Appl Microbiol Biotechnol (2014) 98:4653–4661
DOI 10.1007/s00253-014-5643-5

METHODS AND PROTOCOLS

Bacteriophage-aided intracellular killing of engulfed methicillin-resistant *Staphylococcus aureus* (MRSA) by murine macrophages

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Received: 5 December 2013 / Revised: 15 February 2014 / Accepted: 23 February 2014 / Published online: 18 March 2014

Abstract Phages are known to effectively kill extracellularly multiplying bacteria as they do not have the ability of intracellular penetration within the animal cells. However, the present manuscript focuses on studying the impact of surface-adsorbed phage particles on the killing of engulfed *Staphylococcus aureus* inside phagocytic cells. Mouse peritoneal macrophages were isolated and cultured, followed by evaluation of their ability of bacterial uptake and killing. The intracellular killing potential of macrophages in the presence of unadsorbed free phage as well as phage adsorbed onto *S. aureus* 43300 was studied. Phage added alone to macrophage preparation did not influence intracellular killing of engulfed *S. aureus* by macrophages. However, phage adsorbed onto host bacterial cells (utilizing host bacteria as a vehicle to carry the lytic phage into the phagocytic compartment) brought about time-dependent and titre-dependent significant reduction in the number of viable intracellular cocci. Phage particles that shuttled inside the macrophage along with bacteria also significantly reduced cytotoxic damage caused by methicillin-resistant *S. aureus* (MRSA). This in turn enhanced the bactericidal killing potential of phagocytic cells. In earlier studies the inability of phages to kill intracellular bacteria has been thought to be a major drawback of phage therapy. For the first time results of this study confirm the killing ability of the broad host range lytic phage MR-5 of both extracellular as well as intracellular engulfed *S. aureus* inside macrophages. This approach shall not only restrict intracellular proliferation of staphylococci within the myeloid cells but also protect the host from further relapse of infection and treatment failures.

Keywords Bacteriophage · Macrophage · *S. aureus* · Cytotoxicity · Phagocytosis

Introduction

Staphylococcus aureus has become an important source of both community- and hospital-acquired infections worldwide. The treatment of staphylococcal infections is difficult due to its intrinsic ability to develop resistance to the deployed antibiotics. The emergence of resistant strains referred as methicillin-resistant *S. aureus* (MRSA), glycopeptide intermediate *S. aureus* (GISA) and vancomycin intermediate *S. aureus* (VISA) have made management of such infections a major challenge (Zetola et al. 2005). In fact, appearance of vancomycin-resistant strains (VRSA) in recent years may complicate the matter further (Appelbaum 2006). Hence, alternative options for the treatment of such invasive life-threatening MRSA infections are essentially needed.

Bacteriophage therapy that includes the use of lytic phages or their products or both represents an alternative tool in the treatment of MRSA infections that are refractory to the action of antibiotics. The abilities of phages to kill and lyse the infecting pathogenic bacteria, their ability to self-replicate and proven safety in various animal models (Biswas et al. 2002; Bogovazova et al. 1991; Bull et al. 2002; Cerveny et al. 2002; Chhibber et al. 2008; 2013; Kumari et al. 2009) make this therapy worth considering. Moreover, past workers have shown successful use of phages in treating various infections caused by *S. aureus.* However, not much effort has been made to study the intracellular killing potential of such lytic phages. *S. aureus* initially considered to be an extracellular, pyogenic pathogen is also capable of intracellular survival in a variety of

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5643-5) contains supplementary material, which is available to authorized users.

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cells including phagocytic cells, keratinocytes, fibroblasts, endothelial and epithelial cells, enterocytes and osteoblasts (Bayles et al. 1998; Fowler et al. 2000; Hess et al. 2003; Kubica et al. 2008; Menzies and Kourteva 1998; Nair et al. 2003). These intracellular reservoirs of *S. aureus* may contribute to persistence and relapse of infection even after successful completion of antimicrobial therapy (Kubica et al. 2008). Most of the antibiotics show poor penetration within eukaryotic cells, leading to persistence of pathogens. Moreover, little is known about the intracellular pharmacodynamics and release of available antibiotics (Qazi et al. 2004). In the present study, an already characterized broad host range lytic phage MR-5 (Kaur et al. 2012) has been studied for its ability to kill already invaded and engulfed *S. aureus* persisting within the macrophages using ex vivo experiments. The study concludes that lytic phages eliminated the extracellularly multiplying *S. aureus* and also attacked the already engulfed bacteria surviving within phagocytic cells to the levels that can be taken care by phagocytes themselves.

Materials and methods

Ethical statement

Experimental protocols were approved by the Institutional Animal Ethics Committee (Approval ID: IAEC/346-356) of Panjab University, Chandigarh, India and performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation. All efforts were made to minimize the suffering of animals.

Bacterial strains and phage used

S. aureus ATCC 43300 (MRSA) and *S. aureus* ATCC 29213 (MSSA) from ATCC, Mannasse, USA were used in this study. Bacteriophage MR-5, a broad host range lytic phage isolated from sewage outlet near Chandigarh, belonging to family *Myoviridae*, order *Caudovirales*, active against standard and panel of clinical MRSA isolates was used in the present study (Kaur et al. 2012).

Phage MR-5 is available in our laboratory, and the corresponding author can be contacted, if required.

Thermal and pH profiling

pH stability and thermal stability tests were carried out as described by Yang et al. (2010). Briefly, a fixed titre of phage particles was subjected to a range of thermal and pH conditions. Samples were taken at different time intervals, and supernatant following centrifugation was monitored directly for the number of phages as per the modified double layer agar (DLA) technique (Kaur et al. 2012).

Host lysis test and BIM frequency determination of phage MR-5

Host challenge test was carried out as per the method of Hsieh et al. (2011), using exponential cultures of *S. aureus* 43300 grown to an optical density (O.D.) of 0.3 and then re-diluted to 1:10. To this, phage was added at 0.1 and 1.0 multiplicity of infection (MOIs) along with calcium at a final concentration of 5 mM. The tubes were incubated at 37 °C at 150 rpm. Samples were then withdrawn at an interval of 15 min for measuring the changes in O.D. at 600 nm.

Bacteriophage insensitive mutant (BIM) frequency was determined as per the method of O'Flynn et al. (2004) and Park et al. (2012). Plaque assay was performed using an overnight culture of *S. aureus* 43300 containing known cell numbers and phage added at a MOI of 1 and 10 (with 5 mM $CaCl_2$). The plates were incubated overnight at 37 °C. All resulting colonies were counted, and BIM frequency (number of surviving colonies/original bacterial titre) was determined at both MOIs.

Animals

Female BALB/c mice, 6–8 weeks old weighing 20–25 g, bred in the Central Animal House of Panjab University, Chandigarh, were used. Animals were acclimatized to laboratory conditions before experimentation. The animals were kept in polycarbonate cages housed in well-aerated rooms with a 12-h light/12-h dark cycle at 25±2 °C, fed with standard rodent diet and water ad libitum.

Isolation and culturing of mouse peritoneal macrophages

Five female BALB/c mice were injected with 3 % thioglycollate broth (HiMedia, Mumbai, India), and after 4 days, mice were euthanized by cervical dislocation. The abdominal skin was retracted manually to expose the intact peritoneal wall. Five millilitres of ice cold Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 5 % fetal calf serum (FCS) (Gibco Invitrogen, Paisley, United Kingdom) was injected into peritoneal cavity. This was followed by gentle massaging of peritoneal cavity for 5 min. Using the same syringe, fluid was aspirated from the cavity. The fluid collected from all the mice was pooled, centrifuged and washed twice using ice cold phosphate buffered saline (PBS, pH 7.4), finally suspended in DMEM and kept on ice. The cells were counted in a haemocytometer using trypan blue (Sigma-Aldrich, St. Louis, MO, USA) for determining the viability of cells. Approximately, 10^5 cells were seeded per well (12-well

culture plate), and the plate was incubated at 37 °C in 5 % CO_2.

Macrophage uptake assay and percent killing activity

Macrophage cells (10^5 cells/ml) suspended in DMEM (5 % FCS) were incubated with bacterial suspension of *S. aureus* 43300 (pellet washed with PBS and cell density adjusted to 10^6 cells/ml) in a test tube marked as test. Similarly, the tube with only bacteria suspended in the same volume of DMEM without macrophages was marked as control. The tubes were vortexed gently and kept at 37 °C, 5 % CO_2. Aliquots were taken regularly at 0, 30, 60, 90 and 120 min of incubation and immediately transferred to 2 ml of cold DMEM in separate tubes. Macrophages were pelleted by centrifugation (1,800 rpm/10 min). Viable count of bacteria (expressed in colony forming units/ml (CFU/ml)) in the supernatant was determined by plating the respective serial dilutions (in triplicates) on nutrient agar plates, and percent uptake was calculated.

To determine the killing of engulfed *S. aureus* 43300 by macrophages, peritoneal mouse macrophages were distributed in a 12-well plate (10^5 cells/well), incubated overnight (37 °C, 5 % CO_2) in DMEM supplemented with 5 % FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Sigma, St. Louis, MO, USA). Next day, media were carefully aspirated without disturbing the cell monolayer and washed once with PBS and suspended in fresh media without antibiotics. The macrophages were then infected with *S. aureus* cells (10^6 bacteria/well). The plates were incubated for 3 h at 37 °C in 5 % CO_2 to allow phagocytosis. Phagocytic uptake was stopped, and extracellular bacteria were killed with gentamicin (25 μg/well for 1 h) (Sigma, St. Louis, MO, USA).The cell suspension was later centrifuged at 1,800 rpm/10 min at 4 °C to remove the non-phagocytosed bacteria and subsequently washed with PBS. The cell pellet containing macrophages and engulfed bacteria was again re-suspended in 1.0 ml of DMEM (5 % FCS) and incubated at 37 °C in 5 % CO_2. At 0, 30, 60, 90, 120, 150 and 180 min, cells were lysed by adding Tween 20 (final concentration, 0.03 %) (Sigma, St. Louis, MO, USA) to recover intracellular bacteria, and each lysate was serially diluted in saline and plated to find viable bacteria.

Intracellular killing activity of phage

Peritoneal mouse macrophages were distributed in 12-well plates (10^5 cells/well), incubated overnight (37 °C, 5 % CO_2) in DMEM (5 % FCS). Next day, after washing the cell monolayer and suspending it in DMEM without antibiotics, the macrophages were then infected with S. *aureus* cells (10^5 bacteria/well) and incubated for 3 h at 37 °C in 5 % CO_2 to allow maximum uptake. This was followed by addition of gentamicin (25 μg/well for 1 h) to kill extracellular bacteria. Cells were later washed with PBS and finally suspended in DMEM and incubated for 3 h to allow the killing of engulfed cocci to occur. After this, different treatments were given to wells (in triplicates) as shown below:

Treatment 1	Macrophage cells were infected with 0.1 ml of phage MR-5 (5×10^5 PFU/ml) alone at MOI-0.1.
Treatment 2	Macrophage cells were infected with 0.1 ml of phage MR-5 (5×10^6 PFU/ml) alone at MOI-1.
Treatment 3	*S. aureus* cells (10^4 CFU/ml) were pre-adsorbed with 10^3 PFU/ml of phage MR-5 (MOI-0.1) for 20 min at 37 °C, and such phage-adsorbed *S. aureus* cells (0.1 ml) were added to macrophage cells.
Treatment 4	*S. aureus* cells (10^4 CFU/ml) were pre-adsorbed with 10^4 PFU/ml of phage MR-5 (MOI-1) for 20 min at 37 °C, and such phage-adsorbed *S. aureus* cells (0.1 ml) were added to macrophage cells.
Treatment 5	Only *S. aureus* cells (10^4 CFU/ml, 0.1 ml) not pre-adsorbed with any phage were added to macrophage cells.
Treatment 6 (control wells)	0.1 ml of plain media (DMEM) was added to three wells.

The plate was again incubated for 3 h at 37 °C to allow maximum uptake, and later extracellular bacteria were killed by adding gentamicin (25 μg/well for 1 h). Cells were washed with DMEM and incubated in DMEM (5 % fetal calf serum) at 37 °C in 5 % CO_2. However, gentamicin was not added to control wells (treatment 6) nor the cells were given any additional washing. The plate was incubated, and after 3, 6 and 24 h, samples were withdrawn from all the treatment wells and lysed with Tween 20 (final concentration, 0.03 %) to recover intracellular bacteria. Each lysate was then serially diluted in saline and plated.

Fluorescent microscopy

Cell lysates obtained at different time intervals were subjected to staining using the BacLight LIVE/DEAD bacterial viability kit (Molecular Probes, Invitrogen, USA) for a brief period of 30 min in the dark. The engulfed bacteria within the macrophages could be visualized using this staining method as it incorporates two different fluorescent dyes, i.e. SYTO9 and propidium iodide (PI). SYTO9 stains live bacteria green, and PI stains dead bacteria red. Stained cells were visualized under an epi-fluorescence microscope (Nikon Eclipse 80i, Nikon Instruments Inc., USA).

MTT assay

To determine the cytotoxic damage inflicted by *S. aureus* on macrophages, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) [MTT] assay was performed that measures the reduction of MTT into an insoluble formazan product in viable cells. The amount of colour produced is directly proportional to the number of viable cells. The test was done according to the method of Kubica et al. (2008). *S. aureus*-infected macrophages at different times post-phagocytosis were incubated with MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) for 3 h at 37 °C. Following this, a detergent solution was added to lyse the cells and solubilize the crystals of formazan produced by mitochondria. The samples were read using an ELISA plate reader at a wavelength of 570 nm. All assays were performed in triplicate, and control wells (containing only macrophage cells with no bacteria) were also processed simultaneously.

Statistical methods

The data is expressed as mean±standard deviation of replicated values where indicated. The statistical significance of differences among groups was determined by Student's *t* test (two groups) using Sigma Stat, Graph pad prism (Graph pad software, San Diego, CA). *p* value of less than 0.05 was considered as statistically significant.

Results

pH and thermal stability of phage

Phage MR-5 was stable at near neutral pH range (pH 6.0–8.0). However, at pH 5.0 and below as well as at pH 9.0 and above, there was significant reduction in phage titre ($p<0.05$). Similarly, phage MR-5 exhibited good thermal stability up to a temperature of 42 °C till 24 h. However, it showed a significant drop in titre at a temperature above 42 °C (Table S1 and Fig. S1 in the Supplementary Material).

In vitro host challenge test and BIM generation

To an initial inoculum of 10^7 CFU/ml of host bacteria, the phage was added at different MOI to study its impact on the growth profile of *S. aureus* 43300 with time. There was a time-dependent decrease in bacterial counts as seen in Fig. 1. At 0.1 MOI, the count dropped from an initial 7.11 log CFU/ml to 2.47 log CFU/ml (significant decrease of 4.5 log cycles) by 4 h ($p<0.05$), and no surviving bacteria were seen thereafter. The phage was able to resist the growth of host bacterium for additional 5–6 h after which bacterial counts (by 12th hour) started to increase again. Similarly, at higher MOI-1, the same

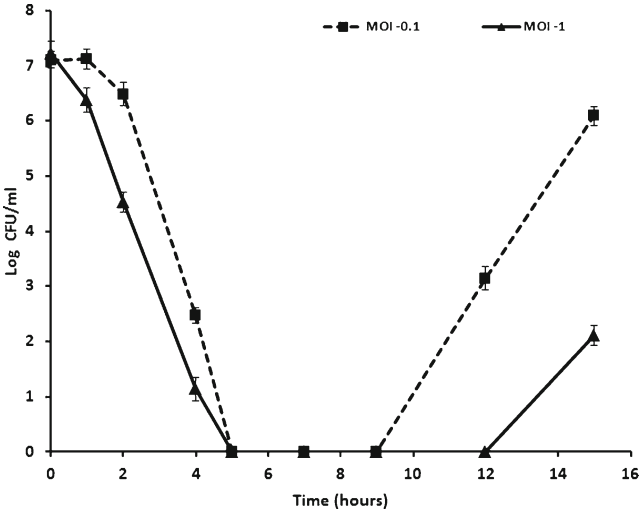

Fig. 1 Effect of phage MR-5 (added at different MOI to exponentially growing culture) on the growth kinetics of *S. aureus* 43300. Error bars represent S.D., and each value represents mean±S.D. of three independent values

effect was more pronounced with a significant decrease of 2.7 log cycles ($p<0.05$) just within 2 h of addition of phage. Bacterial counts were negligible at 4 h, and by 5 h post addition, bacteria were detected. However, phage added at higher MOI was able to suppress the growth for longer period with a minimal increase of 2.11 log CFU/ml seen by 15 h post addition. The frequency of generation of bacteriophage insensitive mutants (BIM) was found to be low with an estimated value of $(1\pm0.64)\times10^{-7}$ at MOI-10 and $(7.5\pm1.1)\times10^{-6}$ at MOI-1. BIMs were isolated as distinct colonies on agar plates, and the isolated colonies (ten individual colonies at each MOI) were incubated in BHI broth. The overnight growth was then used for performing plaque assay to determine their phage susceptibility. All the colonies showed sensitivity to phage lysis.

Macrophage uptake and killing

Peritoneal macrophages were isolated from female BALB/c mice ($n=5$) and cultured on DMEM (5 % FCS) for 24 h to allow formation of monolayer. Uptake assay and intracellular killing were performed to study the ability of isolated macrophages to engulf and kill host bacteria (*S. aureus* 43300). Uptake of bacteria increased in a time-dependent manner with a total uptake of ~33 % seen at 180 and 240 min, respectively (Fig. 2). While studying the killing kinetics, macrophages showed a killing of 23.7 and 14.2 % at bacteria: macrophage ratio of 1:1 and 10:1, respectively, after 24 h. The fluorescent images of bacteria engulfed within macrophages at various stages are shown in Fig. 3. Staining of the macrophages (lysed with tween 20) with LIVE/DEAD staining solution showed that macrophages took up the red stain of PI whereas engulfed

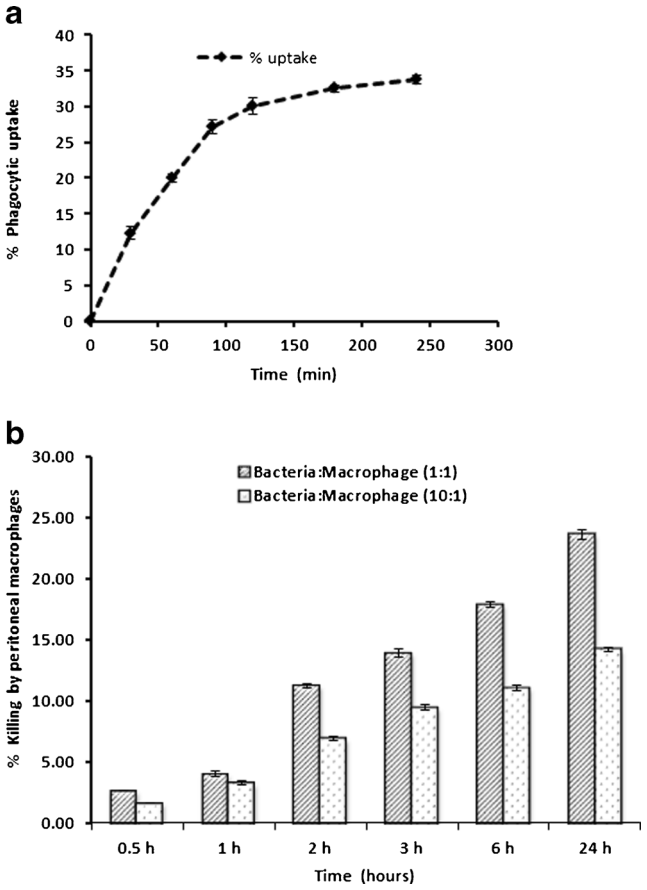

Fig. 2 Phagocytic uptake **(a)** and killing kinetics **(b)** by isolated peritoneal macrophages acting on *S. aureus* 43300. Error bars represent S.D., and each value represents mean±S.D of three independent values

bacteria either stained green (live engulfed) or red (dead engulfed).

Intracellular killing activity of phage

The effect of phage alone as well as phages adsorbed onto *S. aureus* cells on the killing activity of macrophages was evaluated, and results in terms of percent killing are depicted in Fig. 4. Five different treatments (T-1 to T-5) were given to study this effect. The phage added alone (T-1 and T-2) at both MOI was not able to enhance the killing activity of macrophages. The killing was similar to that obtained in control wells (23.2 % in control wells with treatment 6), i.e. 21.4 % when phage MR-5 was added at a MOI of 0.1 and 21.8 % when phage MR-5 was added at a MOI of 1.0. However, phages adsorbed onto bacteria showed a significant increase ($p<0.05$) in the killing of bacteria upon engulfment by macrophages at all time points. At 24 h, phage MR-5 showed a total killing of 38.7 % at a MOI of 0.1 and at a MOI of 1.0, killing was still higher with a calculated value of 54.2 %. The results demonstrate that phage MR-5, once shuttled inside the host cells by *S. aureus*, significantly reduced the population of intracellular staphylococci by ~54.2 %. Whether this enhancement in percent killing upon addition of phage-adsorbed bacteria was solely attributable to the lytic activity of shuttled phage was tested when additional *S. aureus* cells (only) were added to wells (treatment 5). The killing was not significantly higher ($p>0.05$) and was similar to results obtained with wells receiving treatment 6 (i.e. control wells), indicating that adding more bacteria alone did not enhance the killing output by macrophages. Fluorescent images (Fig. 3e, f) also showed increased killing of bacteria as majority of red stained cocci were seen in samples taken from wells to which phage-adsorbed *S. aureus* 43300 was added.

MTT assay

Cytotoxic damage caused by bacteria on macrophages post 12, 24 and 48 h was compared with damage inflicted upon by bacteria on macrophages to which phage alone as well as phages adsorbed onto host bacteria was added. MTT assay revealed a time-dependent increase (Fig. 5) in cytotoxic damage brought about by *S. aureus* on macrophages. A damage corresponding to 45 % cytotoxicity was inflicted by *S. aureus* 43300 on macrophages by the end of 48 h. There was a time-dependent increase in cytotoxicity, increasing from 15.8 % at 12 h to 45 % at 48 h, respectively. Phage added alone at a MOI of 1.0 was not able to significantly decrease ($p<0.05$) the cytotoxic damage. However, phage adsorbed onto host bacteria (MOI -1.0) when added to wells brought a significant decrease ($p<0.05$) in the levels of cytotoxicity. The cytotoxicity measured at 24 and 48 h corresponded to 11.5 and 21 %, respectively.

Discussion

Though *S. aureus* is a known extracellular pathogen, recent evidence suggests that it is also capable of surviving successfully within different host cells including professional (neutrophils, macrophages) and non-professional phagocytic cells (fibroblasts, epithelial, endothelial, keratinocytes, osteoblasts) for a longer period of time. This helps bacteria to evade host immune attack (Bayles et al. 1998; Fowler et al. 2000; Hess et al. 2003; Kubica et al. 2008; Menzies and Kourteva 1998; Nair et al. 2003; Nuzzo et al. 2000). Although little is known about interaction of *S. aureus* with macrophages, however, *S. aureus* is able to survive well within mouse and rat macrophages (Baughn and Bonventre 1975; Hebert et al. 2000; Jakab and Green 1976). In addition, Elliott et al. (1982) have demonstrated short-term survival of *S. aureus* inside human alveolar macrophages. Macrophages with longer life are capable of carrying *S. aureus* from the site of localized infection to invade vascular system leading to further complications (Bellingan et al. 1996; Harmsen et al. 1985; Petti and Fowler 2003).

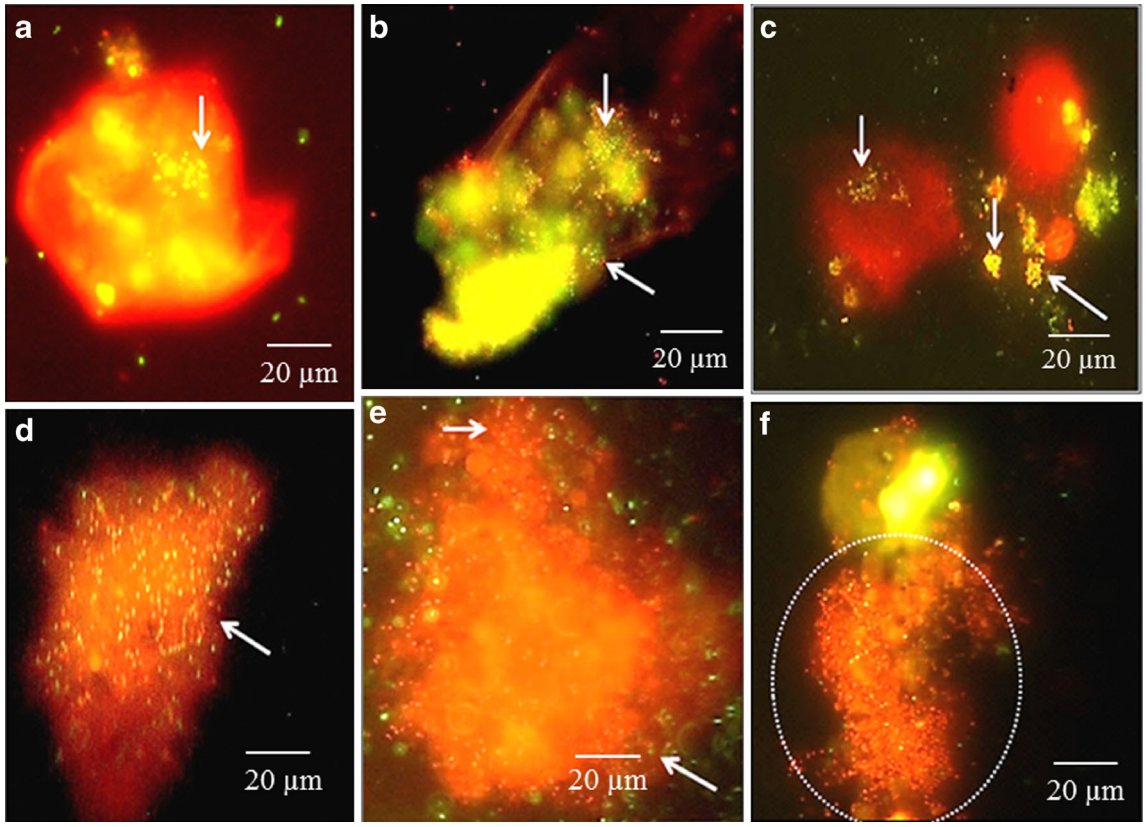

Fig. 3 The samples were stained with SYTO9/PI dyes for clear visualization of live (green cocci) and dead bacteria (red cocci) present within macrophages (seen as *red stain*). **a** and **b** Phagocytic uptake of *S. aureus* 43300 by macrophages post 3 h. **c** Phagocytic killing with live/dead populations seen in control samples at 24 h. **d** Clumps of engulfed cocci containing mixtures of live and dead bacteria as seen in phage-treated samples. **e** and **f** Increased killing as higher number of red stained dead cocci were seen within macrophages in samples taken from wells to which phage-adsorbed *S. aureus* 43300 was added. *White arrows* in the figure represent the engulfed cocci (Color figure online)

Phage therapy appears to be a potent and safe alternative tool for treating such bacterial infections. The therapeutic success of phages against different pathogens has been demonstrated in our laboratory (Chhibber et al. 2008, 2013;

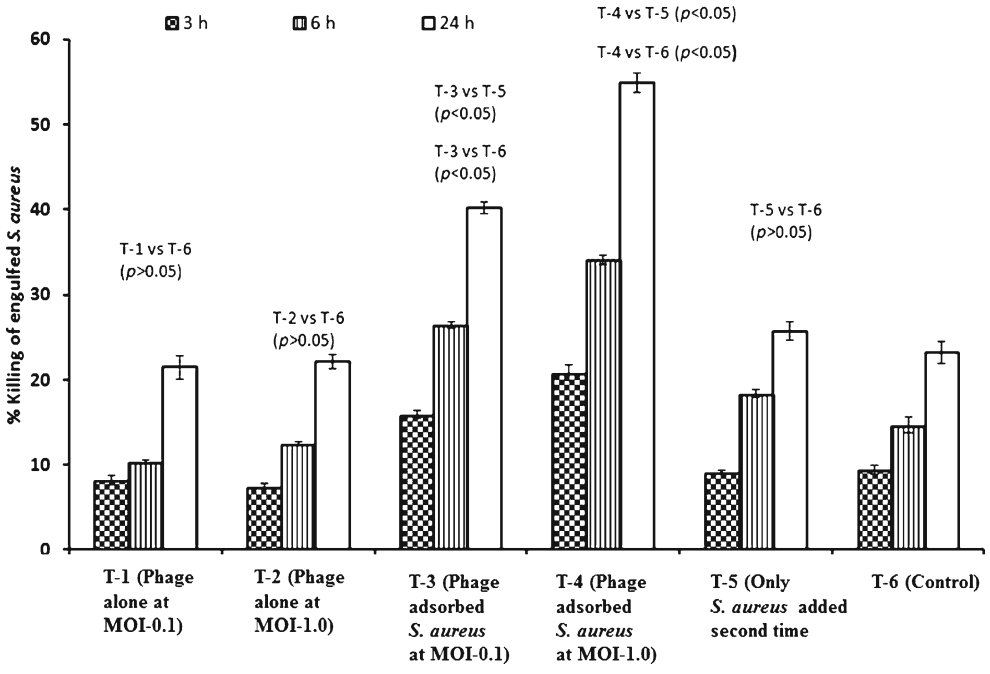

Fig. 4 Intracellular killing activity (percent killing) of peritoneal macrophages against *S. aureus* 43300 engulfed within macrophages in the presence of unadsorbed (phage only) and adsorbed phage (phage adsorbed onto *S. aureus*) at MOI-0.1 and 1. T-1, T-2…T-6 represent different treatment groups which are explained in the figure and elaborated in the respective method section. Error bars represent S.D. (n=3). Statistical analysis (p value) was done between values (at 24-h time point) of the respective treatment groups

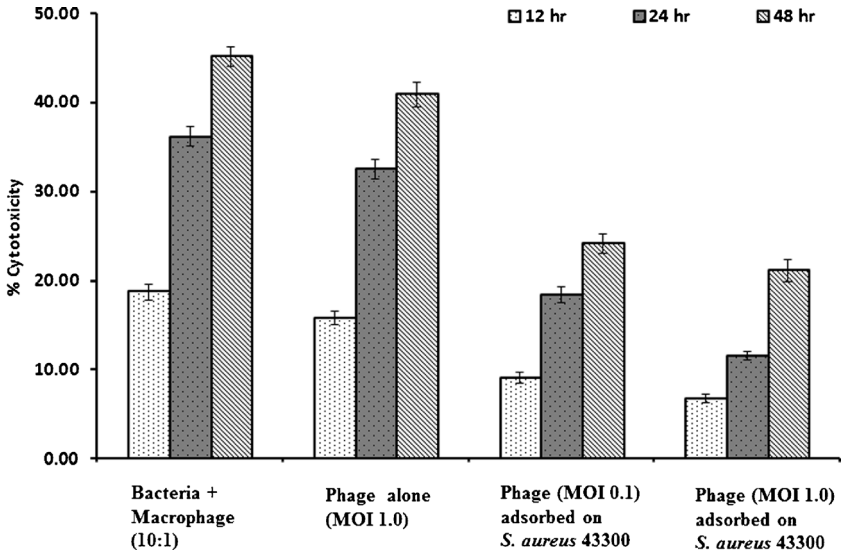

Fig. 5 Cytotoxicity (%) damage caused by *S. aureus* 43300 on macrophages in the presence of unadsorbed phage and phage adsorbed onto host bacteria. Error bars represent S.D., and each value represents mean±S.D. of three independent values

Kumari et al. 2009). Lytic phages specific against *S. aureus* have also been isolated and characterized (Chhibber et al. 2013; Kaur et al. 2012). Also, lytic *S. aureus* phage has been demonstrated to successfully eradicate MRSA from diabetic foot in an acute hindpaw model in diabetic mice (Chhibber et al. 2013). However, the effect of these phages in eradicating intracellular bacteria persisting within the animal/human cells has not been studied. Capparelli et al. (2007) have briefly studied the intracellular killing ability of phage-K, but a detailed study on this aspect was needed to be conducted. Hence, with this aim, phage MR-5, a broad spectrum lytic phage belonging to family *Myoviridae*, isolated and characterized in our laboratory was used in the present study.

Phage MR-5 was highly stable up to a temperature of 42 °C and at near neutral pH range. While studying the pH profile, it was observed that even at low pH (4.0–5.0), phage MR-5 retained 50 % of its titre even at 6 h of sampling. This is an advantage as in the present study the focus was on the activity of phage in enhancing the killing of intracellular bacteria within the phagocytic cell where pH is as low as 4–4.5 (Geisow et al. 1981; Griffiths and Mayorga 2007). From in vitro challenge experiments performed to study the relative ability of phage MR-5 to lyse *S. aureus* MRSA 43300, it was proved that this phage was able to significantly reduce the bacterial population within 2 h by 2.5 log cycles from an initial count of 10^7 CFU/ml. Phage MR-5 reduced the bacterial counts to nil by the 5th hour and suppressed further growth for the next 6 h with regrowth occurring with minimal counts thereafter. The frequency of development of bacteriophage insensitive mutants (BIM) is a factor that compromises the efficacy of a phage treatment. Such phage-resistant mutants are generated because of different bacterial resistance mechanisms. These include adsorption inhibition, restriction–modification, point mutations in genes encoding receptors on the bacterial cell surface, phase variable changes in some receptor proteins, CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) systems and abortive infection (Forde and Fitzgerald 1999; Labrie et al. 2010; Samson et al. 2013). A high frequency of emergence of BIM may pose a negative effect on the effectiveness of phage therapy. The BIM frequency for phage MR-5 was low at both the MOI (1 and 10), and all the emerged BIMs reverted back to phage sensitivity. Recently, Kim and Ryu (2012) and Sørensen et al. (2011) have revealed that one of the reasons for reverting back to phage susceptibility is due to reversion of transient phase variable modifications in some specific receptor/receptor modification gene(s) in host bacteria. Given the low BIM frequency and propensity of these mutants to revert to phage sensitivity, phage MR-5 represents a potent bio-control agent. Finally, efficacy of phage MR-5 to tackle the intracellular load of *S. aureus* surviving well within murine peritoneal macrophage cells was studied. The killing kinetics of engulfed cocci by murine macrophages and cumulative cytotoxic damage posed by engulfed cocci onto phagocytic cells in the presence of adsorbed and unadsorbed phage was thus studied.

First, uptake and killing of isolated macrophages against *S. aureus* 43300 were performed. Isolated macrophages showed 33 % uptake of the total population added and exhibited reduced killing with a maximum of 23.7 and 14.7 % at a bacteria/macrophage ratio of 1:1 and 10:1 at the end of 24 h. The killing was higher at 1:1 than at 10:1 at all time points determined. The findings were similar to the observation made by Baughn and Bonventre (1975), who also showed that the rate and extent of killing of *S. aureus* by mouse macrophages were greater at lower MOI. On this basis these workers concluded that an increased number of intracellular organisms deplete the cells of its bactericidal capabilities, leading to decreased percentage of inoculum clearance. The fluorescent images of lysed macrophage samples taken at 24 h

showed majority of engulfed bacteria stained as green within the macrophage. These findings confirmed the previous observations that *S. aureus* can not only persist for several days inside macrophages but ultimately is able to escape the intracellular confinement, leading to its rapid proliferation (Kubica et al. 2008).

In view of the evidence that *S. aureus* can adopt an intracellular lifestyle (Clement et al. 2005; Kubica et al. 2008), complete eradication of infecting staphylococci would require phage MR-5 to kill both extracellular and intracellular staphylococci. For this, isolated peritoneal macrophages were cultured to form a monolayer. After allowing phagocytic uptake of host bacteria for 3 h, killing was allowed to occur of the engulfed bacteria for an additional 3 h after which different treatments were given to macrophage cells (addition of the phage alone, phage absorbed onto *S. aureus* cells and additional *S. aureus* cells added again). The killing kinetics of macrophages was then studied in comparison to control wells (without any additions). The results demonstrated that phage alone was unable to penetrate within the macrophages, and hence killing rate was not enhanced as percent killing was almost similar to that observed in control wells. However, when phage particles adsorbed onto *S. aureus* cells (MOI of both 0.1 and 1.0) were allowed to be engulfed by macrophages, such wells showed a significant increase in killing of bacteria (38.7 and 54.2 %, respectively) at all time points. Fluorescent images of such samples also demonstrated red stained cocci within the macrophage. The results clearly indicate that at high dose phage (MOI of 10 and above) showed pronounced killing. The enhancement in killing activity of macrophages was due to the presence of shuttled phage taken inside the phagocytic cell by its own host bacteria. Addition of a similar load of *S. aureus* cells again (treatment 5) did not increase the overall killing effect.

S. aureus here simply acted as a delivery vehicle to carry the phage particles inside the macrophage as a result of engulfment of host bacteria. Following pre-absorption on host cells, the phage soon injects its genetic material into the host cell. This is then followed by the intracellular phase of phage growth (in which it uses host machinery for its own progeny production), eventually leading to the release of a large number of progeny phages from lysed bacteria into the phagocyte. These phages start another round of lysis by attacking the surrounding host bacteria engulfed by macrophages. Thus, phage MR-5 once shuttled inside the macrophage cell undergoes self-multiplication (also referred to as auto-dosing) within the phagocyte at the expense of its specific bacteria, thus enhancing the overall killing effect of phagocytic cell. They decrease the population of intracellular staphylococci to levels that can easily be handled by the oxidative killing mechanism of phagocytic cell itself.

Last, the cytotoxic damage implicated by invading *S. aureus* onto viable macrophages was assessed by MTT assay. The cytotoxicity was not too high (with values less than 50 %) even up to 48 h post incubation with bacteria. *S. aureus* uses macrophage as an intracellular niche posing minimum damage to it during its short-term persistence. It uses these mobile phagocytic cells as vehicles for the dissemination of bacteria (Kubica et al. 2008). However, in wells to which phage-adsorbed *S. aureus* was added, a significant reduction ($p<0.05$) in cytotoxic damage at all time points was calculated. This was evident because of the increased killing of the engulfed *S. aureus* responsible for inflicting cytotoxic damage onto the metabolically active macrophages.

The extensive armamentarium of virulence factors that the organism expresses, its ability to successfully evade host immune mechanisms, its intracellular survival within the host cells and its inherent ability to acquire resistance to vast range of antibiotics make this organism a life-threatening pathogen (Foster and Hook 1998; Foster 2005; Gordon and Lowy 2008; Lindsay and Holden 2004). The present study highlights the possibility of using the broad host range lytic phage MR-5 in treating MRSA infections. The phage exhibited potent intracellular activity against engulfed bacteria, thus taking care of both extracellular as well as intracellular populations. This shall lead to the arrest of intracellular staphylococcal growth within the host cells that possibly is responsible for the relapse of infection. This study provides solid evidence to suggest that phage MR-5 appears to be a potential therapeutic candidate, and its efficacy in the treatment of staphylococcal infections should be evaluated in vivo in different animal models. The work in this direction is in progress in our laboratory.

Acknowledgments The financial assistance provided by the Council of Scientific and Industrial Research (CSIR), India in the form of a research fellowship (09/135/(0632)/2011/EMR-I) is gratefully acknowledged.

Conflict of interest No conflict of interest associated with the present manuscript.

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