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Disruption of Biofilms by the action of Peptides from Human Placental Extract

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Abstract
A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA that cause two thirds of all infections and demonstrate a 10 to 1000 fold increase in adaptive resistance to conventional antibiotics as well as resisting phagocytosis and other components of the body's defense system.
Currently, there are no approved drugs that satisfactorily and specifically target bacterial biofilms.
Human placental extract (HPE 100µg/ ml) containing biomolecules like peptides and nucleotides completely prevented biofilm formation and led to the eradication of mature biofilms in representative strains of both Gram-negative and Gram-positive bacterial pathogens including ***Pseudomonas aeruginosa*** *and* ***Staphylococcus aureus****.*
Since peptides from plant and animal origin were reported to haveanti-microbial and anti-biofilm properties, an attempt was made to segregate the peptides identified as Fibronectin type III and Ubiquitin like moietyfrom HPE (hence known as HPE peptides) and study their anti-biofilm properties. An initial study was done with HPE containing these peptides and was shown to possess potent anti-biofilm activity.
Biofilm was produced by a modification of the tube method originally described by Christensen. First the organisms were inoculated in BHI broth in Eppendorf tube followed by incubation. The growth of biofilm, when formed, was detected by staining with crystal violet and taking the OD values of the dissolved materials after eluting it with ethyl alcohol.
When the results were analyzed, it was observed that the HPE peptides inhibited the formation of biofilm by ***Enterococcus faecalis Candida albicans****and****Escherichia coli*** *and these inhibitions were statistically significant. In case of* ***Candida albicans*** *the HPE peptide was found to be comparable to Chlorhexidine which was used as a known inhibitor for biofilm production. Inhibition was a little less for* ***Staphylococcus aureus*** *in the said dose of HPE peptides*

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I. Introduction

Infectious diseases are a leading cause of death worldwide and hence a great challenge for every nation. World Health Organization (WHO) forecasts 13 million deaths attributed to these causes by 2050[6]. Antibiotics are powerful medicines that fight bacterial infections yet in a data released in 2013, CDC estimates that in the United States, more than two million people are sickened every year with antibiotic-resistant infections, with at least 23,000 dying as a result of it. Taking an antibiotic as directed, even after symptoms disappear, is key to curing an infection and preventing the development of resistant bacteria. Antibiotic resistance is a worldwide problem and few urgent threats are ***Clostridium difficile***, Carbapenem-resistant ***Enterobacteriaceae***(CRE), drug-resistant ***Neisseria gonorrhoeae***. Serious threats are posed due to Multidrug-resistant ***Pseudomonas aeruginosa*** and Methicillin- resistant ***Staphylococcus aureus*** (MRSA). Antibiotic resistance of bacteria are often attributed to the formation of "biofilms". The bacteria in a biofilm are up to 1,000-fold more resistant to antibiotic treatment than the same organism that are grown planktonically [2]. The EPS matrix prevents access of certain antimicrobial agents restricting diffusion of compounds from the surrounding into the biofilm[7], [8]. The classes of antibiotics that are hydrophilic and positively charged, such as

aminoglycosides are more obstructed than others. There may be inactivation of the antibiotics by extracellular polymers or modifying enzymes.As most of the bacteria in natural settings reside within biofilms, conjugation is one of the most likely mechanisms by which bacteria in biofilms transfer genes within or between populations. A study of dental plaque have shown that ***Bacillus subtilis***strain that was tetracycline resistant transferred the resistance to ***Streptococcus***speciesin biofilm bacteria[9]. Clinically, infections with biofilm have shown that treatment with antibiotics is not a complete solution as symptoms usually recur even after repeated treatments. The antibiotic therapy eliminates the planktonic cells, but the sessile forms are resistant and continues to propagate within the biofilm. There is continuous release of antigens and production of antibodies that eventually causes more damage to the surrounding tissue.Chronic wound infections as a result of pressure sores, venous leg ulcers and diabetic foot ulcers are typically caused by multiple genera of bacteria, including ***Staphylococcus aureus***and ***Pseudomonas aeruginosa***, which are notorious for biofilm formation. A biofilm-centric approach to reduce the ability of these pathogens to form biofilms is urgently needed to enable more effective treatment with antibiotics or subsequent healing by the body.Some agents have already been identified to possess anti-biofilm activity. A new substituted 2-aminoimidazoles that make up a family of compounds, which are efficient anti-biofilm agents, both against mono and multi-species biofilms, including both yeasts and bacteria, has been identified. A bacterial extract has been derived from a novel bacterial species of ***Paenibacillus*** strain 139SI. The crude extract and its three identified compounds exhibit strong anti-biofilm activity[10].Some plant extracts were studied for their anti-biofilm property as well. The only human member of cathelicidin (CAP18) family, a peptide named LL-37, has been identified whose fragmented part KR-12 has been found to have anti-biofilm activity. Wang reported that the fragment KR-12 (fragment LL18-29) was toxic for bacteria but not for human cells[11].Meanwhile, de la Fuente-Núñez et al. identified, in a library of synthesized fragments, a novel 9-amino-acid peptide with anti-biofilm activity superior to that of LL-37.Human placental extract has already been documented to directly facilitate the process of wound healing and tissue regeneration through its active biomolecules namely Fibronectin Type III like peptides, Ubiquitin peptide, PDRN, and NADPH[12]. These accelerate almost all the steps of wound healing procedure with minimum scar formation. Expedited healing has been observed in clinical studies with HPE and antibiotics with considerable shortening in recovery time improved MAGS score, neoangiogenesis and healthy collagen formation [12], [13].In a preliminary study, human placental extract containing fibronectin type III (FN3) like peptide and Ubiquitin like peptide in a concentration of 100µg/ml has shown promising anti-biofilm activity. HPE has been shown to inhibit quorum sensing dependent biofilm formation in both Gram-positive and Gram-negative organisms. It attenuates pyoverdin and pyocyanin formation and reduced eDNA concentration[3].If peptides from HPE exhibited similar or enhanced activity against bacterial/ microbial biofilms it would be a major breakthrough and could open up new avenues in the management of antibiotic resistant infected wounds. This finding shall be immensely helpful in limiting burn induced sepsis-like situations, chronic and indolent infected wounds that are a menace to treat. The present study targets to segregate the peptides from HPE and investigate their anti-biofilm activity in the concentration of 100µg/ml against bacteria and fungus known to produce biofilms.

II. Materials and Methods

There are various methods to detect biofilm production, e.g. Congo red agar, tube method, tissue culture plate, bioluminescent assay, piezoelectric sensors and fluorescent microscopic examinations [15]. In this study, a modification of the original tube method as described by Christensen et al. was used[15], [16].

Four biofilm producing organisms[3] were selected for the study. One standard bacterial strain namely ***Enterococcus faecalis*(ATCC 29212)** a Gram-positive cocci involved in biofilm formation in dental plaque[17], clinically virulent strains of***Staphylococcus aureus*** a Gram-positive cocci, ***Escherichia coli*** a Gram-negative bacilli and a fungal agent ***Candida albicans***,a fungus, were taken from patients at KrishnadevarayaCollege of Dental Sciences, Bengaluru, India.

The organisms were inoculated in BHI broth for two to three hours at 37^0 C. The growths were compared with 0.5 McFarland opacity tube to make the standardized inoculum.

Each of these were inoculated in three tubes of brain-heart infusion broth (HIMEDIA M210), with 50 g/L sucrose, as a modification to tripticase soy broth that was originally used,which was dispensed into 2 mL Eppendorf tubes. If the organisms are biofilm producers, a slimy layer will be produced on the inner side and the bottom of the tube after incubation. This biofilm producing activity can be qualitatively demonstrated after decanting the supernatant, drying and staining with a suitable stain. For quantitave estimation, after staining with 0.1% crystal violet as in this study and decanting the stain, alcohol was added to each tube to dissolve the layered biofilm, if any, and the solution was put in the cuvette of a spectrophotometer and its optical density was measured at 540 nm.

In this current study inhibitory effect of Human Placental Extract (HPE) peptides as the test agent was determined by statistical analysis.

For each organism 50 µL of chlorhexidine [18], which was considered as positive control to demonstrate inhibition of biofilm formation, 50 µL normal saline which served as a negative control for the study and 50 µL of peptide solution desiccated out from Human Placental Extract as the test agent to observe its biofilm inhibitory activity were added to the three tubes respectively.

Peptides were desiccated out from of the commercially available injectable form of human placental extract (Injection Placentrex; Albert David Ltd). For this 10 mL of the injectable solution was first poured in a beaker and placed in a vacuum desiccator. Drying agents, like silica gel and sodium hydroxide pellets, are used to accelerate the process of desiccation. After a period of time, the extract dries up and the protein component is eluted with water. The final eluent is a yellow-coloured liquid and is centrifuged at 10,000 rpm for 10 minutes. This separates any residual non-protein component and the supernatant liquid is collected for further testing[4].

The Eppendorf tubes were incubated overnight. The effects of these agents on biofilm formation were determined after staining with crystal violet and addition of absolute alcohol and then recording their OD values at 540 nm. Lower OD value corresponded with higher inhibitory effect. (Fig-1)

The values were analyzed statistically through ANOVA model using SPSS software.

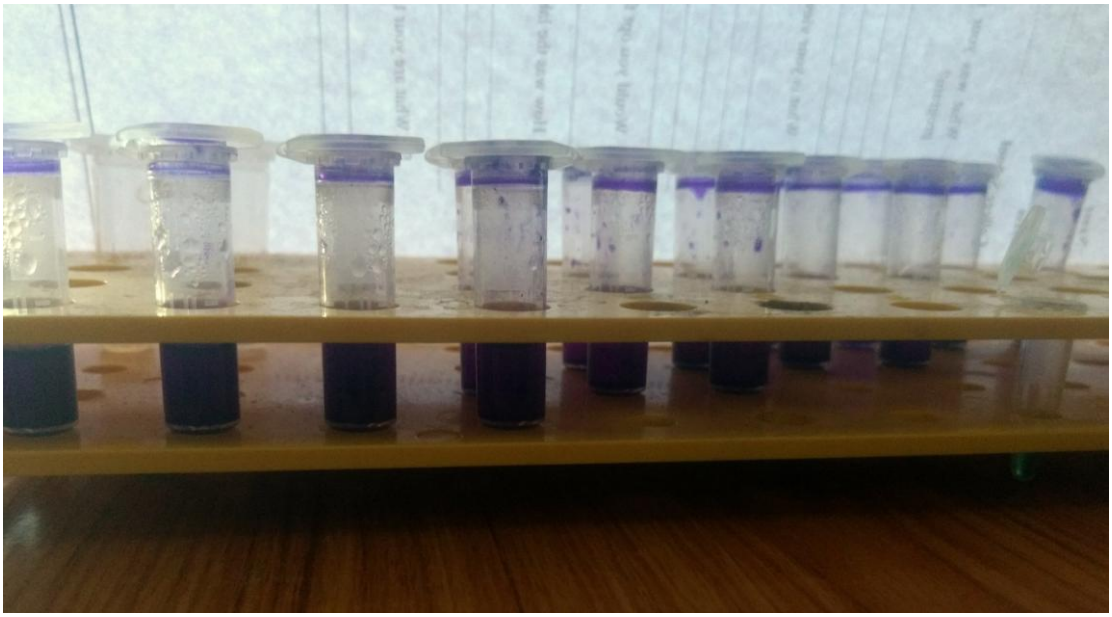


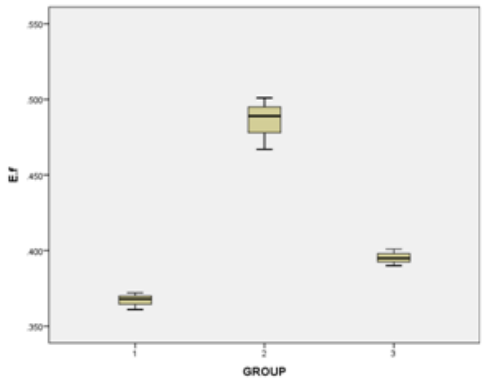
Fig-1 : Eppendorf tubes after incubation, staining with Crystal violet and addition of absolute alcohol for recording of OD values.

III. Results and Discussion

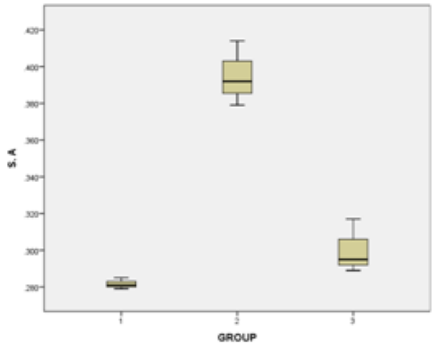
Preliminary observations from the present study indicate that there is a significant reduction in biofilm formation with HPE Peptides in a concentration of 100 µg/ml. The negative control,normal saline, did not show any such activity.

	Chlorhexidine			**Normal Saline**			**HPE peptide**		
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Enterococcusfaecalis	0.372	0.361	0.368	0.467	0.501	0.489	0.390	0.401	0.395
Staphylococcus aureus	0.300	0.242	0.208	0.504	0.380	0.375	0.364	0.267	0.225
Candida albicans	0.157	0.116	0.149	0.487	0.567	0.454	0.291	0.317	0.323
Escherichia coli	0.311	0.336	0.385	0.570	0.551	0.516	0.431	0.394	0.371

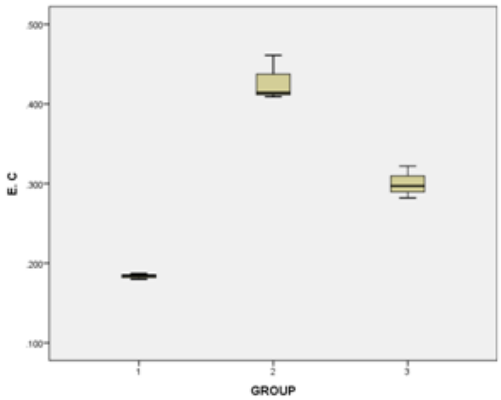
Table-1: Showing OD values of four organisms after grown in presence of Chlorohexidine, Normal saline and HPE peptide.



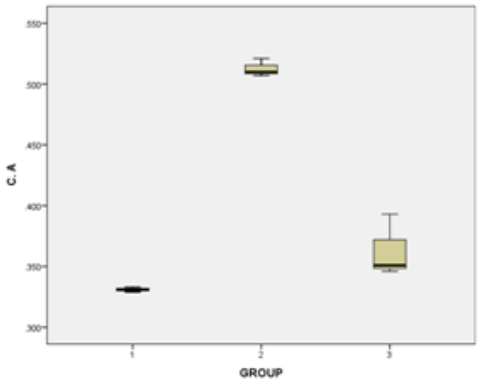
Q-Q plot: ***Enterococcus faecalis***



Q-Q plot***Staphylococcus aureus***



Q-Q plot ***Escherichia coli***



Q-Q plot***Candida albicans***

Table-1 shows OD values for chlorhexidine, normal saline and peptides from Human Placental Extract (HPE peptide) while incorporated in the growth of ***Enterococcus faecalis***, and clinically virulent strains of ***Staphylococcus aureus***, ***Candida albicans*** and ***Escherichia coli***in BHI broth. Their effect on biofilm formation was noted and interpreted through the OD readings.

In the case of ***Enterococcus faecalis*** both 2% chlorhexidine and HPE peptide in concentration of 100µg/ml were found to inhibit biofilm formation in comparison to normal saline and both of these inhibitions were statistically significant. The effect of HPE peptide in said concentration was comparable to that of chlorhexidine.

There was an inhibitory effect of HPE peptide in concentration of 100µg/ml on ***Staphylococcus aureus***.Chlorhexidine was observed to inhibit biofilm significantly in comparison to normal saline.

Both 2% chlorhexidine and HPE peptide in concentration of 100µg/mlhad shown significant inhibition on biofilm formation by ***Candida albicans*** and effect of chlorhexidine was also found to be superior to HPE peptide of that concentration.

With ***Escherichia coli*** the observations were like that of ***Enterococcus faecalis***. Both chlorhexidine and HPE peptide in concentration of 100µg/ml had significant inhibitory effect on biofilm formation in relation to normal saline but no significant difference was found between these two.

The above observations are also depicted in the Q-Q plots of the four organisms.

IV. Discussion

Production of biofilms is one of the important tools of microorganisms to combat lethal effect of antimicrobials. Many attempts were made to interfere the process of biofilm formation so that antimicrobials can reach the site of microbial growth directly and could exert a microbicidal activity to target population.

The wound healing property of Human placental extract has already been proven clinically and results were published.Recently, the biofilm breaking property of the extract were also published[3].The components of Human Placental Extract is still under study and certain biomolecules like peptides and nucleotides have been identified. These biomolecules help in various process of wound healing and tissue regeneration.

Since some peptides have been implicated as having anti-biofilm properties, attempts have been made to study HPE peptides for the same in a concentration of 100µg/ml.

In the present study anti biofilm effect of HPE peptides were seen against **Enterococcus faecalis(ATCC 29212)** and clinically virulent strains of **Staphylococcus aureus**, **Candida albicans** and **Escherichia coli** in BHI broth.It was found that HPE peptides inhibited biofilm formation of **Enterococcusfaecalis,Escherichia coli and Candida albicans** corroborating the finding of Goswami et al [3].

In case of **Enterococcus faecalis** and **Escherichiacoli**, the HPE peptide in a concentration of 100µg/ml was found to be as effective an inhibitor as chlorhexidine. Biofilm formed by **Staphylococcus aureus**and**Candida albicans** were also inhibited by HPE peptides at a concentration of 100µg/ml but less when compared to chlorhexidine.

V. Conclusion

The study was done with virulent clinical strains of ***Staphylococcus aureus***, ***Candida albicans*** and ***Escherichia coli***,while***Enterococcus faecalis*** which was a standard strain. HPE peptides in a concentration of 100µg/ml were found to have significant inhibitory effect on biofilm production by all the organisms.
Effect on ***Staphylococcus aureus*** was less as compared to chlorhexidine.

It is required that the experiment be repeated with higher concentration to optimize the effective dose of inhibition of biofilm production.

The inhibition of biofilm formation by HPE peptides is a novel finding and may serve as the platform of overcoming antibiotic resistance due to biofilms in life threatening infections.

The peptides as components of human placental extract is in use for long time and no toxicity has ever been reported.

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