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EXPERIMENTAL THERAPEUTICS

Transfersomal Phage Cocktail Is an Effective Treatment against Methicillin-Resistant *Staphylococcus aureus*-Mediated Skin and Soft Tissue Infections

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ABSTRACT The emergence of drug resistance has rekindled interest in phage therapy as an alternative treatment option; its potency, safety, and proven efficacy are worth noting. However, phage therapy still suffers from issues of poor stability, narrow spectra, and poor pharmacokinetic profiles. Therefore, it is essential to look into the use of drug delivery systems for efficient delivery of lytic phages *in vivo*. The present study evaluated the use of nanostructured lipid-based carriers, i.e., transfersomes, as transdermal delivery systems for encapsulating a methicillin-resistant *Staphylococcus aureus* (MRSA) phage cocktail. Furthermore, the therapeutic potential of the encapsulated phage cocktail in resolving experimental soft tissue infections in rats was studied. Results from *in vitro* stability and *in vivo* phage titer experiments indicated that the transfersome-entrapped phage cocktail showed better persistence and stability than did free phages. Rats treated with the transfersome-entrapped phage cocktail resolved the experimental thigh infections within a period of 7 days, unlike the 20-day period required for untreated animals. The findings of the present study support the use of transfersomes as delivery agents to enhance the stability and *in vivo* persistence of the encapsulated phages. In addition, this study highlights the advantages offered by transfersome-encapsulated phages in providing better therapeutic options than free phages for treating skin and soft tissue infections. The transfersome-entrapped phage cocktail was able to protect all test animals (with no deaths) even when administered with a delay of 12 h postinfection, unlike free phages, thus making this treatment option more suitable for clinical settings.

KEYWORDS linezolid, transfersome, bacteriophage therapy

Staphylococcus aureus has emerged as one of the major pathogens involved in skin and soft tissue infections (SSTIs). With the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) on the rise (1, 2), a major concern is the increasing prevalence of community-associated MRSA (CA-MRSA) strains, which cause complicated soft tissue infections in young and healthy individuals and are resistant to commonly deployed antibiotics. In a study of 422 patients with SSTIs attending emergency rooms across the United States, 59% (range, 20% to 74%) of the cases were due to CA-MRSA (3, 4). Such infections are difficult to treat and are associated with longer hospital stays, major complications, increasing resistance, and greater economic burdens for patients (5); this calls for the evaluation of newer and more effective therapeutic regimens to treat such infections. To supplement antibiotic therapy, an alternative, which is currently being reexamined, is the use of bacterial viruses (bacteriophages). Their ability to kill infected bacteria and their specificity, proven clinical safety, and self-replicating nature make bacteriophages ideal, safe, and robust antimicrobial agents of the near future (6, 7). However, there are still some concerns related to the use of phages as therapeutic agents, including (i) poor stability of lytic phages, which makes it essential to determine

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their infectivity and titer every time before use, and (ii) rapid clearance from the body by cells of the reticuloendothelial system (RES), and possibly by neutralizing antibodies, after injection into the bloodstream. Therefore, issues pertaining to their short half-life and poor *in vivo* pharmacokinetics need to be addressed for successful use of phages as biocontrol agents (8–10).

Drug delivery systems such as liposomes and transfersomes help to improve the pharmacological properties of conventional ("free") drugs by altering the pharmacokinetics and biodistribution of the associated drugs or function as drug reservoirs, leading to sustained release of the desired molecules. Controlled drug delivery improves bioavailability by preventing premature degradation and enhancing uptake of the desired agent. This also helps maintain drug concentrations within the therapeutic window by controlling the drug release rate and reduces side effects by targeting the drug to the disease site and target cells (11). Transfersomes have gained much more attention than other systems because of their specific characteristics, such as their lack of toxicity, biodegradation, and ability to encapsulate both hydrophilic and lipophilic molecules. Other attributes include their capacities to prolong the existence of the drug in the systemic circulation through encapsulation in vesicular structures, to target organs and tissues, to reduce drug toxicity, to reach deeper skin layers, and to increase drug bioavailability (12). Due to these advantages, transfersomes are preferred over liposomes for treating deep skin infections. Hence, the present study was designed to evaluate the therapeutic efficacy of a transfersome-entrapped cocktail of *Staphylococcus aureus*-specific phages in treating MRSA-mediated thigh infections in experimental rats.

RESULTS

Formulations. Formulations of phosphatidylcholine (PC), Tween 80 (T-80), and stearylamine (SA) at three ratios, i.e., 8:2:1, 8:2:0.5, and 9:1:0.5, were prepared and observed for size, uniformity, and stability. Of the three formulations, the 8:2:0.5 formulation of transfersomes showed the minimum size, uniformity, high entrapment efficiency, and good stability. Data on the selection, characterization, and stability of the transfersome formulations are given in the supplemental material. The 8:2:0.5 formulation was used for the entrapment of both of the phages, i.e., MR-5 and MR-10, for use in further experiments.

Efficacy studies. The therapeutic potential of the transfersome-entrapped phage cocktail (TPC) was compared with that of the free phage cocktail (FPC) (not entrapped) in resolving *S. aureus* ATCC 43300-induced thigh infections in rats. The bacterial dose that allowed the development of acute thigh infections (mimicking skin and soft tissue infections) was optimized. With an inoculum size of 10^7 CFU/ml, acute thigh infections with visible abscess pockets full of pus and dead tissue developed; at this dose, no deaths occurred. The infections were sustained for a period of 15 days and resolved thereafter, with no load being seen at day 20. At higher bacterial doses, i.e., 10^8 CFU/ml and 10^9 CFU/ml, mortality rates of 50% and 75 to 100%, respectively, occurred within 24 to 48 h. Thus, 10^7 CFU/ml was selected as the optimal dose for efficacy study 1, in which the therapeutic effects of free phage and transfersome-encapsulated phage in resolving experimental thigh infections (with bacterial load as the primary indicator of efficacy) were studied. The therapeutic effect of delayed phage administration on thigh infections was studied in study 2, in terms of death versus survival (with a higher bacterial inoculum, i.e., 10^9 CFU/ml, injected into rats).

Phage efficacy study 1. The transfersome-entrapped bacteriophage (MR-5 and MR-10) cocktail was evaluated for its ability to resolve experimental thigh infections in rats, on the basis of the following factors.

(i) Thigh bacterial burdens. Bacterial burdens were determined on different days after infection and treatment. As shown in Fig. 1, the peak bacterial load of 8.48 log CFU/ml was obtained on day 3 in the infection control group. The load remained high (~6 log units) up to day 8, followed by a decline, and minimal counts were obtained by day 15. In all treatment groups, however, bacterial reduction of ~3 log

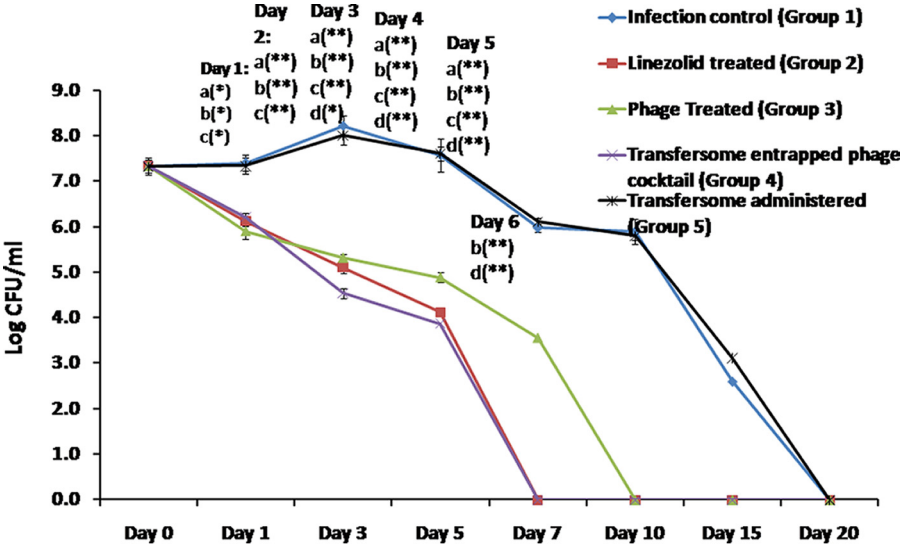

FIG 1 Thigh bacterial burdens in various groups of rats on different days following treatment. Each data point represents the mean of three values, and error bars represent SDs. *P* values among groups have been determined, as follows: a, group 2 versus group 1; b, group 3 versus group 2; c, group 4 versus group 1; d, group 4 versus group 3. *, $P < 0.05$; **, $P < 0.01$.

units ($P < 0.05$) was observed by day 2 onward, compared to the untreated group. The FPC group showed a consistent load of ~5 log units from day 2 until day 5, followed by a gradual decline, with sterile tissue being obtained on day 9. In the case of TPC-treated animals, a highly significant reduction (>3 log units; $P < 0.01$) occurred from day 2 onward, compared to the infection control group. The peak bacterial load of 6 log CFU/ml was obtained on day 1, followed by a consistent time-dependent decline thereafter. Minimal counts were seen on day 5, with sterile tissue by day 6. The bacterial counts were significantly lower on all days in TPC-treated rats, compared to FPC-treated rats ($P < 0.05$). The entire thigh infections were resolved in a comparatively shorter time, unlike the 9-day period required by free phage to contain the infection process. The TPC-treated bacterial load showed a trend similar to that seen in the linezolid-treated rat group. However, animals that received the plain transfersome preparation did not show any significant decrease in the bacterial burden in the thigh tissue on all days, compared to the infection control group ($P > 0.05$). The peak bacterial load (~8 log units) was observed on day 3 and the loads remained high (~6 log cycles) until day 8, just like in the untreated control group. Thereafter, minimal counts (2.8 log units) were obtained on day 15.

(ii) Phage titers. Results shown in Fig. 2 indicate that, although a titer equivalent to ~10^8 PFU/ml (multiplicity of infection [MOI] of 10) was injected into the thigh tissue, the phage titer of FPC-treated rats reached ~5 log units on day 1, indicating a highly significant loss (2.6 log units) within 24 h ($P < 0.01$).The titer showed a significant increase from 5.65 log PFU/ml on day 1 to 6.14 log PFU/ml on day 3 ($P < 0.05$). Thereafter, the phage titer showed a consistent decline until day 8. No phage activity was seen on day 9, corresponding to sterile tissue obtained by day 9.

In the case of TPC-treated rats, three main points are noteworthy. (i) Although there was a decrease of 1 log unit in the phage titer obtained on day 1, compared to day 0, it was comparatively less than the decline seen in free-phage-treated animals. A 1-log decrease in phage titer occurred by day 1 (compared to the day 0 titer), with a peak phage titer of ~7.3 log units being seen on day 1. The phage titer remained consistently high (~7 log PFU/ml) even on day 5, with no visible phage being detected on day 7. (ii) The phage titer of TPC-treated animals was significantly higher (on all days), compared to the FPC group, although similar amounts (in terms of PFU per milliliter) were injected immediately after infection. (iii) In the case of TPC-treated rats, sterile

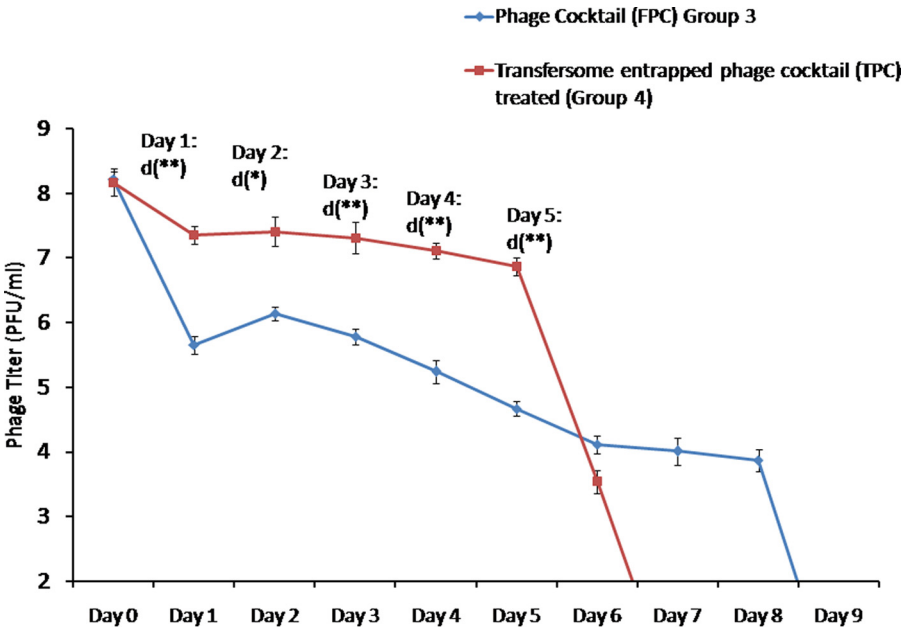

FIG 2 Phage titers measured in thigh homogenates of *S. aureus* ATCC 43300-infected rats on different days after treatment. Error bars represent SDs. *P* values among groups have been determined, as follows: d, group 4 versus group 3. *, $P < 0.05$; **, $P < 0.01$.

tissue with no bacterial burden occurred by day 6, but a phage titer of 3.54 log PFU/ml was still detected on day 6. This indicates longer persistence and viability of transfersome-encapsulated phage in the body, despite complete clearance of its bacterial host.

Histopathological analysis of specimens from various groups was also performed as an additional study. The study indicated that the animal group treated with TPC showed comparatively less infiltration and tissue damage, compared to untreated animals (data not shown).

Phage protection study 2. As depicted in Fig. 3, a larger inoculum of MRSA 43300, i.e., 10^9 CFU/ml, injected into the thigh tissue led to an initial mortality rate of 66.6% on day 1, which increased to 100% by day 3 after infection. However, rats that received FPC (group 3) or TPC (group 4) 30 min after infection showed no deaths during the experimental period (2 weeks).

When free phage was administered at 12 h after infection (group 6), a mortality rate of 50% was seen on day 1, which increased to 83.3% by day 3. This was unlike TPC-treated rats that received treatment with a delay of 12 h (group 7); no deaths were observed for any of the test animals.

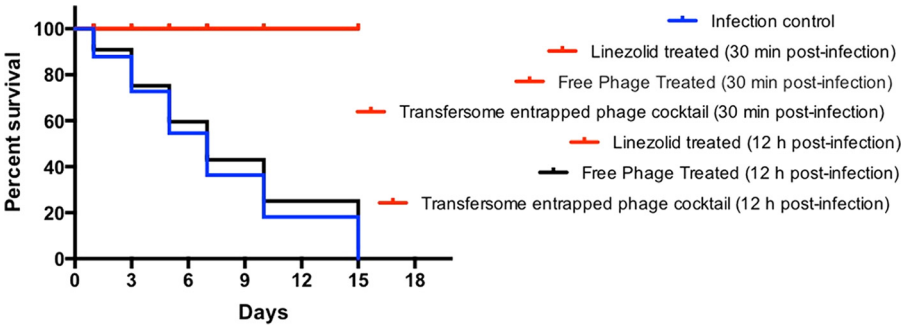

FIG 3 Death versus survival on different days after treatment in various groups of rats. Each data point represents the mean of three values.

DISCUSSION

Our laboratory has reported the successful entrapment of phages within a suitable lipid-based delivery system, i.e., liposomes that are biocompatible and help to maintain the phage titer within the *in vivo* system (13). In the present study, the focus was on a different drug delivery system, i.e., transfersomes, which are most apt for the transdermal delivery of antimicrobial agents, especially in cases of skin and soft tissue infections.

Transfersomes differ vastly from commonly used liposomes, as they are much more flexible and adaptable. The extremely high flexibility of their membrane permits transfersomes to squeeze themselves even through pores much smaller than their own diameter (14–16). They are more stable and exhibit a better ability to penetrate the skin and to reach the deeper layers spontaneously, compared to liposomal formulations (12). Also, they enjoy generally recognized as safe (GRAS) status and are nontoxic, biocompatible, biodegradable, and easy to scale up. Thus, transfersomes were used for phage entrapment, to study their therapeutic use in the treatment of MRSA-mediated soft tissue infections.

For drug delivery applications, transfersomes are usually unilamellar and range from 200 to 500 nm in diameter. Large transfersomes are rapidly removed from the blood circulation (17). Therefore, it is very important to optimize the formulation in terms of size, unilamellar nature, and entrapment efficiency, to maximize phage loading and to minimize leakage. Transfersomes with entrapped phage cocktail were 450 nm in diameter, of unilamellar nature, and devoid of aggregation and exhibited entrapment efficiency of 93%. Transfersomes were highly stable at 4°C over a 9-week period.

In the rat thigh infection model, transfersome-entrapped phage cocktail produced faster infection clearance than did free phage. The improved performance appears to be the result of greater phage infectivity or persistence. The phage titers of TPC-treated animals were significantly higher (on all days) than those of FPC-treated animals, although similar amounts (in terms of PFU per milliliter) were injected immediately after infection. This might explain the greater reduction and faster resolution of infections in TPC-treated animals than in FPC-treated animals.

We speculate that transfersome encapsulation protected the phage from clearance via the innate immune system. Also, slow release of phage from transfersomes might have led to improved kinetics, relative to free phage. Persistence of transfersome-administered phage, even after elimination of the bacterial burden, is consistent with this idea. This was seen in the case of TPC-treated rats, for which the phage titer of 3.5 log PFU/ml was still present on day 6, when no bacterial load was detected (i.e., 24 h after bacterial clearance). Evidence for enhanced therapeutic effects and longer persistence of phages encapsulated within lipid-based drug delivery systems has also been provided by the findings of Singla et al. (18). Moreover, it has been found that such encapsulated phages are well protected (up to 100%) from phage-neutralizing antibodies, which further increases their *in vivo* retention time within the body (13). Also, workers in the past (19, 20) have shown that the use of such a delivery system improves the pharmacokinetics and biodistribution of the associated drug. Apart from the higher phage titer, which accounted for a greater degree of protection in TPC-treated animals, we speculate that the properties of transfersomes also allowed deeper tissue penetration of encapsulated phage, making the entire process of delivery more effective.

Past workers showed that delaying phage intervention for more than a few hours after infection rendered the entire treatment ineffective in resolving the infection process (21–23). Since transfersomes increase the persistence and viability of phages, the effect of delayed administration of treatment on the infection outcomes was studied. In study 2, a higher bacterial dose (10^9 CFU/ml) was used to establish the thigh infections, and death versus survival was used as the treatment outcome for test animals.

The 12-h delay in treatment in the second study rendered the treatment ineffective in FPC-treated rats. These observations are in line with the findings of previous workers (24, 25). However, a 12-h delay in the case of TPC-treated rats did not lead to any deaths

among the test animals during the 2-week observation period. A similar observation was reported by Singla et al. (18); liposome-encapsulated phages given with a delay of 24 h were still able to effectively resolve *Klebsiella pneumoniae* infections in mice. Timely administration of phage before the pathogenic bacteria reach large numbers is not possible on all occasions. Therefore, the use of such delivery agents may provide an effective solution to the existing problems associated with phage therapy.

The present study highlights the benefits of a new approach to the treatment of skin and soft tissue infections caused by MRSA. The use of transfersomes as a delivery system helped to increase phage infectivity and stability over a longer time, thus potentiating faster clearance of MRSA *in vivo*. It can be concluded that the use of transfersome-entrapped phage cocktail is an attractive therapeutic approach against MRSA infections that is worth exploring.

MATERIALS AND METHODS

Ethics statement. Experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University (Chandigarh, India) (approved identification no. IAEC/S/14/129) and performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, regarding animal experimentation. All efforts were made to minimize the suffering of animals.

Bacterial strain. A standard strain of *Staphylococcus aureus* ATCC 43300 (MRSA) was used in this study. Disk diffusion assays were performed to confirm its resistance to penicillin, methicillin, and oxacillin, followed by determination of the oxacillin MIC value by the broth microdilution assay, as recommended by the Clinical and Laboratory Standards Institute (33). The strain was stored in 60% glycerol at −80°C and, when necessary, was maintained on nutrient agar slants at 4°C.

Bacteriophages. Bacteriophages specific for *S. aureus*, i.e., phages MR-5 and MR-10, were used. Both phages were isolated previously in our laboratory and were characterized as lytic, double-stranded DNA, tailed phages belonging to the family *Myoviridae*, order *Caudovirales* (19, 26, 27).

Bacteriophage concentration and purification. High-titer stocks of each phage were prepared from a single plaque of bacteriophage by the plate lysate method, as described by Sambrook et al. (28). The bacteriophage titers were estimated by the double agar overlay method, as described by Adam (29), and were expressed as PFU per milliliter.

Both phages were concentrated by filtering an initial volume of 400 ml of amplified phage sample on a Millipore Labscale TFF system (Pellicon), fitted with a 10-kDa pore-size polyethersulfone membrane, for a period of 3 to 4 h, until the sample volume reached 25 to 30 ml. The sample was then dialyzed against 10% polyethylene glycol (PEG) 8000 at 4°C for 3 to 4 h, followed by dialysis against fresh phosphate-buffered saline (PBS) (pH 7.2) at 4°C for 24 to 48 h. After dialysis, the sample was centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was filtered through a 0.45-μm syringe filter into a sterile flask and stored at 4°C. The sample was then assayed to determine the final phage titer achieved after amplification. For long-term storage, the bacteriophages were stored at −70°C in PBS containing 60% glycerol as a preservative. Phages for routine use were stored at 4°C in PBS (pH 7.2).

Preparation of transfersomes. Formulations were prepared using the rotary evaporation technique (30). Various ratios of PC, T-80, and SA were used. PC, T-80, and SA (total mass of 100 mg) were dissolved in 10 ml of a chloroform-methanol mixture (2:1 [vol/vol]) in a beaker at room temperature. The dissolved lipids (10 ml) were transferred into six separate round-bottom flasks of 25 ml each. The solvent was evaporated under high vacuum, with rotation at 100 rpm, at 40°C for 20 min on a Heidolph rotary evaporator. The round-bottom flasks, containing the deposited thin lipid film on their inner walls, were removed from the rotary evaporator and left at room temperature for an additional period of 18 h, to remove traces of solvent in a desiccator under vacuum. Ten milliliters of bacteriophage suspension was added to the thin film at 40°C, and the flasks were rotated for 10 min to detach the film from the glass wall. The suspension was left at room temperature overnight for proper swelling of vesicles and then was sonicated for 30 min in a water bath sonicator (Branson ultrasonic cleaner) prior to further treatment.

Characterization of transfersomes. (i) Morphology and structure of vesicles. The morphological characteristics (shape, uniformity, and structure) of transfersomes were monitored by using an optical microscope (Olympus CH20i) at suitable magnification.

(ii) Average size and polydispersive index. The particle size and polydispersive index of freshly prepared transfersomes were measured by the dynamic light scattering (DLS) technique, using a Beckman Coulter Delsa Nano C instrument, at the Department of Chemistry, Panjab University. Prior to analysis, the samples were diluted 1:10. Each run consisted of two measurements with 70 acquisitions per sample.

(iii) Transmission electron microscopy. The morphology and structure of the phage-loaded transfersomes were determined with transmission electron microscopy (TEM). TEM was performed according to the method of Goodridge et al. (31). Drops of ultracentrifuged phage samples (centrifuged at 100,000 × *g* for 2 h at 4°C in a Beckman L-80 centrifuge) were dropped onto nitrocellulose-coated grids (diameter, 3 mm; 300 meshes), stained with 2% (wt/vol) potassium phosphotungstate (pH 6.8 to 7.2) for 10 s, and examined with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan) at 80 kV.

(iv) Entrapment efficiency. The phage entrapment efficiency of transfersomes was determined by the method of Gulati et al. (32), using calcium chloride aggregation. The total phage load was determined by adding Triton X-100 (0.02% [vol/vol]) at a ratio of 1:1 (vol/vol), to disrupt the transfersomes and to release the entrapped phage. The mixture was incubated at room temperature for 1 h and centrifuged at 10,000 × *g* for 10 min at room temperature, and the total number of phage particles in the supernatant was determined using the plaque assay. Free phage was separated from transfersome-associated phage by the calcium chloride aggregation method. In this method, an aqueous solution of calcium chloride was added to the transfersome dispersion at a final concentration of 20 mM. The mixture was incubated at room temperature for 24 h, the supernatant was collected, and the number of free phages was determined using the plaque assay.

The phage entrapment efficiency was calculated using the following equation: entrapment efficiency = $[(E_0 - E_1)/E_1] \times 100$, where E_0 is the total number of phages, E_1 is the number of free phages, and $(E_0 - E_1)$ represents the number of phage particles entrapped in the transfersome formulation.

Stability and leakage studies. For stability studies, 30-ml plain and phage-loaded transfersome preparations were prepared. The initial sizes of the plain and phage-loaded transfersome formulations were determined. Phage entrapment in phage-loaded transfersomes was also determined.

The two transfersome formulations were distributed in 3 parts (10 ml each), and the glass ampoules were sealed. The sealed ampoules were stored at different temperatures, i.e., 4°C, 37°C, or room temperature (30°C), for a period of at least 3 months. Samples kept at different temperatures were withdrawn periodically from each batch, at regular intervals of 1 week. Phage entrapment and the average size were determined for both formulations (plain and phage-loaded transfersomes) on each occasion.

Animals. Female Wistar rats (4 to 6 weeks of age, weighing 120 to 150 g) were used in the study. The animals were obtained from Central Animal House, Panjab University. The animals were kept in polycarbonate cages, housed at 25 ± 2°C in well-aerated rooms with a 12-h light/12-h dark cycle, and fed standard rodent diet and water *ad libitum*.

Experimental thigh infections in rats. *S. aureus* ATCC 43300 was cultivated for 24 h at 37°C in brain heart infusion broth. After 24 h, the cells were pelleted and washed twice with PBS. The bacterial suspension prepared in PBS was adjusted to achieve a cell density corresponding to a range of bacterial inocula (10^7, 10^8, or 10^9 CFU/ml). The number of CFU per milliliter was confirmed by quantitative plate counting. Wistar rats were distributed into four groups, with 24 rats in each group. The posterior portion of the left thigh of each rat was disinfected with 70% alcohol, and 100 μl of a suspension of *S. aureus* ATCC 43300 was injected intramuscularly into the left thigh muscle. Three groups received different inoculum doses. Animals from the fourth group received the same volume of PBS, injected in the thigh muscle. Four animals from each group were killed on day 1, 3, 5, 7, 10, and 15 after bacterial challenge, and tissue was homogenized, diluted, and plated to determine the bacterial burden.

Phage protection studies. The FPC and TPC, along with a known antibiotic, were evaluated for their ability to resolve MRSA 43300-induced thigh infections in female Wistar rats in two protection studies, i.e., study 1 and study 2. In study 1, a total of five groups, with 33 rats/group, were included. At each time point, three rats were sacrificed and the left and right thighs of each rat were subjected to processing for bacterial load estimation, as described below.

In study 1, the following treatments were given to the groups. In group 1 (infection control), rats were infected with *S. aureus* ATCC 43300 (10^7 CFU/ml) by intramuscular injection of inocula (0.1 ml each) in both the left and right thighs. In group 2, rats were infected followed by treatment with two doses of linezolid (10 mg/kg) given orally with a 12-h interval (the first dose was administered 30 min postinfection). In group 3, rats were infected followed by intramuscular administration of plain phage cocktail (FPC), at an MOI of 10, into the thigh tissue 30 min after bacterial challenge. In group 4, rats were infected followed by intramuscular administration of TPC, at an MOI of 10, into the thigh tissue 30 min after bacterial challenge. In group 5, rats were infected followed by intramuscular administration of plain transfersome preparation into the thigh tissue 30 min after bacterial challenge. The progress of infection was monitored on the basis of bacterial loads and phage titers in the different groups.

Three rats from each group were sacrificed on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15, by cervical dislocation. Left and right thighs of the rats were disinfected with 70% alcohol, and the thigh muscle was dissected. Tissues were homogenized in PBS (pH 7.2), and dilutions of homogenates were plated on nutrient agar. The same homogenates were subjected to centrifugation (15,000 rpm for 20 min at 4°C), followed by filtration of the supernatants through 0.22-μm-pore filters prior to phage titration.

In study 2, the protective effects of immediate and delayed treatment were assessed in terms of death versus survival. A total of seven groups, with six rats/group, were included. The following treatments were given to the groups. In group 1 (infection control), rats were infected with *S. aureus* ATCC 43300 (10^9 CFU/ml) in the left thigh muscle. In group 2, rats were infected followed by treatment with two doses of linezolid (10 mg/kg) given orally with a 12-h interval (the first dose was administered 30 min after infection). In group 3, rats were infected followed by intramuscular administration of FPC, at an MOI of 10, into the thigh tissue 30 min after bacterial challenge. In group 4, rats were infected followed by intramuscular administration of TPC, at an MOI of 10, into the thigh tissue 30 min after bacterial challenge. In group 5, rats were infected followed by treatment with two doses of linezolid (10 mg/kg) given orally with a 12 h-interval (the first dose was administered 12 h after infection). In group 6, rats were infected followed by intramuscular administration of FPC, at an MOI of 10, into the thigh tissue 12 h after bacterial challenge. In group 7, rats were infected followed by intramuscular adminis-

tration of TPC, at an MOI of 10, into the thigh tissue 12 h after bacterial challenge. All animals were monitored daily for death after bacterial challenge.

Statistical analysis. Statistical analysis of the data was performed using Microsoft Excel 2007. Most of the data are expressed as the mean ± standard deviation (SD) of more than two experimental values for every variable. Student's *t* test was used to compare different variables. *P* values of <0.05 were considered significant, whereas *P* values of <0.01 were considered highly significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02146-16.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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