Journal of Advanced Veterinary Research

https://advetresearch.com

Evaluation of Short Synthetic Antimicrobial Peptides against *Staphylococcus pseudintermedius*

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ARTICLE INFO

Original Research

Received:
04 January 2021

Accepted:
06 March 2021

Keywords:

S. pseudintermedius, antimicrobial peptides, dogs, biofilm, ophthalmic lesions

ABSTRACT

Staphylococcus pseudintermedius infections present a serious challenge because of the emergence of resistance to numerous conventional antibiotics. Antimicrobial peptides are novel alternatives to traditional antibiotics due to their unique mechanism of action. In this study, we investigated the antibacterial activity of 12 short synthetic peptides against two clinical isolates of *S. pseudintermedius* isolated from two dog cases with ophthalmic lesions. In addition, the ability of the peptides to disrupt the established biofilm of these two *S. pseudintermedius* isolates was investigated. RRIKA and 5RHH showed good antimicrobial activity with MICs 2 and 4 µg/ml, respectively. IK8aIID and Indolicidin showed antimicrobial activity with MICs 8 and 16 µg/ml, respectively. Indolicidin, 5RHH and IK8aIID showed a significant biofilm mass % reduction up to 90%. Taken together, these results support the potential use of antimicrobial peptides for the treatment of *S. pseudintermedius* infections.

Introduction

Staphylococcus pseudintermedius has become an animal pathogen of veterinary importance (Grönthal *et al.*, 2014). It is an opportunistic pathogen recognized as the leading cause of skin, ear, and post-operative bacterial infections in dogs and cats (Devriese *et al.*, 2009; Bannoehr and Guardabassi, 2012). Infections caused by *S. pseudintermedius* are difficult to treat due to its resistance to many antimicrobials (van Duijkeren *et al.*, 2011). One of the important virulence factors, which led to *S. pseudintermedius* multi-drug resistance is its ability to form a biofilm (DiCicco *et al.*, 2012). Biofilm forming bacteria surround itself with an extracellular polysaccharide layer, which makes it highly resistant to antimicrobials and difficult to control (Cohn and Middleton, 2010). Biofilm forming strains of *S. pseudintermedius* isolated from canine eye infections often show resistance to many antibiotic classes (Hamed *et al.*, 2017), which has made it increasingly difficult to treat these infections (Eckholm *et al.*, 2013). Therefore, there is an urgent need for alternative antimicrobial treatment.

Antimicrobial peptides (AMPs) have been detected in a wide range of organisms (Jenssen *et al.*, 2006). AMPs have the potential to be used as an alternative therapy in the treatment of multi-drug resistant bacterial infections. AMPs usually bind to teichoic acid of Gram-positive bacteria, leading to the destabilization of the lipid head groups and the production of cell membrane pores, which results in the leakage of cytoplasm and death of the bacteria. Moreover, AMPs can kill bacteria through inhibition of certain molecular synthesis pathways (Brogden, 2005). The high production cost of natural AMPs in addition to its host toxicity led to the development of short synthetic peptides (Mohamed *et al.*, 2016). The efficacy of AMPs has previously been evaluated against *Staphylococcus aureus* (*S. aureus*) in multiple reports (Mohamed *et al.*, 2014a); however, limited studies have been conducted to evaluate their efficacy against *S. pseudintermedius* (Santoro and Maddox, 2014). In this study, we investigated the antimicrobial activity of 12 short synthetic peptides against two isolates of biofilm forming *S. pseudintermedius* obtained from two clinical cases of infected domestic dog eyes.

Materials and methods

Bacterial strains and media

The two isolates of *S. pseudintermedius* included in this

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ISSN: 2090-6277/2090-6269/

study were previously identified by Hamed *et al*. (2017) from specimens collected from two dog cases with history of recurrent bacterial conjunctivitis that were submitted to Purdue University for bacterial culture and sensitivity testing (Hamed *et al*., 2017). The first case: a 10-year-old female neutered Cairn terrier initially presented to the North Central Veterinary Emergency Clinic, Westville, IN, for ophthalmic examination for chronic severe keratoconjunctivitis sicca. The second case: a 5-year-old female neutered Yorkshire terrier was initially presented to Animal Eye Care, Bellingham WA, for ophthalmic examination. Bacterial colonies harvested from conjunctival swabs from both dogs and the grown isolates identified at the Indiana Animal Disease Diagnostic laboratory to the species level using the Vitek 2 (BioMérieux). Identification of the isolated bacterial pathogen via amplification of 16S rRNA gene region of bacterial DNA by conventional PCR resulted in successful identification of *S. pseudintermedius*. The two *S. pseudintermedius* isolates were also tested for their ability to form a biofilm and showed enhanced biofilm formation.

Peptides

Peptides (RRIKA, IK8allD, KFF3K, ll-37, Indolicidin, 5RHH, Apidaecin IB, Buforin ll, Antennapedia, Tat1, Tat2 and Tat3) were synthesized by GenScript (Piscataway, NJ) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and were purified to a purity of 98% using reverse-phase high-performance liquid chromatography (HPLC) (Äkta Explorer; GE Healthcare). Peptide mass was confirmed by mass spectrometry (Applied Biosystems) (Table 1).

Antibiotics

Hamed *et al*. (2017) found that isolate one was susceptible to ticarcillin, ceftiofur, cefazolin, moxifloxacin, amikacin and rifampicin with MICs ranging 0.03125–4 µg/ml. Meanwhile it showed intermediate susceptibility to tobramycin, gatifloxacin, ofloxacin, trimethoprim, polymyxin B, neomycin, tetracycline, and chloramphenicol with MICs ranging 8-32 µg/ml. Meanwhile, isolate two was susceptible to ceftiofur, gatifloxacin, cefazolin, moxifloxacin, tetracycline, doxycycline, and rifampicin with MICs ranging <0.0625–4 µg/ml. Meanwhile it showed intermediate susceptibility to ticarcillin, tobramycin, ofloxacin, polymyxin B, amikacin and chloramphenicol with MICs ranging 8–32 µg/ml. So, Gatifloxacin (15156; CHEM-IMPEX INT'L INC), Ofloxacin (O8757; Sigma) and Amikacin (ALX-380-045; Enzo Life Sciences) was selected as control antibiotics for both isolates.

Antibacterial assays

Minimum inhibitory concentration (MIC) of peptides and antibiotics were evaluated using the broth microdilution technique in Mueller-Hinton broth (MHB) with an initial inoculum of 5 ×10^5 CFU/ml in non-treated Polystyrene microtiter plates (CELLTREAT Scientific Products, 229596) in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2010). The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 16 h of incubation at 37°C. Each agent was tested in triplicate in at least two independent experiments. The highest MIC value was reported.

Efficacy of peptides on bacterial biofilm

The two biofilm-forming clinical isolates of *S. pseudintermedius* were grown overnight in TSB. After incubation, cultures were diluted 1:100 in TSB supplemented with 1% glucose. Diluted bacteria were inoculated into 96-well flat-bottom cell culture plates (polystyrene) and incubated at 37°C for 24 h to permit the formation of an adherent biofilm. The culture medium was subsequently removed, and wells were carefully washed with PBS three times to remove planktonic bacteria before refilling wells with fresh TSB. Peptides and antibiotics were added at different concentrations, and plates were incubated at 37°C for 24 h. After the removal of medium at the end of incubation, wells were rinsed by submerging the entire plate in a tub containing tap water. Biofilms were stained with 0.1% crystal violet for 30 min. After staining, the dye was removed, and the wells were washed with water. The plates were dried for at least 1 h prior to the addition of ethanol (95%) to solubilize the dye bound to the biofilm. The optical density (OD) of biofilms were measured at 595-nm absorbance using a microplate reader (Spectra Max i3x, 363701003). Minimum Biofilm Eradication Concentration (MBEC) was calculated as the minimum concentration of tested antibiotic able to eradicate preformed biofilm.

Statistical analyses

The mean absorbance of crystal violet in biofilm reduction were compared between the groups using the two-tailed Student t test ($P < 0.05$). All statistical analyses were performed using Graph Pad Prism 6.0 (Graph Pad Software, La Jolla, CA).

Results

Antibacterial assays

As shown in Table 2, the two isolates of *S. pseudintermedius* were resistant to Buforin II, Apidaecin IB, Antennapedia, Tat1, Tat2, KFF3K and II-37 peptides with MIC ranged from 32 to >128 µg/ml. Moreover, isolate 2 showed resistance to

Table 1. Amino acid sequence and physicochemical properties of peptides used in this study.

Peptide designation	Amino acid sequence	Length	Molecular weight
ll-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	4493.28
RRIKA	WLRRIKAWLRRIKA	14	1866.32
KFF3K	KFFKFFKFFK	10	1413.7
IK8 all D	IRIKIRIK	8	1039.37
indolicidin	ILPWKWPWWPWRR	13	1907.28
5RHH	VLTTGLPALISWIRRRHRRHC	21	2542.04
Apidaecin IB	GNNRPVYIPQPRPPHPRL	18	2108.42
Buforin ll	TRSSRAGLQFPVGRVHRLLRK	21	2434.86
Antennapedia	RQIKIWFQNRRMKWKK	16	2246.74
Tat1	GRKKKRRQRRRYK	13	1816.18
Tat2	XRRXRRXRRXRXB	13	1748.21
Tat3	RFRRFRRFRRFRXB	14	2040.44

Tat3 peptide with 32 µg/ml MIC. Meanwhile, the two isolates were susceptible to 5RHH, Indolicidin, RRIKA and IK8aIID peptides with MIC ranged from 2 – 16 µg/ml. Biofilm MICs of both isolates with the active peptides ranged from 32 - >128 µg/ml. However, the minimum biofilm eradication concentration (MBEC) of these isolates with all active peptides was 128 µg/ml or more.

Efficacy of peptides on bacterial biofilm

At 1X MIC, only Indolicidin showed significant reduction in biofilm mass % of Isolate 1 by more than 50%, while peptides 5RHH and RRIKA showed significant reduction in biofilm mass % of Isolate 2 by 60 – 70%.

At 2X MIC, Indolicidin, 5RHH and IK8aIID peptides showed significant reduction in biofilm mass % of Isolate 1 by 40 -60 %. While Indolicidin, 5RHH and RRIKA showed significant reduction in biofilm mass % of Isolate 2 by 67 -86 %.

At 4X MIC, all peptides showed significant reduction in biofilm mass % of both isolates by 47 -68 % for isolate 1 and 76 – 91 % for isolate 2 (Fig. 1).

Only gatifloxacin showed significant reduction in biofilm mass % of both isolates at 1X, 2X and 4X MIC, while amikacin and ofloxacin did not show any significant reduction in biofilm mass % except for isolate 2 at 4X MIC (Fig. 2).

Discussion

Antimicrobial resistance is rapidly developing within *S. pseudintermedius*, this is particularly prevalent within clinically identified biofilm producing isolates, thus alternative treatment approaches are urgently needed. AMPs have been identified as a possible solution, however only a few reports have shown antimicrobial activity against *S. pseudintermedius* (Fazakerley *et al*., 2010; Santoro and Maddox, 2014). Twelve short synthetic peptides were selected for the study to investigate their ability to inhibit the two clinical isolates of *S. pseudintermedius* isolated from two dog cases with ophthalmic lesions by Hamed *et al*. (2017).

Peptides RRIKA and 5RHH have shown good antimicrobial activity against both isolates of *S. pseudintermedius* in the planktonic form with MICs 2 and 4 µg/ml respectively. IK8aIID and Indolicidin have also displayed antimicrobial activity against both isolates, with MICs 8 and 16 µg/ml respectively. These finding were similar to Mohamed *et al*. (2014b) who found that RRIKA had antimicrobial activity against clinical isolates of *S. pseudintermedius* with MIC 1 µM. Additionally, previous studies of IK8aIID have shown activity against *S. aureus* (Deslouches *et al*., 2013; Ong *et al*., 2014), and potent activity against MRSA (Ong *et al*., 2014). The remaining 8 peptides (Buforin II, Apidaecin IB, Antennapedia, Tat1, Tat2, Tat3, KFF3K

Table 2. MIC of bacteria and biofilm (µg/ml), and MBEC of biofilm (µg/ml) of the two isolates of *S. pseudintermedius* against peptides and antibiotics.

	Isolate 1			Isolate 2		
	MIC of bacteria	MIC of Biofilm	MBEC of Biofilm	MIC of bacteria	MIC of Biofilm	MBEC of Biofilm
Buforin ll	>128	>128	-	>128	>128	-
5RHH	2	64	128	4	128	128
Apidaecin IB	>128	>128	-	>128	>128	-
Indolicidin	16	32	128	16	64	128
RRIKA	2	128	-	4	64	>128
Antennapedia	64	>128	-	64	>128	-
Tat1	>128	>128	-	>128	>128	-
Tat2	>128	>128	-	128	>128	-
Tat3	2	128	-	32	128	-
KFF3K	32	128	-	32	128	-
IK8allD	8	64	>128	8	64	>128
WR-12	<1	>128	-	<1	>128	-
II-37	>128	>128	-	>128	>128	-
Gatifloxacin	8	64	>128	4	64	>128
Ofloxacin	32	128	>128	16	32	>128
Amikacin	2	>128	>128	8	8	>128

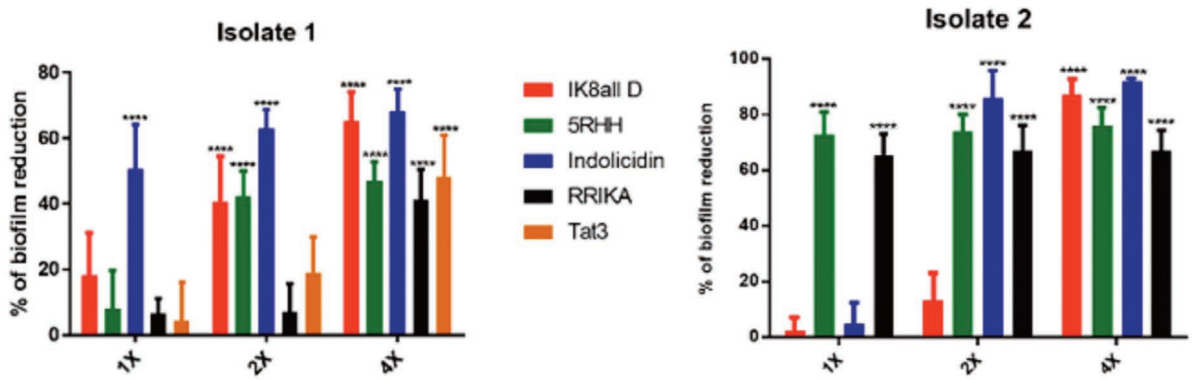

Fig. 1. The effect of active peptides (IK8aII D, 5RHH, Indolicidin, RRIKA and Tat3) on established biofilms of the two isolates of *S. pseudintermedius* with 1X, 2X and 4X MIC. The adherent biofilm was stained by crystal violet, and then the dye was extracted with ethanol, measured at a 595-nm absorbance, and percentage of biofilm reduction compared to untreated wells ("control"). All experiments were done in triplicate for statistical significance.

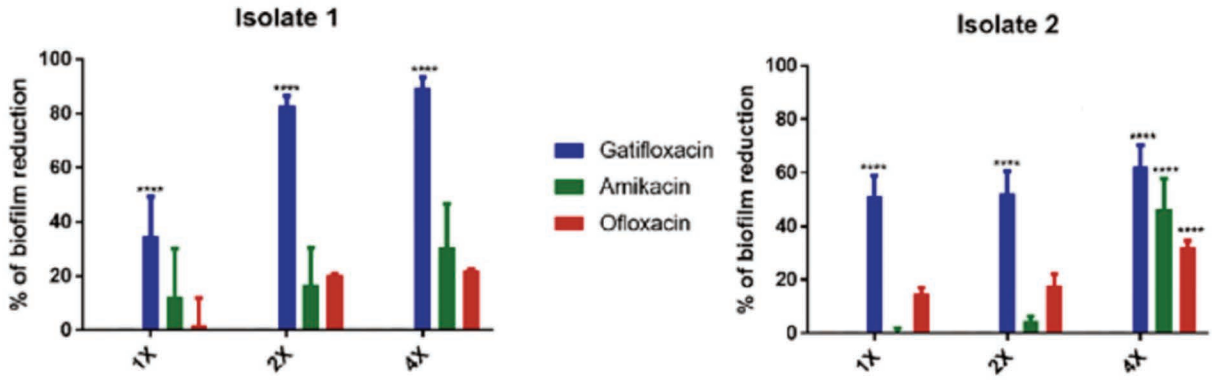

Fig. 2. The effect of control antibiotics (Gatifloxacin, Amikacin and Ofloxacin) on established biofilms of the two isolates of *S. pseudintermedius* with 1X, 2X and 4X MIC. The adherent biofilm was stained by crystal violet, and then the dye was extracted with ethanol, measured at a 595-nm absorbance, and percentage of biofilm reduction compared to untreated wells ("control"). All experiments were done in triplicate for statistical significance.

and II-37) were inactive against Isolate 1. Isolate 2 was also resistant to the remaining peptides with exception of Tat3. Tat3 displayed a good level of antimicrobial activity with MIC of 2 µg/ml. Active peptides with good antimicrobial activity against *S. pseudintermedius* in the planktonic form showed high Biofilm MICs and MBECs, which ranged from 32- >128 µg/ml.

Bacterial biofilm usually protects the bacteria against the host defense mechanisms and prevents antimicrobial drugs penetration, resulting in the failure of treatment (Mohamed *et al*., 2014a). Moreover, the biofilm acts as an infectious recess with continuous release of bacteria which develops into chronic infection (Archer *et al*., 2011). The ability of active peptides (IK8aIID, 5RHH, Indolicidin, RRIKA and Tat3) to disrupt the biofilm of both isolates of *S. pseudintermedius* at 1x, 2x and 4x MIC was investigated. Indolicidin, 5RHH and IK8aIID displayed a significant biofilm mass % reduction of up to 60% in Isolate 1, with a 2x MIC. Indolicidin, 5RHH and RRIKA demonstrated a significant reduction in biofilm mass % of up to 86% in Isolate 2 with 2x MIC. Furthermore, all peptides displayed up to a 90% reduction in biofilm mass % of both isolates with 4x MIC. The peptides in this study demonstrated a superior reduction in biofilm mass % when compared to the control antibiotics (amikacin and ofloxacin). This is potentially due to the amphipathic nature of the peptides and their high cationic charge, which facilitates their penetration to biofilm matrix (Mohamed *et al*., 2016).

Conclusion

IK8aIID, 5RHH, Indolicidin, RRIKA and Tat3 peptides significantly disrupted established in vitro biofilms of *S. pseudintermedius* which support the potential use of these peptides for treatment of infections caused by this bacterium.

Conflict of interest

No competing financial interests exist

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