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# Liposome loaded phage cocktail: Enhanced therapeutic potential in resolving *Klebsiella pneumoniae* mediated burn wound infections

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ABSTRACT

*Background:* *Klebsiella pneumoniae* is one of the predominant pathogens in burn wound infections, and prevalence of multidrug resistant strains has further complicated the situation. An increased interest in phage therapy as a means of combating infection has been accruing in recent years. In order to overcome the drawbacks associated with phage therapy, the present study was conducted to evaluate the potential of liposomes as a delivery vehicle for phage in the treatment of burn wound infection.

*Methods:* Burn wound infection with *Klebsiella pneumoniae* B5055 was established in BALB/c mice. The therapeutic efficacy of free phage cocktail in comparison to liposome entrapped phage cocktail in resolving the course of burn wound infection in mice was evaluated.

*Results:* The results depicted that mice treated with liposomal entrapped phage cocktail showed higher reduction in bacterial load in blood and major organs. This was accompanied with faster resolution of the entire infection process as compared to non-liposomal free phage cocktail. The liposomes increased phage retention time *in vivo* thus potentiating efficacy. Liposomal phage preparation was able to protect all the test animals from death even when there was a delay of 24h in instituting the therapy.

*Conclusion:* The results showed the potential of liposome entrapped phage cocktail for treating *Klebsiella pneumoniae* mediated infections. Thus, this strategy can serve as an effective approach for treating *Klebsiella* mediated burn wound infections in individuals who do not respond to conventional antibiotic therapy.



## 1. Introduction

Nosocomial infections due to *Klebsiella pneumoniae* are a major cause of morbidity and mortality among burn patients [1]. It is an important pathogen that accounts for 15.2% of all burn wound infections caused by gram-negative bacteria [2]. The propensity of this organism to disseminate promptly leaves little time to institute effective antimicrobial treatment. Moreover, with its ability to develop resistance to commonly employed antibiotics, management of *K. pneumoniae* especially in burn patients has become challenging [3]. Due to the inexorable spread of antibiotic resistant-bacteria and lack of development of new antibiotics, there is an urgent need to explore newer therapeutic options against this pathogen.

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Phage therapy represents a safe and potent alternative strategy against drug resistant pathogens [4,5]. Although success in the therapeutic use of bacteriophages for treating various bacterial infections has already been substantiated by many workers [6–9] in the past but till date, none of the phage based therapies have successfully made it to the market. The intrinsic disadvantages associated with phage therapy need to be examined for its clinical success in near future. The major drawbacks include: high specificity, making them narrow range antibacterial agents [10], rapid clearance of phages by reticuloendothelial system of our body, poor pharmacokinetic profile that negatively affects the potency of treatment [11,12]. Thirdly, most of the phage protection studies in animals have been performed with phages being administered immediately following bacterial infection, which is not the situation in a clinical setting [13].

In the present study, two major issues associated with phage therapy i.e. narrow host range and rapid clearance have been addressed by employing (i) phage cocktail (rather than monophage therapy) and (ii) suitable lipid based phage delivery system i.e. liposomes for enhancing the viability and stability of bacteriophages, both *in vitro* as well as *in vivo*. Among the plethora of delivery systems, liposomes are one of the most intensively employed system due to their nontoxic nature, biocompatibility with phages, non-immunogenic effect and GRAS status (generally regarded as safe) [14]. The present study for the first time provides information on the application of liposome encapsulated lytic phage cocktail for treating burn wound infection.

## 2. Materials and methods

### 2.1. Bacterial strains

*K. pneumoniae* B5055 was used in the present study. It was procured from Dr. Matthias Trautmann, Department of Medical Microbiology and Hygiene, Ulm, Germany. Clinical isolates of *K. pneumoniae* were procured from Post Graduate Institute of Medical Education and Research (PGIMER) and Government Medical College and Hospital (GMCH-32) Chandigarh, India. Morphological and biochemical properties of the isolates were identified according to Bergey's Manual of Systematic Bacteriology [15]. The strains were stored in 60% glycerol and sub-cultured periodically.

### 2.2. Bacteriophages isolation and host range determination:

Isolation of bacteriophages against *Klebsiella pneumoniae* B5055 and clinical isolates was done following the enrichment technique of Cerveny et al. [16]. Sewage effluents were collected from different localities in and around Chandigarh. Untreated sewage samples were filtered and centrifuged (10,000 g for 10 min) after which the supernatant was filter sterilized (0.45 mm pore size Millipore filter). Equal volume of overnight grown bacteria and filtered sewage were incubated at 37°C under shaking condition (180 rpm). After 24 h, sample was centrifuged (10,000 rpm/10 min) and lytic activity was evaluated in the supernatant. Phage titer was checked by double agar overlay technique of Adams [17] and expressed in terms of plaque forming units/ml. The isolated phage was purified by successive single plaque-isolation technique until homogeneous plaques were obtained [18]. All the isolated phages were evaluated for their lytic spectrum against 32 clinical strains of *Klebsiella pneumoniae* by spot assay [19]. Five phages showing lytic activity against *K.pneumoniae* B5055 were selected on the basis of broad host range [ability to infect 29/32 clinically relevant and diverse isolates of *Klebsiella pneumoniae*; Table S1 (supplementary data)]. All were tailed dsDNA phages belonging to family *Myoviridae* and referred as KØ1, KØ2, KØ3, KØ4 and KØ5 (as mentioned in supplementary data; Fig. S1). Growth characteristics (latent phase, eclipse period and burst size) of all the five phages were studied as depicted in supplementary data [File S1; Table S3].

### 2.3. Preparation of phage cocktail and phage loaded liposomal formulation

The bacteriophage cocktail used in the present study was prepared by mixing five different purified bacteriophage preparations (KØ1, KØ2, KØ3, KØ4 and KØ5) in an equal proportion (1:1:1:1:1). The method of Bhatia et al. [20] was used for the preparation of phage loaded liposomes. For preparation of cationic liposomal formulation of bacteriophage, Phosphatidyl choline: Cholesterol: Tween 80: Stearylamine (8:2:1:0.5) were dissolved in a mixture of chloroform: methanol (2:1 v/v). Thin film was prepared by rota-evaporator using hydration temperature of 40°C. Phage cocktail (10 ml prepared in PBS [pH 7.2]) was added to thin film at 40°C and rotated for 10 min to detach the film from the glass wall. The suspension was left overnight at room temperature for proper swelling. Cationic liposomal formulation of phage was used in the present study for *in vitro* as well as *in vivo* experiments.

[Note: Liposomes with entrapped and unentrapped phage cocktail has been referred as **CP** and **LCP** respectively]

### 2.4. Characterization of liposomal formulation loaded with phage cocktail (LCP)

*In vitro* studies were performed to study the uniformity, shape, size, entrapment efficiency and phage elution profile by the following methods:

a) Morphology and structure of vesicles

The morphological characteristics of liposomes namely shape, uniformity and structure were evaluated by optical microscopy (OlymusCH20i) at suitable magnification (100×).

b) Transmission electron microscopy (TEM)

The morphological analysis of liposome loaded phage cocktail was done using TEM according to the method of Goodridge et al.[21]. Drops of liposome loaded phage sample were put on carbon coated grids, negatively stained with 2%