ORIGINAL ARTICLE INFECTIOUS DISEASES

Enhanced antimicrobial activity of peptide-cocktails against common bacterial contaminants of *ex vivo* stored platelets

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Abstract

Bacterial contamination of blood components such as *ex vivo*-stored platelets is a major safety risk in transfusion medicine. We have recently shown that synthetic antimicrobial peptides named PD1–PD4 derived from the thrombin-induced human platelet-derived antimicrobial proteins, and repeats of Arg-Trp (RW1–RW5) demonstrate microbicidal activity against selected bacteria and viruses. In the present study, we selected PD3, PD4, RW2, RW3 and RW4 and evaluated each individual peptide and their various combinations to see whether the cocktail regimen enhances the antimicrobial activity above and over the individual peptides. Stored platelet or plasma samples spiked with known titres of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus cereus* were treated with either individual peptides or with peptides in various combinations. Analyses revealed that individual peptides show moderate microbicidal activity (10- to 100-fold reduction) against the tested bacteria relative to their combined regimen. The peptide combinations (RW2 + RW4, RW2 + RW3 + RW4 and PD4 + RW3 + RW4) on the other hand enhanced the microbicidal activity (*c.*10 000-fold reduction) and revealed a minimal inhibitory concentration of 5 μM. Time-kill kinetics indicated that these three peptide combinations exhibited enhanced antimicrobial activity bringing about a 100-fold reduction of bacterial titres within 20 min of incubation. The present study therefore demonstrates the synergistic effect of antimicrobial peptides when used in combinations and provides a proof-of-concept of its potential application as a molecular tool towards pathogen reduction and further extends the possibility of using peptide combinatorial therapeutics as broad-spectrum antibiotics or as alternatives to combat drug-resistant bacteria.

Keywords: Antimicrobial peptides, bactericidal, colony-forming units, pathogen reduction, platelet-derived peptides
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Introduction

Antibiotic misuse and overuse have led to the emergence of multiple antibiotic-resistant strains [1], which necessitated the need for new classes of antimicrobial agents. Natural and synthetic antimicrobial peptides (AMPs) are gaining considerable attention because of their novel drug targets and the low frequency of developing resistance to the AMPs [2,3]. These peptides are 8–50 amino acids in length and mostly have positive net charge, though anionic AMPs also exist [3–5]. The exact mechanism of action of AMPs has not been fully understood. However, extensive studies on the mode of action of cationic AMPs have shown that positively charged peptides interact with negatively charged membranes, thereby causing increased permeability of the membrane and eventually leading to bacterial cell death [3–5]. Development of resistance to AMPs may occur, but to a lesser degree as it may require alterations in the lipid composition of the membrane of microorganisms [6].

Naturally occurring AMPs are components of the innate defence mechanism of most life forms [6–8]. Cathelicidins and

defensins are the best-characterized peptides among the host defence peptides [7–9]. Platelets liberate platelet microbicidal proteins (PMPs) when activated, thereby contributing to the host defence against infection. PMPs from thrombin-induced rabbit, and human platelets, also called the kinocidins (hPF-4), were isolated and characterized and were shown to have potent microbicidal activity against pathogens of the bloodstream, such as *Staphylococcus aureus*, *Streptococcus viridans*, *Escherichia coli*, *Candida albicans* and *Cryptococcus neoformans* [10–16].

Short synthetic peptides containing Arg (R) and Trp (W) residues have been shown to display significant antimicrobial activity and low cytotoxic activity [17–19]. Longer chains of RW repeats are effective against both gram-positive and gram-negative bacteria though the peptide chain length increases the haemolytic activity. Positively charged Arg residue interacts with the bacterial membrane through electrostatic attraction whereas the non-polar Trp residue interacts with the lipid bilayer through hydrophobic interactions. These two activities in a single peptide aid the peptide to interact with the bacterial membrane, which ultimately results in membrane destabilization and pore formation, leading to bacterial cell death [17–20].

Contamination of *ex vivo* stored platelets in bags at room temperature mostly by bacteria, and rarely by viruses, and parasites is a safety issue in transfusion medicine. Development of methods to reduce the growth of medically important bacteria in platelets would enhance the safety of these transfusion products. We have recently demonstrated that peptides derived from thrombin-induced human platelet antimicrobial proteins (PD1–PD4) and Arg-Trp (RW1–RW5) repeats exhibit microbicidal activity against bacteria and vaccinia virus in plasma and in platelet concentrates [21,22]. In this study we have selected PD3, PD4, RW2, RW3 and RW4 peptides and tested their efficacy either individually or in combinations to see whether the combined regimen enhances the antimicrobial activity of these peptides.

Materials and Methods

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 25923, *Staphylococcus epidermidis* ATCC 35983, *E. coli* ATCC 700928, *Pseudomonas aeruginosa* ATCC 12121, *Klebsiella pneumoniae* ATCC 10031 and *Bacillus cereus* ATCC 11778 were used in this study. All bacterial strains were grown routinely at 37°C on nutrient agar or Luria–Bertani (LB) broth (Mediatech Inc, Herndon, VA, USA). For long-term storage, all strains were stored in tryptic soy broth with 10% glycerol at −80°C.

Peptide synthesis

Antimicrobial peptides described in this study were synthesized at the Center for Biologics Evaluation and Research, the Food and Drug Administration core facility, as previously described [21,22]. PMP-1 consensus sequence-derived peptides (PD3 and PD4) were 15 amino acids in length and Arg-Trp peptides (RW2, RW3 and RW4) were two to four repeats of Arg-Trp amino acids (Table 1) [21,22]. Peptides were all reconstituted in PBS, pH 7.4 (Invitrogen, Carlsbad, CA, USA) and stock solutions were made to 10 mM concentration in the same buffer.

Antibacterial assays

The test bacteria mentioned in the Materials and Methods were grown in LB broth to mid-logarithmic phase and centrifuged at 3000 **g** and resuspended in 1 × PBS. Human plasma and platelet samples were acquired from the National Institutes of Health Blood Bank (Bethesda, MD, USA). All antibacterial assays were performed as described previously with slight modifications [21–24]. Approximately 10^6 CFU/mL (0.1 mL) of each bacterial strain was spiked into 0.9 mL of plasma or platelets and incubated with each PD and RW peptide individually or in different combination cocktails as shown in Table 2. The treated samples and controls were all incubated at room temperature for 90 min on an orbital shaker. At the end of the 90-min exposure period, 0.1 mL of the suspension was plated on nutrient agar plates and the colonies were counted after 18–24 h of incubation. Bactericidal activity of the peptides and their combinations were measured by log-reduction by viable bacteria. Experiments were repeated at least five times.

Minimal inhibitory concentration analyses

Mid-logarithmic phase cultures of bacteria grown in LB broth were centrifuged at 3000 **g** and resuspended in 1 × PBS [21–24]. Approximately 10^6 CFU/mL (50 μL) of each bacterial strain was incubated with 50 μL of serial, double-fold dilutions of the four different combination cocktails: RW2 + RW4, RW2 + RW3 + RW4, PD3 + RW3 + RW4 and PD4 + RW3 + RW4. The peptide serial dilutions were calibrated such that upon adding the bacterial suspension the final concentration of

TABLE 1. Sequence of antimicrobial PD and RW peptides used in the study

S. No.	Peptide	Sequence
1	PD3	KNGRKLCLDLQAALY
2	PD4	AALYKKKIIKKLLES
3	RW2	RWRW
4	RW3	RWRWRW
5	RW4	RWRWRWRW

TABLE 2. Various combinations of peptides that were tested for antibacterial activity

S. No.	Peptides and combinations
1	Control (bacteria without peptides)
2	PD3
3	PD4
4	RW2
5	RW3
6	RW4
7	PD3 + PD4
8	RW2 + RW3
9	RW3 + RW4
10	RW2 + RW4
11	RW2 + RW3 + RW4
12	PD3 + RW3
13	PD3 + RW4
14	PD4 + RW3
15	PD4 + RW4
16	PD3 + RW3 + RW4
17	PD4 + RW3 + RW4

each peptide combination in the 0.1-mL sample series were 10 μM, 5 μM, 2.5 μM, 1.25 μM and 0.6 μM. Bacterial titres were estimated at the end of incubation by colony counting as described above.

Time-kill assay

Kinetics of the bactericidal activity of the peptides were evaluated both for single peptides and for the combinations using the time-kill assay on two bacterial species, *S. aureus* and *E. coli* representing gram-positive and gram-negative bacteria [23–26]. These two bacterial cultures were treated with either the individual peptides or combinations of PD4, RW2, RW3 and RW4 peptides at 10 μM concentrations. Samples were collected and plated at 0, 20 min, 60 min, 90 min and 120 min of incubation at 37°C. Positive controls for the assay included ampicillin for *S. aureus* and Polymyxin B for *E. coli*. Bacterial cultures without any peptide were included as negative controls.

Transmission electron microscopy

As above, the same two bacterial species, *S. aureus* and *E. coli*, were selected for this study. Log-phase cultures of bacteria were centrifuged and the bacterial pellet was washed three times and resuspended in PBS. Ten-fold serial dilutions of the *S. aureus* and *E. coli* cultures were incubated with a 10 μM concentration of each peptide or PBS for 1 h. The bacteria–peptide complex was washed three times with PBS and the samples were fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS, pH 7.3 for 3–24 h. The samples were then further fixed in 1% osmium tetroxide in PBS. Finally, the samples were dehydrated before embedding in resin. After microtomy, the samples were transferred to copper grids and stained with uranyl acetate and lead citrate. Sections were observed by using a Zeiss 912 transmission electron microscope and images were captured digitally. Test samples without any peptides were included as controls.

Statistics

Assays described here were performed at least four times independently. Mean values ± standard deviation (SD) was calculated using Microsoft Excel®. Statistical analyses were performed using Student's *t* test and values were considered significant when $p < 0.05$.

Results

Peptides in combination exhibit enhanced microbicidal activity

Analyses of PD3, PD4, RW2, RW3 and RW4 peptides both individually and in various combinations against bacteria spiked in plasma revealed that individually each peptide was able to bring about a 10- to 100-fold reduction in bacterial titres. Comparatively the RW peptides exhibited a higher antimicrobial activity than the PD peptides (Fig. 1). Among the various combinations, the RW2 + RW4, RW2 + RW3 + RW4, PD3 + RW3 + RW4 and PD4 + RW3 + RW4 were the most potent ones in bringing about ≥1000-fold reduction in bacterial titres and in some cases almost about a 10 000-fold reduction. A distinct variability in action of the combined peptides against the different bacteria was also observed (Fig. 1).

Additional evaluation of antimicrobial activity of the above peptides in spiked platelets revealed that the peptides were equally active in this biological matrix. The peptide combinations, RW2 + RW4, RW2 + RW3 + RW4, PD3 + RW3 + RW4 and PD4 + RW3 + RW4 demonstrated similar antimicrobial activities to that observed in the case of spiked plasma, with a 1000- to 10 000-fold reduction in bacterial titres. Though the antimicrobial activity in spiked-platelets was slightly lower than the peptide activity in plasma, the difference was not significant.

Four most effective peptide combinations against all tested bacteria

None of the PD and RW peptides were individually active in affecting a ≥1000-fold reduction in bacterial titres against all six tested bacteria, so we tested whether this could be achieved by combining one or more peptides in cocktail combinations. Our analyses revealed that of the 11 different cocktail combinations, four peptide combinations: (i) RW2 + RW4, (ii) RW2 + RW3 + RW4, (iii) PD3 + RW3 + RW4 and (iv) PD4 + RW3 + RW4, were the most potent in bringing about a 1000- to 10 000-fold reduction of bacterial titres (Fig. 2). In addition there were two other peptide combinations (RW2 + RW3 and RW3 + RW4) that were effective in bringing about at least a 10- to 10 000-fold reduction against

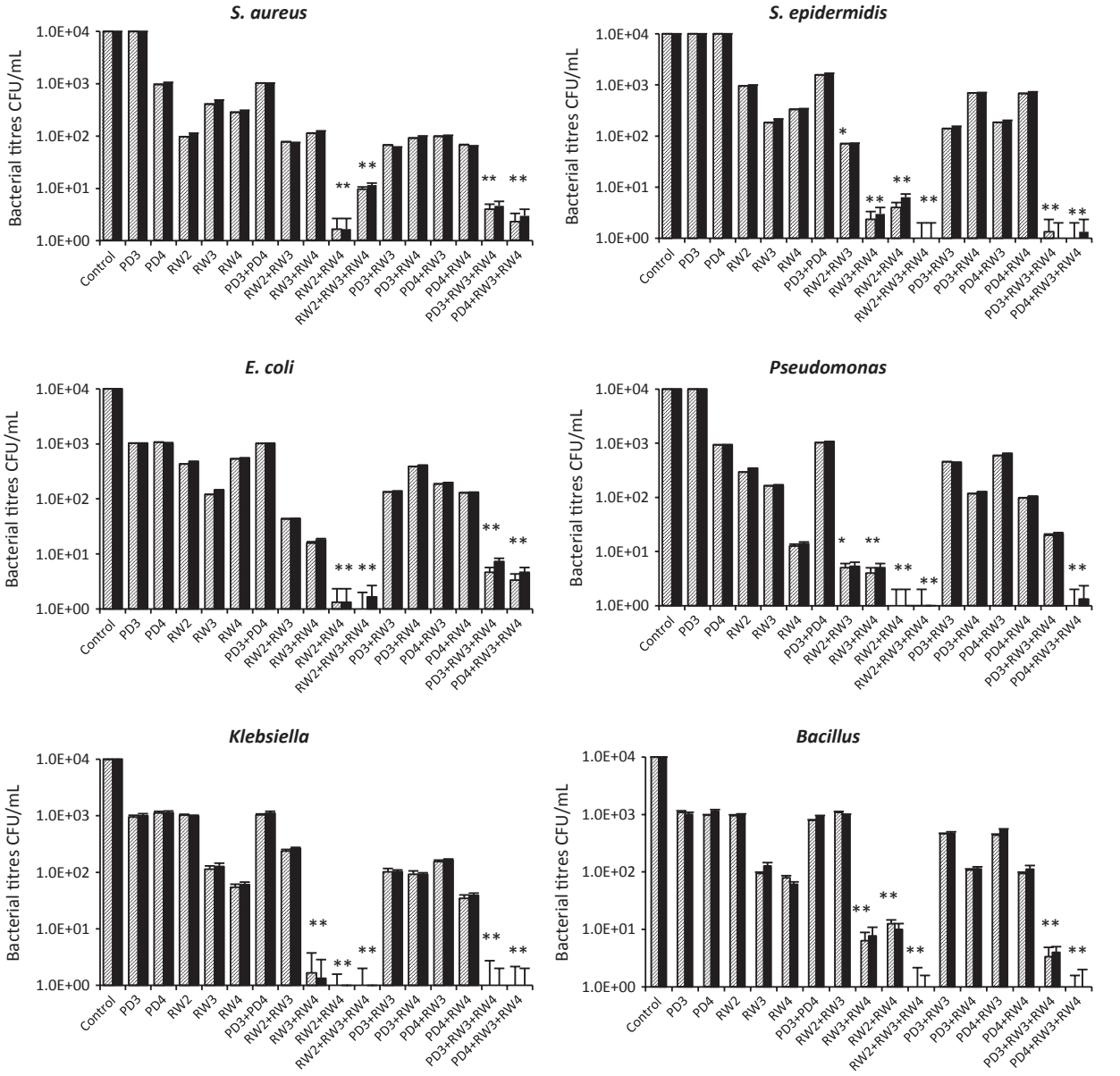

FIG. 1. Illustration of antimicrobial activity of PD and RW peptides and their combinations. Plasma (striped columns) and platelet (filled columns) samples were spiked with six different medically important bacteria and co-incubated with either PD or RW peptides individually or in combinations at 10 μM concentrations. Bacterial titres were estimated by plating a fixed volume of the incubation mix and performing a colony count. Samples without any peptides but spiked with individual bacterial strain were treated as controls. The peptide combinations demonstrate enhanced microbicidal activity against most of the bacteria tested in both plasma and platelet samples. Results are presented as mean ± SD. (**p <0.0001, *p <0.001).

the six tested bacteria (Fig. 2). Certain combinations, such as PD3 with RW3 or RW4 and PD4 with RW3 or RW4 did not exhibit any significant increase in microbicidal activity when compared with individual activity of the peptides (Fig. 2).

Combined peptides exhibit a MIC of 5 μM

Once it was evident that the peptide combinations RW2 + RW4, RW2 + RW3 + RW4, PD3 + RW3 + RW4 and PD4 + RW3 + RW4 were the most potent, we evaluated the MIC of these combinations that would bring about at least a ≥1000-fold reduction of all the six tested bacteria. Serial, double-fold dilutions of the cocktail peptide combinations were prepared (range: 10–0.6 μM) and tested against all six bacteria. Our analysis revealed that of the four tested peptide combinations, three: (i) RW2 + RW4, (ii) RW2 + RW3 + RW4, and (iii) PD4 + RW3 + RW4, were effective even at

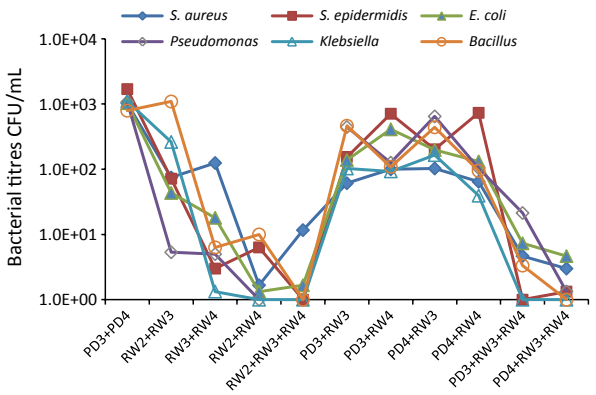

FIG. 2. Analyses of antimicrobial activity of combinatorial peptides against various bacteria. The most potent combinations are RW2 + RW4, RW2 + RW3 + RW4, PD3 + RW3 + RW4 and PD4 + RW3 + RW4 exhibiting a 1000- to 10 000-fold reduction of bacterial titres. Also note two other peptide combinations (RW2 + RW3 and RW3 + RW4) that effectively resulted in a 10- to 10 000-fold reduction of the six tested bacteria.

5 μM concentration and brought about a ≥1000-fold reduction of all six tested bacteria (Fig. 3). The PD3 + RW3 + RW4 combination at 5 μM concentration was effective in bringing about only a 2-log reduction. Lower dilutions of the various combinations on the other hand were selectively effective against some bacteria and resulted in *c.*10- to 1000-fold reduction of bacterial titres (Fig. 3).

Time-kill kinetics reveals a rapid killing potential of the peptide combinations

The results so far indicated that the combined peptides were active in both plasma and platelets with three of the combinations being most potent with an MIC of 5 μM. Hence, it was critical to further evaluate the rapidity at which these peptide combinations brought about the microbicidal effects; for this reason the time-kill assay was performed at various time points between 0 and 120 min of peptide treatment on two selected bacterial species, *S. aureus* and *E. coli* representing gram-positive and gram-negative bacteria, respectively. Findings from this assay revealed that all three peptide combinations were effective the instant they were added to the bacterial culture resulting in *c.*ten-fold reduction in bacterial numbers of both *S. aureus* and *E. coli* and, by 20 min the combined treatment resulted in a 100-fold reduction of bacterial titres (Fig. 4). By the end of 90-min incubation, the three peptide combinations effectively resulted in *c.*1000- to 10 000-fold reduction of bacterial titres. Interestingly, individual peptide treatments took 60–90 min to bring about a *c.*10- to 100-fold reduction in numbers of *S. aureus* and *E. coli* (Fig. 4).

Electron microscopy reveals structural changes in peptide-treated bacteria

To evaluate structural changes elicited by the peptides on bacteria two bacterial species were selected, *S. aureus* and *E. coli* representing gram-positive and gram-negative bacteria, and treated individually with PD4, RW2, RW3 or RW4 peptides. Samples were analysed using a Zeiss 912 transmission electron microscope and images were captured. Results indicated that all four peptides were able to bring about a multitude of structural changes in the treated bacteria (Fig. 5). PD4 treatment resulted in loss of structural integrity, ballooning of cells, loss of cytoplasmic content and cytoplasmic granulation (Fig. 5). Similarly, treatment with RW2, RW3 or RW4 resulted in all of the above cytopathic effects both in *S. aureus* and *E. coli*. Additionally, certain bacterial cells also exhibited formation of bulbar structures or protrusions from the cell surface (Fig. 5).

Discussion

We had previously shown that PD and RW peptides exhibit antimicrobial properties against bacteria and vaccinia virus spiked in blood components such as plasma and platelets [21,22]. Our previous findings indicated that there was no single peptide that was active against all bacteria in bringing about a ≥1000-fold reduction in bacterial titres [21,22]. Hence, we theorized that perhaps by combining these individual peptides, as a cocktail, an additive or synergistic effect could be imparted to the cocktail mixture that could then have a broad-spectrum antimicrobial effect and bring about a significant reduction in bacterial titres. In the present study we demonstrate that by testing 11 different peptide combinations of PD3, PD4, RW2, RW3 and RW4 we were able to identify the three most potent peptide combinations as RW2 + RW4, RW2 + RW3 + RW4 and PD4 + RW3 + RW4. These three cocktails were able to bring about *c.*10 000-fold reduction in the bacterial titres of all six bacteria tested, which was at least 10- to 100-fold higher than the action of individual peptides acting alone.

Combination therapy, which uses a mixture of two drugs, has been extensively used to treat many infectious diseases [8,27]. More recently, experimental evidence is gaining importance with regards to combination therapy in controlling bacterial infections as well [23,25,28,29]. So far, there have been a few reports that demonstrate the efficacy of combi-

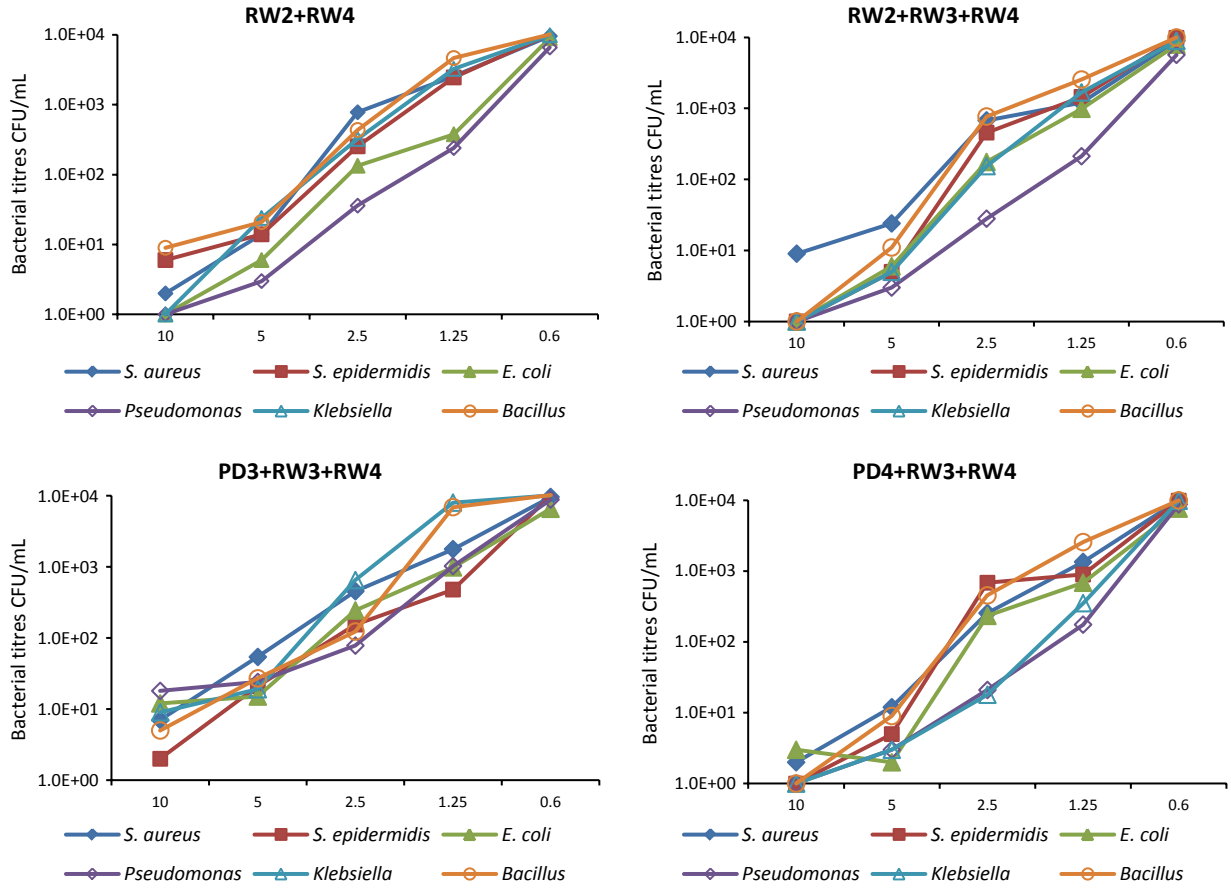

FIG. 3. Demonstration of MIC of four selected peptide combinations based on their potent action. Serial two-fold dilutions of the peptide combinations were carried out with final concentration ranging among 10, 5, 2.5, 1.25 and 0.6 μM and incubated with the respective bacteria. As illustrated in the figure, peptide combinations RW2 + RW4, RW2 + RW3 + RW4, and PD4 + RW3 + RW4 were effective even at 5 μM concentration and brought about a $\geq$1000-fold reduction of all six tested bacteria. The PD3 + RW3 + RW4 combination, however, was effective in bringing about only a 2-log reduction at 5 μM concentration.

nation therapy using AMPs in combination with a conventional antibiotic [23,27,28,30]. Such studies have clearly demonstrated the enhanced effectiveness of the combined therapy over single peptide or drug treatment especially for combating drug-resistant bacterial strains or other hard-to-treat scenarios such as persister cells [23,25,27,30]. Chen *et al.* [30] had elegantly demonstrated that by combining peptides RW2, RW3 and RW4 with ampicillin or ofloxacin, a synergistic increase in microbicidal activity against persister cells in biofilms was achieved. Furthermore, it was observed that even if the peptides did not directly kill the bacteria, the peptide treatment itself increased the susceptibility of the bacteria to both antibiotics. Similarly, other studies have reported synergistic effects of AMPs that enhance or potentiate susceptibility of microorganisms that were resistant to a previously known drug or antibiotic. Findings from these studies included *Pseudomonas* susceptibility to multiple antibiotics, increase in therapeutic efficacy of miltefosine for treating experimental leishmaniasis or enhanced synergistic effect of AMPs against gram-positive bacteria [23,27,28]. The present study provides further advancement of or an alternative to the above scenarios and suggests that by combining various AMPs as a mode of treatment, the microbicidal potential of some of the individual peptides could be enhanced. An additional advantage of combinatorial therapy is the lower minimal inhibitory concentration or the lower dose requirement of drug/peptides to bring about a significant treatment effect.

Our findings also present for the first time visual evidence of the membrane-damaging effects of PD and RW peptides on bacteria such as *S. aureus* and *E. coli* by means of transmission electron microscopy. Though the cytopathic effects of PD and RW peptides are not peptide-specific, they do indicate a broader mode of action that encompasses effects such as loss of structural integrity, ballooning of cells, loss of cytoplasmic content, cytoplasmic granulation, and formation of bulbar structures or protrusions from the cell surface of bacteria.

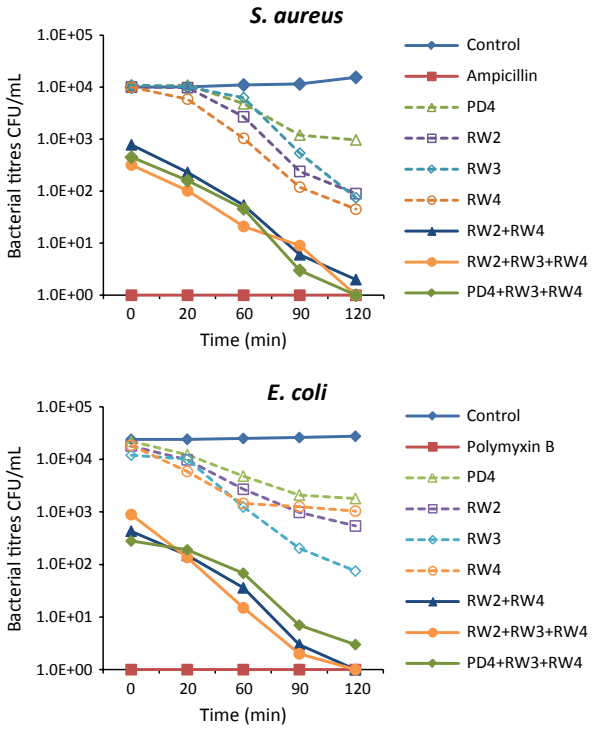

FIG. 4. Illustration of time-kill kinetics of three most potent peptide combinations. Log-phase cultures of two bacterial species, *Staphylococcus aureus* and *Escherichia coli* representing gram-positive and gram-negative bacteria, were treated with peptide combinations at a concentration of 10 μM and bacterial numbers were estimated at time-points 0–120 min. As observed in the figure the peptide combinations exhibited enhanced antimicrobial activity resulting in a 100- and 1000-fold reduction of bacterial titres within 20 and 60 min of incubation, respectively.

Antimicrobial peptides have been reported to control infections in various biological situations. Attempting to further extend the varied application of AMPs the present study demonstrates a proof-of-concept of the potential applicability of these AMPs as molecular tools for pathogen reduction in stored blood components against common bacterial contaminants. This is a first step in moving towards preclinical evaluation of these peptides and their combinations in an appropriate small animal model.

In conclusion, our findings demonstrate that by combining the PD and RW peptides the following advantages can be clearly achieved: (i) a broad-spectrum antimicrobial activity, (ii) increased microbicidal activity in terms of fold reduction of bacteria, (iii) lower MIC of the cocktail AMPs, and (iv) much more rapid time-kill kinetics. Using our studies as a model, similar synergistic AMP combinations may be designed using other novel peptides and/or antibiotics to test difficult-to-treat situations. Such combinations may result in the discovery of more potent combinations of drugs that exhibit a broad-spectrum

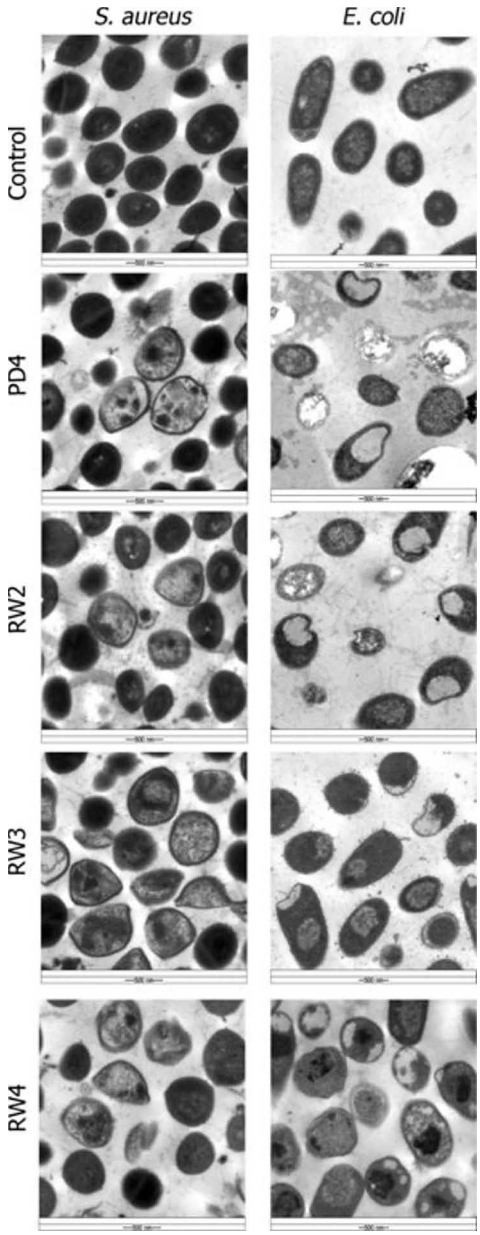

FIG. 5. Transmission electron microscopy to illustrate the effects of peptide treatment on bacteria. Structural changes elicited by the peptides on bacteria were evaluated on two bacterial species, *Staphylococcus aureus* and *Escherichia coli* representing gram-positive and gram-negative bacteria that were treated individually with PD4, RW2, RW3 or RW4 peptides. Controls included bacteria without peptide treatment and electron microscopy analysis reveals a normal morphology and structural integrity of both *Staphylococcus aureus* and *Escherichia coli* cells. Peptide treatment on the other hand revealed a multitude of structural changes in the treated bacteria such as loss of structural integrity, ballooning of cells, loss of cytoplasmic content, and cytoplasmic granulation. Certain bacterial cells also exhibited formation of bulbar structures or protrusions from the cell surface. Scale: 500 nm.

activity not just against bacteria but also against viruses, fungi and parasites.

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Authors Contribution

KVM conceived and designed the study; KVM, SSR and YG performed experiments and collected data; KVM, SSR, and YG analysed data; KVM, SSR and CDA wrote the manuscript.

Transparency Declaration

The authors have no conflict of interest to declare.

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