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Original article

ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF NOVEL ANTIMICROBIAL PEPTIDES AGAINST *PSEUDOMONAS AERUGINOSA* ISOLATES FROM BOVINE MASTITIS

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Summary

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Pseudomonas aeruginosa is one of the causative pathogens of bovine mastitis. Most of *P. aeruginosa* cells can form biofilm, thereby reducing antibiotic efficacy which has become a significant public health challenge nowadays. In the present study, biofilm formation of 50 isolates was assessed. The inhibitory effect of three designed antimicrobial peptides (FASK, YDVD, WSF) on *P. aeruginosa* planktonic growth and biofilms was evaluated. The minimum inhibitory concentration (MIC) of all the peptides under study was 1600 µg/mL. The synthetic compounds had a significant inhibitory effect at concentrations of 1/2 MIC (800 µg/mL) and 1/4 MIC (400 µg/mL) on biofilm formation of these isolates, and showed anti-biofilm activity at a lower concentration than MIC. Antibacterial peptides FASK, YDVD with 70% antibiofilm effect and WSF with 60% effect prevented the formation of biofilm by mastitis isolates at a concentration of 1/2 MIC. The peptides of this study are promising candidates for inhibiting *P. aeruginosa* biofilm formation.

Key words: anti-bacterial peptides, anti-biofilm peptides, bovine mastitis, *Pseudomonas aeruginosa*

INTRODUCTION

Mastitis is inflammation of the parenchymal tissue of the mammary glands, which causes pathological changes in the mammary glands and physical and chemical changes in the milk (Martins *et al.*, 2019). Today, mastitis is one of the three diseases that cause great economic losses to herds, and is more common in cattle (Van Soest *et al.*, 2016). Economic losses are due to reduced milk production and quality, treatment costs, livestock losses etc. Transfer of antibiotic resistance genes from isolates of animal origin to humans creates strains resistant to conventional therapies. The prevalence of chronic and refractory diseases in the herd makes the

management system difficult for veterinarians. Also, chronic mastitis prevents the identification of effective treatments to control the disease in the herd (Sharma & Jeong, 2013).

P. aeruginosa is one of the most important opportunistic pathogens that cause sub-clinical mastitis refractory to therapy in dairy herds. This bacterium is important as a Gram-negative pathogen in peripheral mastitis which is transferred to the cow during milking through milking equipment (Bannerman *et al.*, 2005). Mastitis infection by *Pseudomonas* sp. is a treatment barrier that is resistant to most antibiotics (Park *et al.*, 2014). The ability of this bacterium to produce biofilm reduces the effective treatments and leads to chronic mastitis (Mpatswenumugabo *et al.*, 2017). The biofilm is a community of several microorganisms in which cells are enclosed within an extracellular matrix of exopolysaccharides (Lyczak *et al.*, 2000; Flemming *et al.*, 2016). *Pseudomonas* biofilm formation leads to survival in undesirable environmental conditions and provides resistance to traditional antibiotics (Olszewska *et al.*, 2016). Despite the significant knowledge about mastitis, the truth is that mastitis by *P. aeruginosa* is very difficult to control because it is caused by an opportunistic bacterium that is often transmitted to cattle through surfaces, devices, and environments (Ganda *et al.*, 2016). Frequent use of antibiotics and their attendance, in the environment, due to increased antimicrobial resistance and their resultant adverse effects on veterinary medicine and human health, have raised many concerns (Aga *et al.*, 2016; Holko *et al.*, 2019). Major concerns are related to the possible presence of resistant genes stores in bacterial populations of food-producing animals due to continuous antibiotic prescription. These bacterial populations can release genes resistant to other bacteria that are potentially perilous to humans (Oliver *et al.*, 2010). Due to the fact that bacteria in the biofilm state are more resistant to conventional antibiotics, therapies should be improved. Therefore, there is a need for new alternative therapies to reduce antibiotic treatments or reduce the dose of antibiotics. In recent years, antimicrobial peptides have received much attention. Small synthetic peptides with anti-biofilm activity are a new way to treat biofilm-related infections (Gopal *et al.*, 2014).

The purpose of this study was to evaluate the antibacterial and anti-biofilm impacts of three synthesised peptides on *P. aeruginosa* isolates from cows with mastitis infection.

MATERIALS AND METHODS

Clinical isolates

A total of 50 isolates of *Pseudomonas aeruginosa (P. aeruginosa*) from bovine mastitis were used in this study, obtained from Department of Microbiology, School of Veterinary Medicine, Tehran University, Tehran, Iran. The isolates were maintained on cryostat beads at –80 °C. They were grown in tryptic soy broth and nutrient agar, and were validated by PCR assay targeting PA-16S rDNA; PA-SS-F (5′-GGG GGA TCT TCG GAC CTC A-3′) and PA-SS-R (5′-TCC TTAGAG TGC CCA CCC G-3′) gene-specific for *P. aeruginosa* species (Spilker *et al.*, 2004). The reference strain studied in this research was *P. aeruginosa* ATCC 27853.

Antimicrobial peptides

In this project, amino acid libraries, which included various amino acid compounds, were evaluated after the necessary preparations against the intended target. Evalu-

ations included developing a pharmacophore model and examining it to identify the first binders, peptide docking using several algorithms with low-up accuracy, examining the free binding energy to identify the primary candidates, and molecular dynamics to realistically analyse compounds within target compounds. The obtained final results showed three final candidate peptides (FASK, YDVD, and WSF) (unpublished data).

Peptides used in this study (FASK, YDVD, WSF) were synthesised by Mimotopes company (Australia) and made >95% pure using reverse-phase high-performance liquid chromatography (HPLC).

Biofilm quantification assay

The biofilm formation of 50 *P. aeruginosa* isolates from mastitis was evaluated by microtiter plate method (O'Toole, 2011; Azeredo *et al.*, 2017). For this purpose, samples were cultured on tryptic soy agar containing 0.1% glucose and incubated at 37 °C for 24 hours. Colonies were inoculated into wells of the 96-well plates U-bottom polystyrene containing 100 µL LB broth. The bacteria were grown overnight at 37 °C with shaking. After incubation, the exponential growth culture was pipetted. The turbidity of the bacterial suspension was adjusted to match turbidity comparable to that of the 0.5 McFarland standard (10^8 CFU/mL) for biofilm cultivation (Gui *et al.*, 2014). The bacterial suspensions were diluted 1:100 and cultured in 96-well plates U-bottom polypropylene containing TSB w/0.1% glucose medium and incubated under aerobic conditions 24 hours at 37 °C.

After 24 hours of biofilm formation, all wells were aspirated, and washed three times with sterile phosphate-buffered saline (PBS) to remove all cells not attached to the surface of the well. In the next step, for biofilm quantification, 125 µL of 0.1% crystal violet solution was added to each well and biofilm-forming isolates were allowed 20 minutes to attach to the wells in order to be stained. The wells were then washed three times with sterile distilled water to remove excess crystal violet. The amount of 125 µL 70% ethanol was added to each well to dissolve the remaining dyes completely. The plates were placed on a shaker for 20 minutes at room temperature, and finally, the absorbance was read by Epoch2-microplate reader (BioTek company) at 595 nm. This test was performed with three replications for each bacterial sample and a well containing TSB without bacterium was considered as negative control. Positive control in this experiment was *P. aeruginosa* ATCC 27853.

Biofilm assays were performed on four consecutive days to evaluate the biofilm formation on different days. Separate plates were considered for four consecutive days. For this purpose, the culture medium of all wells was discarded by a sampler every day, and 100 µL fresh culture medium was added to each well and incubated at 37 °C.

For biofilm formation ability, the isolates were classified into four categories based on the average optical density and the optical density cut-off value (Odc) results (Stepanovic *et al.*, 2007). ODc was obtained from the relationship between the average OD of negative control plus three times the standard deviation of the negative control (ODc = average OD of negative control): OD (isolate) ≤ ODc = non-biofilm-producers; ODc ≤ OD (isolate) ≤ 2 ODc = weak producers; 2 ODc ≤ OD (isolate) ≤ 4 ODc = moderate producers; 4 ODc ≤ OD (isolate) = strong producers.

Antibacterial activity assay

The microdilution method was used to determine the minimum inhibitory concentration (MIC) of compounds. For this purpose, the peptides were first dissolved in sterile distilled water, and their antimicrobial activity was investigated on *P. aeruginosa* isolates. The bacteria were cultured in tryptic soy broth (TSB) and kept overnight at 37 ° C with shaking at 180 rpm to achieve exponential growth (optical density between 0.2 and 0.6 at 600 nm). MIC assays were performed on 96-well U-bottom polypropylene plate (Sigma-Aldrich, Germany). The amount of 100 µL of Mueller Hinton broth was poured into each well and two-fold dilutions of each peptide were created (1600 µg/mL, 800 µg/mL, 400 µg/mL to 12.5 µg/mL concentrations) and added to each well.

The exponentially grown culture was diluted 1:10 in Mueller Hinton broth and then inoculated with a final concentration of 5×10^5 CFU/mL in each well. A well was considered as sterility control well when it contained sterile medium (100 µL) and as growth control well when it contained 100 µL medium with bacterial suspension at a concentration of 5×10^5 CFU/mL. The plates were incubated overnight at 37 °C. Then, the absorbance was read at 600 nm with the Epoch2-microplate reader (BioTek Company, America) to assess the growth of bacterial isolates in confrontation with the designed peptides. The MIC was defined as the lowest peptide concentration at which no growth was observed (Wiegand *et al.*, 2008; De La Fuente *et al.*, 2012).

Antibiofilm activity assay

Assessing the inhibition of biofilm formation was evaluated using the methods described previously. The antibiofilm activity of peptides on isolates was evaluated using the same plates used for planktonic growth. Briefly, an overnight culture of *P. aeruginosa* cells was prepared in a fresh TSB medium (1:100 dilution). Different bacterial cultures were prepared at a final density of 5×10^5 CFU/ mL and inoculated into 96-well U-bottom polypropylene plates containing peptide solution in inhibitory concentrations (1/2× MIC to 1/128×MIC). Stock solutions of the peptides were prepared by dissolving each peptide in sterile distilled water (dH_2O), and the various peptide working concentrations used in the microtiter broth dilution assay were prepared in two-fold serial dilutions starting at a concentration of 1600 µg/mL to 12.5 µg/mL. Plates were incubated at 37 °C for 24 h.

After staining with crystal violet (in the same manner as described in the biofilm assay), the absorbance was read by the Epoch2-microplate reader (BioTek company) at 595 nm to determine the inhibitory effect of peptides at different concentrations on biofilm formation. The quantity of biofilm inhibition was measured as 100% biofilm (biofilm produced in the absence of peptide) and 0% biofilm (media sterility control).

Statistical analysis

All statistical analyses were performed with SPSS software version 26. The distribution of quantitative variables was investigated by the Shapiro-Wilk test, and the normality of isolates was confirmed at levels of >95% ($P<0.05$ value). The Mann-Whitney U test was used to compare the amount of biofilm produced at different times. Kruskal-Wallis test was used to compare the effect of different concentrations of peptides studied.

RESULTS

Measurement of biofilm formation by P. aeruginosa isolates

The ability of biofilm formation by *P. aeruginosa* isolates was evaluated by the crystal violet method. According to OD values, isolates were classified into four groups compared to ODc: OD600 (isolate) ≤0.123 = non-biofilm producers; 0.123≤ OD (isolate) ≤0.246 = weak producers; 0.246 ≤OD (isolate) ≤0.492 = moderate producers; 0.492 ≤OD (isolate) = strong producers. According to the analyses performed, the majority of isolates were able to form strong biofilms (46%), moderate biofilms (46%), and weak biofilms (8%) (Fig. 1).

All isolates showed a significant increase in biofilm production over four consecutive days (P<0.05). On the other hand, this trend of changes has increased significantly after 48 hours (P<0.005) (Fig. 2).

Evaluation of minimum inhibitory concentration (MIC) of synthetic peptides

Different inhibitory concentrations of the designed peptides on the isolates was evaluated by microdilution method, and compared to the result of *P. aeruginosa*

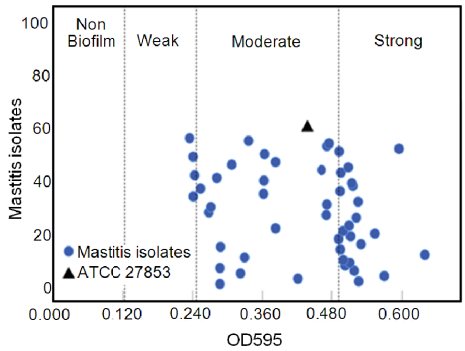

Fig. 1. Distribution of *Pseudomonas aeruginosa* isolates based on biofilm formation.

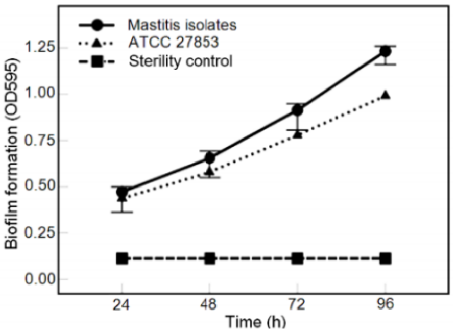

Fig. 2. Evaluation of biofilm production on mastitis isolates and *Pseudomonas aeruginosa* ATCC 27853 during four consecutive days. Sterility control = TSB without bacterium; P <0.005 vs the previous day.

ATCC 27853 as positive control. According to the results, MICs of all three peptides on mastitis isolates were 1600 µg/mL to inhibit the planktonic growth of isolates and positive control samples. Descriptive analysis of average OD600 values at different concentrations of peptides is displayed on Fig. 3.

Evaluation of minimum biofilm inhibitory concentration (MBIC) of synthetic peptides

The results showed that all three peptides led to inhibition of biofilm formation at concentrations of 1/2 MIC (800 µg/mL) and 1/4 MIC (400 µg/mL). The concentrations were lower than those needed to prevent the growth of *Pseudomonas* isolates. Descriptive analysis of average OD595 values at different concentrations of antibiofilm peptides is presented on Fig. 4.

DISCUSSION

One of the main objectives of this study was to evaluate the effect of anti-biofilm peptide compounds against *P. aeruginosa*

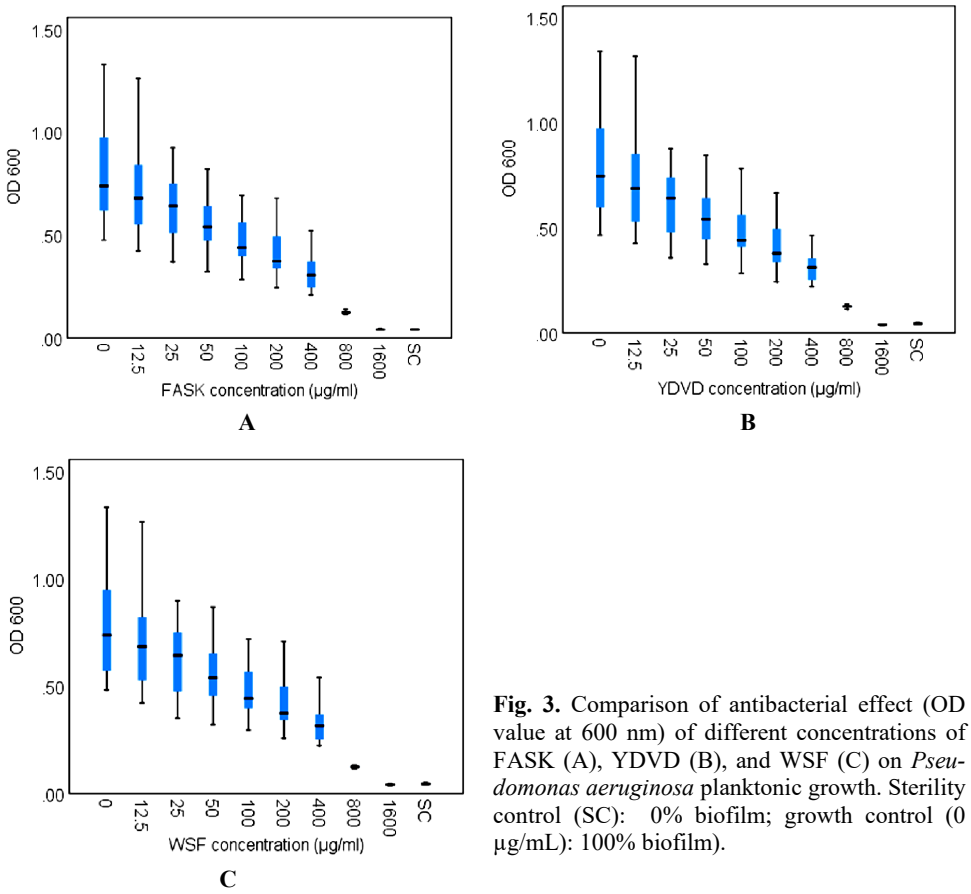

Fig. 3. Comparison of antibacterial effect (OD value at 600 nm) of different concentrations of FASK (A), YDVD (B), and WSF (C) on *Pseudomonas aeruginosa* planktonic growth. Sterility control (SC): 0% biofilm; growth control (0 µg/mL): 100% biofilm).

mastitis isolates. *P. aeruginosa* is an opportunistic bacterium that has received a great deal of attention in recent years because of the problems it has created in the livestock industry. Treatment of infections caused by this bacterium has become a complex dilemma due to its widespread antibiotic resistance (Cobirka *et al.*, 2020). Regarding that biofilm formation by this bacterium is one of the protective ways against the host immune system during chronic and secondary infections (Melchior *et al.*, 2006; Tremblay *et al.*, 2014), inhibition of biofilm formation by antibiofilm components is relevant as a potential therapeutic approach (Jacques *et al.*, 2010). The use of antibiofilm compounds also improves the performance of antibiotics.

The majority of the 50 studied *P. aeruginosa* bovine mastitis isolates (92%) were able to form strong and medium biofilms. This high rate of biofilm formation is very common in human isolates (da Costa Lima *et al.*, 2017), while the study of biofilm formation power in animal isolates has received very little attention (Clutterbuck *et al.*, 2007). The frequency of *P. aeruginosa* isolation in animals has not been well recorded but appears to be increasing (Pye *et al.*, 2013). The results of a study that estima-

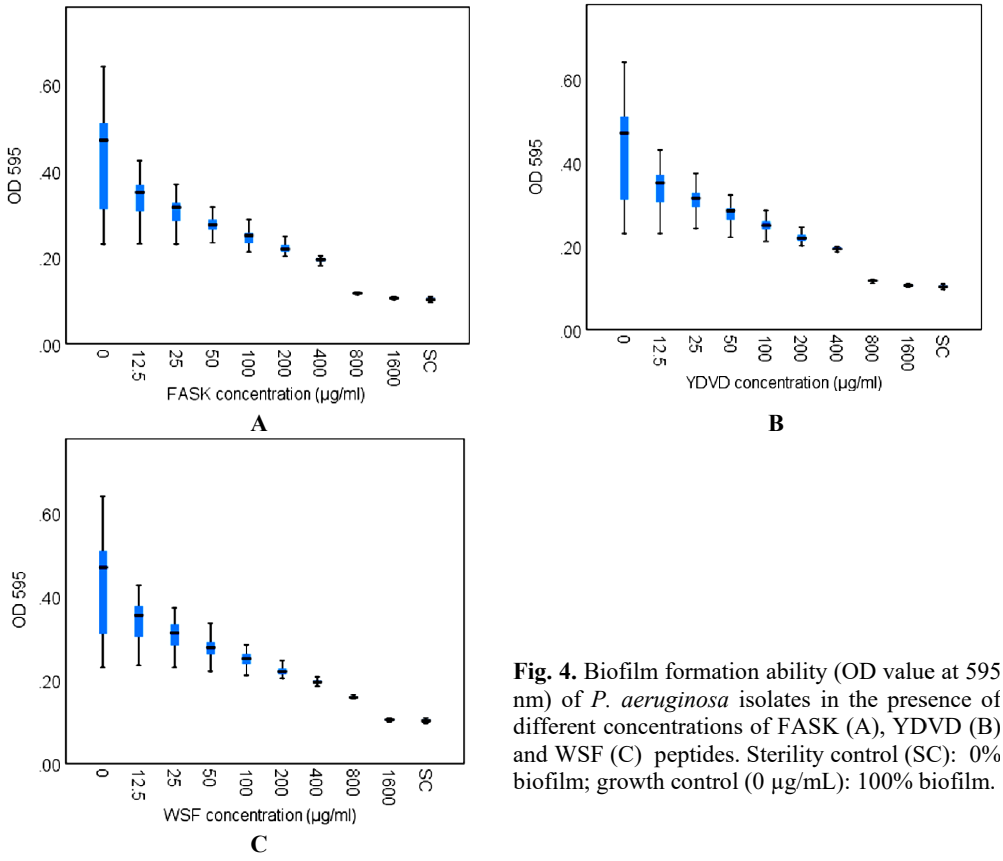

Fig. 4. Biofilm formation ability (OD value at 595 nm) of *P. aeruginosa* isolates in the presence of different concentrations of FASK (A), YDVD (B) and WSF (C) peptides. Sterility control (SC): 0% biofilm; growth control (0 µg/mL): 100% biofilm.

ted the significance of *Pseudomonas* biofilm formation as an indicator of the infection ability showed that 93% of animal isolates were able to form biofilms (Milivojevic *et al.*, 2018) in line with the results of this study.

Three synthetic peptides were evaluated by the microdilution method on *P. aeruginosa* isolates from bovine mastitis in two-fold dilutions (concentrations 1600 µg/mL, 800 µg/mL, 400 µg/mL to 12.5 µg/mL). The MIC rate showed that the three peptides FASK, YDVD, WSF at high concentrations (1600 µg/mL) had extensive antimicrobial activity against the planktonic *P. aeruginosa* forms. Such results are comparable to what has been reported for echinoderm *Holothuria tubulosa* (Schillaci *et al.*, 2013), and the lactoferrin protein *in vitro* (de Andrade *et al.*, 2014). The activity of these peptides at high concentrations may be due to the small sequences and low stability of these compounds. However, in anti-biofilm assays, our results showed that peptides at concentrations of 1/2 MIC, or concentrations that did not kill planktonic bacteria, clearly inhibited the biofilm formation in mastitis isolates. What is more, a complementary treatment can inhibit microbial growth and delay the appearance of chronic infections.

As observed in other studies, a small number of anti-microbial peptides have exhibited bactericidal activity or inhibition of *P. aeruginosa* biofilm formation

(Junker & Clardy, 2007; de la Fuente-Nunez *et al.*, 2012; Lin *et al.*, 2018). Studies have shown that LL-37 prevented the formation of *Pseudomonas* biofilm at a concentration lower than MIC (64 µg/mL), without destroying the bacterial membrane, but its mechanism of action was unclear (de la Fuente *et al.*, 2014).

Based on the research, the probable mechanism of the anti-biofilm peptides is mostly related to its cationic nature. The positive charge of cationic peptides is anticipated to react with the negatively charged biofilm ingredients such as DNA and extracellular polymeric substance resulting in an inhibitory effect on bacterial biofilm (Khan *et al.*, 2020).

Although this study did not illustrate a potential mechanism, further studies could confirm the antimicrobial effect at the genotypic levels, biofilm-producing genes, and quorum sensing system. Additionally, cytotoxicity in eukaryotic cells should be determined.

It is essential to look for new treatment options that are effective in controlling and treating bovine mastitis (Jacques *et al.*, 2010). New efficient antimicrobial agents are needed against *P. aeruginosa* to treat wound infections, and topical usage of this synthetic peptides could be considered. Combining new anti-biofilm agents and common antibiotics can be a promising approach to treating biofilm-associated infections such as mastitis.

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