Eradication of Biofilm Produced by *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Wound Infection by Using Proteinase K Enzyme

Estabraq A. Mahdi[1], Sura S. Hasan[2]

[1]Assist. Lect., [2]Lecturer, Department of Dentistry, Al Hadi University College, Baghdad, Iraq

Abstract

This research is aimed to eradicate the biofilm formed by bacteria causing wound infection through using proteinase K enzyme. For this purpose six different concentrations of proteinase K were used for the degradation of biofilm produced by Staphylococcus aureus and Pseudomonas areuginosa. These two species are the most common abundant bacteria causing infection by biofilm. Each of the concentrations was kept in contact with the pathogenic bacteria for 1, 2 and three hours. After 3 hours of incubation period the concentration (4 µg /ml) resulted in the highest eradication ability against S. aureus biofilm, while the same concentration was no significant in the eradication of Pseudomonas aeruginosa biofilm.

Keywords: *Biofilms, Eradication, Proteinase K, Wound Infection, Pseudomonas aeruginosa, Staphylococcus aureus.*

Introduction

There are different causative-agents of chronic wound infection caused by bacteria, biofilm one of the most agent that is difficult to eradicate by treatment with antibiotics. Regardless of living-style of bacteria as free-living (planktonic) or as biofilms for causing infection to wounds, presence of biofilms in wound cause chronic infection due to delaying in healing of wounds, biofilms are more than 1000 times difficult to treat by antibiotics than free-living bacterial cells, so biofilm need to new and special ways in order to eradicate biofilm [(1)(2)]. The difficulty of eradication by using antibiotics due to resistance, for example, Staphylococcus aureus are microorganisms which acquire resistance rapidly, on the other hand Streptococcus pyogenes are less resistance. Prevalence of bacteria that resist antibiotics is a big trouble in antibiotics-therapy [(3)]. Degradation of biofilms by enzymes is consider effectively way for eradication, by degradation of biofilm contents, which consider potential way to degrade biofilm, for example, the degradation of extracellular polymeric substances (EPS) one of biofilm contents. The effects of antibiotics on cells which embedded in the biofilm enhanced by protease-treatment, lead to disruption of biofilm-matrix, due to releasing of components and planktonic-cells which then cleared by the immune system [(4)]. Proteases are a large-class of enzymatic-molecules that catalyze the cleavage of peptide bonds, all living organisms have these enzymes, proteases enzymes have important physiological functions ranging from generalized-protein-degradation to very specific-regulatory-activity. In nature, proteases divided to intracellular and extracellular. Although, substrate-recognition of extracellular enzymes is little and cleave in equal efficiency both of self- and non-

self-molecules, extracellular enzymes are essential enzymes and consider as zymogens, or in their inactive form, to prevent premature of proteolytic activity which hurt the producer cell itself [5].

According to researches findings, it is believed that biofilm matrix consists of polysaccharides, in addition to surface and secreted proteins and extracellular-DNA (eDNA) are also important factors in the formation, the stability and the regulation of biofilm. Interestingly, according to recent researches, proteases role becomes very clear, that the application of different proteases on the bacterial-cultures result in reduction of formation of biofilm and in the dispersal of established biofilms [6].

The aim of the research was eradication and degradation of biofilm produced by wound infection bacteria using different concentrations of proteinase K enzyme.

Materials and Methods

Specimen's Collection

Swabs samples were taken from 90 surgical wounds of patients attending Medical City Hospital in Baghdad for the period from July to September 2018. The samples were taken by the attending physician of the hospital through using sterile applicator stick with cotton swabs moistened in test tubes were used to collect them.

Isolation and Identification of Staphylococcus aureus and Pseudomonas areuginosa

All collected-swabs were transported to the laboratory in an appropriate medium. Bacteria were isolated by using the routine laboratory techniques. Each specimen was streaking on the surface of nutrient Agar, mannitol salt agar & blood agar. Thereafter, all plates incubated for 24 hours at 37°C. The isolates were identified depending on the microscopic characterization, colony morphological features, Gram staining, the biochemical tests including the catalase test, coagulase test, and others were perform according to Bergey 's Manual [7].

Detection of the bacterial ability to produce slime layer and Biofilm formation

The tissue culture plate assay described by Mathur and et al. [8] is the most widely used and was considered as standard test for the detection of biofilm formation. This method was applied on isolates of Staphylococcus aureus and Pseudomonas aeruginosa the bacterial cells were grown in Nutrient broth overnight at 37°C under aerobic conditions. A suspension of bacterial isolate that equivalent to the McFarland No.0.5 turbidity standard were inoculated in nutrient broth and incubated for 18- 24 hours at 37°C in individual wells of sterile, polystyrene, 96-well, flat bottomed tissue culture plate stationary condition. Nutrient broth supplemented with 1% glucose. After that, 200 µl of the inoculum were transferred to the assay wells of a sterile 96-well assay plate, which corresponds to an inoculum of approximately 5×10^6 cells/well. Each plate was covered with the lid supplied by the manufacturer. Subsequently, inoculated assay plates were transferred to an incubator set at 37°C for 18–24 hrs. without shaking. Negative control wells contained sterile Nutrient broth only; while, positive control wells contained nutrient broth with bacterial cells without glucose. After incubation, assay plates were uncovered. The optical density (OD) was measured at 630 nm of each well using a multi-well plate reader to quantify overall growth of biofilm. This step is achieved to identify strains that are defective in overall growth or conditions that inhibit overall growth, resulting in decreased biofilm growth. Liquid culture was removed from each well, and non-adherent bacteria were removed by washing each well 3-4 times with phosphate-buffered saline (PBS; pH 7.4). To staining fix adherent cells prior, washed plates were incubated at 60ºC for at least 60

min. This step reduces variability caused by loss of biofilm during the staining process. Biofilms can be detected and quantified using various stains. Biofilms were stained by adding 200 µl of 0.1% crystal violet dissolved in distilled water to each well and allow at least 15 minutes for staining. After the staining reaction has been completed, excess stain was removed by repeated washing (3-4 washes) with phosphate-buffered saline (PBS). As described above. The wash solution should be clear after final last washing step and dried. Afterwards, 200 µl of 95% ethanol was added to each well for 10 minutes. All assays were done in triplicates. The amount of crystal violet extracted by the ethanol in each well can be directly quantified spectrophotometrically by measuring the OD 630 using an appropriate microplate reader [9]. As shown in (table 1) Classification based on OD values obtained for individual strains of Staphylococcus spp. were used for the purpose of data simplification and calculation.

(Table 1): Classification of bacterial adherence by tissue culture plate method

Mean OD 630 nm	Biofilm Construction Adherence
OD ≤ ODc	Non–adherent
ODc < OD & OD ≤ 2ODc	Weakly
2ODc < OD & OD ≤ 4ODc	Moderately
4 ODc < OD	Strongly

Biofilm dispersal by different concentrations of proteinase K 4

Biofilm stability was investigated against proteinase K treatment was tested as described by Rohde et al. [10], with the following modifications. Bacteria were grown at 37°C overnight in nutrient broth supplemented with 1 % (w/v) glucose in 96-well microtitre plates as descripted in Tissue culture plate method. Supernatants were carefully removed and each well was washed with 200µl phosphate-buffered saline (PBS). Biofilms were treated with different concentrations of proteinase K (2, 4, 8, 16, 32, 64µg/ml) in distilled water Afterward, plates were incubated at 37°C for 1, 2, 3hrs.the control wells contained media with bacteria only. Following incubation, each well was carefully washed with 200 µl (PBS), and dried for 30 second at 60°C prior to staining with 0.1% (w/v) crystal violet solution. The A630 of the adhered, stained biofilms were measured using ELISA reader. Each strain was tested at least three times and means results are presented. After staining, the plates were again washed three times with PBS. After that, the micro titer plate was thoroughly air dried at room temperature, the dye bound to the cells should be re-solubilized, i.e. eluted from attached cells with 200 µl of 95% ethanol per well. Ethanol should be gently added and thereafter the microtiter plate covered with the lid (to minimize evaporation) should be left at room temperature for at least 10 min without shaking and measured as above [11].

Results and Discussion

Bacterial isolation and identification

From a total of 90 samples were collected, only 30

isolates (33%) had the ability to grow on the Mannitol salt agar which considered selective and differential media for genus Staphylococcus [12]. On culture media, appearance of colonies was round, smooth, raised, mucoid and glistening. Consequently, these isolates were belonging to the genus Staphylococcus. Colonies of bacteria on mannitol salt agar appeared large golden colonies with luxuriant growth and medium-color turned from pink to yellow, because some isolates able to ferment mannitol [7][13] reported that the carotenoid pigment (Staphyloxanthin) is responsible for S. aureus characteristic golden color and plays a role in the environmental fitness of S. aureus. Golden-pigment is a trait of human-pathogen S. aureus, which protect the bacterial cell from clearance by oxidation [14]. Microscopic examination was applied to all 30 isolates after staining by Gram stain to detect their response to stain, the cells appeared as Gram positive cocci mostly arranged in grape-like irregular clusters. All 30 isolates gave negative result to the oxidase test, which preformed to differentiate Staphylococcus from genus Micrococcus that usually gives positive result. Moreover, all isolates gave positive catalase test, which was done in order to differentiate Staphylococcus species from Streptococcus species that normally gave a negative result [15]. Noticeably, all mannitol fermenters were coagulase and DNase positive; Moreover, all MRSA isolates were able to give positive results for DNase and developed beta hemolysis behavior on blood agar as shown in table 2.

(Table2): The characterization of Staphylococcus aureus

Test	*Staphylococcus aureus*
Gram stain	Gram-positive
Blood agar medium	β-hemolysis
Mannitol salt agar	Yellow colony
Catalase	Positive
Oxidase	Negative
Coagulase	Positive
Bacitracin susceptibility	Resistance
Deoxyribonuclease (DNase agar)	Positive

A total of 25 isolates were able to grow on cetrimide agar plates, which may be suspected to be Pseudomonas sp., where further, identified according to morphological characteristics and biochemical tests. Colonies of each isolate were plate on nutrient agar showed different morphological characteristics of Pseudomonas sp. Such as mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color and has fruity odor and most of them were produce pyocyanin. While colonies of Pseudomonas areuginosa grown on MacConky agar medium appeared pale in color, with irregular edge, oval and large. On blood agar Pseudomonas areuginosa was able to hemolyse blood agar completely, these

results are reasonable with the results demonstrated by Collee et al., [16]. Microscopical examination of Pseudomonas sp. showed that the cells were gram negative, bacilli, appeared single, pairs or short chain and non-spore forming. These results are comparable to the reported morphological characteristics of Pseudomonas aeruginosa. These results were agreed with Holt et al., [17] who certify the identification. Biochemical tests for Pseudomonas sp. were made also. Results indicated in (table 3) showed that these isolates gave a positive result for oxidase and catalase which indicate that these isolate belongs to Pseudomonas aeruginosa.

(Table 3): Morphological and biochemical characteristics of the isolated Pseudomonas aeruginosa.

Test	Result
Colony color	Green
Cell shape	Rod or Bacilli
Gram stain	Negative
Catalase	Positive
Oxidase	Positive
Growth on King A	Positive
Growth on King B	Positive
Growth on cetrimide	Positive
Citrate utilization	Positive
Growth at 4ºC	Negative
Growth at 42ºC	Positive

Detection of the bacterial ability to produce slime layer and Biofilm formation

For detection formation of biofilm, microtiter palte assay (MtP) is the most common way and consider as standard test for detection [18]. As reported, MtP-method is most sensitive, accurate and reproducible screening method in the determination of biofilm-production by clinical isolates of Staphylococci and Pseudomonas in addition to MtP advantage as a quantitative tool which used in comparing the adherence of different strains [8]. Using MtP-method for the detection of biofilm formation by S. aureus and P. aeruginosa isolates. When grown in nutrient broth without any supplementation, 100% S. aureus isolates were able to form weak biofilm. In the presence 1% glucose lead to enhanced biofilm forming capacity in for 66% of S. aureus isolates moderate producers. While P. aeruginosa isolates were able to form moderate biofilm in presence1% glucose.

Eradication of biofilm by Proteinase K enzyme

To investigate the factors that contribute to biofilm degradation, we performed an enzyme treatment on the formed biofilm. Enzymatic solutions with concentrations (2, 4, 8, 16, 32,64µg/ml) of

proteinase K. Proteinase K treatment hampered the biofilm development of S. aureus isolates. All isolates, (weak biofilm-producing strain), showed significant inhibition in biofilm growth when treated with 2 µg/ml proteinase K after 2hr. as shown in (Fig. 1). 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 1, 2, 3, hrs. Proteinase K treatment of S. aureus biofilms caused a significant disruption of all S. aureus biofilm. The proteinase K can be used in biofilm dispersion. Interestingly, significant; but not 100%; removal of biofilm was achieved by treatment with the proteinase K which has a wide specificity as other proteases enzymes in biofilm degradation [19]. The proteinase K have frequently been used as efficient biofilm removal agents that hinder bacterial adherence and biofilm formation in S. aureus presumably through degradation of surface structures, also reported that proteinaceous-biofilms formed by S. aureus with the help of Bap proteins were susceptible to proteinase K mediated detachment and dispersal. Our findings showed that (4 µg/ml) the best concentration of enzyme have a degrading effect against S. aureus biofilm. Among various surface proteins in S. aureus, has been reported to have a major role in early adhesion, as well as in the biofilm development [20]. After proteinase K treatment, a significant decrease in the protein and extracellular DNA (eDNA) but not in the carbohydrate content in extracellular polymeric substance (EPS). Extracellular DNA (eDNA) is also known to play very important role in S. aureus biofilm stability [21]. This enzyme targeting peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids [22]. It is proved that proteinase K degrade effectively the EPS of P. aeruginosa biofilms, by binding and hydrolysis of the protein molecules due to converting them into smaller units and been transported to through cell membranes, the metabolized. In most cases proteins seem to be the main constituents of the biofilms EPS and are found mostly at the outer layer of the P. aeruginosa biofilms [23]. So that, breaking of EPS in addition to proteins of outer layer by proteinase K, will make proteinase K better than other enzymes like amylase which only degrade the proteins of EPS, these enzymes are less efficient than proteinase K in degradation of P. aeruginosa biofilms [24].

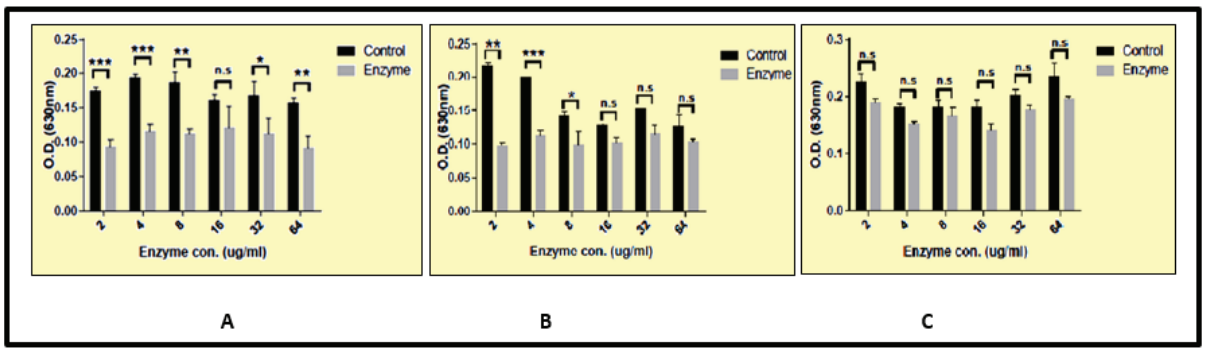

Figure (1): Effect of different concentration of proteinase K on the formation of Staphylococcus aureus biofilms after 1hour of treatment.

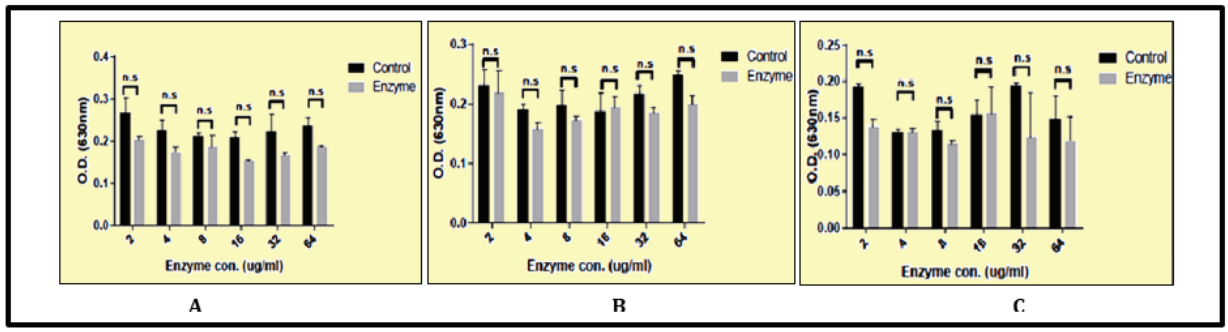

Figure (2): Effect of different concentration of proteinase K on the formation of Staphylococcus aureus biofilms after 2hours of treatment.

Figure (1): Effect of different concentration of proteinase K on the formation of *Staphylococcus aureus* biofilms, (A) show degradation activity of proteinase K after one hour of treatment (B and C) are the influence of proteinase K after two and three hours of treatment respectively.

Figure (2): Effect of different concentration of proteinase K on the formation of Pseudomonas aeruginosa biofilms, (A) show degradation activity of proteinase K after one hour of treatment (B and C) are the influence of proteinase K after two and three hours of treatment respectively.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

Funding: Self-funding

References

1. Zhao G., Usui M., Lippman S., James G., Stewart P., Fleckman P. Biofilms and inflammation in chronic wounds. Adv. Wound Care.2013; 2, 389–399.
2. Lebeaux D., Ghigo J., Beloin C. Biofilm-Related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol. Mol. Biol. Rev. 2014; 78, 510–543.
3. Schaffer A., Lee J. Vaccination and passive immunisation against *Staphylococcus aureus*. Int. J. Antimicrob. Agents. Suppl. 2008; 1: S71-8.
4. Xavier JB., *et al.* Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix a modeling study. Microbiol. 2005;151: 3817-3832.
5. Frees D., Brøndsted L., Ingmer H. Bacterial proteases and virulence. Subcell Biochem. 2013; 66: 161-192.
6. Chaignon P., Sadovskaya I., Ragunah Ch., Ramasubbu N., Kaplan JB., *et al*. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl Microbiol Biotechnol. 2007;75: 125-132.
7. Schleifer K.,Bell J A. Staphylococcusea p:392-420 *.In:* Bergey's Manual Of Systematic Bacteriology. Parte AC., Whitman WB., Vos P. De GM. Garrity, Dorothy Jones, Krieg NR., Ludwig W., Rainey FA., Schleifer K. and Whitman WB. (eds) Biological Sciences Building University of Georgia Athens. 2009;GA –USA.
8. Mathur T., Singhal S., Khan S., Upadhyay DJ., Fatma T., Rattan A. Detection of Biofilm Formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J. Med. Microbiol.

2006;24: 25-9.

9. Kwasny SM., Opperman TJ. Static biofilm cultures of gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. Curr Protoc Pharmacol. 2010; Chapter 13:Unit 13A.8.
10. Rohde H., Burdelski C., Bartscht K., Hussain M., Buck F., Horstkotte MA., Knobloch JK., Heilmann C., Herrmann M., Mack D. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation associated protein by staphylococcal and host proteases. Mol. Microbiol. 2005; 55:1883-1895.
11. Holland LM., Conlon B., O'Gara JP. Mutation of *tagO* reveals an essential role for wall teichoic acids in *Staphylococcus epidermidis* biofilm development. Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland. 2011;157: 408–418.
12. Benson JH. Microbiological Application, Laboratory Manual in General Microbiology 8th ed. The McGrath-Hill companies, inc. 2002.
13. Clauditz A., Resch A., Wieland KP., Peschel A.G., ootz F. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. Infect. Immun.2006; 74:4950-4953.
14. Liu GY., Nizet V. Color me bad: microbial pigments as virulence factors. Trends. Microbiol. 2009;17:406-413.
15. Schneewind O., Missiakas D. *Staphylococcus aureus* and Related Staphylococci. in:Goldman E. andGreen L. H.(ed) .(.Practical handbook of microbiology. 2009,Taylor and Francis Group, an informa business.
16. Collee JG., Franser AG., Mormion BP., Simmons A. Mackie and McCartney Practical Medical Microbiology. 14th ed. Churchill Livingstone. 1996;New York.
17. Holt JC., Krieg NR. Bergey 's Manual of Systemic Bacteriology. (4th ed.). Williams and Wilkins, Baltimore.1994; USA.
18. Gad GF, El-Feky MA., El-Rehewy MS., Amin M., Hassan, Abolella H.,Abd El-Baky RM. Detection of *icaA, icaD* genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. J. Infect. Dev. Ctries. 2009; 3:342-351.
19. Mootz JM., Malone CL., Shaw LN.,Horswill AR. Staphopains modulate Staphylococcus aureus biofilm integrity. Infect Immun .2013; 81:3227-3238.
20. Vautor E, Abadie G, Pont A, Thiery R. Evaluation of the presence of the bap gene in *Staphylococcus aureus* isolates recovered from human and animal's species. Vet. Microbiol. 2008;127, 407–411.
21. Sudhir KS, Subba RT. *Staphylococcus aureus* biofilm removal by targeting biofilm-associated extracellular proteins. Indian J Med Res .2017;146, pp 1-8.
22. Shukla SK, Rao TS. Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K. J. Antibiot. 2013; 66, 55–60.
23. Lequette Y, Boels G, Clarisse M, Faille C. Using enzymes to remove biofilms of bacterial isolates samples in the food industry. Biofoul. 2010; 4: 421-431.
24. Bhaskar PV, Bhosle NB. Microbial extracellular polymeric substances in marine biogeochemical processes. Cur. Sci. 2005; 88: 47-53.

.