INTRODUCTION

Antimicrobial resistance has been a growing threat to the effective treatment of ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. Bacterial resistance is closely associated with the use of antimicrobials in clinical settings (Archer et al., 2011). Prolonged or incomplete therapy can lead to development of resistance in bacteria, which were earlier sensitive but gradually became resistant to antibiotics (Bansal et al., 2014). Due to exploitation of antibiotics, emergence of a wide range of antibiotic resistant organisms has been seen in the past. Moreover, there is an increased incidence of microorganisms developing resistance against more than one antibiotic termed as multidrug resistant strains (MDR). Multidrug resistance has been demonstrated both in gram-negative and gram-positive bacteria such as Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae, S. aureus, Klebsiella pneumoniae and Acinetobacter baumannii (Alekshun et al., 2007). Antibiotic resistance results in reduced efficacy of antibiotics, making the treatment of patients difficult, costly or even impossible. Its effect on vulnerable patients is most obvious, resulting in prolonged illness and increased mortality. The magnitude of Antimicrobial resistance (AMR) worldwide and its impact on human health/cost sand wider societal impact are still largely unknown (http://www.cdc.gov/drugresistance/threat-report-2013). Some estimates of the economic effects of antibiotic resistance have been attempted and the findings are disturbing. For example, in year 2014 an estimated amount of $34 billion dollars has been spent by the US health system alone to treat infections caused by multidrug resistant strains (http://www.cdc.gov/drugresistance/threat-report-2014).

Antibiotic resistance is mainly acquired by four mechanisms: 1) Drug inactivation or modification (e.g. production of β-lactamases) 2) Alteration of target site (e.g. alteration of penicillin-binding site (PBP 2a) in MRSA) 3) Alteration of metabolic pathway (non-requirement of p–aminobenzoic acid) 4) Reduced drug accumulation (e.g. active efflux pumps) (Hawkey, 1998). The first report of S. aureus acquiring resistance to methicillin and oxacillin (termed as Methicillin resistant S. aureus) was reported in 1960s (Hartman and Tomasz, 1981; Crisosomoto et al., 2001; Appelbaum, 2006). The resistance was associated
with integration of a mobile genetic element—“staphylococcal cassette chromosome mec” (SCCmec) into the chromosome of S. aureus that contained resistance gene mecA. mecA gene encodes for PBP 2a protein, a new penicillin binding protein, which alters the native staphylococcal PBP (Hartman and Tomasz, 1981; Katayama et al., 2000; Hiramatsu, 2004). Hence, mutation in PBP results in increased resistance to β-lactam antibiotics. In addition to this, β-lactam resistant strains are known to develop cross-resistance against streptomycin and tetracyclines and in some cases to erythromycin as well (Sakoulas et al., 2006; LaPlante et al., 2007).

Methicillin resistant S. aureus (MRSA) is a leading cause of nosocomial infection worldwide as it is an etiologic agent of a wide range of diseases, relatively from benign skin infections to potentially fatal systemic disorders (Kazakova et al., 2005). Among various nosocomial pathogens, the prevalence of methicillin-resistance among S. aureus isolates in intensive care units in the United States is 60 percent (Niami et al., 2003) In addition, MRSA accounts for more than 90,000 invasive infections in the United States (Center of Disease Control and Prevention, 2003). In recent years, prevalence of MRSA in India has increased from 12% in 1992 to 80.83% in 2007 (Raghunath, 2008). The problem of drug resistance in S. aureus gets further compounded as this organism has the ability to form potent biofilm. Majority of the diseases, including endocarditis, osteomyelitis and foreign body related infections appear to be caused by biofilm-associated S. aureus (Ruben et al., 1999; Wenzel and Edmond, 2001; Archer et al., 2011).

Biofilms, which are a sessile microbial communities embedded in a self-produced extracellular polymeric matrix are known to be of special clinical relevance (Costerton et al., 1995; Donlan and Costerton, 2002; Archer et al., 2011). Biofilm associated bacteria show an innate resistance to antibiotics, disinfectants and host defenses. Such properties are likely to contribute towards persistence and recalcitrance of staphylococcal related biofilm infections. Based on in-vitro experimental models, the complex mature staphylococcal biofilm formation can be described into a four-step process 1) initial attachment of bacterial cells; 2) Accumulation of cells in multiple layers; 3) maturation of biofilm and 4) detachment of cells from the biofilm (Arciola et al., 2012).

During the first step, a non-specific initial interaction takes place between the material surface and bacteria. The non-specific interactions are driven by hydrophobic, electrostatic or
Materials and Methods

Van der Waals forces (Legeay et al., 2006). Previous studies have incriminated that the degree of hydrophobicity of the staphylococcal cell surface and that of the matching biomaterial surface are highly important for initial attachment. In addition to this, specific proteins have been identified that mediate the binding to the abiotic surfaces (Arciola et al., 2012). According to findings of Heilmann et al. (1998), the adhesive and enzymatic properties of bacterial autolysins (AtlA in S. aureus and AtlE in S. epidermidis) facilitate the initial attachment of staphylococci to biomaterial surface by ionic or hydrophobic interactions. In the second step, the accumulation of biofilm structure in the form of multiple bacterial layers occurs with the help of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) and by intercellular adhesion (Patti et al., 1994; Foster, 1995; Hirschhausen et al., 2010). During this phase the biofilm is progressively established on the colonized surface. Further, in the subsequent step the maturation of biofilm takes place and characteristic structural features of the biofilm, specific for the bacterial species, are developed (Speziale et al., 2009; Arciola et al., 2012). During the fourth step, the bacteria previously enclosed and protected in the biofilm structure return to their initial planktonic form of life and gets dispersed for a new invasive phase. The bacterial detachment and dispersion is the characteristic feature of this final step of bacterial life cycle (Arciola et al., 2012).

The challenge of developing a potential therapeutic agent against staphylococcal biofilm requires an understanding of multiple biofilm forming mechanisms. Previously, it was known that icaADBC genes encode for the production of polysachharide intercellular adhesion (PIA) or polymeric N-acetyl-glucosamine (PNAG), which is responsible for biofilm forming ability of MRSA. However, recently it has been reported that methicillin resistant S. aureus (MRSA) strains express an icaADBC-independent biofilm phenotype, which is dependent on the fibronectin binding proteins (FnBP and FnBPB) and the major autolysin (Atl). In such strains, the Atl associated autolytic activity helps in the initial attachment of biofilm followed by role played by FnBPs to promote subsequent intercellular accumulation and biofilm maturation. (Cramton et al., 1999; Mack et al., 2006; O’Neil et al., 2008; Houston et al., 2011). Other staphylococcal surface proteins implicated in biofilm formation include the biofilm-associated protein (Bap, in bovine S. aureus isolates), accumulation-associated protein SasC (homologue of Aap present in S. epidermidis) (Schroeder et al., 2009).

Although biofilm is known to play a major role in device-related infections, but in recent
years its importance in the pathogenesis of burn wound infections has also been confirmed.
Studies have shown that mature biofilm formation takes place on the burnt and infected area within 48 h of infection (Harrison-Balestra et al., 2003). Not only this, methicillin-resistant Staphylococcus aureus strains (MRSA) have become increasingly prevalent in burn wounds. The rate of methicillin-resistant Staphylococcus aureus (MRSA) infection (23.4%) is marginally higher than that of Pseudomonas spp. (17.6%), β-hemolytic streptococci (1.7%), Enterobacteriaceae (11.5%) and Acinetobacter (7.1%) (Bagdonas et al., 2004). Many of these MRSA isolates are becoming multidrug-resistant and show susceptibility only to tetracyclines and glycopeptides (Ruhe and Menon, 2007). The mortality rate is much higher in case of burn wound infection caused by MRSA (42%) compared to MSSA (18%) (Kandati et al., 2015). Strategies to eliminate MRSA from colonized wounds are therefore essential and should include the use of simple, low-cost and effective treatments (Altoparlack et al., 2004).

Presently burn wound infections can be treated either by instituting systemic or localized treatment options (Church et al., 2006). The commonly deployed antibiotics (tigecycline, vancomycin) to treat MRSA mediated burn infection systemically are associated with large number of side effects. Moreover, S. aureus strains resistant to these antibiotics have already emerged in hospital and community settings (American Burn Association, 2000). In addition, administration of antibiotics systemically might suppress symptoms of infections by killing the free-floating bacteria shed from the attached population, but it fails to eradicate those bacterial cells which are still embedded in the biofilm matrix resulting in emergence of antibiotic resistant strains. Also, use of high dose of antibiotics results in neutropenia in patients, which in turn delays the wound healing process. This leads to persistence of biofilm-mediated infections, which can be cured only if colonized burnt area is surgically removed from the body (Ruhe and Menon, 2007). Another alternative of treatment is the use of various antimicrobial agents, which are available commercially (such as Silver sulfadiazine, Mafenide acetate, Silverlon, Mupirocin, Gentamicin sulphate, Nystatin, Betadine etc.) applied topically on the burnt and infected area. Though this treatment showed significant results but it has limitations such as inability to penetrate the eschar, electrolyte imbalance, leukopenia, metabolic acidosis and toxicity (Church et al., 2006). Inspite of various limitations, both the treatment options are being used worldwide depending upon the severity of infection.
In recent years, enzymes have been identified as useful adjunct to antibiotics for acute as well as chronic infections. They are known to minimize mortality rates and lead to an early recovery from infection. They enhance the action of antimicrobials and assist in prompt and better control of infections (Donelli et al., 2007; Bansal et al., 2014). A commercially available Dispersin B® is the only yet known anti-biofilm agent having the ability to degrade the polymeric matrix in ica-dependent biofilm (Ramasubbu et al., 2005; Donelli et al., 2007). On the other hand, ica-independent biofilm can be treated using Proteinase K. However, no such agent has been reported that can target the common moiety present in both types of biofilm. Hence, further research is needed to find a potential candidate, which can take care of formed biofilm, irrespective of biofilm-phenotype.

Phage and phage products have emerged as a promising therapeutic approach in recent years (Garcia et al., 2004; Malik and Chhibber, 2009; Fischetti, 2010). During the course of evolution, phages have developed two mechanisms for the release of their phage progeny from host bacterial cells. Filamentous phage are released through bacterial cell wall without causing cell lysis whereas non-filamentous phage use specific lysins to either inhibit the synthesis of peptidoglycan, (ssRNA or ssDNA phages) or hydrolyze the peptidoglycan using a holin-endolysin system (dsDNA phages) (Wang et al., 2000; Young et al., 2000; Bernhardt et al., 2001; Wang et al., 2003).

Phage lysins, which are produced by dsDNA lytic bacteriophage, have various advantages over antibiotics such as 1) rapid killing efficacy 2) low probability of development of resistance 3) lower chance to disrupt normal micro flora due to its host specificity (Schuch et al., 2002; Lossener et al., 2005; Fischetti, 2005; Pastagia et al., 2011). Previously, our laboratory has established the therapeutic efficacy of bacteriophages in treating both gram-positive as well as gram-negative infections (Chhibber et al., 2008; Kumari et al., 2011; Chhibber et al., 2013; Singla et al., 2015).

However, use of endolysin has various advantages over using whole bacteriophage. In case of usage of whole phage, resistance arising from adsorption, restriction modification or abortive infection has been reported in various genera (Schuch et al., 2013). Bacteriophages have maintained the equilibrium for co-existence by developing resistance and counter resistance (Fenton et al., 2010). On the contrary, there have been no reports of development of resistance in bacteria even after growth in presence of sub-lethal doses of endolysin and after repeated exposures (Schuch et al., 2002). In addition, the risk of horizontal gene transfer