Discussion
7. Discussion

*S. aureus* is commonly found on the skin and anterior nares of healthy individuals. However, with any defect in host immune system, they gain entry into the tissue and cause infections ranging from localized abscess to invasive infections such as SSTIs, endocarditis, osteomyelitis, bacteremia and pneumonia. This pathogenicity reflects its ability to produce a variety of exotoxins and adherence to medical devices by production of biofilm. The development of resistance to methicillin among *S. aureus* was reported in late 1960s. It posed a serious therapeutic challenge and still continues to do so. Currently, MRSA is an important pathogen associated with both hospital and community-acquired infections. HA-MRSA has been a serious problem in health care facilities worldwide including India.

7.1 Prevalence of MRSA isolates

In India, MRSA emerged as a public health problem from 1980 onwards, the prevalence rate varying from 25% in western part of India to 50% in south India. In the present study, prevalence rate of MRSA is found to be 34.2% and the majority of these (66%) isolates were collected from pus and burn wound swabs. Rajaduraipandi et al., 2006 reported that out of 906 strains of *S. aureus* isolated from clinical samples, 31.1% were found to be methicillin resistant. Recent surveillance report from a network of microbiology laboratories (INSAR) at premier medical colleges and hospitals in India indicated an overall prevalence of MRSA infections to an extent of 41%. Majority of these isolates were from patients with SSTIs followed by blood stream and respiratory infections. Other studies have also shown high MRSA prevalence in various parts of the country ranging from 25 to 60%. Reports from Europe-wide survey showed the most common organism in SSTIs was *S. aureus* (71% cases) with 22.5% of them were
MRSA isolates. The occurrence of MRSA varied among countries, ranging from 0.4% in Sweden to 48.4% in Belgium. Pakistan reported a higher rate of isolation up to 83% of MRSA from pus. Tracy et al., 2011 reported that, over the span of 10 years (1999-2008) there was an increase in the overall incidence of S. aureus infection and out of which 61% was caused by MRSA. Among these MRSA isolates, 69% were community acquired and 31% were hospital acquired.

7.2 Antibiotic resistance in MRSA isolates

In the present study, 42.4% of MRSA isolates were found to be multidrug-resistant i.e. resistant to more than three classes of antibiotic. In the earlier reports from other parts of the country, the burden of such strains has been found to be 32 to 63.6%. It can be seen that our findings are well within the above range. Detection of inducible clindamycin resistance among clinical isolates plays a crucial role in choosing macrolides for the treatment. Inducible clindamycin resistance is found significantly high in MRSA compared to MSSA isolates. Thirty three percent of MRSA isolates from clinical specimens were positive for inducible clindamycin resistance. This is in concordance with the studies reported earlier. Precise susceptibility reports are important for therapeutics. Providing susceptible report to clindamycin without testing for inducible resistance may result in therapeutic failure. On the other hand, specifying the test results as negative for inducible clindamycin resistance confirms clindamycin susceptibility and provides a very good therapeutic option. Since the iMLS_B resistant phenotypes are not recognized by using standard susceptibility test methods, D-test can be used as a simple, supplementary and reliable method to detect inducible and constitutive clindamycin resistance in routine clinical laboratories.
7.3 Bacteriophage typing of MRSA isolates

Bacteriophage typing method helps to differentiate and evaluate the circulating strains in hospital and community. Therefore, phage typing is recommended as first line of approach in epidemiological investigations of MRSA and MSSA strains. In the present study MRSA isolates were subjected to phage typing using 23 international sets of bacteriophage. Out of which, 82.6% isolates were found to be typable and the remaining 17.4% were non-typable. These non-typable isolates (75%) were from SSTIs with multidrug-resistant profile and the remaining isolates were collected from nasal swabs. In a recent report, higher percentage (39%) of MRSA was non-typable. The non-typability of *S. aureus* strains is a major problem with the available sets of bacteriophages in India and other developing countries.\(^{268}\) Egyptian investigators reported that 25 to 40% of *S. aureus* strains isolated from wound infections were non-typable and also there is an increase in the number of non-typable *S. aureus* isolates from nosocomial infections.\(^{292-294}\) Among typable MRSA isolates, we observed that predominant (64.9%) of them belonged to phage group III. It was observed that 51.3% strains of phage group III were found to be multidrug-resistant. These findings are in consistence with study conducted by Mehndiratta et al., 2010.\(^{268}\) Studies conducted during 1999, the most common phage group was found to be mixed group followed by phage 85.\(^{295}\) Recent studies suggest that there is a deviation in circulating phage group and phage group III found to be predominant among MRSA isolates.\(^{268, 296, 297}\)

7.4 Biofilm production among MRSA isolates

Biofilm production has been identified as the most important virulence factor which promotes persistent infection. It also decreases the antibiotic penetration which leads to therapeutic failure, particularly with prosthetic devices such as indwelling catheters and
endotracheal tubes. Obtaining clinical samples from such devices for laboratory testing to identify biofilm production can help in preventing the persistent infections. In the present study, MRSA clinical isolates were screened for biofilm production by CRA and biofilm plate assay. It was observed that 16.4% of MRSA isolates produced strong biofilm. The results of CRA are in accordance with the biofilm plate assay method for strong biofilm producing isolates. However, it was difficult to differentiate moderate and weak biofilm producers. Similar findings were reported by Mathur et al., 2006.

Earlier studies suggest that the polysaccharide intracellular adhesins/poly N-acetyl glucosamine produced by *S. aureus* in low amount cannot be detected by the CRA method. Therefore, biofilm plate assay found to be an appropriate method for screening of biofilm production among *S. aureus* isolates.

7.5. **SCCmec typing of MRSA isolates**

The emergence of methicillin resistance in *S. aureus* has been a serious problem worldwide since 1980s. As already discussed in review of literature, SCCmec typing is an important tool to differentiate hospital and community acquired MRSA strains. In the present study multiplex PCR was performed for SCCmec typing. SCCmec type of MRSA isolates included types III (4.1%), IVa (7%) IVc (17.8%) and V (67.1%). However 4.1% of MRSA isolates were non-typable with the primers used. The non-typable isolates were shown positive for *mecA* gene. Almost the same percentage of non-typable MRSA isolates were found by the other researchers. Zhang et al., in 2005 reported 2.8% non-typable clones among 453 local clinical randomly selected isolates. Certain MRSA isolates are non-typable possibly because of the presence of new structural types and subtypes or structural rearrangements and recombination of the *mec* element.
investigations, including sequencing the mec element are needed in order to characterize these non-typable isolates. SCCmec type III is the predominant MRSA in Asian continent except Korea and Japan which had type II. In our study, we observed a low percentage (4.1%) of SCCmec type III MRSA isolates compared to 24.5% reported by D’Souza et al., 2010. Whereas, SCCmec type V was found to be 67.1% in our study against a low (4%) reported by D’Souza et al., 2010. Recent studies suggest that SCCmec type V and IV seems to be an emerging MRSA strains in India. SCCmec types IV and V have recently been associated with community-acquired infection, detecting type V and discriminating type IV into subtypes IVa, b, c, and d may play an important role in the understanding of the epidemiology, prevention and control of currently emerging community MRSA clonal outbreaks.

7.6 PVL toxin production among MRSA isolates

PVL toxin gene commonly (~85%) seen among CA-MRSA isolates. It is rarely found in hospital acquired infections. PVL is highly prevalent in strains associated with necrotizing infections. In the present study PVL toxin gene was detected in 43.8% of the MRSA isolates. We have observed that majority (83.3%) of SCCmec type IV MRSA isolates carried PVL toxin gene. PVL toxin producing MRSA isolates showed significant association with SSTIs. Our finding is more or less in the agreement with the earlier reports. In a survey of 117 CA-MRSA isolates from the United States, France, Switzerland, Australia, New Zealand, and Western Samoa, all CA-MRSA strains carried a type IV SCCmec cassette and the PVL locus. In San Francisco, PVL gene was detected in 70% of MRSA isolates which were collected from jail inmates.
7.7 Antistaphylococcal activity of lysostaphin

The emergence of multiple-drug resistant MRSA strains has frequently caused treatment failure. There is a need for developing new antibacterial agent to treat MRSA and VISA/VRSA infections. The availability of recombinant lysostaphin, have rekindled the interest in using lysostaphin as a therapeutic agent for staphylococcal infections.

7.7.1 In vitro activity of lysostaphin

The present study evaluated in vitro and in vivo activity of r-lysostaphin. Lysostaphin showed promising activity against MRSA and VISA isolates; no resistance was found among them. Lysostaphin 50 µg/disc showed the zone of inhibition between 14 to 17 mm and there was no growth of small variant colonies within the zone of inhibition. The zone size increased (3 to 4 mm) on continued incubation for 48 hours. It can be attributed to the continued diffusion of lysostaphin through the agar. It also testifies that lysostaphin retains its bactericidal activity for longer time at 37ºC. Interestingly, lysostaphin could give a reasonable good zone of inhibition of about 15 mm at 50 µg potency even against VISA strain. Lysostaphin 25 µg/disc showed proportionally smaller zones of inhibition (11 to 13 mm). Therefore, it was difficult to discriminate sensitive and resistance isolates. Our results are in good conformity with reports of Kusuma et al., 2005. The ideal disc concentration was found to be 50 µg, which gives a zone of inhibition ranging from 15 and 25 mm for most susceptible strains, smaller (<11 mm) or no zone of inhibition with resistant strains. 233 Von Eiff et al., 2003 tested 429 MRSA clinical isolates obtained from blood and nasal swabs and reported the same zone of inhibition (15 to 21 mm) at 50 µg of lysostaphin disc, lending further support to the above findings. 234 Despite of lysostaphin being bactericidal in action, 50 µg of lysostaphin per disc was required to produce a usable zone of inhibition for assay
purposes. It is a well-known fact that antibiotics with a low molecular weight quickly diffuse through agar medium and gives a measurable zone of inhibition at lower concentration. In spite of good bactericidal activity, 50 µg of lysostaphin required for effective testing using discs on agar. Which can be explained on the basis of its higher molecular weight (~27kDa), could be preventing it from a faster diffusion through medium; this was also noticed by Von Eiff et al.\textsuperscript{234}

MRSA and VISA (ATCC 700699) strains were found to be sensitive to lysostaphin. Lysostaphin MIC was ranged from 0.25 to 2 µg/ml. Ninety percent of the isolates were inhibited at <1 µg of lysostaphin concentration. No tolerance was observed against lysostaphin, MBC/MIC ratio was found to be 1. Recent studies have examined lysostaphin activity against various strains of \textit{S. aureus} and reported MIC of 0.001 to 2.0 µg/ml.\textsuperscript{31, 232-234} Kusuma et al., 2005 found defined genetic mutant strains of \textit{S. aureus} and small colony variants were highly susceptible to the lytic activity of lysostaphin.\textsuperscript{233} Lysostaphin is showed rapid bactericidal activity and maintained >99.99% killing of MRSA and VISA isolates at their respective MIC. In support of this, the MIC and MBC for \textit{S. aureus} strains are found to be the same. The MIC assay is commonly used for the determination of antibiotic susceptibility. However it may not be the most appropriate assay for a rapidly acting lytic enzyme like lysostaphin, since the MIC assay measures the growth inhibition activity of an antimicrobial agent while lysostaphin would likely to kill the initial inoculum without further growth of bacteria in MIC wells.\textsuperscript{233} Based on the findings of this study, the disc diffusion assay appeared to be the simple method for testing lysostaphin susceptibility. The MBC assay can be used as a follow-up assay for questionable strains to determine actual lysostaphin susceptibility concentrations, since it measures the bactericidal activity of
Discussion

lysostaphin. Assignment of *in vitro* susceptibility criteria for lysostaphin in accordance with CLSI guidelines will require more research and may require adaptations in the guidelines to accommodate this unique rapidly bactericidal protein.

7.7.2 Synergistic activity of lysostaphin

Antimicrobial combination therapy affords broad-spectrum activity against microorganisms and prevents the emergence of resistant mutants. Synergistic activity of lysostaphin with linezolid/vancomycin/oxacillin against MRSA and VISA isolates were investigated. Lysostaphin showed synergistic activity with oxacillin as well as with linezolid against all the isolates tested; however with vancomycin only, 41.6% of the isolates showed synergistic activity and the remaining 58.4% isolates showed additive effect. Earlier studies on lysostaphin also have confirmed the same additive effects with vancomycin, gentamicin, tetracycline and erythromycin.\textsuperscript{236} The additive effect of lysostaphin and vancomycin was also seen *in vivo* animal models.\textsuperscript{239, 242}

It has been observed that there is eight-fold decrease in MIC of linezolid when combined with lysostaphin. A combination therapy that includes linezolid and lysostaphin could be used in future life threatening infections like endocarditis. This can increase the *in vivo* activity of the drugs and prevent emergence of linezolid or vancomycin resistant mutants. Similarly, synergistic activity was also seen with oxacillin when combined with lysostaphin. It is noteworthy that there was four-fold decrease in lysostaphin concentration and sixteen-fold reduction in oxacillin concentration when combined. To suppress the emergence of lysostaphin resistant mutants, combination of β-lactam drug could be a better choice. Studies conducted on characterization of lysostaphin resistant strains, reported that
the lysostaphin resistant staphylococcal variants actually become more susceptible to β-lactam antibiotics than their parental strains. Resistance to lysostaphin among *S. aureus* is mediated by the changes in the muropeptide cross bridge. Mutations of the *femA* gene, which controls the addition of the second and third glycine to the pentaglycine cross bridge, result in the formation of a new cross bridge structure composed of a mono or tri-glycine. This cannot be used as a substrate for transpeptidation by PBP2a. Strains with this monoglycine cross bridge are lysostaphin resistant but also become hyper-susceptible to β-lactam antibiotics and resistance to both lysostaphin and β-lactams is unlikely to coexist. This supports that oxacillin and lysostaphin combination would be a good choice clinically for treatment of staphylococcal infections.256, 259

### 7.7.3 Biofilm inhibitory actions of lysostaphin

Increased usage of medical devices and intravenous catheter has led to increase in biofilm associated infection in health care setup. Biofilm producing strains are difficult to eradicate due to slime layer formation. This inhibits the access of required concentration of antibiotics to eradicate them. Clinicians are facing challenge to select appropriate antibiotic to eradicate biofilm and prevent systemic infections.88, 97 Currently, vancomycin is used as drug of choice for MRSA infections; however clinical trials reported failure of vancomycin in the treatment of biofilm associated infections.311-313

Current research studies are focusing on the development of alternative therapeutic agents against biofilm associated infections. Recombinant lysostaphin has shown some promise in inhibiting formation of biofilm. In the present study, four-fold lesser concentration of lysostaphin was required to inhibit the biofilm formation in comparison to
linezolid and vancomycin. Biofilm inhibition was observed under AFM, 8 µg/ml of lysostaphin was able to reduce height and roughness of the biofilm. To achieve similar biofilm inhibiting activity of vancomycin and linezolid; the concentration was found to be 64 µg/ml. The above observation with lysostaphin gets ample support from the studies carried by other researchers.\textsuperscript{31, 226} Wu et al., 2003 reported the effective role of preventing biofilm formation on lysostaphin coated catheter, on polystyrene and polycarbonate surface.\textsuperscript{226} Lysostaphin was also reported to be a potent therapeutic agent for \textit{S. aureus} infections in various animal studies which includes eradication of biofilm in jugular vein catheterized mouse model and aortic valve infective endocarditis in rabbit model.\textsuperscript{238, 239} The bactericidal activity of lysostaphin on biofilm producing MRSA isolates suggests that lysostaphin is a potential antimicrobial agent compared to vancomycin and linezolid. Lysostaphin may be a promising antistaphylococcal agent in case of life-threatening situations arising due to biofilm associated infection of \textit{S. aureus}.

\subsection*{7.7.4 Lysostaphin topical application in burn wound infection}

Burn wound infections, especially those caused by gram positive and gram negative bacteria, such as MRSA and multidrug-resistant \textit{Pseudomonas aeruginosa}, are immensely concerning according to the NNISS report from October 2004.\textsuperscript{314} Currently, for the treatment of wound infection is systemic antibiotic therapy; however systemic antibiotics are often ineffective due to reduced tissue availability and most often due to the limited peripheral blood supply found in patients with chronic wounds. Additionally, the systemic application of antibiotics to treat peripheral wound infection must be critically examined for adverse events.\textsuperscript{315-317} Topical agents are more appealing since they lack systemic side effects, like nephropathy, allergic reactions and disturbances within the intestinal flora. In
addition, application of the antibacterial agent directly to the infected wound site will result in higher local concentration, bypassing the necessity for sufficient vascularization. If a wound is not severely infected, systemic administration should not be preferred. Instead, local application of the drug will be useful in preventing infection or sepsis.\textsuperscript{317, 318}

Appropriate topical antibacterial agents are very important for burn wound treatment management. Mupirocin ointment is the most widely used topical antibiotic for MRSA decolonization. It is currently the only topical agent with FDA approval and its use is restricted to nose.\textsuperscript{316} However, its resistance to \textit{S. aureus} has already been identified.\textsuperscript{124, 250}

Although commonly used antistaphylococcal antiseptics and topical agents have bactericidal activity against MRSA, a significant number of these organisms are not eliminated from the site of infection.\textsuperscript{252, 319, 320} The progressive reduction in therapeutic options of available antibiotics and the need for topical application underlines the urgency for the development of new therapeutic options for the treatment of infected wounds.

In this context, the efficacy of lysostaphin topical gel was evaluated against MRSA and VISA infection in burn wound model. Lysostaphin topical gel was prepared with Carbopol, which is a known mucoadhesive agent and propylene glycol to improve permeability of the drug.\textsuperscript{274} Lysostaphin gel was found to reduce bacterial count as well as serous fluid discharge considerably with remarkable improvement in the burn wound healing process. This was very well supported by histological findings. While lysostaphin had biological impact on both epidermal and connective tissue components of the wound healing process, mupirocin lacked the same. This improved efficacy of the former could not be explained by the reduction of bacterial count alone. At this stage, it is interesting to bring the results of a comparative study of moxifloxacin with mupirocin in burn wound healing
study carried out by Jacobsen et al., 2008.\textsuperscript{321} In spite of significant reduction in bacterial load, mupirocin couldn’t improve wound healing, while moxifloxacin could do it by reducing the production of pro-inflammatory cytokines, like interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α) or IL-1β.\textsuperscript{322-324} This goes well with the results of systemic administration of lysostaphin with the attendant reduction in the expression of TNF and IL-6 in response to \textit{S. aureus} challenge.\textsuperscript{245} Overall, the results garnered from the present study indicates that lysostaphin do warrant further investigation as a topical treatment choice for MRSA and VISA infected wounds.

7.7.5 Lysostaphin topical application on nasal colonization in rat model

The nasal carriage of \textit{S. aureus} has been repeatedly shown to have a significant epidemiological link with subsequent development of staphylococcal disease, particularly for those requiring major surgery, implanted devices, hemodialysis, or treatment in ICU.\textsuperscript{250} Currently, 2\% mupirocin calcium ointment is the most widely prescribed and has been used successfully to eradicate nasal and hand staphylococcal colonization in patients and healthcare workers.\textsuperscript{325} Although the optimal regimen is not known, 2\% topical mupirocin applied in conjunction with 4\% aqueous chlorhexidine is commonly used to decolonize patients with MRSA. However, this approach is being compromised by the increasing frequency of resistance to mupirocin, indicates that development of new interventions for \textit{S. aureus} nasal decolonization are clearly needed.\textsuperscript{122, 124, 129, 132, 134, 250, 251}

Earlier, nasal colonization model used mouse as the animal, but consistent and reproducible levels of colonization could not be achieved.\textsuperscript{281} Therefore, the model was changed to rats which could give satisfactory results.\textsuperscript{34, 326} Following this method, lysostaphin potential was investigated for nasal decolonization of MRSA in comparison with mupirocin
Discussion

gel. In the present study, Wistar rat model was consistent for the nasal colonization of MRSA, colonization was between 3 to 4 log\textsubscript{10} CFU/nasal tissue, which is similar in magnitude to other S. aureus strains reported in previous studies.\textsuperscript{34, 326} A very low concentration, as low as 0.25% of lysostaphin could bring about complete decolonization with an eight-fold concentration (2%) of mupirocin. In a earlier study, 0.5% lysostaphin concentration was reportedly needed for complete eradication when petroleum base was used,\textsuperscript{34} we could achieve the same results with 0.25% concentration by incorporating it into a thermoreversible in situ gel (Poloxamer). The latter could import gelation property at the nasal temperature though at instillation temperature it was liquid. However it didn’t have appreciable mucoadhesive property. This could be bestowed by incorporation of NaCMC mucoadhesive agent. Nasal mucus comprises of mucin which is an anionic polyelectrolyte rich in sulphate groups. NaCMC and other polymers such as Chitosan, Carbopol 934P, Hydroxypropyl methylellulose have the ability to interact electronically or to form hydrogen bonds, thus acting as good candidates for mucoadhesion.\textsuperscript{327} NaCMC in the above formulation gave good mucoadhension, thus probably contributing to the better efficacy of lysostaphin formulation. Resistance to antibiotics often arises when concentrations of the drug fall below the minimum therapeutic value for extended periods of time. If lysostaphin gel were to be applied daily for the course of treatment, with the increased retention time, intranasal concentrations of the drug would never fall below this threshold, and the potential for emergence of lysostaphin resistant S. aureus would be minimized. However, a thorough pharmacokinetics investigation is needed to assess the amount of drug retained in the nasal mucosa at different intervals of time. In view of the positive inference gathered in our studies it will be worthwhile to use lysostaphin topical gel as an alternative to mupirocin for nasal decolonization of MRSA, after sufficient clinical evaluation.