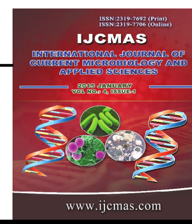

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 1 (2015) pp. 822-830**
http://www.ijcmas.com

Original Research Article

In-vitro bactericidal activity of recombinant lysostaphin on biofilm producing methicillin resistant *Staphylococcus aureus*

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A B S T R A C T

Bacteria embedded in biofilms are often difficult to eradicate with standard antibiotic regimens. Hence the current antimicrobial therapies have largely proven unsuccessful in the treatment of many chronic *S. aureus* biofilm associated infections. Lysostaphin is a glycyl-glycine endopeptidase for which there has been a recent interest renewed to use as anti-staphylococcal agent. In the present study we determined the in-vitro bactericidal activity of lysostaphin against twelve strong biofilm producing methicillin resistant *Staphylococcus aureus* clinical isolates. The minimum biofilm inhibitory concentration (MBIC) of lysostaphin was found between 4 to 8 µg/ml. Vancomycin and linezolid showed anti-staphylococcal activity on biofilm producing MRSA isolates at the concentration between 16 to 64 µg/ml, which is four fold higher compared to lysostaphin. Atomic force microscopy revealed production of strong biofilm on coverslip had height of 681 nm with a maximum surface roughness was around 103 nm. Lysostaphin treated at 8 µg/ml concentration showed decrease of biofilm height to 88 nm with 7.3 nm roughness, whereas the vancomycin showed similar biofilm inhibiting results at high concentration of 64 µg/ml. This result indicates that lysostaphin is a potential antimicrobial agent compared to vancomycin and linezolid, which are the present drug of choice for MRSA infections.

Keywords

Biofilm, Lysostaphin, Methicillin resistant *Staphylococcus aureus* (MRSA)

Introduction

Staphylococcus aureus is wide spread in nature and colonizes the skin and anterior nares of the individuals. The colonization is a risk factor for infection from which bacteria can invade to cause localized abscess to serious, invasive infections (Ryu *et al*., 2014). Its pathogenicity reflects ability to produce a variety of exotoxins,

adherence to medical devices by production of biofilms and an extraordinary ability to develop antimicrobial resistance. Treatment options for serious invasive infections of *S. aureus* are limited because of increasing antimicrobial resistance to routine antibiotic classes (Evans *et al*., 2003). Vancomycin is widely used as an empirical therapy in MRSA infections, however issues related to its poor effect on biofilms producing bacteria, current limitations of susceptibility testing, and infection due to strains with reduced susceptibility to vancomycin raises fears of increased treatment failure (Liu *et al*., 2011). The increasing use of indwelling intravascular catheters for diagnosis of the diseases and for therapeutic procedures has resulted in increase in the number of medical device-related biofilm infections. Bacteria embedded in biofilms are often difficult to eradicate with standard antibiotic regimens.

Hence the current antimicrobial therapies have proven unsuccessful in the treatment of many chronic *S. aureus* biofilm associated infections (Kloos *et al*., 1994; Donlan *et al*., 2001; Gordon *et al*., 2008). There is a need for development of new therapeutic agents for *S. aureus* with special reference to the treatment of biofilm associated infections.

Scientists have renewed interest in enzybiotics, which are catalytic protein with therapeutic potential as antimicrobial agents. One such anti-staphylococcal agent for which there has been a recent interest is lysostaphin. It is a 27 kDa Zinc containing glycyl-glycine endopeptidase produced by *S. simulans* biovar *staphylolyticus*. It exhibits a high degree of anti-staphylococcal activity, which cleaves specifically between the third and the fourth glycine residues of the peptidoglycan cross bridges of the staphylococcal cell wall, leading to rapid lysis of the bacteria (Wu *et al*., 2003; Bastos *et al*., 2010). Previous studies from 1960s to 1970s demonstrated anti-staphylococcal activity of crude extract of lysostaphin (Schindler *et al*., 1964). However, study of lysostaphin as an anti-staphylococcal agent was discontinued due to lack of homogenous preparations of lysostaphin and the availability of other effective treatments. With rapidly decreasing effectiveness of current antibiotics for treatment and the availability of recombinant lysostaphin, studies on investigating lysostaphin as a therapeutic agent for staphylococcal infections have re-emerged (Kusuma *et al*., 2005). The objective of this work was to determine the in-vitro bactericidal activity of recombinant lysostaphin against biofilm producing MRSA clinical isolates.

Materials and Methods

Bacterial strains

A total of seventy-three clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from pus, burn wound swab, blood, urine, sputum, endotracheal aspirate, catheters tips, skin surfaces and nasal swabsreceived from Kasturba hospital, Manipal, India were used for the present study. Clinical specimens were processed as per standard operating procedure at Department of microbiology. Methicillin resistance was determined by using Kirby-Bauer disk diffusion method according to recommendation of Clinical and Laboratory Standards Institute. *Staphylococcus aureus* sub sp. *aureus* ATCC 43300 and ATCC 25923 were used as controls. The isolates which showed a zone diameter of ≤19 mm for 30 µg disc of Cefoxitin and growth on MRSA selective chrom agar (Hi-media) were confirmed as methicillin resistant *Staphylococcus aureus* (MRSA). All the strains were preserved as glycerol stocks at -70 ºC.

Antimicrobial agents

Recombinant lysostaphin (Sigma Aldrich L9043), vancomycin (Sigma Aldrich 861987), linezolid (Sigma Aldrich PZ0014) and oxacillin (Hi-Media CMS5372-5G) were used in present study.

Screening for biofilm production

Congo red agar method

Congo red agar (CRA) method was used to detect slime production among the isolates. Congo red agar medium was prepared with brain heart infusion broth (BHI) supplemented with 2.5% sucrose, 0.08 % Congo red and 2 % agar (Freeman et al. 1989). Cong red agar plates were inoculated with test strains and incubated at 37ºC for 24 h aerobically. Strains which produced black colonies considered as slime producer and strains with red colonies indicated as non-slime producers.

Microtitre plate assay

The ability of MRSA isolates to produce biofilm was determined by 96-well microtitre plate assay (Christensen et al. 1985 & Wu et al. 2003). MRSA isolates from a blood agar plate was grown overnight at 37°C in 5ml of Soyabean Casein Digest broth (SCDB, Hi-Media) supplemented with 0.25 % D-(+)-glucose. The culture was diluted 1:50 in SCDB plus 0.25 % glucose to a final volume of 200 µL in each wells of sterile, polystyrene, 96 well-flat bottom microtitre plates and only broth served as control to check sterility and non-specific binding of media. The microtitre plates were incubated at 37°C with shaking at 120 rpm for 24 hours. These plates were placed in the stationary incubator at 37°C and the growth was allowed to continue for an additional 24 hours. After 48 hours of growth, the wells were washed twice with 200 µl of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. The assay plates were inverted and allowed to dry at 37°C for approximately 1 hour. The wells of the 96-well plate were stained with 200 µl of 0.1 % Gram safranin for 1 minute. The stain was removed, and the wells were gently washed twice with 200 µl of PBS and plates were kept for drying. Quantitative analysis of biofilm production was performed by adding 200 µl of 33 % glacial acetic acid to destain the wells. The amount of safranin dye adhered to biofilm was measured at 540 nm using a microtitre plate reader. The experiment was performed in triplicate. The interpretation of biofilm production was done according to the criteria of Stepanovic *et al.* 2007 (Table I). *Staphylococcus aureus* MTCC 1430 was used as positive control for strong biofilm production.

Determination of minimum biofilm inhibitory concentration

The experiments were done in 96-wells polystyrene microtiter plates with flat bottoms. Microtitre plate wells containing biofilms of *S. aureus* were incubated with serial dilutions of lysostaphin (0.25 to 16 µg/ml), oxacillin (1 to 256 µg/ml), vancomycin (0.25 to 128 µg/ml) and linezolid (0.25 to 128 µg/ml) for 24 hours. The quantification of biofilm inhibition by antibiotics was carried out as mentioned above for the microtitre plate method.

Atomic Force Microscopy (AFM)

The 12 strong biofilm producing isolates of MRSA were inoculated into tubes containing 5 ml of SCDB and allowed to grow to overnight. The overnight cultures were diluted 1:50 in SCDB plus 0.25% glucose to a final volume of 2 ml in each

well of sterile flat-bottom 6-well plates. Sterile glass coverslips were inserted into each well. Plates were incubated at 37°C with shaking at 120 rpm for 24 hours. After completion of incubation, biofilms are treated with lysostaphin (4 to 8µg/ml), vancomycin (16 to 64 µg/ml) for further 24 hours. Wells without antibiotic served as biofilm positive control.

Free floating 'planktonic' bacteria were rinsed off by washing coverslip with PBS twice and coverslips were allowed to air dry. The surface topology of the biofilm was measured using a BrukerInnova® SPM Atomic Force Micro-scope. The system is capable of scanning a maximum cross sectional area of 100 µm × 100 µm and a minimum area of 1 µm × 1 µm.

Result and Discussion

A total of seventy-three MRSA clinical isolates were screened for biofilm production by two conventional methods. Out of 73 MRSA isolates 12 (16.4 %) showed strong biofilm production, 12 (16.4 %) were moderate and 49 (67.1 %) isolates were weak biofilm production was observed by microtitre plate method. By Congo red agar method only 12 (16.4%) were positive for slime production. The results are depicted in Fig. 1-4. The microtitre plate showed good correlation with the Congo red agar method for strong biofilm forming strains. However, it was difficult to discriminate between moderate and weak biofilm producing isolates on Congo red agar method.

Twelve strong biofilm producing MRSA isolates were tested for determining minimum biofilm inhibiting concentration (MBIC) by lysostaphin, vancomycin, linezolid and oxacillin. The capacity of antibiotics to disrupt the biofilms was determined by comparing the differences of gram safranin stain absorbance (OD 540nm) intensity between treated and untreated biofilms. MBIC was defined as the minimum concentration of antibiotics required to reduce sessile growth by ≥99.9 %. Lysostaphin was successful in eradicating ≥99.9% of sessile growth of *S. aureus* within the range 2–8 µg/ml. Lysostaphin activity was compared with vancomycin, linezolid and oxacillin.

The lysostaphin treatment could drop the absorbance value to baseline 0.17 at the concentration of 4 to 8 µg/ml, whereas linezolid and vancomycin at 16 to 64 µg/ml and oxacillin128 to 256 µg/ml in 24 h (Fig. 5). The minimum inhibitory concentration of planktonic bacterial cells of the same isolates ranged from 0.5 to 1 µg/ml with lysostaphin, vancomycin and linezolid.

To confirm inhibitory action of lysostaphin and vancomycin on biofilm producing isolates, coverslip were observed under Bruker-Innova atomic force microscopy with the area of 50 μm^2. Height and roughness of biofilm on glass surface in tapping (non-contact) mode was recorded. Data was analyzed using nano-drive software. Biofilm formed on coverslip had height of 681 nm with a maximum surface roughness was around 103 nm. Lysostaphin treated at 8 µg/ml concentration showed decrease of biofilm height to 88 nm with 7.3 nm roughness, whereas the vancomycin showed similar biofilm inhibiting results at high concentration of 64 µg/ml (Fig. 6).

Increased usage of medical devices and intra-venous catheter has led to increase in biofilm associated infection in health care setup. Biofilm producing strains are difficult eradicate due to slime layer formation which inhibits the access of required concentration of antibiotics to kill them.

Table.I Classification of biofilm production abilities by microtitre plate method

Average OD value at 540nm	Adherence	Biofilm production	Results obtained
≤ OD c (Cut-off value)	Nil	Non producer	≤ 0. 17
≥ OD c to ≤ 2 x OD c	Weak	Weak producer	≤ 0. 34
≥ 2 x OD c to ≤ 4 x OD c	Moderate	Moderate producer	≤ 0. 68
≥4 x OD c	Strong	Strong producer	≥ 0. 69

Optical density cut-off value (ODc) = mean of OD of negative control + 3 x standard deviation (SD) of negative control.
Average OD negative control – **0.132**
OD cut-off value = **0.132** + **3 x 0.01293**
OD cut- off value obtained = **0.17**

Fig 1:- Screening for slime production by MRSA clinical isolates on Congo red agar medium

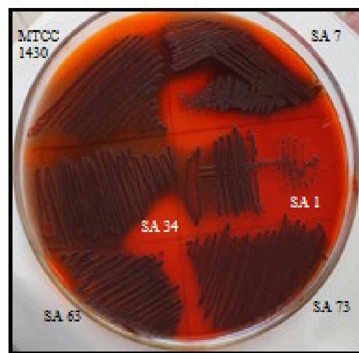


Fig 2:- Percentage of MRSA isolates of slime producers on Congo red agar

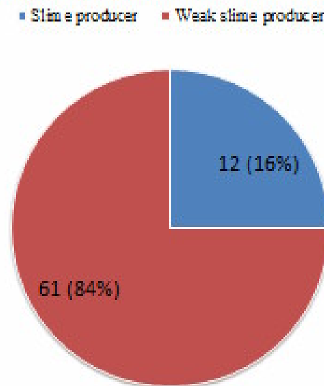


Fig 3:- Screening of MRSA isolates for biofilm Productions in microtitre plate method

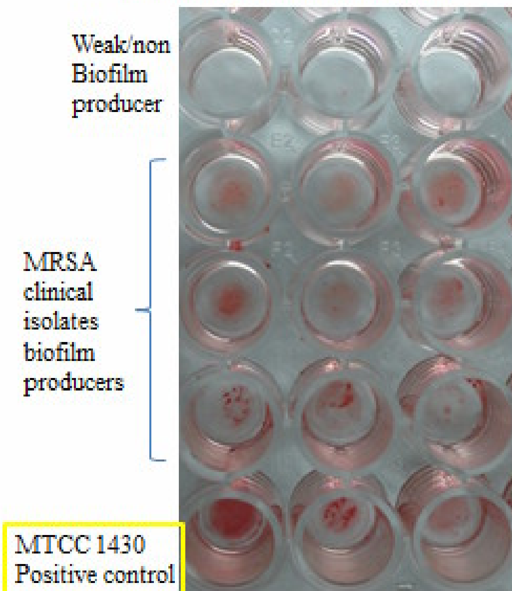
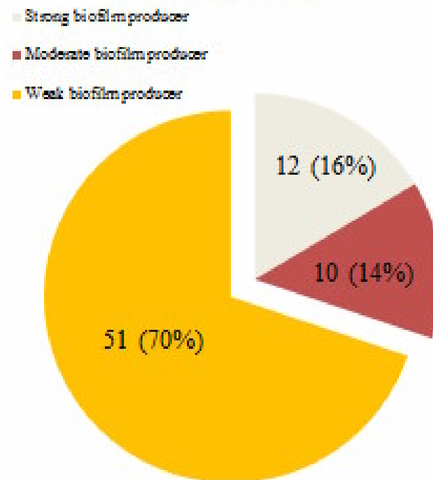


Fig 4:- Percentage of MRSA isolates producing biofilm by microtitre plate method



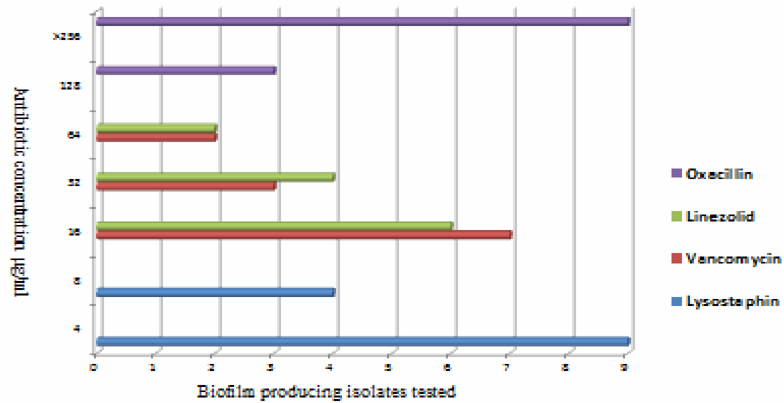
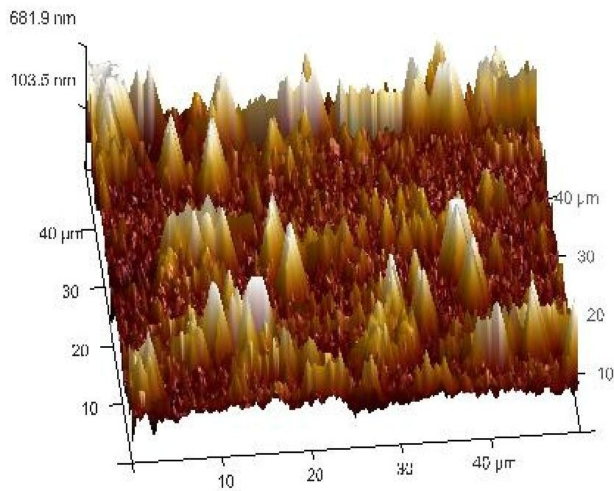
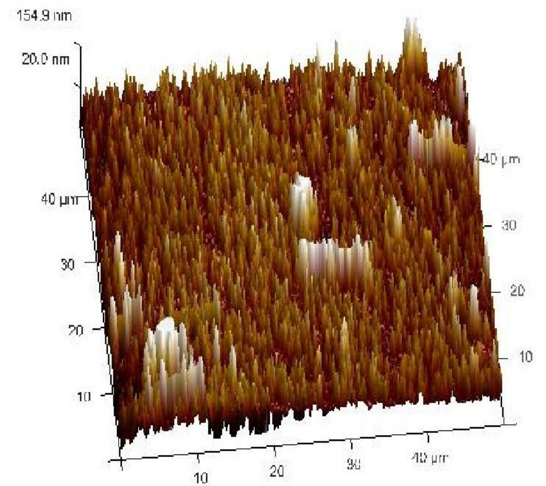


Fig 5:- Graphical representation of MRSA isolates biofilm inhibitory concentration of Antibiotics used

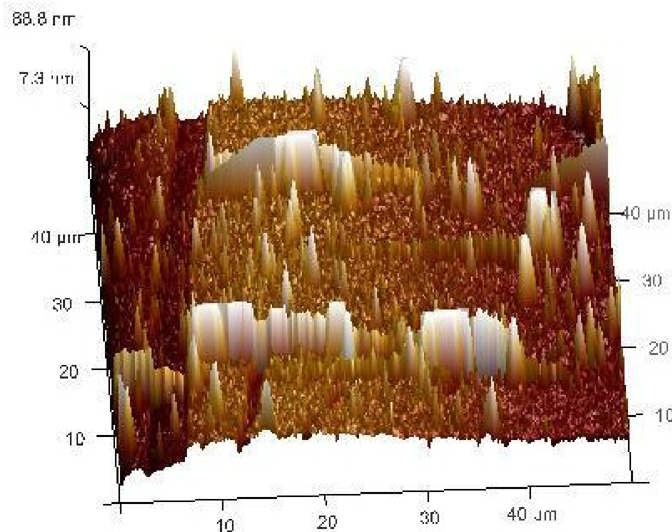
Fig.6 Atomic force microscopy images of biofilm after treatment with lysostaphin (8 µg/ml) and vancomycin (64 µg/ml)



Biofilm without antibiotic treatment



Biofilm treated with vancomycin at 64 µg/ml



Biofilm treated wth lysostaphin at 8 µg/ml)

Clinicians are facing challenge to select appropriate antibiotic to eradicate biofilm and prevent systemic infections. Biofilm associated infection is affecting the treatment outcome among millions of people (O Gara *et al*., 2001; Donolan *et al*., 2001). Biofilm production has been identified as most important virulence factors in *S. aureus* infections in clinical settings. *S. aureus* is a medically important organism associated with a vast variety of diseases; some strains can cause chronic infections and gain increased resistance to antimicrobial agents through biofilm formation. In the present study 12 (16.4 %) out of 73 MRSA isolates showed strong biofilm production by microtitre plate method. The results of microtitre plate assay are in accordance with the Congo red agar method for strong bio-film producing isolates. The Congo red agar method is not recommended as a medium for biofilm production study of *S. aureus*, as researchers have recently found that it is difficult to demonstrate the production of polysaccharide intracellular adhesins/poly N-actyl glucosamine of *S. aureus* (Taj *et al*., 2012). Mathur *et al*., 2006 have observed that microtitre plate method is helpful to detect strong and moderate biofilm production and our study findings correlates with it.

Currently, vancomycin is used as drug of choice to treat MRSA infections; however several clinical trials reported failure of vancomycin to treat biofilm associated infections. Few studies have investigated the efficacies of the newer antibiotics like daptomycin, linezolid, and tigecycline, in comparison with those of vancomycin, minocycline and rifampin against MRSA embedded in biofilm and the results showed minocycline, daptomycin, and tigecycline were more effective on biofilm producing MRSA isolates than linezolid, vancomycin (Raad *et al*., 2007).Current research studies are focusing on development of new therapeutic agents against MRSA infections and recombinant lysostaphin one of the agent (Kumar, 2008). In the present study lysostaphin showed significant effect on inhibiting biofilm production at concentration of 4 to 8μg/ml. Vancomycin and linezolid showed anti-staphylococcal activity on biofilm producing MRSA at the concentration between 16 to 64 μg/ml, which is four fold higher compared to lysostaphin. Atomic force microscopy confirmed that lysostaphin inhibited both the height and roughness of the biofilm at 8 μg/ml concentration. The finding of the present study on in-vitro activity of r-lysostaphin is found to be comparable with the other recently reported studies on anti-staphylococcal activity of lysostaphin (Von Eiff *et al*., 2003; Walencka *et al*., 2005). There are few studies reported the effective role of preventing biofilm formation on lysostaphin coated catheter, on polystyrene and polycarbonate surface (Wu *et al*., 2003) and has been demonstrated to be a potent therapeutic agent for *S. aureus* infections in various animal studies (Climo *et al*., 1998; Dajcs *et al*., 2001) The bactericidal activity of lysostaphin on biofilm producing MRSA isolates suggests that lysostaphin is a potential antimicrobial agent compared to vancomycin and linezolid, which are the present drug of choice for MRSA infections. Lysostaphin may be a promising anti-staphylococcal agent in the case of life-threatening situations arising due to biofilm-associated infection of *S. aureus*.

Acknowledgment

We thank Dr. Chiranjoy Mukhyopadhya, Head of Department of Microbiology, Kasturba Medical College, Manipal and Dr. J Venkta Rao, Head of Department of Pharmaceutical Biotechnology, Manipal

College of Pharmaceutical Sciences, Manipal for their encouragement and facilities provided to carry out this work.

Conflict of interest statement

The authors wish to state that there is no conflict of interest in the work presented in this article. None of the authors of this paper have any financial or personal relationship with other people that could inappropriately influence or bias the content of the paper.

Ethical committee Approval

Institutional ethical committee approval was obtained from Kasturba Hospital, Manipal. (Reference number – IEC 197/2011).

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