Indian J Med Res 146 (Supplement), July 2017, pp 1-8
DOI: 10.4103/ijmr.IJMR_410_15

Quick Response Code:

Staphylococcus aureus biofilm removal by targeting biofilm-associated extracellular proteins

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Received March 16, 2015

Background & objectives*: Among cell surface proteins, biofilm-associated protein (Bap) promotes biofilm development in *Staphylococcus aureus* strains. The aim of this study was to investigate proteinase-mediated biofilm dispersion in different isolates of *S. aureus.

***Methods:* Biofilm assay was done in 96-well microtitre plate to evaluate the effect of proteinase K on biofilms of bovine mastitis *S. Aureus* isolates. Extracellular polymeric substances were extracted and evaluated for their composition (protein, polysaccharides and extracellular DNA), before and after the proteinase K treatment.**

***Results:* Biofilm assay showed that 2 µg/ml proteinase K significantly inhibited biofilm development in *bap*-positive *S. aureus* V329 as well as other *S. aureus* isolates (SA7, SA10, SA33, SA352), but not in *bap*-mutant M556 and SA392 (a weak biofilm-producing strain). Proteinase K treatment on *S. aureus* planktonic cells showed that there was no inhibition of planktonic growth up to 32 µg/ml of proteinase K. Proteinase K treatment on 24 h old preformed biofilms showed an enhanced dispersion of *bap*-positive V329 and SA7, SA10, SA33 and SA352 biofilms; however, proteinase K did not affect the *bap*-mutant *S. aureus* M556 and SA392 biofilms. Biofilm compositions study before and after proteinase K treatment indicated that Bap might also be involved in eDNA retention in the biofilm matrix that aids in biofilm stability. When proteinase K was used in combination with antibiotics, a synergistic effect in antibiotic efficacy was observed against all biofilm-forming *S. aureus* isolates.**

***Interpretation & conclusions*: Proteinase K inhibited biofilms growth in *S. aureus* bovine mastitis isolates but did not affect their planktonic growth. An enhanced dispersion of preformed *S. aureus* biofilms was observed on proteinase K treatment. Proteinase K treatment with antibiotics showed a synergistic effect against *S. aureus* biofilms. The study suggests that dispersing *S. aureus* by protease can be of use while devising strategies against *S. aureus* biofilms.**

Key words Antibiotic treatment - bacterial surface proteins - biofilm-associated protein - biofilm dispersal - extracellular DNA - microbial biofilms - proteinase K - *Staphylococcus aureus*

Most bacteria in nature execute definite development stages in biofilm formation; (*i*) adherence of cells to a substratum, (*ii*) development of microcolonies, (*iii*) maturation of microcolonies into biofilms, and (*iv*) detachment of bacteria and acquisition of motile phase, known as biofilm dispersal[1]. Biofilm disassembly/

dispersion is believed to play an important role in pathogenicity, in environmental distribution and also in phase transition[2]. The dispersal phenomenon can also be triggered by several environmental signals or unfavourable condition[3]. Biofilms aid many advantages to microorganisms such as higher resistance to adverse environmental conditions, higher resistance to antimicrobial agents and enhanced protection from immune response in case of persistent infections[4].

Staphylococcus aureus is a universal pathogen which causes mild to severely life-threatening diseases. This bacterium also constitutes a major cause of hospital-acquired/healthcare-associated infections (HAIs). According to Center for Disease Control and Prevention, *S. aureus* strains are associated with 15.6 per cent of the total HAIs reported between 2009 and 2010[5] and 12.3 per cent between 2011 and 2012 in Europe[6]. Commonly, a mature biofilm consists of polysaccharides, proteins, extracellular DNA (eDNA)[7] and amyloid fibres as matrix. There are several reports on the role of surface proteins in *S. aureus* biofilm formation and its stability[8,9]. Among various surface proteins, biofilm-associated protein (Bap) was first reported as a large, multi-domain, cell surface-anchored protein, which plays a crucial role in *S. aureus* biofilm development, architecture and in the pathogenesis of bovine mastitis[10-13]. A study carried out in Brazil showed the presence of *bap* gene in all the coagulase-negative *Staphylococcus* spp. strains isolated from the nosocomial infections[14]. Another report showed a higher frequency of occurrence of *bap* gene (56.6%) in *Staphylococcus* spp. (189 samples) isolated from bovine subclinical mastitis. Apart from this, frequency of *bap* gene occurrence was significantly higher in coagulase-negative strains as compared with coagulase-positive[15]. The involvement of polysaccharide intercellular adhesin (*ica*-dependent) component of the *S. aureus* biofilm matrix has been studied comprehensively[16]. However, role of *ica*-independent mechanisms, which is predominantly mediated by biofilm-associated surface proteins [Bap, accumulation-associated protein (Aap), fibronectin-binding protein, *etc*.] in the stability of Staphylococci biofilm matrix, is poorly understood[9,17,18]. Previous reports on *ica*-independent biofilm formation in staphylococci showed a strong link between biofilm formation and cell wall-associated proteins, in particular, Bap[11], Aap[18] and Bap-homologue protein[19]. Repeated domains contain an amyloidogenic peptide motif (-STVTVTF- derived from the C-repeat of the Bap), which is responsible for cell-cell interaction[20]. Therefore, Bap and Bap-like surface proteins could be an important target for biofilm dispersal studies.

Dispersal mechanisms vary in different bacteria and this event is considered as a novel approach to treat drug-resistant *S. aureus* which is common in body implants and catheter-related infections[21]. Among the natural ways of *S. aureus* biofilm dispersal, Agr-mediated biofilm dispersal and secretion of major extracellular proteases, SspA, SspB, Aur and Scp as pro-enzymes, were reported[22]. Theoretically, these enzymes may contribute to biofilm detachment, but very little is known about their role with regard to Staphylococci. An extracellular serine protease, Esp secreted by a subset of *S. epidermidis*, has shown to inhibit biofilm formation and nasal colonization by *S. aureus*[23]. Esp has proteolytic activity specifically towards biofilm-specific proteins that are associated with *S. aureus* biofilm formation and host-pathogen interaction[24]. Studies on Staphylococci chronic infections and biofilms as well as discovery of major dispersal mechanisms shifted the focus on the development of dispersal-mediated treatment options for *S. aureus* biofilm infections[25]. In an earlier report, we have shown that proteinase K can emulate the naturally produced proteases and can be used to enhance the biofilm dispersal through cleavage of surface proteins *i.e.* Bap-dependent *S. aureus* biofilm establishment[17]. In this study, we investigated whether this approach would be useful in general and has wider applicability using five other *S. aureus* mastitis isolates.

Material & Methods

Microorganisms and culture conditions: A *bap*-positive *S. aureus* V329 and its isogenic-mutant *S. aureus* M556 were used along with five other mastitis isolates of *S. aureus viz*., SA7, SA10 SA33, SA252 and SA392. Bovine mastitis *S. aureus* strains SA7, SA10 and SA33 were procured from Karnataka Veterinary College, Bengaluru, whereas SA352 and SA392 were procured from Madras Veterinary College, Chennai. All bovine mastitis *S. aureus* strains used in the study were isolated from infected site of bovine mastitis. M556 was generated by transposon insertion in the downstream part of *bap* gene of *S. aureus* V329 in such a way that Bap is synthesized but remains non-functional as cell wall anchoring region is truncated[11]. For each experiment, single colonies were picked from Tryptic Soy Agar (TSA) culture plates and inoculated in Tryptic Soy Broth (TSB) medium supplemented with 0.25 per cent

glucose (TSB-G) and incubated at 37°C at 150 rpm. Overnight grown cultures were used for all experiments after checking for culture purity. All experiments were performed at Biofouling and Thermal Ecology Section, Water and Steam Chemistry Division, BARC Facilities, Kalpakkam, from January 2013 to December 2014.

Quantitative biofilm assay: Biofilm assay was performed in 96-well microtitre plates to estimate the inhibitory/dispersion action of proteinase K. The working concentration of proteinase K was chosen as 2 µg/ml in all the experiments. The overnight grown cultures of the *S. aureus* cells in TSB-G were diluted 1:40 in sterile TSB-G medium and added to the pre-sterilized 96-well flat bottom polystyrene microtitre plates. To estimate the inhibitory action, *S. aureus* biofilms were grown in the presence of 2 µg/ml of proteinase K. To study dispersion, biofilms were grown on microtitre plates and washed after prescribed time and 200 µl of fresh TSB-G amended with 2 µg/ml of proteinase K was added to the wells and the plates were incubated at 37°C for 24 h. To study the effect of Ca^{2+} on proteolytic cleavage of Bap in terms of biofilm formation, V329 biofilms were grown at 37°C for 24 h in the presence of 2 µg/ml of proteinase K along with increasing concentration of Ca^{2+} in the range of 1.56-50 mM. V329 biofilm grown in the presence of only Ca^{2+} in the similar concentration range acted as control for Ca^{2+}. After 24 h of incubation, biofilm growth was quantified. Biofilm quantification was done by classical crystal violet assay as described previously[2]. To dissolve the bound crystal violet, 33 per cent acetic acid was used[26]. Biofilm growth was monitored in terms of absorbance at 570 nm using a multimode microplate reader (BioTek, USA).

Planktonic growth studies: Overnight grown bacterial cultures were harvested and washed twice with phosphate buffered saline (PBS) and OD_{600} of each culture was set to 0.1. One hundred microlitres of each re-suspended culture was inoculated in 1900 µl of TSB-G and different concentrations of proteinase K. Cultures were incubated at 37°C and 150 rpm. Absorbance of each culture was recorded at different time intervals after vortexing for five seconds, to re-suspend the settled cells.

Extracellular polymeric substance (EPS) extraction and quantification of biofilm matrix components: *S. aureus* biofilm was grown for 48 h on glass slides immersed in 20 ml TSB-G. After 48 h, planktonic cells were aspirated and biofilm was gently washed twice with PBS. *S. aureus* biofilms were treated with 2 µg/ml proteinase K in TBS-G for 4 h at 37°C. After four hours, biofilm was gently washed with PBS and then remaining biofilm was scrapped and collected in 5 ml of PBS. Biofilm was disintegrated by gentle vortexing using glass beads. Five millilitres of the biofilm sample was centrifuged at 8000 rpm and 4°C for 30 min. Supernatant was collected and mixed with double volume of 90 per cent chilled ethanol and kept at 4°C for overnight. EPS was collected by centrifugation at 10,000 rpm and 4°C for 10 min. The supernatant was discarded and pellet was collected and dried at 60°C to remove ethanol. Pellet was re-suspended in 100 µl PBS buffer. The protein and eDNA content in the re-suspension was quantified using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA); the quantization protocol was followed as published by the vendor[27,28]. Glucose concentration as a measure of polysaccharide content was quantified by the method as described elsewhere[29].

Anti-biofilm activity of antibiotic-proteinase K treatment: To investigate the effect of proteinase K on the efficacy of antibiotics, proteinase K treatment was given a combination of gentamicin against biofilm forming *S. aureus* isolates *viz.,* SA7, SA10, SA33 and SA352. The antibiotic concentrations were chosen as 10 and 50 times of the minimum inhibitory concentration (indicated as X and 5X, respectively) against *S. aureus* planktonic cells. Proteinase K treatment was given in combination with X concentration of antibiotics. *S. aureus* biofilms were grown in microtitre plates at 37°C and 150 rpm. After 24 h, planktonic cells were aspirated by pipette and the biofilms were gently rinsed twice with sterile PBS. After rinsing, the biofilms were treated with antibiotics alone and antibiotic-proteinase K combinations. After 24 h, planktonic cells were aspirated and biofilms were gently rinsed twice with PBS. Two hundred microlitres of PBS was added to each well, and the biofilm cells were dislodged by ultra-sonication for five minutes. Cells released from the biofilms were harvested, and the viable cell count was obtained by plating on TSA media and incubated at 37°C overnight.

Statistical analysis: Two-tailed Student's *t* test was used to determine the differences in biofilm formation between the groups.

Results

Effect of proteinase K on biofilm development and planktonic growth of S. aureus: Proteinase K treatment

hampered the biofilm development of most *S. aureus* isolates *viz.,* SA7, SA10, SA33, SA352 and *bap*-positive V329. All *S. aureus* isolates, except SA392 (weak biofilm-producing strain), showed significant inhibition in biofilm growth when treated with 2 µg/ml proteinase K (Fig. 1). SA7, SA10, SA33, SA352 biofilms showed 84, 71, 83 and 68 per cent reduction in biofilm growth in the presence of 2 µg/ml proteinase K. On the contrary, strains M556 and SA392 were found to be weak biofilm producers and there was no significant inhibition of biofilm formation in the presence of proteinase K. Planktonic growth studies of *bap*-positive V329 and *bap*-mutant M556 and other *S. aureus* strains were carried out in the presence of different concentration of proteinase K. There was no effect of proteinase K on the planktonic growth of *S. aureus* isolates when tested up to 32 µg/ml (Fig. 2).

Effect of different concentrations of Ca^{2+} on proteolytic degradation of biofilm-associated protein (Bap) and biofilm development of V329: Biofilm assay in the presence of proteinase K with increasing concentrations of Ca^{2+} was carried out. Addition of increasing concentrations of Ca^{2+} had no effect on proteinase K-mediated inhibition of biofilm development (Fig. 3). In other words, Ca^{2+} did not affect the proteolytic degradation of surface protein Bap by proteinase K. On the other hand, lower concentration of Ca^{2+} (up to 6.25 mM) had no significant effect on V329 biofilm formation, but higher concentrations showed an inhibitory effect.

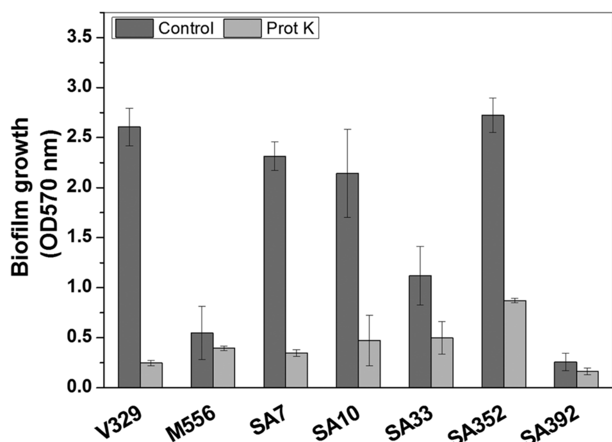

Fig. 1. Effect of proteinase K on the growth of *Staphylococcus aureus* biofilms. Values are mean±standard deviation (n=3).

Proteinase K enhances dispersal in S. aureus biofilms: To investigate the biofilm dispersal activity of proteinase K against *S. aureus* biofilms, proteinase K treatment was given to 24 h old *S. aureus* biofilms. Proteinase K treatment of *S. aureus* biofilms caused a significant disruption of all *S. aureus* biofilms except M556 and SA392 (Fig. 4). Upon 2 µg/ml proteinase K treatment for 24 h, approximately 92.5, 23.4, 90.1, 95.8, 81.9 and 60 per cent biofilm dispersal was observed in V329, M556, SA7, SA10, SA33 and SA352, respectively. Although proteinase K enhanced the biofilm dispersal, 100 per cent biofilm removal could not be achieved in any case. Moreover, SA392 formed a weak biofilm as compared to V329 after 24 h; hence, estimating SA392 biofilm dispersal was difficult and inappropriate, and therefore, SA392 was not involved in further experiments.

S. aureus V329 biofilm had significantly higher amount of carbohydrate as well as eDNA ($P<0.05$, n=3) as compared to Bap-mutant M556 biofilm. On the other hand, M556 biofilm was comprised significantly higher amount of biofilm matrix proteins ($P<0.05$, n=3), (Fig. 5). There was significant decrease in the protein as well as eDNA content in V329 and M556 biofilms after proteinase K treatment; however, there was no significant decrease in the carbohydrate content of biofilm matrix in either case.

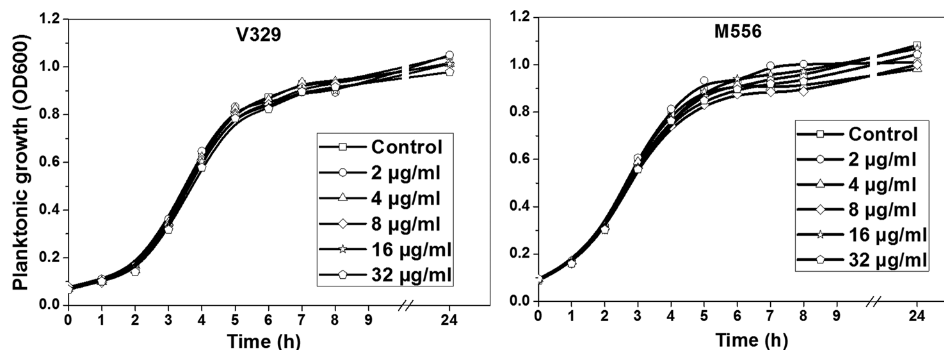

Fig. 2. Effect of different concentrations of proteinase K on planktonic growth of *Staphylococcus aureus* V329 and M556.

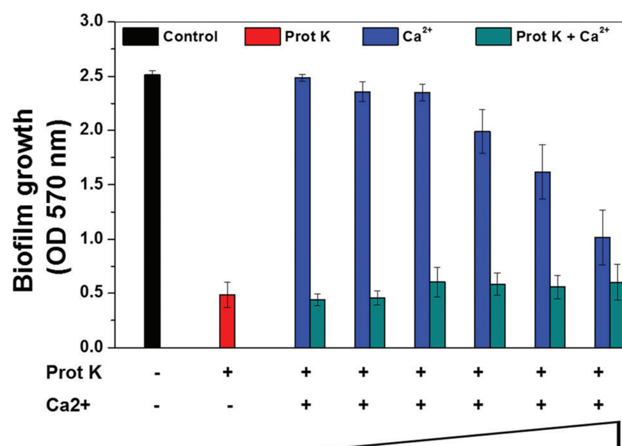

Fig. 3. Effect of Ca^{2+} on proteinase K-mediated biofilm inhibition in *bap*-positive *Staphylococcus aureus* biofilm. Results are shown as mean±standard deviation (n=5).

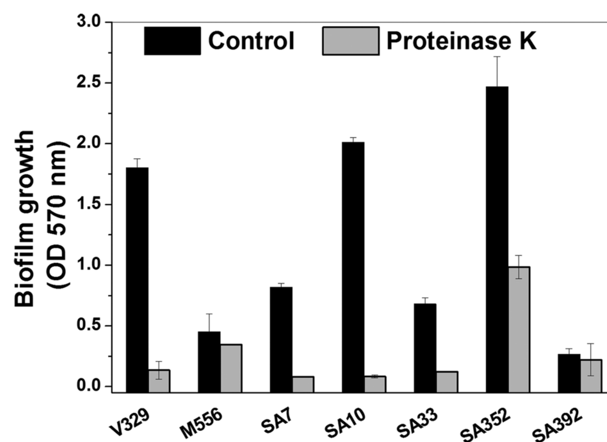

Fig. 4. Dispersal of pre-grown 24 h-old *Staphylococcus aureus* biofilms by proteinase K. Results are shown as mean±standard deviation (n=5).

Proteinase K enhances antibiotic efficacy against S. aureus biofilms: Fig. 6 shows the reduction in colony forming unit (cfu) count in *S. aureus* biofilms (SA7, SA10, SA33 and SA352) upon the treatment of gentamicin in various combinations. It was observed that addition of proteinase K in combination with gentamicin had more impact against *S. aureus* biofilm cells when compared to gentamicin alone. When the antibiotic concentration was increased by five times, there was no significant increase in log reduction of cfu count in any case. On the other hand, the addition of 2 µg/ml proteinase K in combination with antibiotics resulted in significant log reductions in each case. Proteinase K treatment caused an increase in the reduction in cfu in biofilms by 3.85, 5.0, 3.03 and 3.76 logs unit in the case of SA7, SA10, SA33 and SA352, respectively. Only 0.45, 1.27, 0.88 and 0.79 log units enhancement was noticed when gentamicin concentration was increased five times.

Discussion

In clinical settings, *S. aureus* biofilms impose resistance to host immune/defence mechanisms and antimicrobial therapy, thus enabling the bacterium to persist[30]. It was anticipated that proteolytic cleavage of these biofilms would hamper the initial adhesion process and in turn progression of biofilm. Proteinase K treatment significantly impacted the biofilm development of most *S. aureus* isolates. To investigate if the biofilm inhibition was due to the hampered growth or due to the switch-over of lifestyle of the cells to planktonic form, the effect of different concentrations of proteinase K on planktonic cells was tested. There was no impact of proteinase K on cell viability; hence, biofilm inhibition was due to proteolytic cleavage of surface proteins. These observations also reemphasized the important role played by Bap-like surface proteins in biofilm development as similar effect was not observed in *bap*-mutant M556.

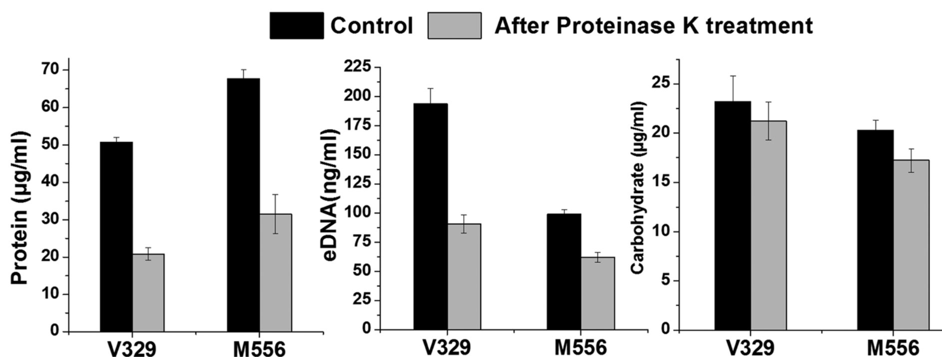

Fig. 5. Constituents of *Staphylococcus aureus* extracellular polymeric substance before and after treatment of proteinase K. Results are shown as mean ± standard deviation (n=3).

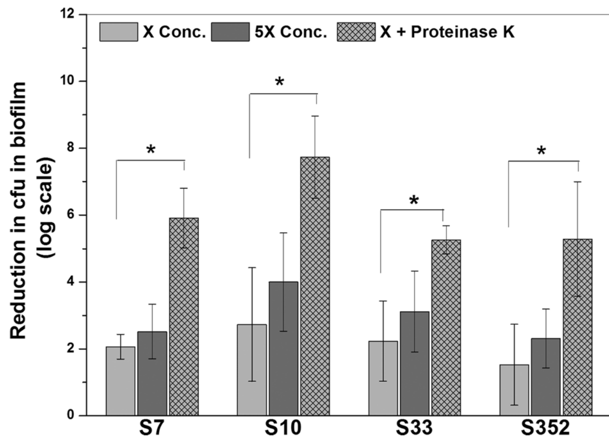

Fig. 6. Antimicrobial efficacy of antibiotics in combination with proteinase K against 24 h old *Staphylococcus aureus* biofilms. Proteinase K was used at 2 µg/ml. Two concentrations of gentamicin were used; X=150 µg/ml and 5X=750 µg/ml. $^{*}P<0.05$.

Bap contains four putative Ca^{2+} binding EF-hand motifs, thus, it was evaluated whether binding of Ca^{2+} to Bap would confer any resistance against proteinase K-mediated degradation and in turn biofilm dispersion. The result showed that there was no apparent difference between biofilms grown in the presence of proteinase K alone and proteinase K along with increasing concentrations of Ca^{2+}, whereas another set of increasing concentrations of Ca^{2+} alone showed inhibition of V329 biofilm at higher concentrations. This result demonstrated that binding of Ca^{2+} ions to Bap had no effect on proteinase K-mediated inhibition of V329 biofilm. In other words, biofilm assay using Ca^{2+} alone and Ca^{2+} with proteinase K showed that Ca^{2+} did not confer any immunity against proteolytic degradation of Bap.

Proteinase K treatment caused a significant dispersal of all pre-grown (24 h) *S. aureus* biofilms, except M556 and SA392. Since, in the case of M556, Bap does not remain attached to the cell wall, it remains non-functional and does not contribute to biofilm stability. M556 harbours functional *ica*-operon and hence could produce significant amount of biofilm after 48 h as shown in earlier report[11,17]. Therefore, it can also be speculated that in weak biofilms by M556 and SA392, polysaccharide intercellular adhesin (PIA) might play a predominant role as their biofilms were not affected by proteinase K treatment.

After proteinase K treatment, a significant decrease in the protein and eDNA but not in the carbohydrate content in EPS was observed. eDNA is also known to play very important role in *S. aureus* biofilm stability[31]. As there was a significant decrease in eDNA content along with the biofilm matrix protein content, it was speculated that matrix proteins might also be involved in eDNA retention in the biofilm. Since Ca^{2+} binds with Bap[12] as well as eDNA[32], it is speculated that Ca^{2+} might act as a cross-linking agent between Bap and eDNA, thereby the presence of Bap can assist in retention of eDNA. Therefore, upon proteinase K treatment that degraded Bap, a significant amount of eDNA was also lost along with the proteins. M556 biofilm comprised significant amount of eDNA and carbohydrate, which suggested that in the absence of functional Bap, eDNA and carbohydrate *i.e.* polysaccharide polymers in matrix might play a crucial role in *S. aureus* biofilms. In M556 biofilm, matrix proteins do not contribute to biofilm stability despite having higher amount of protein content. The results obtained also indicate that matrix proteins were neither protected by sugars and DNA nor resistant to proteinase K and hence degraded by proteinase K. It is speculated that carbohydrate polymers retain the biofilm structure and do not allow the M556 biofilm to get dispersed upon proteinase K treatment.

It is known that biofilm cells are extremely (1000 times or more) resistant to antibiotics as compared to planktonic cells due to physical as well as genetic reasons[33,34]. The proteinase K-mediated dispersal of *S. aureus* biofilms suggested its potential use in enhancing the susceptibility of bacterial cells towards antibiotic treatment. Our earlier report showed that proteinase K treatment in combination with different antibiotics had a synergistic effect on efficacy of antibiotics[17]. On similar lines, gentamicin efficacy in combination with proteinase K was investigated against four other biofilm-forming *S. aureus* isolates in this study. The result showed that proteinase K treatment significantly enhanced the efficacy of gentamicin against all *S. aureus* biofilm tested *i.e.* SA7, SA10 SA33 and SA352. As shown in our previous report[17], proteinase K treatment increases the surface to volume ratio and roughness coefficient of biofilm. Thus, the enhanced values of surface to bio-volume ratio and roughness coefficient after proteinase K treatments leave more biofilm surface available for antibiotics action. Moreover, it was also shown that proteinase K treatment significantly decreased the average diffusion distance as well as maximum diffusion distance[17]. This enhances the antibiotics penetration in biofilms and in turn its efficacy. Thus, proteinase K shows synergistic effect when associated with antibiotics for biofilm removal.

Enzyme-based *S. aureus* biofilm disruption has emerged as a promising strategy to combat biofilm-related persistent infections as enzyme-based antibiotic treatment enhances the antibiotic sensitivity of microbial biofilm[25,35]. Among such enzymes, DNase I, dispersin B and proteinase K are commercially produced. Dispersal mechanisms using such enzymes could be utilized in the prevention of biofilm formation associated with implanted medical devices[25]. Several studies have found that pre-treatment of polymeric surfaces[36] or local delivery of dispersal agents from the implanted device[7] should prevent biofilm development. Studies done with recombinant human DNase I to reduce the antigenicity of DNase I enzyme have shown promising results in terms of anti-biofilm activity against *S. aureus* biofilm[37]. While these approaches sound promising, there are several concerns that need to be thoroughly addressed before clinical trials. For example, if the antibiotic treatment along with the proteinase K fails to fully eradicate the dispersed microbial cells, it might result in acute infections. Thus, more studies need to be performed to confirm dispersal mechanisms in relevant animal models of infection before treating *S. aureus* biofilm infections in clinical set-ups.

The present study showed a wider applicability of proteinase K treatment against *S. aureus* biofilms, and that antibiotics in combination with proteinase K could be more effective in controlling *S. aureus* biofilm-mediated infection.

***Conflicts of Interest*:** None.

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