Antimicrobial and Antibiofilm activity of Enzybiotic against *Staphylococcus aureus*

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Background: Biofilms are communities of microorganisms embedded in a self produced polymeric matrix composed of polysaccharide, protein and DNA, adhered to inert or living surfaces. Researchers have focused on understanding the complex nature of biofilm and their reduced susceptibility to antibiotics. The use of biomaterials and indwelling medical devices like catheters increase probability of biofilm formation and hamper complete eradication of infection. Current treatment modalities of antimicrobial agents often fail to eradicate biofilm which can result in chronic infections. In recent years, high prevalence of antibiotic resistance has drastically narrowed the spectrum of available therapeutic options thereby increasing the demand of improving existing treatments and development of new therapeutic targeted drug delivery systems. Treatment with antibiotics has given apparent success in eliminating bacteria but unable to prevent the remission of the infection, likely due to remnant of persister cells in biofilm structure resulting in reseeding of the infection. One of the characteristic properties of biofilm is its tolerance to very high concentrations of antibiotics. The apparent antibiotic resistance of biofilm associated cells is not due to mutations, since sensitivity reappears when the biofilms are disrupted and the cells return to the planktonic state. Recent studies have indicated that the disruption of biofilm structure could be achieved via enzymes leading to resurgence of interest in enzymatic therapy. A treatment would be of great benefit to mankind if it could rapidly eliminate free-living as well as the ones present in the biofilms. Enzymes possess antibiofilm property by degrading the individual components of the biofilm. Thus enzymes complemented with antibiotics can significantly enhance the effectiveness of antibiotics against bacteria. We have termed this combination system of enzyme and antibiotic as "enzybiotic" wherein enzyme acts as a nano-driller to disrupt the biofilm and paving the way for the antibiotic to elicit its action. We will also discuss our recent work on improved biofilm activity of antibiotic in presence of proteolytic enzymes.

Methods: Fluoroquinolones are effective against a wide range of bacterial infections and their combination with enzymes may help in enhancing bactericidal effect. Comparative screening for antimicrobial activity of Ciprofloxacin (CPX) with enzymes such as Serratiopeptidase (SRP), Lysozyme (LYS), Catalase (CAT) alone and in varying combinations were performed for determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Staphylococcus aureus* (*S. aureus*) by 96 well microtiter plate method. Minimum biofilm inhibitory concentration (MBIC) of CPX and SRP, LYS, CAT alone and in combination was assayed on preformed biofilm grown in a static condition on the bottom of the microtitre plate for 24 hours. CPX was screened alone (0.25 to 8 µg/ml) as well as in combination with SRP, LYS, CAT (50 to 400 µg/ml) for MBIC. The bacterial biofilms dwell within the biomaterial such as catheter thereby promoting bacterial growth. Combinations of SRP and CPX were tested on catheter segments coated with *S. aureus* biofilm. Sterile polyethylene catheter segments of approximately 1 cm length were suspended in Luria Bertani broth containing 1% glucose and *S. aureus*. The catheter segments were then exposed to CPX-SRP combination and plated on Muller Hinton agar plate for colony count. *S. aureus* was sensitive to CPX in combination with enzymes.

Results: MIC of CPX was found to be 4 µg/ml. SRP, LYS and CAT alone failed to show antimicrobial activity. CPX at sub-MIC concentrations (1/16, 1/8, 1/4 and 1/2) in combination with SRP at 50 µg/ml showed 85-90% eradication of preformed biofilm whereas antibiofilm activity decreased at higher concentration of SRP. Conversely, CPX at sub-MICs (1/4 and 1/2) in addition to LYS at 300-400 µg/ml showed more than 90% eradication of preformed biofilm. Similarly, CPX at sub-MICs (1/4 and 1/2) with CAT at 50-100 µg/ml showed 80 to 90% eradication of preformed biofilm. The catheter study revealed that the use of CPX at sub-MIC concentration reduced the bacterial adherance to non-living surface.

Conclusion: Combination of CPX with SRP was capable of killing biofilm cells due to synergistic effect of SRP possibly degrading the exoploysaccharide sheath and enhancing effectiveness of CPX at sub-MIC concentrations. Further, the combination not only kills the bacteria present in the biofilm but also interfers with the process of biofilm formation.

Keywords: biofilm degrading enzymes; antibiotic resistance

1. Introduction

Biofilm is a complex structure comprising of dense, highly hydrated communities of microorganisms adhering to a biotic or abiotic substrate, enclosed in a thin yet robust mucilagenous layer of self-produced exopolysaccharide sheath that binds cells and microcolonies together, protect itself from the influx of antimicrobial agent [1, 2]. Biofilm formation occurs via bacterial adherence to a surface leading to growth of a multifaceted cell clusters. The process of biofilm formation can occur in natural as well as artificial environment under varied conditions. It enables cells to bond to each other at solid/liquid and liquid/liquid interfaces. In floating liquid bacteria exist in planktonic form and at solid/liquid interface they are present in aggregates as microcolonies known as sessile form [3]. The traditional approach for treating bacterial infection with antimicrobials is relatively simple as they exist in planktonic bacteria. The

infections caused by sessile bacteria are difficult to treat as they undergo changes to form biofilms which harbour notorious cells tolerant in nature. Once a mature biofilm is formed it may move collectively as a single organism or detach themselves to colonize on new surfaces [4, 5].

1.1 Stages of biofilm development [1, 2, 4, 6]

The life cycle of a biofilm includes adhesion, aggregation, maturation and detachment or dispersion. The primary step involved in the initiation of biofilm formation is the attachment of small number of free bacterial cell known as planktonic cells, to an animate or inanimate surface with the help of pili and flagella. The attachment of cells to the substrate is known as adhesion stage which plays a vital role in growth cycle of a biofilm. Adhesion process of the planktonic cells can be carried out by primary adhesion or reversible binding and secondary adhesion or irreversible binding. As the bonding between the cells and the substrate becomes stronger, it turns into irreversible attachment to the surface resulting in production of extracellular polymer or biofilm matrix.

Aggregation is the second stage of biofilm formation; the cells undergo cell division resulting in growth, accumulation and multiplication of cells leading to formation of microcolonies. The adherence of new planktonic cells to the already bound cells surrounded by biofilm matrix leads to further development of biofilm. The free and bound forms of bacterial cells are held together within the microcolonies by intercellular bonding, enclosed in an exopolysaccharide sheath which forms the basis of biofilm matrix, responsible for adhesion and cohesion of biofilm to the substrate.

Biofilm maturation is the third stage in process of biofilm formation. A mature biofilm is a heterogenous slimy multilayered cells embedded in an extracellular polymeric substance composed of polysaccharide (45-95%), proteins (1-60%), nucleic acids (1-10%), lipids (1-40%) and water channels. These highly hydrated channels acts as a filter allowing the transportation of nutrients and elimination of waste metabolic products across the cells that are multiplying within the biofilm. The third stage of biofilm development is maturation, a slow process and which undergoes remarkable changes in the microenvironment surrounding the bacterial cells.

Dispersion or detachment of bacteria from the biofilm structure is the last stage involved in development of biofilm. This stage can occur by different mechanisms namely seeding, erosion, sloughing and abrasion. The reason behind the detachment of cells might be the change in environmental condition, limited supply of nutrients or oxygen, cell growth or division, release of large number of individual cells or small aggregates. The rate of detachment increases with the biofilm formation.

1.2 Biofilm resistance [5, 7]

Microbial resistance can be defined as the ability of the microorganism to multiply in presence of increased concentration of antimicrobials. Minimum inhibitory concentrations (MIC) for such microorganisms are elevated resulting in resistant strain thus overcoming the lethal effect of the antimicrobial agents. Several mechanisms by which microbes develop resistance includes, target modification by mutation or enzymatic changes or substitution, reduced or restricted permeability of antimicrobial agent across the membrane, increased efflux or inactivation of the antimicrobial agent. Biofilm matrix shows resistance to antimicrobial agents as it provides strong protection to the bacteria embedded in it. This extracellular biofilm matrix acts as a diffusion barrier or adsorbent that prevents the influx of the antimicrobial agent across the barrier thereby leading to surface exposure of its lethal concentration or produces enzymes that inactivate the antimicrobial agent. The hypothesized biofilm resistance mechanism includes slow or incomplete penetration of antibiotic.

1.3 Strategies to control biofilm [6]

Table 1 Various antimicrobial strategies.

Antimicrobials	**Nature of compounds**	**Mechanism of Action**
Quorum sensing inhibitors	Furanones	Inhibitional production of signal, reception or dissemination and disruption of quorum sensing process and biofilm formation.
Bacteriophages	Dispersin B polymerase	Production of depolymerases that hydrolze biofilm extracellular polymers.
	Lysins and endolysins	Antibacterial activity against Gram positive bacteria.
Enzymes	Glycosidases, proteases, deoxy nucleases	Degradation of biofilm matrix.
Surfactants and Biosurfactants	Surfactin Rhamnolipids	Modification of hydrophobicity of surfaces and prevention of microbial adhesion.
Nanoparticles	Magnesium fluoride	Generation of reactive oxygen species.
Phytochemicals	Polyphenolics, Catechins, Aqueous extracts, Milk protein (casein and lactoferrin)	Inhibition of enzymes such as glucosyltransferases involved in extracellular polymeric substance production.

1.4 Enzybiotic Approach to control biofilm

Therapeutic enzymes are those enzymes which can be used safely as an adjuvant to potentiate the effectiveness of other agent [8]. Our research work explores the influence of enzymes when combined with a Fluoroquinolone antibiotic in eradicating bacterial biofilms. We screened three enzymes and one Fluoroquinolone antibiotic in combination against *S. aureus* biofilm. Selected enzymes possess different activity which probably contributed in providing protection against bacterial infection as well as biofilm. Firstly, Serralysin or Serratiopeptidase, a proteolytic enzyme, perform proteolysis by hydrolysing the peptide bond that links amino acids together in a peptide chain [9]. Secondly Muramidase or Lysozymes, hydrolyses 1,4-beta glycosidic bond between N-acetylmuramic acid, N-acetyl-D-glucosamine residues in peptidoglycan of bacterial cell wall leading to breakdown of structural integrity of cell wall resulting in cell lysis [10]. Lastly, Catalase, a common enzyme found in nearly all living organisms that are exposed to oxygen, where it function to catalyze the decomposition of hydrogen peroxide to water and oxygen. Hydrogen peroxide is a harmful byproduct of many normal metabolic processes, to prevent damage to cells and tissues it must be converted to less dangerous substances. Thus Catalase protects the cell from the oxidative damage by reactive oxygen species [11]. These enzymes can play a vital role in degrading or disrupting the biofilm layer thereby enabling influx of Fluoroquinolone antibiotic in an optimum concentration required for bactericidal action. Fluoroquinolone antibiotics such as Ciprofloxacin functions by inhibiting two enzymes namely DNA gyrase and topoisomerase IV. Enzyme bacterial DNA gyrase nicks double-stranded DNA, introduces negative supercoils and then reseals the nicked ends. This is necessary to prevent excessive positive supercoiling of the strands during replication and transcription. The DNA gyrase consists of two A and two B subunits. The A subunit carries out nicking of DNA, B subunit introduces negative supercoils and then A subunit reseals the strands. Fluoroquinolones, like Ciprofloxacin, binds to subunit A, interfere with its strand cutting and resealing function. In Gram-positive bacteria, it acts on a similar enzyme topoisomerase IV. The ultimate result, in either cases, is digestion of DNA by exonuclease [12]. Both the mechanisms attribute to kill bacterium, thus Ciprofloxacin acts as a bactericide. Hence, combination of antimicrobial enzyme and Fluoroquinolone antibiotic is anticipated to result in synergistic effect leading to disruption of biofilm layer and bactericidal action. This combination strategy will help in overcoming biofilm resistance of antimicrobial agents when complemented with a suitable enzyme.

2. Material and methods

2.1 Bacterial isolate

Test bacterial strain *Staphylococcus aureus* (*S. aureus*, ATCC No. 6538) was purchased from American Type Culture Collection, Pune, India. The inoculum of *S. aureus* was prepared using a 24 h old culture density of which was adjusted to match with the turbidity of 0.5 McFarland standard.

2.2 Antimicrobial agents

Ciprofloxacin hydrochloride was obtained as a gift sample from Dr. Reddy's, India. Serratiopeptidase (2300 Units mg^{-1}) and Lysozyme (22,000 Units mg^{-1}) was obtained as a gift sample from Advanced Enzyme Technologies Ltd, India. Catalase (5000 Units mg^{-1}) was purchased from Sigma Aldrich, USA. Stock solution of Ciprofloxacin was prepared in phosphate buffer, pH 5.0. Stock solutions of enzymes namely, Serratiopeptidase was prepared in tris buffer pH 7.0, Lysozyme in phosphate pH 6.24 and Catalase in phosphate buffer pH 7.0.

2.3 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of Fluoroquinolone antibiotic in combination with enzymes and *per se*

Ciprofloxacin hydrochloride (CPX, 0.25-64 µg/ml), Serratiopeptidase (SRP, 50-6800 µg/ml), Lysozyme (LYS, 50-6800 µg/ml) and Catalase (CAT, 50-6800 µg/ml) varying combinations of CPX with 2500 µg/ml of SRP, LYS and CAT were screened for MIC against *S. aureus* using the 96 well microtiter plate method [13, 14]. The combination was tested by Checkerboard method and Fractional inhibitory concentration (FIC) of CPX was determined [15]. MBC of CPX in combination with SRP and LYS was determined by addition of triphenyltetrazolium dye (TTC) to microtiter plates followed by incubation for 3 h at 37°C. After incubation the color change of the dye from colorless to red was noted visually [16, 17]. The wells showing no color change were plated on the Muller Hinton agar (MHA) plates, incubated for 24h at 37°C to confirm the bacteriostatic or bactericidal effect of CPX in combination with SRP, LYS and CAT.

2.4 Disc diffusion test

Antibiotic sensitivity screening of CPX *per se* in combination with SRP was done by disc diffusion method, which was performed as per Clinical and Laboratory Standards Institute guidelines (CLSI) [18]. *S. aureus* was inoculated on nutrient agar plates and incubated for 18-24 h at 37ºC. Two to four well isolated colonies from the plates were suspended in sterile saline and the turbidity of the suspension was adjusted so as to be equivalent to 0.5 McFarland standard. The bacterial suspension was spread on the surface of MHA plates using a sterile cotton swab. CPX *per se* as

well as combination with SRP and blank phosphate buffer impregnated discs were then dispensed onto the surface of inoculated MHA plates, with slight pressure to ensure complete contact of the disc with the agar surface. The studies were performed in duplicates, with two plates tested for each CPX concentration *per se* and in combination with SRP. The plates were incubated for 18-24 h at 37ºC. After the incubation period, the diameters of the inhibited zones were measured using a ruler. Zone of inhibition of CPX alone as well as in combination with SRP and SRP alone was compared.

2.5 Confirmation of formation of biofilm

The biofilm forming bacteria was identified on the basis of Gram character, colony appearance, biochemical tests. Gram character of *S. aureus* was determined by Gram staining method. The colony appearance included colony shape and colour. Biochemical tests included catalase and coagulase test; selective media test namely Mannitol salt agar and Vogel-Johnson agar. *S. aureus* was tested *in-vitro* for their ability to form a biofilm by two methods. Screening was done on the basis of visual appearance and qualitative microtitre plate based biofilm assay.

2.5.1 Visual appearance

The bacterial isolate was enriched in sterile Luria Bertani (LB) broth containing 1% glucose to enhance biofilm formation. The media tubes were inoculated for 24 h at 37°C. The biofilm formation was observed visually as layered growth (pellicle formation) on the top of the medium.

2.5.2 Qualitative microtiter plate based assay

Qualitative microtiter plate based biofilm assay was carried out to confirm the biofilm forming isolate. *S. aureus* was grown in a sterile LB broth containing 1% glucose overnight at 37°C. To each well of microtitre plate, 20 µl of *S. aureus* suspension along with 180 µl of sterile LB broth containing 1% glucose was added. The microtiter plate was covered with its lid and incubated overnight at 37°C. After overnight incubation period, medium was removed from the wells and microtiter plate wells were washed five times with sterile distilled water to remove the loosely associated bacterial cells. Plates were air dried for 45 min and each well was stained with 200 µl of 1% aqueous crystal violet for 45 min. After staining, the plates were gently washed with sterile distilled water for five times. The qualitative analysis of biofilm production was performed by addition of 200 µl of 95% ethanol to destain the wells. At this stage biofilm formation is visible in the form of the blue ring at the sidewall or at the bottom of the well [19].

2.6 Determination of Minimum biofilm inhibitory concentration (MBIC) of CPX in combination with SRP, LYS and CAT

MBIC of CPX, SRP, LYS and CAT *per se* and in combination on preformed biofilm in static condition at the bottom of the microtitre plate. The effect of 2MIC, MIC, 1/2MIC, 1/4MIC, 1/8MIC, 1/16MIC of CPX on the preformed biofilm of *S.aureus* was tested on the microtiter plate. Also different concentrations of SRP, LYS and CAT (50, 100, 200, 300 µg/ml) were used for this study. The methodology is based on developing biofilm enriched with *S. aureus* in LB broth containing 1% glucose for 24 h, subjecting it to test compounds alone and in combination followed by staining the biofilm remnant with crystal violet [19, 20]. The crystal violet taken up by the biofilm was quantified by reading the absorbance at 600 nm using microtitre plate reader (Turner Biosystems Modulus microplate, USA).

2.7 Determination of antibiofilm activity of CPX in combination with SRP, LYS and CAT on Catheter associated biofilm

The catheter associated biofilm experiment was performed in qualitative manner to study the influence of enzymes on the effectiveness of the antibiotic [20, 21]. Sterile polyethylene catheter segments were suspended in LB broth containing 1% glucose and *S. aureus* strain and were incubated overnight at 37°C. After overnight incubation, the catheter segments were removed and rinsed three times with sterile phosphate buffer saline (PBS) pH 7.4 in order to remove the non-adherant bacteria. LB broth containing 1% glucose and *S. aureus* strain was discarded and replaced with drug solutions, incubated overnight at 37°C. After overnight incubation, the catheter segments were removed from the drug solutions with the help of sterile forceps rinsed three times with sterile PBS, pH 7.4 to remove the sessile adherent cells. The presence of viable cells on the catheter segments were detected by embedding the segments into the MHA plates. The plates were incubated overnight at 37°C and examined for the growth around the catheter segment.

3. Results & Discussion

3.1 Minimum inhibitory concentration and Minimum bactericidal concentration of Fluoroquinolone antibiotic in combination with enzymes and *per se*

MIC of CPX *per se* was found to be 4 µg/ml as shown in figure 1. SRP, LYS and CAT did not show any inhibition *per se* even at higher concentrations. Papa, R. *et al* 2013, showed that SRP did not affect the growth of *S. aureus* at concentration of 1000 Units/ml. Lee, S. *et al* 2013, reported that LYS exhibited mild antimicrobial effect on bacteria at concentrations upto 10 mg/ml. Similar observation is reported by Cisani G. *et al* 1982, suggesting that LYS *per se* has low bactericidal activity and its MIC was 15 mg/ml, whereas Chung W. *et al* 2000, proved by time kill kinetic experiment that LYS at 500 µg/ml killed bacteria at a very slow rate [22].

CPX in combination at 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 µg/ml with SRP at 2500 µg/ml showed 80-90% inhibition of *S. aureus* as shown in figure 2. Bhagat S. *et al* 2013, stated that SRP when given with antimicrobials such as cefotiam, cephalexin, sulbenicillin, increased tissue penetration of antibiotics due its proteolytic effect [23]. Penta J. *et al* 2010, investigated the effect of antibiotics combined with enzymes. They studied combinations of SRP (at concentration 10-100 µg/ml) with gatifloxacin (0.03-0.05 µg/ml), rifampicin (0.2-0.3 µg/ml), streptomycin (22-25 µg/ml) and ampicillin (5-8 µg/ml). Combination of these antibiotics with SRP exhibited synergistic effect and combination of ampicillin with SRP, most effectively inhibited the growth of *S. aureus*. Our results revealed that combination of LYS with CPX showed 70-80% inhibition of *S. aureus* as shown in figure 3. Bhagat S. *et al* 2013 also reported that, LYS at concentration 1-5 mg/ml in combination with gatifloxacin at concentration 0.01-0.03 µg/ml failed to show any bacterial inhibition and was an ineffective combination. Combination analysis of LYS and nisin by Chung W. *et al* 2000, demonstrated that LYS at 375 µg/ml with nisin at 125 µg/ml resulted in bacterial killing similar to that of nisin alone at 500 µg/ml. Results of work by Pellegrini A. *et al* 1992, showed that the LYS in combination possessed bactericidal property and in combination with aprotinin was successful in killing Gram positive and Gram negative bacteria *in vitro* [24]. Lee, S. *et al* 2013, also reported that ternary mixture of spray dried ciprofloxacin, gatifloxacin and lysozyme as well as binary mixture of spray dried ciprofloxacin and gatifloxacin failed to exhibit synergy against *S. aureus*. CPX in combination at 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 µg/ml with CAT at 2500 µg/ml failed to show and inhibitory activity on *S. aureus*.

FIC of CPX was derived by dividing the MIC of tested combination by the MIC of tested antibiotic alone against *S. aureus.* FIC of CPX at concentrations lower than 4 µg/ml when combined with SRP and LYS was found to be < 0.5 [15]. FIC for SRP, LYS and CAT individually could not be determined, as the enzymes within the experimental condition did not show >80% inhibition of *S. aureus*. TTC dye, a redox indicator which is reduced to red formazan in presence of bacteria indicating viability of the cells. The test was carried out to confirm and enhance precision of MIC determination. On addition of TTC dye to MIC microtitre plates, plates remained colourless. The results confirmed bactericidal activity of the combinations. A loopful of colourless test samples when plated on MHA plates did not show growth and confirmed the bactericidal activity of the combinations. The observation highlights the potential synergistic application of various combinations in treating *S. aureus* infections.

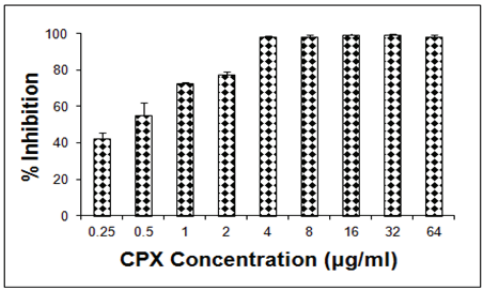

Fig. 1 Effect of different concentrations of CPX on *S. aureus* strain (n=3, ± SD)

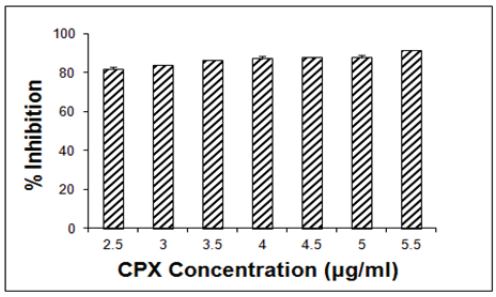

Fig. 2 Effect of different concentrations of CPX in combination with SRP at 2500 µg/ml on *S. aureus* strain (n=3, ± SD)

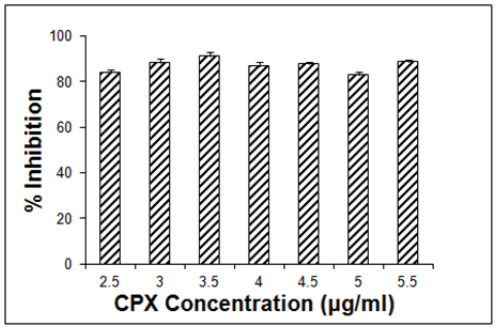

Fig. 3 Effect of different concentrations of CPX in combination with LYS at 2500 µg/ml on *S. aureus* strain (n=3, ± SD)

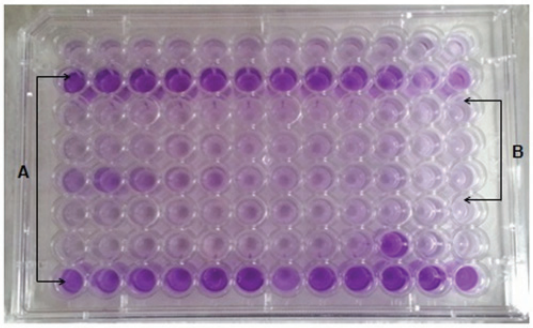

Fig. 4 Qualitative biofilm test for *S. aureus* by 96 well microtitre plate assay A indicates wells with biofilm formation and B indicates wells with no biofilm formation

3.2 Disc diffusion test

The objective of disc diffusion test is to determine susceptibility of *S. aureus* to CPX *per se*, CPX in combination with SRP and blank phosphate buffer. The absence of *S. aureus* growth around the disc is a direct measure of the ability of the test compound to inhibit that organism. Zone of inhibition of CPX at 5 µg/ml in combination with SRP at 2500 µg/ml was comparable to that observed for CPX alone at 5 µg/ml. Statistical analysis was carried using student t-test, no statistical significant difference was observed in the zone diameter of CPX alone and in combination with SRP. The zone diameter for blank phosphate buffer and SRP alone was similar to that of positive control (untreated disc). Thus CPX in combination with SRP showed similar zone of inhibition as shown by CPX alone thereby indicating equivalent effectiveness to that of CPX alone against *S. aureus*.

3.3 Biofilm test

S. aureus is a Gram positive bacterium, yellowish golden colour colonies with grape like structure which possess an ability to ferment mannitol. The blue ring formed at the bottom of the 96 well microtiter as shown in figure 4 confirmed the biofilm forming property of *S. aureus* [19].

3.4 Minimum biofilm inhibitory concentration of CPX in combination with SRP, LYS and CAT

Literature reveals that disruption of biofilm structure which is composed of DNA, proteins and exopolysaccharides, can be achieved by degrading the individual components of biofilm [25]. In biofilm eradication experiments, we investigated the combined effect of CPX *per se* and when used in combination with either of SRP, LYS and CAT against *S. aureus* associated biofilm. The earlier studies confirmed sensitivity of *S. aureus* to CPX in combination with enzymes at MIC and sub-MIC concentrations. It has been reported in literature that different biofilm associated cells resist exposure to CPX [26]. The resistance to CPX was not attributed to low diffusion of CPX but due to failure of CPX to kill bacteria in the biofilm since the bacteria either do not grow or grow very slowly [27]. CPX at sub-MIC concentrations (1/16, 1/8, 1/4 and 1/2) in combination with SRP at 50 µg/ml showed 85-90% as shown in figure 5, eradication of preformed biofilm whereas antibiofilm activity decreased at higher concentration of SRP. Also the antibiofilm activity of SRP was proportional to biofilm forming capacity of *S. aureus* [28]. The results suggest synergism at low concentration of SRP with CPX at sub-MIC concentrations. Papa R. *et al* 2013, screened SRP alone at 200 to 800 Units/ml and demonstrated that SRP was capable of removal of *S. aureus* biofilm at lower concentration of 0.39 Units/ml, disaggregation of biofilm at concentration of 200 Units/ml but was less effective at higher concentration of 800 Units/ml. Exposure of CPX (at 1 µg/ml) and SRP (at 50 and 100 µg/ml) in combination on *S. aureus* biofilm resulted in significant inhibition of biofilm when compared with CPX alone at 1 µg/ml. This significant inhibition was dependent on the concentration of both CPX as well as SRP. These results were similar to those described by Selan *et al* 1993, who reported that SRP greatly enhanced efficacy of ofloxacin against planktonic biofilm. It has been shown by an *in-vitro* studies that proteolytic enzymes are capable to inhibit or suppress the basic activities of bacteria [3]. Based on the evidence from literature as well as our results, the possible mechanism for CPX-SRP combination might be weakening of bacterial attachment by removal and dispersal action of SRP thereby increasing susceptibility to CPX [26, 28].

Lysozyme also known as muramidase possess natural antibiotic property [29]. It hydrolyzes the bond between N-acetyl glucosamine and N-acetyl muramic acid leading to degradation of peptidoglycan in bacterial cell wall resulting in rupturing of bacterial cell wall and rapid killing of *S. aureus* [29, 30]. LYS at high concentration possessess bactericidal property by hydrolysing beta 1-4 linkages of the mucopolysaccharide of the bacterial cell wall [31, 32]. Samaranayake Y.H. *et al* 2009, reported that antifungal agents such as nystatin, amphotericin B and ketoconazole at 6MIC, 12MIC, 18MIC and 24MIC in association with LYS at 100 µg/ml exhibited synergistic killing of *Candida* with increasing

concentration of antifungal agents in comparison to drug-free controls. Similarly, study on imidazole lanoconazole in combination with LYS showed synergistic antifungal activity. They also noted that LYS at 100 µg/ml showed 45% antifungal activity against *Candida* biofilm whereas at increasing concentration upto 240 µg/ml was found to be ineffective to completely eradicate the biofilm. Our results revealed that CPX at sub-MICs (1/4 and 1/2) in addition to LYS at 300-400 µg/ml showed more than 90% eradication of preformed biofilm as shown in figure 6. Reported literature as well as our results suggested that LYS supports lysis of bacteria and thereby potentiates antimicrobial activity of CPX. CPX at sub-MICs (1/4 and 1/2) with CAT at 50-100 µg/ml showed 80 to 90% eradication of preformed biofilm as shown in figure 7. Based on literature available for enzymes in combination with antibiotics, the possible synergistic mechanism of all the combinations can be concluded as double attack phenomenona; wherein the enzyme loosens and disrupts the exopolymeric sheath, weakens bacteria increasing permeability and thereby bacterial susceptibility to antibiotic. The rationale of combination treatment was to eliminate biofilm by synergistic effect of enzyme and antibiotic at lower concentration. Eradicating biofilm can help in regaining the sensitivity of bacterial biofilm to the antibiotic.

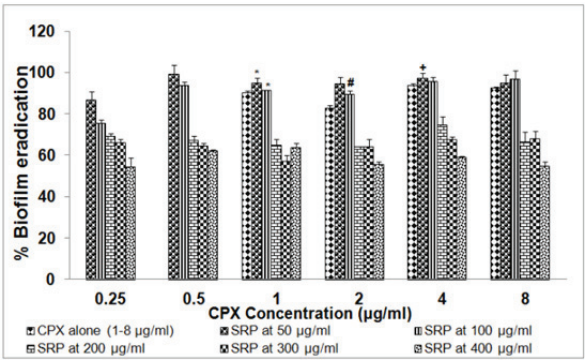

Fig. 5 Effect of different concentrations of CPX and SRP in combination against *S. aureus* biofilm (n=3, ± SD)

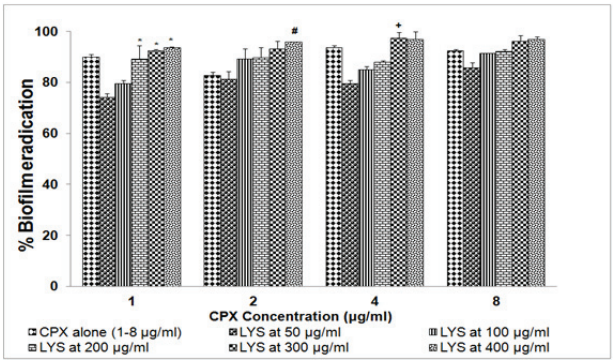

Fig. 6 Effect of different concentrations of CPX and LYS in combination against *S. aureus* biofilm (n=3, ± SD)

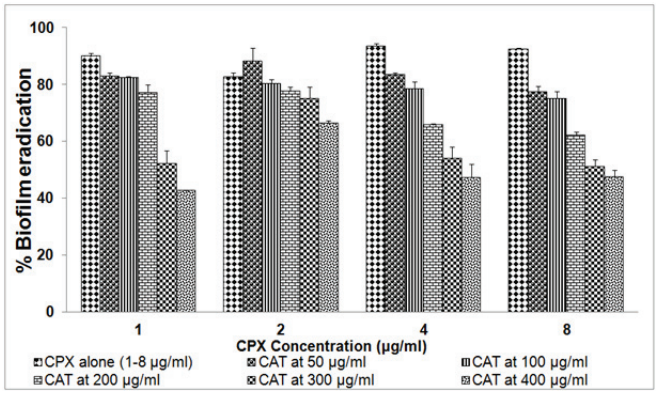

Fig. 7 Effect of diffeent concentrations of CPX and CAT in combination against *S. aureus* biofilm (n=3, ± SD)

Data is expressed as mean ± standard deviation (n = 3): Statistical analysis was carried using one way ANOVA followed by Newman-Keul's Multiple comparison post hoc test, statistically significant difference was observed compared to treatment with CPX alone, $^{*}P < 0.05$, $^{\#}P < 0.05$ and $^{+}P < 0.05$ was considered as statistically significant. Statistically significant difference was observed in eradication of preformed biofilm after treatment with

- CPX at 1 µg/ml in combination with SRP at 50, 100 µg/ml and LYS at 200, 300, 400 µg/ml as compared to treatment with CPX alone at 1 µg/ml.
- CPX at 2 µg/ml in combination with SRP at 100 µg/ml and LYS at 400 µg/ml as compared to treatment with CPX alone at 2 µg/ml.
- CPX at 4 µg/ml in combination with SRP at 50 µg/ml and LYS at 300 µg/ml as compared to treatment with CPX alone at 4 µg/ml.
- No significant difference was observed in eradication of preformed biofilm after treatment with CPX at 8 µg/ml in combination with SRP, LYS and CAT at 50, 100, 200, 300 and 400 µg/ml as compared to treatment with CPX alone 8 µg/ml.

3.5 Antibiofilm activity of CPX in combination with SRP, LYS and CAT on Catheter associated biofilm

The treatment of catheter associated infections caused by biofilm producing bacteria be can considered as one of the serious problems for overcoming biofilm resistance. In this model *S. aureus* biofilms were formed on the surface as well as in the lumen of the catheter. It was performed in qualitative manner to study the influence of SRP, LYS and CAT on the effectiveness of CPX against bacterial biofilm. Preformed biofilm on the catheter segment were exposed to CPX and enzyme solutions in combination. Our study revealed that the combination of CPX at sub-MIC concentration with SRP and LYS was capable to reduce the adherance of *S. aureus* both present in the lumen and on the external surface catheter. The absence of *S. aureus* colonies in the vicinity of catheter indicated that the combination possessed antibiofilm property. Observations were graded as growth, thin layer growth and no growth surrounding the catheter as mentioned in table no 2 & 3. No viable cell count was recorded. The CPX at sub-MIC concentrations with SRP at concentration 50 and 100 µg/ml cleared *S. aureus* present on the catheter. It has been shown that SRP complemented with ofloxacin improved activity of ofloxacin in prosthetic device related infection due to its ability enhance antibiotic penetration a the infected site [3]. Combination of CPX at sub-MIC concentrations with LYS at concentrations 200 and 400 µg/ml showed promising result in eliminating *S. aureus* biofilm. LYS possesses antimicrobial activity due its ability to breakdown carbohydrate chain in the bacterial cell wall [31]. An *in-vitro* study showed that, LYS which is a muralytic enzyme was capable of detaching and also disintegrating the *S. aureus* cell wall. This detaching activity of LYS plays a vital role in eradicating *S. aureus* biofilm bound on the catheter. Catheter biofilm associated experiment for CPX in combination with CAT was not conducted due to limited solubility CAT for preparing its sub stock solutions. Thus Catheter associated biofilm study revealed that CPX in combination with SRP and LYS reduced bacterial adherence to biotic surface.

Table 2 Observations for Catheter associated biofilm experiment for CPX-SRP combination.

Combinations (concentration in µg/ml)	Observation for No growth	Conclusion
CPX at 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2MIC, MIC, 2MIC + SRP at 50	✓	Effective
CPX at 1/16 MIC + SRP at 200	✓	Effective
CPX at 1/16 MIC + SRP at 200	✓	Effective

Table 3 Observations for Catheter associated biofilm experiment for CPX-LYS combination.

Combinations (concentration in µg/ml)	Observation for No growth	Conclusion
CPX at 1/2 MIC + LYS at 200, 300, 400	✓	Effective
CPX at MIC + LYS at 200, 300 and 400	✓	Effective
CPX at 2MIC + LYS at 50, 100, 200, 300 and 400	✓	Effective

4. Conclusion

Enzymes have a greater prospective to treat biofilm resistant disease when combined with drugs having the prospective to induce synergistic effects [28, 33]. In this chapter, we have demonstrated that Serratiopeptidase and Lysozyme both would be helpful in treating and eliminating established *S. aureus* biofilms when combined with Ciprofloxacin. The combination could be utilised in protecting prosthetic devices from *S. aureus* infections. Our studies have shown that SRP forms a good team with CPX in order to deliver required concentration of antibiotic to the infecting bacteria. The experimental results demonstrate that Fluoroquinolone antibiotic in combination with enzymes is an effective strategy in eradicating preformed biofilms.

Acknowledgements The support by Indian Council of Medical Research (ICMR), India is gratefully acknowledged.

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