificity nature. Phages can be used as a natural antimicrobial agent to reduce bacterial pathogens in the food supply.[1] Since phage discovery, these biological agents have been applied to humans only in few countries (Georgia, Poland, Russia). Bacteriophages have long been known to be clinically effective and safe antimicrobials in animals and humans.[2] This has once again drawn attention to phage therapy to treat several diseases such as abscesses, wounds, vaginitis, acute and chronic infections of the respiratory tract, etc. Bacterial virus, in particular marine forms have a great potential for their applications especially in phage therapy, nanotherapy, phage display, food decontamination, surface disinfection and bio-detection.[3] The phages are environmentally friendly. Their production cost is also very low as compared to antibiotic production.[4] However, the mangrove-derived phages were never attempted in phage therapy. Hence, the present study was undertaken to analyse the effect of mangrove-derived phages on biofilm forming bacteria in the mice bearing lung cancer.

MATERIALS AND METHODS

In this study, the potential use of bacteriophages was examined as therapeutic agents against two biofilm forming bacteria (BFB) namely *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* in the mouse model (*Mus musculus*) in which pulmonary carcinoma (lung cancer) was induced by benzo (amino) pyrene B[a]P.[5]

ISOLATION AND IDENTIFICATION OF BFB

Sampling and Plating: Qualitative screening of BFB was made from mice induced with pulmonary carcinoma. Severely injured lethargic (fatally infected) cancer-induced animals were sacrificed under sterile condition. The mucus samples were collected under laminar flow hood from the upper respiratory tracts and cancerous cell lesions of the mice, and transferred to a solution consisting of 500 µL of PBS (phosphate buffered solution) and 500 µL of ultra pure water. The samples were processed within 2 hours**.** The collected samples were weighed, suspended in 2 ml of filter-sterilized PBS (pH 7.2), and homogenized with sterile mortars and motor-driven Teflon pestles. The numbers of bacteria (cfu.g^{-1} tissue) from homogenized samples were determined by serial dilution and plating on nutrient agar

medium and incubated for 24-48 hrs at 37°C. BFB were selected based on biofilm formation in nutrient broth.

Identification: Molecular identification of bacterial isolates was done at species level by DNA extraction and amplification using PCR with universal bacterial primers.[6, 7]

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES

Phage Screening, Isolation and Purification: Mangrove soil samples from Pichavaram, Tamil Nadu, India were screened for the presence of phages against the BFB derived from cancer-induced animals[5] by liquid enrichment cultures using the host of interest[8] and isolated the phages by plaque assay (double-layer agar overlay).[9] The plaques were cut out from the plate and inoculated to fresh culture of BFB to enrich the phages. After incubation at 30±2°C for 24 h, the enrichment was centrifuged to remove bacteria and filtered through 0.22 µm filter and the presence of lytic activity on BFB lawn was confirmed. Phage plaques were harvested from the agar plate and single phage plaques were purified three times on a host strain by the standard procedure as described by Sambrook *et al.*[10] The phages derived against *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* were referred to as PS Phage and VPH phage respectively. Once clonal isolates were obtained, a series of characterization and experimental studies were carried out to determine the impact of these viruses. Some of these include electron microscopic (TEM) observation, epifluorescence microscopy (EFM), one-step growth experiments, determination of optimal multiplicity of infection (MOI) [the ratio of infectious agent (e.g. phage or virus) to infection target (e.g. bacterial cell)][11] and *in vitro* biofilms clearance study.

Electron microscopy of phage: The phage suspension was concentrated to a concentration of 10^9 pfu.ml^{-1}. After dialysis against 50 mM Tris-HCl buffer (pH 7.2) containing 0.2 M NaCl overnight, phage samples were viewed under TEM. The phages were transferred to 300 µm-mesh carbon-coated copper grids by floating the grids on drops of filtered lysate for 30 min. The grids were stained with 2% uranyl acetate and photographed at an acceleration voltages of 80 kV using TEM (Philips EM).

Phage adsorption rate and one-step growth curve: The adsorption rate of phages PS and VPH was determined by following the method of Foschino *et al.*[12] and Abedon *et al.*[13] A one-step growth curve was performed in order to determine the burst size, eclipse period, rise

period and latent period of the phage.[14, 15, 16, 17] It was designed to observe one cycle of adsorption, multiplication and lysis of PS and VPH phages.

***In vitro* biofilms clearance study under static conditions:** A modified method of Reisner *et al*.[18] and Sillankorva *et al*.[19] was followed. In this work, *P. stutzeri* and *V. parahaemolyticus* biofilms were allowed to form into glass test tubes containing 4 ml of nutrient broth and treated with phage at final concentrations of 10^5, 10^6, 10^7 and 10^8 pfu.ml^{-1} and incubated at 37°C for 48 h without shaking. Biofilm formation was assayed by staining of test tube-attached cells with crystal violet (CV). After removal of medium and two washes with 6 ml of 0.9% NaCl solution, surface-attached cells were covered with 6 ml of 0.1% CV for 5 min. Following two subsequent washes with 7 ml of 0.9% NaCl solution, surface-bound CV was observed.

Epifluorescence microscopic (EFM) studies: The disruption of bacterial cell membrane with respect to incubation time (before and after treatment of phages) was visualized by Epi-Fluorescence microscopy (EFM). Fluorescein isothiocyanate (FITC) and Propidium iodide (PI) dual stains were used for identifying living/dead cells on bacterial population. About 0.5 µl of dual stain (FITC-PI; 1:1%) was added to bacterial culture and to bacterial culture infected with phages. The stained samples were homogenized with a tip pipette to break up biofilms, if any and distributed the cells evenly across the surface of the coupon. They were incubated under dark conditions for 15 min at room temperature. The excess stain was rinsed with sterile distilled water and examined under 40× objective of Epi-fluorescence microscope (E200 Coolpix -Nikon, Tokyo, Japan).

ANIMAL STUDY

The male albino mice with an age of 8-10 weeks weighing 90-110 g procured from central animal facility house, Annamalai University were used as experimental animals for this study with proper university ethical clearance (Ethical number: 160/1999/CPCSEA). The animals were housed under conditions of controlled temperature (26°C), with a 12-h day–night cycle. They were fed a standard pellet diet (Amrut rat/mice feed; M/s. Hindustan Unilever Ltd, Mumbai, India) and were given free access to water *ad libitum*.

Chemicals Used for the Study: Carcinogen Benzo (a) pyrene (B[a]P) was purchased from Sigma Chemical Company, USA and used for this study. All other reagents used were of analytical grade.

Experimental design

The animals were randomized into experimental, control groups and divided into five groups.

Group I: Normal male mice [olive oil]

Group II: B[a]P (50mg/kg body weight dissolved in olive oil)

Group III: B[a]P + oral administration of *P. stutzeri* and *V. parahaemolyticus* (10^6 cfu.ml^{-1})

Group IV: Cocktail of Phages alone (10^{10} pfu.ml^{-1}) (cytotoxicity if any also tested for phages alone)

Group V: B[a]P + Biofilm bacteria + PS and VPH phages suspension (10^8 pfu.ml^{-1})

Induction of pulmonary carcinoma in mice by oral route: Animals in group I was treated as untreated control (olive oil alone). Experimental pulmonary carcinoma was induced in mice with B[a]P. The animals in group II were administered with B[a]P (50 mg/kg body weight dissolved in olive oil) using a No. 0.6 mm feeding tube twice a week for 4 weeks (8 doses).

Introducing biofilm-forming bacteria in cancer-induced mice by oral route: Animals in group III were given with B[a]P as in group II, orally challenged with 10^6 cfu of *Pseudomonas stutzeri* and *Vibrio parahaemolyticus*. To determine the minimum lethal dose in these mice, doses ranging from 10^2 to 10^8 cfu.ml^{-1} were given orally. The dose (100 µl inoculum containing 10^6 cfu each) giving 99 % infection rate without causing mortality was taken as the optimum dose and animals were observed for 10 days. One animal at 1, 2, 3, 5, 7 and 10 post-infection day were sacrificed. Lungs were aseptically removed, homogenized in 5 ml sterile PBS buffer (pH 7.2) and subjected to bacteriological examination.

Toxicity testing of phage in mice: Male mice of 8-10 weeks weighing 90-110 g were used in this study. The toxicity of PS and VPH phages suspension was investigated in group IV animals according to a modified method of Soothill.[20] Three animals were injected with 0.25 ml phage suspension (10^{10} pfu. ml^{-1}) by the intraperitoneal (i.p.) [21] and intravenous (i.v.) route.[22] Three uninjected mice were retained as sub-controls. The mice were observed for signs of illness, and rectal temperature was taken hourly during the first 5 h after injection and then daily for the next 4 days. The experiment was terminated at the end of 16th week and the samples were analyzed.

Phage half-life in mice: The survival and stability of PS and VPH phages was measured in mice according to the method of Cerveny *et al*.[23] with slight modifications. Uninfected mice were injected intraperitoneally (i.p.) and intravenous (i.v.) with phage preparation at 10^8 pfu.ml^{-1}. At 3, 6, 12, 24, 36, 48 and 72 h post-infection, mice were sacrificed and their peritoneal cavities were subjected to lavage with PBS buffer (pH 7.2).[24] A total of 100 µl of cardiac blood was collected in 0.05 M EDTA. The lungs were aseptically removed, weighed and homogenized in PBS buffer. Phage titre was measured in all the samples by plaque assay.

Animal weight (Data not given)**:** Animal weight was recorded in every week.
Animal weight = initial weight of the animal – final weight of the animal
Tumor volume: Tumor volume was calculated by the water displacement method.
Tumor burden: Tumor burden = number of tumor × volume of tumor.

Phage protection study: The therapeutic potential of PS and VPH phages, specific for *Pseudomonas stutzeri* and *Vibrio parahaemolyticus*, was evaluated in Group V animals induced with pulmonary carcinoma by B[a]P for their ability to reduce the load of biofilm forming bacteria. Optimal phage dose was determined following the method of McVay *et al*.[25] Group V contained 12 animals. Two sets of six animals each were administered by the i.p. route and i.v. route with 100 µl phage preparation at a MOI of 100 each, followed by simultaneous oral bacterial challenge. Group III animals were kept as bacterial infected control for this group V experimental set up. In both the sets, the animals were monitored for 10 days. One animal each at days 1, 2, 3, 5, 7 and 10 post-infection were sacrificed and lungs were aseptically removed and subjected to bacteriological examination as described earlier.

Histopathological analysis: Lung tissue samples preserved in 10% formalin were dehydrated in an ascending series of alcohol (70–100%). The tissue was embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin.[26, 27]

Statistical analysis: Results were analysed statistically by applying Student's t-test for comparing bacterial counts in phage-treated mice and phage-untreated control mice. Differences were considered statistically significant if P values were less than 0.05.

RESULTS

The study was to test the therapeutic potential of *Pseudomonas* phage and *Vibrio* phage in mice induced with pulmonary carcinoma. Two major aspects focused are (i) control of biofilms *in vitro* and (ii) control of BFB *in vivo*.

Isolation and identification of BFB

Two biofilm forming bacterial (BFB) isolates were isolated from mucus of mice bearing lung cancer. Molecular identification of those two BFB isolates was done. The BFB isolates were found to be *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* and were submitted to the NCBI's Genbank through BankIt according to NCBI's procedure with required information and obtained accession numbers as KF886683 and KF886684.

Isolation and characterization of BFB phages from mangrove soil

The mangrove soil samples were tested for the presence of phages by using *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* as indicator hosts for phages. Standardization of the soft agar medium, used for the bacterial lawn in the plaque assays, became a variable which interfered with the isolation of morphologies of plaques by size.

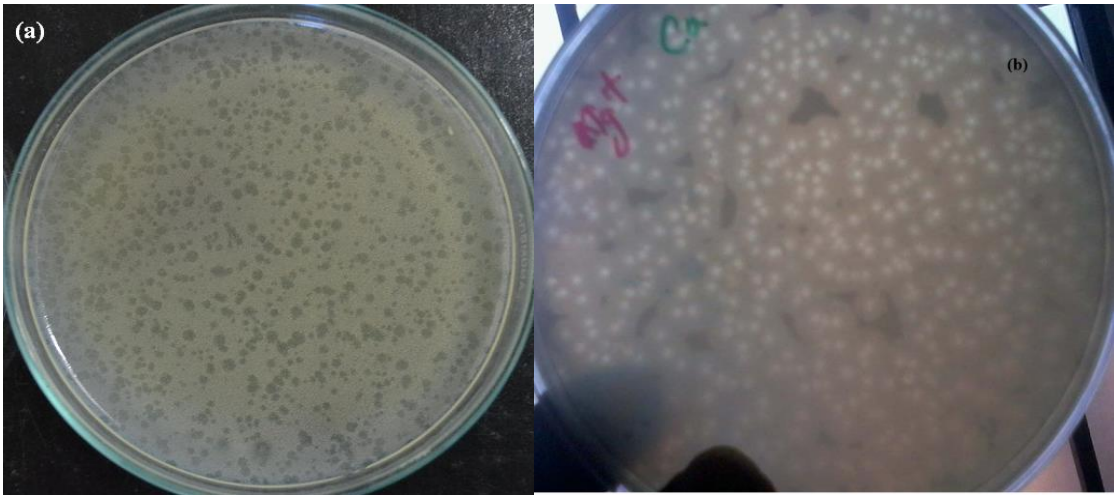

Fig. 6.1. a) Plaques formed on *Pseudomonas stutzeri* plates called PS Phage, b) Plaques formed on *Vibrio parahaemolyticus* plates called VPH Phage.

Plaques were identified from the enriched soil samples. Plaques were found to form on both the *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* plates (Fig. 1a and 1b). The adsorption rate of PS and VPH Phages were determined by mixing phage with an excess of their respective host cells, and then non-adsorbed infectious phages were serially diluted and counted. The adsorption rate of PS and VPH Phages were found to be 96% and 98% respectively. The one- step growth curve of PS on *Pseudomonas stutzeri* and VPH on *Vibrio parahaemolyticus* was produced. The latent period, defined as the time interval between the adsorption and the beginning of the first burst, of the infectious cycle was about 20 and 55 min respectively. The mean burst size was about 102 and 156 pfu per bacterial cell

respectively, calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cell during the latent period (Fig. 1).

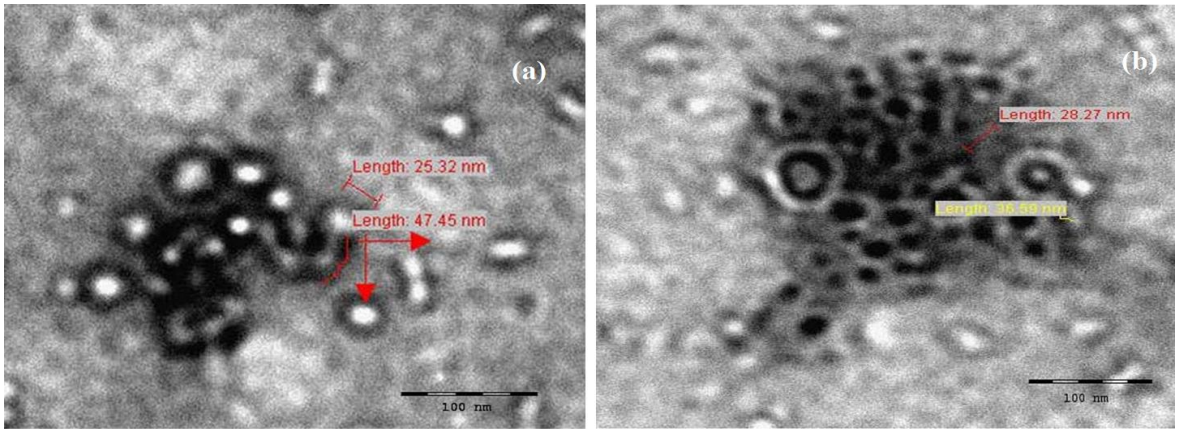

Fig. 2 a and b. TEM image of PS phage particles negatively stained with 2% uranyl acetate

The TEM images of PS and VPH phage particles are shown in Fig. 2a and 2b. PS phage isolates had isometric heads that were 25-28 nm in diameter and contractile tails (47-60 nm) (Fig. 2a). VPH phage consists of a head with a hexagonal to polyhedron shape, 28-30 nm in diameter, and a straight tail, 36-40 nm length (Fig. 2b).

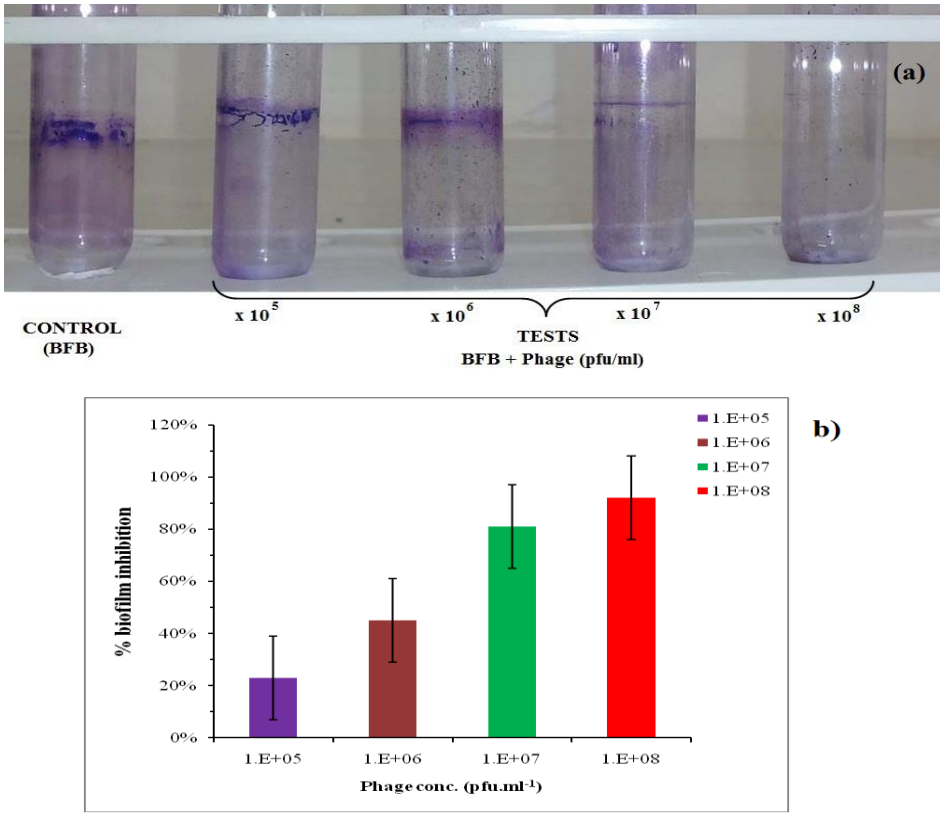

Fig. 3 a) Reduction of biofilm by four different concentrations of phages as evidenced by the clearance of biofilm layer inside the tubes stained by crystal violet. b) Percentage of biofilm inhibition.

***In vitro* biofilms clearance study**

In biofilm quantification assay, the test bacterial pathogens were treated with phage at a final concentration of 10^5, 10^6, 10^7 and 10^8 pfu.ml^{-1}. The biofilm thickness was found to be reduced at four different concentrations of phages as evidenced by the clearance of biofilm layer inside the tubes stained by crystal violet (Fig. 3a).

Biomass or biofilm removal due to phage activity varied between 23 and 92 % (Fig. 3b) depending on the biofilm age and the conditions under which the biofilm had been formed and phages applied. Removal of the biofilm by phage treatment was faster in younger biofilms. Under static conditions, a 3 log higher number of phage progeny remained either inside the biofilm matrix or attached to the substratum surface, showed that the static condition had some efficacy of phage entrapment into the biofilm.

EFM

Images were taken in Epifluorescence microscope (EFM) before and after incubation of phages, added to the host cultures to assess the status of bacterial cells after infection. Propidium Iodide (PI) penetrates only damaged cells and binds to the DNA emitting red colour, whereas FTIC remains exterior to undamaged cell walls giving raise to green emission. Green fluorescence was observed (Fig. 4a) before incubation in the samples that was added with the phages representing the presence of living cells. The simultaneous application of both dyes resulted in green fluorescence of viable cells with an intact membrane, whereas lysed cells showed intense red fluorescence (Fig. 4b).

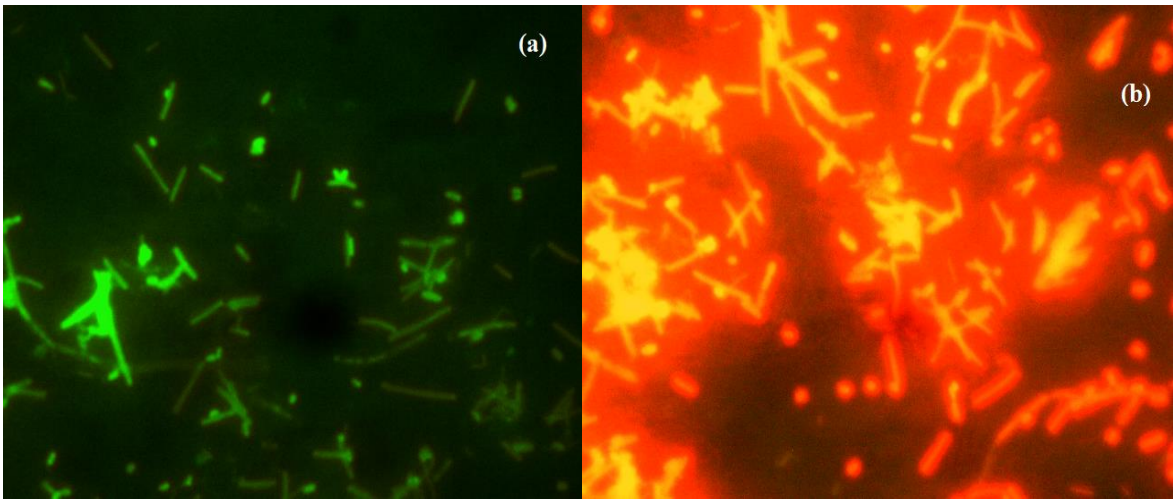

Fig. 4. EFM images of BFB that is subjected to phage infection (a) before incubation; (b) after incubation of 4 hrs.

Toxicity Test

In the present study, the therapeutic potential of PS and VPH phage was evaluated in an acute infection model of pulmonary carcinoma in mice (*Mus musculus*). The injected phages showed no toxicity in mice and thus were considered for further *in vivo* use. The mean rectal temperature of all the mice injected with bacteriophage was 36.7ºC, which was comparable to the temperature of the control group at 37.1ºC. No symptoms of lethargy or sickness were noted in the test group during the period of observation.

Survival and stability of PS and VPH phages were measured in animals after i.p. and i.v. injection of phage preparation containing 3×10^8 pfu.ml^{-1}. The phage count was measured in blood and lungs. Maximum phage counts in lungs and blood were obtained 6 h of post injection (Fig. 5 a and b). The phage count in lungs was higher than that in blood. The phage count showed a significant decrease of 7 log units at 12 h and negligible counts were obtained at above 40 h in lungs and 25 h in blood after injection.

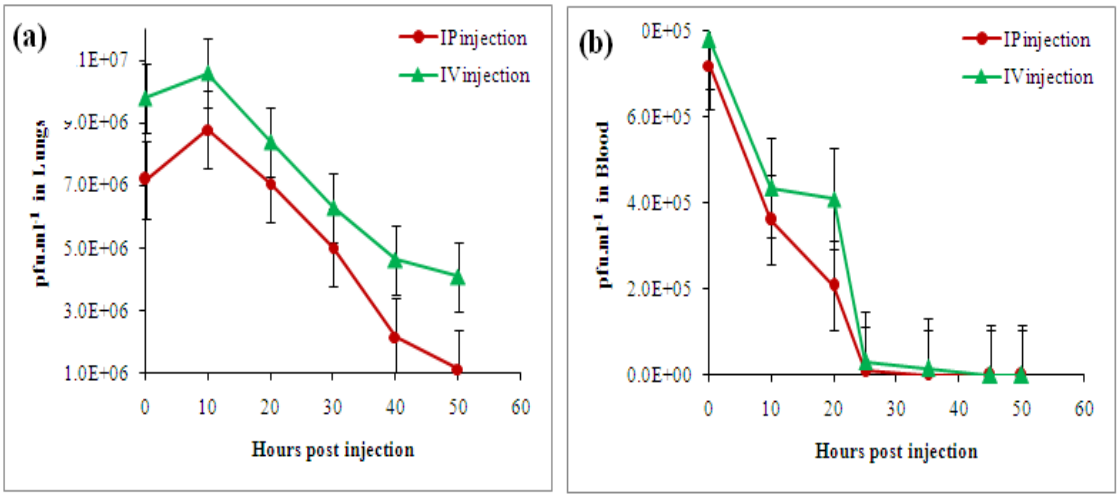

Fig. 5. Phage count (pfu.ml^{-1}) at varying time periods after phage administration (10^8 pfu.ml^{-1}) to *Mus musculus* a) in lung homogenate, b) in blood

No phage could be isolated in lungs and blood at 48 h after phage treatment. The route of administration was particularly important to the efficacy of the treatment. The i.v. route provided the most significant (87%) protection (Fig. 5a, b).

Phage protection study

The Biofilm forming bacteria (BFB) colonized in mice-induced with lung cancer by B[a]P in experimental animals. The BFB were higher in group III than group II in which the animals

were treated with B[a]P. In phage-treated animal groups, as compared with untreated control groups, the BFB got reduced significantly in potentially fatal lung cancer as judged by bacterial counts in the lung homogenates after the animals were sacrificed (Fig. 6). The titers (pfu.gm^{-1} tissue) of the phages recovered from lung tissues of mice were determined by the soft agar overlay technique following the addition of few drops of chloroform (per ml).

Mice intravenously injected with PS and VPH phages in addition to BFB had a much lower death rate than mice administered with BFB alone. Treatment of mice with PS and VPH phages proved effective as measured by reduction in cfu per gram of lung tissues and body temperature and in lesion material and histopathologic analysis (Fig. 7). The two different phages were effective against two different biofilm forming strains. Optimum protection required the phages administration at doses as high as 10^8 pfu.

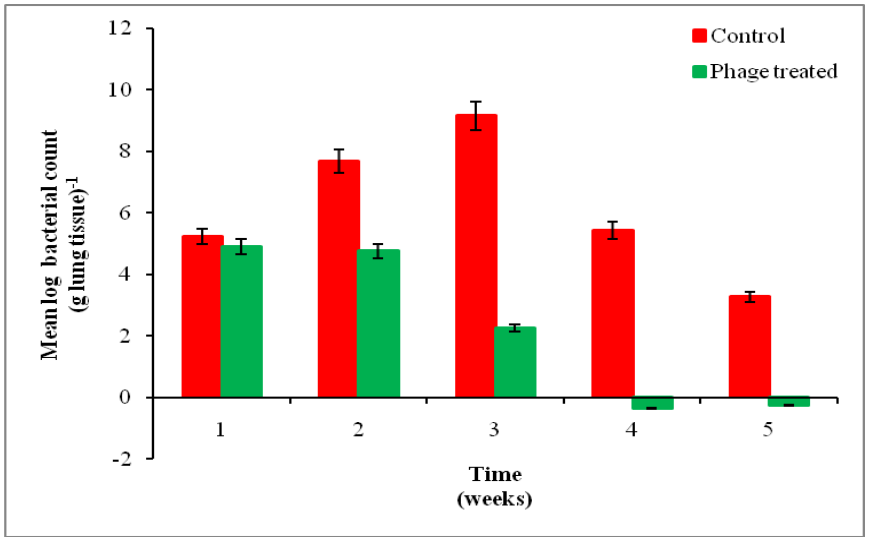

Fig. 6. Bacterial counts in lung tissue homogenate following infection with *Pseudomonas stutzeri* and *Vibrio parahaemolyticus* (10^6 cfu.ml^{-1}) in *Mus musculus* (Control) and treatment with PS and VPH phages (10^8 pfu.ml^{-1}) (Phage treated)

One of the protective phages had a half-life in blood of over 3 h. The results of these studies indicated that a single dose of these two phages cocktail could significantly decrease the load of BFB in aseptically injured mice. These results demonstrated that bacteriophages had therapeutic potential for both localized and systemic infections caused by bacterial pathogens in animals. Potential of phage therapy for controlling biofilm forming bacteria was proven from this model.

Histopathological analysis

Histological examination of lung tissues of Group I animals found to be normal (Fig. 7a) when compared with that of the B[a]P treated animals that showed tissue damage(Fig. 7b). Group III animals treated with B[a]P and simultaneously administered with BFB orally showed serious tissue damage (Fig. 7c). Cocktail of phages treated animal tissues showed no characteristic changes (Fig. 7d) as evident by the images of lung tissues. Reduction in tissue damage was noticed in animals treated with phages (Fig. 7e) and it proved that bacterial load acted as a trigger to induce tissue damage in cancer cells.

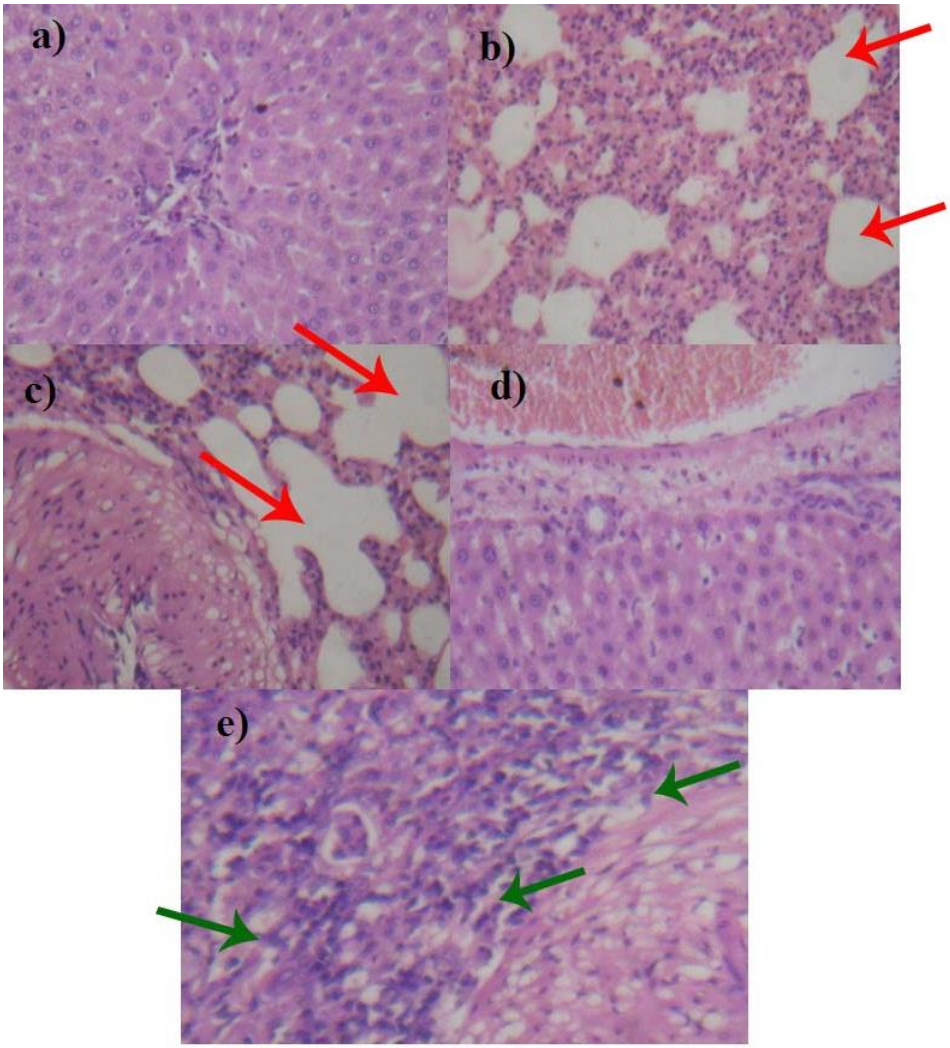

Fig. 7. Histological changes in the lung tissues of *Mus musculus* (Magnification 40 x).

DISCUSSION

Phage therapy has been applied to a variety of infections like bacterial dysentery, wound infections, infections of skin nasal mucosa, and gastrointestinal tract infections caused by other etiologic agents like *Shigella*, *Salmonella*, *Proteus*, *Staphylococcus* and

Streptococcus.[28] Phages have also been used to prevent bacterial disease in fish and to control pathogens of tomatoes.[29, 22] The therapeutic uses of phages in humans have been reviewed by Alisky *et al*.[30]; the overall reported success rate for phage therapy is found to be in the range of 80-95%.

Several pathogenic bacteria, particularly those that can establish a persistent, infection, can promote or initiate abnormal cell growth by evading the immune system.[31] Bacterial species or their toxins can alter host cell cycles or stimulate the production of inflammatory substances linked to DNA damage.[32] Benzo (a) pyrene (B[a]P) is an important carcinogenic chemical abundantly present in the cigarette smoke. Also the Benzo(a)pyrene [B(a)P] is an important toxic polycyclic aromatic hydrocarbon mainly found in cigarette smoke.[33] Metabolites of B(a)P act as carcinogenic by binding with DNA, the event results in mutation leads to formation of range of cancers in physiology of human system and it forms DNA adducts by rigorous ROS generation to facilitate process of carcinogenesis. Also the B(a)P of tobacco smoke causes genetic damage particularly in lungs tissue pertaining to effect of lung cancer.[34] Though the pulmonary carcinoma is induced by the environmental carcinogen B[a]P, our earlier study revealed that the microbial interactions may play a role in the severe cell proliferation and suppressing the apoptosis and further infections. This is in support of the earlier report that *Helicobacter pylori* suppress gastric epithelial cell apoptosis.[35] In addition to this evidence, a substantial number of bacterial pathogens have been putatively linked to cancer. The successive treatment of cancer is not only depending on apoptosis induction. Generally cancer progression and cell death is dependent on balance between cell survival and death signal.[36]

The present study successfully isolated phages from mangrove soil and tested against two biofilm forming bacteria (BFB) isolated from mice bearing lung cancer. The results of survival and stability studies showed that although phage entered into the blood stream after 3 h they reached their maximum concentration at 6 h post injection. These results are in support of earlier findings of Bogovazova *et al*.[37, 38] who have studied immunological properties and therapeutic effectiveness of bacteriophage. According to these workers, phages take a maximum of 2–4 h to reach their peak in the blood stream and approximately 6–8 h to reach their peak in various internal organs.

The phage count showed a significant decrease at 12 h and no phage was detected at 48 h post-injection in all these samples. The pharmacokinetics of phage delivery to the blood and

lungs suggested that the phages administered by the i.v. route at a higher dose, were delivered earlier for a more sustained period of time.

The results of this study suggested that phage therapy had a potential to check the growth of biofilm forming bacteria (BFB) in mice models. However, there are still many technological challenges of phage therapy that need to be looked into before making general rules for phage products.[39] Research on different aspects of phage therapy can help in the evolution of standardized phage banks having a comprehensive library of therapeutically approved phages. This will help in initiating phage therapy immediately after the identification of the pathogen.

Hence, further studies involving multiple phages effective against multi-drug resistant BFB isolates are warranted. Such an approach not only will be important from the point of view of controlling infections caused by multidrug-resistant BFB strains but also will check the spread of infection further to the environment. Many new microbial populations may also competently involve in the DNA adduct formation and cell proliferations and this deserves further study.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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