AAC Accepts, published online ahead of print on 5 May 2014
Antimicrob. Agents Chemother. doi:10.1128/AAC.02578-14

Targeting methicillin-resistant *Staphylococcus aureus* with short salt-resistant synthetic peptides

Mohamed F. Mohamed[1], Maha I. Hamed [1], Alyssa Panitch [2], Mohamed N. Seleem[1] #

Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, USA[1]

Weldon School of Biomedical Engineering, Purdue University, West Lafayette, USA[2]

Running title: Targeting methicillin resistant-*Staphylococcus aureus*

Key words: antimicrobial peptides, MRSA, VRSA, biofilm, lysostaphin

 # Corresponding Author:

Mohamed N. Seleem

Department of Comparative Pathobiology

Purdue University College of Veterinary Medicine

625 Harrison Street, Lynn 1298, West Lafayette, IN 47907-2027

Phone: 765-494-0763 Fax: 765-496-2627

Email: mseleem@purdue.edu

Abstract:

The seriousness of microbial resistance combined with the lack of new antimicrobials have increased the interest in the development of antimicrobial peptides (AMPs) as novel therapeutics. In this study, we evaluated the antimicrobial activity of two short synthetic peptides, namely RRIKA and RR. These peptides exhibited potent antimicrobial activity against *Staphylococcus aureus* and their antimicrobial effects were significantly enhanced by addition of three amino acids in the C terminus, which consequently increased the amphipathicity, hydrophobicity and net charge. Moreover, RRIKA and RR demonstrated a significant and rapid bactericidal effect against clinical and drug-resistant *Staphylococcus* isolates including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), linezolid-resistant *S. aureus* and methicillin-resistant *S. epidermidis*. In contrast to many natural AMPs; RRIKA and RR retained their activity in the presence of physiological concentrations of NaCl and $MgCl_2$. Both RRIKA and RR enhanced the killing of lysostaphin over 1000-fold and eradicated MRSA and VRSA isolates within 20 minutes. Furthermore, the peptides presented were superior in reducing adherent biofilms of *S. aureus* and *S. epidermidis* when compared to conventional antibiotics. Our findings indicate that the staphylocidal effects of our peptides were through permeabilization of the bacterial membrane, leading to leakage of cytoplasmic contents and cell death. Furthermore, peptides were not toxic to HeLa cells at 4 to 8 fold their antimicrobial concentrations. The potent and salt-insensitive antimicrobial activities of these peptides present an attractive therapeutic candidate for treatment of multidrug-resistant *S. aureus* infections.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become a major public health concern in both hospital and community settings (1, 2). Since the late 1990s, community-associated MRSA (CA-MRSA) emerged as a principal cause of skin and soft-tissue epidemics throughout the world (3). It has been estimated that mortality rates from MRSA infections in US hospitals are higher than infections caused by HIV/AIDS and tuberculosis combined (4). Furthermore, there is a considerable medical challenge for treating MRSA infections due to the remarkable ability of *S. aureus* to develop resistance to multiple antibiotics, thus limiting the number of viable therapeutic options (2, 5). Therefore, there is an urgent need to develop novel antimicrobials with unique mechanisms of action to combat MRSA.

Antimicrobial peptides (AMPs) are key components of the host innate defense against infections in most creatures (6, 7). The potential therapeutic applications of AMPs are significant (8). They have rapid and broad spectrum antibacterial activity. In addition, it is extremely difficult for bacteria to evolve resistance against AMPs that disrupt the microbial membrane, as it would necessitate fundamental alterations in the lipid composition of the bacterial membranes. Though AMPs have many important antibacterial properties, several studies have exposed their potential limitations as therapeutic agents.

A significant number of natural AMPs are large in size with high host cytotoxicity and moderate antimicrobial activity. Moreover, their production cost is high (8, 9). In addition, many natural AMPs lose their antimicrobial activity in physiological salt concentrations (10, 11). These characteristics have substantially hindered their pharmaceutical development as new therapeutic agents. Thus, successful development of novel AMPs as future therapeutics requires

identification of short AMPs demonstrating strong antimicrobial activity, salt tolerance and minimal toxicity to host tissues.

Previously, our group has developed short synthetic peptide templates for therapeutic applications against hyperplasia and inflammation (12-14). However their antimicrobial activity was never explored. In the present study, we screened four synthetic peptides and identified two peptides, namely RRIKA and RR, with potent antimicrobial activity against a panel of clinical and drug resistant *Staphylococci* strains. In addition, we investigated the synergistic activity of these peptides in combination with clinically relevant antimicrobials and examined the peptides' ability to disrupt staphylococcal biofilms. Moreover, we performed a series of experiments to explore the antibacterial mechanism of action of RRIKA and RR and examined the toxicity of these peptides toward mammalian cells.

MATERIALS AND METHODS

Bacterial strains and media

All *Staphylococcus* strains tested in this study are presented in table (1). Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Sigma-Aldrich, while trypticase soy broth (TSB) and trypticase soy agar (TSA) were purchased from Becton-Dickinson, Cockeysville, Md.

Reagents, peptides and antibiotics

Nisin (Sigma, N5764), melittin from honey bee venom (Sigma, M2272), magainin I (Sigma, M7152), vancomycin hydrochloride (Gold Biotechnology), linezolid (Selleck Chemicals), ampicillin sodium salt (IBI Scientific), recombinant lysostaphin (3000 U/ mg)

from *Staphylococcus simulans* (Sigma, L9043) and calcein AM (Molecular Probes, Life Technologies) were all purchased from commercial vendors.

Peptide synthesis

Peptides RRIKA, RR, KAF, and FAK were synthesized on Knorr-amide resin (Synbiosci Corp.) using standard FMOC chemistry. Two different chemistries were used to couple each amino acid. The first coupling reagents were N-hydroxybenzotriazole (HoBt)/N, N'-diisopropylcarbodiimide (DIC) and the second coupling reagents were 2-(1Hbenzotriazole-1-yl)-1, 1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and lutidine. For FITC labeled YARA, an aminohexanoic acid spacer was added to the N-terminus to serve as a spacer for the addition of FITC isomer 1 (Molecular Probes). The FITC isomer was solubilized in 12:7:5 pyridine/DMF/DCM and incubated with the deprotected peptide overnight. A ninhydrin test was used to check complete coupling of FITC to the peptide. Following synthesis, the peptide was cleaved from the resin with a trifluoroacetic acid-based cocktail, precipitated in ether, and recovered by centrifugation. The recovered peptide was dried in vacuo, resuspended in MilliQ purified water, and purified using an FPLC (ÄKTA Explorer, GE Healthcare) equipped with a 22/250 C18 prep-scale column (Grace Davidson). An acetonitrile gradient with a constant concentration of 0.1% trifluoroacetic acid was used to achieve purification. Desired molecular weight was confirmed by time-of-flight MALDI mass spectrometry using a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems).

Antibacterial assays

Minimum inhibitory concentrations (MIC) of peptides and antibiotics were evaluated with the broth microdilution technique in MHB with an initial inoculum of 5×10^5 cells non-treated Polystyrene microtiter plates (CytoOne, CC7672-7596) in accordance with the Clinical and

Laboratory Standards Institute (CLSI) (15). The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 16 hr incubation of the plates at 37°C. Each agent was tested in triplicate in at least two independent experiments. The highest MIC value was reported.

Antimicrobial activity in the presence of salts

To investigate the activity of peptides in the presence of high salt concentrations, the MIC was again identified as described above except, fixed concentrations of NaCl or $MgCl_2$ were added to each well of the microtiter plate. For further analysis of the effect of salinity on killing ability of peptides; logarithmic phase of MRSA USA300 and VRSA VRS13 at 5×10^5 colony forming units/ml (CFU/ml) were incubated with 2 × MIC of RRIKA and RR, in MHB, with different concentrations of NaCl (0, 50, 100, 150 mM) or $MgCl_2$ (0, 1, 2, 3 mM) for 4 hr. Bacteria were serially diluted and plated, in triplicate, on TSA plates. CFUs were counted after incubation of plates for 24 hr at 37°C.

Time-kill assay

An overnight culture of MRSA USA300 was diluted in fresh MHB and incubated until optical density (OD_{600}) ≈1, was reached, then washed twice with phosphate buffered saline (PBS) diluted to ~5×10^5 CFU/ml in MHB. Peptides, vancomycin and linezolid were added at concentrations equivalent to 5 × MIC. Peptide diluent (sterile water) served as a negative control. Bacterial cell viability was monitored up to 24 hr. Samples were removed at specific intervals, serially diluted in PBS, and plated, in triplicate, on TSA plates. CFUs were counted after 24 hr incubation of plates at 37°C. To study the effect of serum and salinity on the killing kinetics of the peptides, a time-kill assay was performed as described above with the exception that bacteria

and peptides at 5 × MIC were incubated in MHB alone or with 10% fetal bovine serum (FBS) or incubated in PBS (containing 137 mM NaCl).

Synergy with antimicrobials

The fractional inhibitory concentration (FIC) index was utilized to determine the relationship between antimicrobial agents. Peptide MICs against test microorganisms were determined in triplicate samples. Two-fold serial dilutions of antimicrobials (lysostaphin, vancomycin, linezolid and nisin) were tested in the presence of a fixed concentration of peptide, equals to ¼ × peptide MIC. It is worthy to note that none of the peptides killed the test microorganisms at their quarter MICs. The FIC index was calculated as follows: FIC = 0.25 + MIC (antibiotic in combination)/MIC (antibiotic alone); 0.25 is equal to MIC (peptide in combination)/MIC (peptide alone). An FIC index of ≤ 0.5 is considered to demonstrate synergy. Additive effect was defined as an FIC index of 1. Antagonism was defined as an FIC index of > 4(16).

To further investigate the synergism of peptides with lysostaphin, the time-kill method of determining synergy was utilized as described elsewhere (17). MRSA USA300 and VRSA VRS13 were incubated with ½ × MIC of RRIKA or ½ × MIC of RR alone; or in combination with ¼ × MIC of lysostaphin (MIC of lysostaphin against MRSA USA300 and VRSA VRS13 were 0.04 and 0.08 μM, respectively). Samples were obtained at different time points, then diluted and plated on TSA plates. The plates were incubated for 24 hr at 37°C before CFU were determined. Synergy was defined as a 100-fold or 2-$\log_{10}$ decrease in colony count by the combination of two agents together as compared to each agent tested alone. Additivity or indifference was defined as a 10-fold change in CFU and antagonism was defined as a 100-fold increase in CFU by the combined peptide treatment in comparison to the single treatment.

Bacterial membrane disruption activity (Bacteriolysis)

Cell lysis, as indicated by a decrease in OD_{595}, was determined as described before (18). Briefly, MHB was inoculated with an overnight culture of MRSA USA300 and incubated aerobically at 37°C until an OD_{595}≈0.6 was reached and then diluted to OD_{595}≈0.2 in MHB (equivalent to ≈10^8 CFU/mL). 200 µL of diluted bacteria was added to all wells of a 96-well plate. RRIKA and RR were added in concentrations equivalent to 4 × MIC. Nisin at 4 × MIC was used as a positive control and untreated bacteria served as a negative control. MHB with the same concentrations of drugs served as blanks. Turbidity was monitored at defined intervals up to 10 hr in a Molecular Devices Vmax microplate reader at 595 nm absorption. The assay was carried out in triplicate samples for each treatment regimen.

Calcein leakage assay

Membrane permeabilization of *S. aureus* by peptides was monitored and quantified by the leakage of the preloaded fluorescent dye, calcein, as described before (19). MRSA USA300 was grown in MHB to logarithmic phase at 37°C. Cells were then harvested by centrifugation, washed twice with PBS, and then adjusted spectrophotometrically to an OD_{600} of 1.0 (≈10^9 CFU/ml) in PBS containing 10% (vol/vol) MHB. Then MRSA cells were incubated with 3 µM calcein AM for 1 hr at 37°C. Calcein-loaded cells were harvested by centrifugation (3,000 × *g*, 10 min), suspended in PBS, and diluted to achieve a final inoculum of 10^7 CFU/ml. Aliquots of 100 µL were then added into a sterile black-wall 96-well plate. RRIKA and RR were added in concentrations equivalent to 5 × and 10 × MIC. Bacteria treated with peptide diluent (sterile water) and nisin (10 µg/mL) served as negative and positive controls, respectively. Calcein leakage was measured for 120 min using a fluorescence plate reader (FLx800 model BioTek® Instruments, Inc. Winooski, Vermont). Membrane permeabilization (%) was calculated as the

absolute percent calcein leakage by peptides with respect to calcein-loaded with no-peptide treated cells. Experiments were done in triplicate and repeated independently twice.

DNA binding assay

The ability of RRIKA and RR to bind DNA was investigated by the electrophoretic mobility shift assay as has been previously described (20). In brief, increasing concentrations of RRIKA, RR and magainin were incubated with 250 ng of plasmid DNA (pUC19) in 30 µL binding buffer (10 mM Tris-HCl, 1 mM EDTA buffer, pH 8.0) at room temperature for 30 min. After incubation, the DNA was analyzed by 1% gel electrophoresis. DNA migration was visualized by ethidium bromide staining.

Quantification of activity against biofilms

Biofilm-forming clinical isolates of *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 35984) were grown overnight in TSB. After incubation, cultures were diluted 1:100 in TSB supplemented with 1% glucose. Diluted bacteria were inoculated in either 24- or 96-well flat bottom cell culture plates (polystyrene) and incubated at 37°C for 24 hr. The culture medium was subsequently removed and wells were carefully washed with PBS three times to remove planktonic bacteria before re-filling wells with fresh MHB. Peptides and antibiotics were added at different concentrations and plates were incubated at 37°C for 24 hr. 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% (w/v) crystal violet for 30 min. After staining, the dye was removed and the wells washed with water. The plates were dried for at least 1 hr, prior to addition of ethanol (95%) to solubilize the dye bound to the biofilm. The optical densities (OD) of biofilms were measured at 595 nm absorbance by using a microplate

reader (Bio-Tek Instruments Inc.). An inverted microscope (Vista Vision, VWR), with attached camera and 25X objective was used to photograph the biofilm in 24-well plates.

Hemolysis assay

Human red blood cells (RBCs) (Innovative Research "Novi, MI") were pelleted by centrifugation at 2000 rpm for 5 min followed by washing three times with PBS. An 8% suspension (v/v) of RBCs was prepared in PBS and 50 µL of the solution was transferred to a 96-well plate. Then, 50 µL of different concentration of peptides in PBS were added to give a final suspension of 4% (v/v) of RBCs. PBS served as a negative control. 0.1 % triton X-100 as well as 5 µM melittin served as a positive control. The plate was incubated at 37°C for 1 hr. The plate was subsequently centrifuged at 1000 rpm for 5 min at 4°C. 75 µL aliquots of the supernatants in each well were carefully transferred to a new sterile 96-well plate. Finally the hemolytic activity was evaluated by measuring the optical absorbance at OD_{405} with a microplate reader (Vmax, Molecular Devices). The hemolysis percentage was calculated based on the 100% release with 0.1 % triton X-100 or 5 µM melittin. Experiments were done in triplicates.

Cytotoxicity assay

The toxicity of RRIKA, RR and melittin against HeLa cells was carried out using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit from Promega (21). Briefly, ~ 2×10^4 HeLa cells suspended in 200 µL of DMEM supplemented with 10% FBS, were seeded in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 hr. The HeLa cells were further incubated with different concentration of peptides for 24 hr. At the end of the incubation period, the culture media was discarded and the cells in each well were washed with PBS. 100 µL of cell culture media and 20 µL CellTiter 96® AQ_{ueous} assay reagent were added next (solution reagent

contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]). The plate was returned to the incubator for 2 hr to allow color development. The intensity of color was quantified at 490 nm using a 96-well microplate reader (Vmax, Molecular Devices). Results were expressed as the percentage mean absorbance by cells upon incubation with peptide with respect to incubation with the control (untreated wells).

Statistical analyses

The mean absorbance of crystal violet in the biofilm reduction was compared between the groups using the two-tailed Student *t* test (p □0.05). All statistical analyses were performed with Microsoft Excel's statistical program.

RESULTS:

Antimicrobial activity

The amino acid sequences and characteristics of the synthetic peptides used in this study are shown in Table 2. We screened the synthetic peptides against a panel of clinical and drug-resistant *Staphylococcal* strains (Table 1). RRIKA inhibited the growth of methicillin-sensitive *S. aureus* (MSSA), MRSA, VISA, VRSA, linezolid-resistant *S. aureus*, methicillin-resistant *S. epidermidis* and multidrug-resistant strains from concentrations ranging from 2 to 4 μM. RR inhibited the same isolates with MICs ranging from 8 to 32 μM. In contrast, KAF and FAK peptides were inactive against all strains tested up to 64 μM (data not shown).

Bacterial killing kinetics

Figure 1 presents the rate of microbial killing by RR, RRIKA, vancomycin and linezolid when MRSA USA300 was exposed to 5 × MIC of each treatment over a 24 hr incubation period at 37

ºC. Both RRIKA and RR exhibited a rapid bactericidal effect with 3-$\log_{10}$ reduction (99.9 % clearance) within 60 and 90 min, respectively. In addition, both peptides completely eliminated the inoculum (5×10^5 CFU/mL) within 120 and 180 min, respectively. In comparison, vancomycin achieved a 3-$\log_{10}$ bacterial reduction only after 24 hr; while linezolid (a protein synthesis inhibitor) exhibited a bacteriostatic effect producing only a single log bacterial reduction over the 24 hr incubation. The killing kinetics of RRIKA and RR in PBS (containing 137mM NaCl) did not significantly change when compared to MHB (figure 2). In the presence of 10% FBS, RRIKA retained its bactericidal activity, though it required longer to kill the bacteria completely (6h), while RR lost its antibacterial activity at the same condition.

Antimicrobial activity in the presence of salts

The MIC of RRIKA and RR for MRSA USA300 was determined in the presence of NaCl or $MgCl_2$. There was no increase in the MICs of RRIKA and only a one fold increase in case of RR was observed in the presence of 100 mM NaCl. Moreover, the antimicrobial activity of RRIKA and RR was not impeded in the presence of $MgCl_2$ at a concentration equivalent to reported physiological conditions (2 mM). To study the effect of salinity on the killing ability of the peptides, CFU were counted after incubation of MRSA USA300 and VRSA VRS13 strains with RRIKA and RR (2 × MIC) in increasing concentrations of NaCl or $MgCl_2$. As shown in supplementary figure 1, there was no significant difference in the bactericidal activity of RRIKA in the presence of NaCl up to 150 mM. Similar patterns were observed for RR with the exception of one $\log_{10}$ increase in CFU observed at 150 mM NaCl; however this peptide retained its bactericidal activity at this concentration (producing a 3-$\log_{10}$ reduction). Furthermore, physiological concentrations of $MgCl_2$ (1, 2 and 3 mM) had no effect on the killing ability of both RRIKA and RR.

Synergy with antimicrobial agents

Both RRIKA and RR acted synergistically with lysostaphin against MSSA, MRSA and VRSA strains with a FIC index equal to 0.26 (Table 3). When both peptides were combined together, only one fold decrease in each peptide's MIC was observed (FIC index = 0.75). However, an additive effect was observed when each peptide was combined with vancomycin, linezolid or nisin against tested strains (data not shown). There were no antagonistic effects observed between the peptides and antimicrobials against all strains tested.

Time-kill synergy studies revealed RRIKA and RR, at only ½ × MIC enhanced the killing of lysostaphin (at ¼ × MIC) more than 1000 fold and completely eradicated MRSA and VRSA isolates within 20 minutes (figure 3).

Bacterial membrane disruption activity (Bacteriolysis)

Since evidence of rapid cell lysis of treated cultures is indicative of membrane damaging activity of antimicrobial peptides (22, 23), we assessed whether the bactericidal activity of our peptides was associated with lysis of bacterial cells by measuring culture turbidity over time after treatment. We monitored the turbidity of MRSA cultures treated with 4 × MIC of peptides over a 10 hr period at OD_{595} via a microplate reader. As shown in figure 4 both RRIKA and RR caused a rapid decrease in OD_{595} even with a high inoculum concentration of 10^8 CFU/ml. Peptide RRIKA produced greater than 50% and 86% reduction in turbidity after 2.5 and 6 hr, respectively. RR produced a similar percentage of turbidity as RRIKA albeit at a slower rate (after 4.5 and 10 hr). Nisin at 4 × MIC, a polycyclic antibacterial peptide which is known as a membrane perturbing agent (24), lysed cells at a slightly higher rate than RRIKA with more than 70% reduction in turbidity observed after 2.5 h. In contrast, wells treated with 4 × MIC of

vancomycin (a cell wall biosynthesis inhibitor) had no effect on culture turbidity within the same time frame.

Permeabilization of *S. aureus* membrane (calcein leakage assay)

We monitored the effect of the peptides on MRSA membrane integrity by using the calcein leakage assay as described previously (19, 25). Damage of membrane integrity is indicated by calcein leakage from bacterial cells, leading to a reduction in fluorescence intensity. Figure 5 demonstrates that both peptides perturb the *S. aureus* cell membrane leading to leakage of preloaded calcein in both a concentration- and time-dependent manner. RRIKA was faster and more potent in comparison to RR in membrane perturbation. At 5 × MIC, RRIKA and RR caused more than 60% and 30% leakage respectively within 60 minutes. When the concentration of peptide was increased to 10 × MIC, a significant change in membrane damage was observed for both peptides. There was a more than 75% and 60% reduction in fluorescence intensity measured for RRIKA and RR respectively in the same time frame. Nisin at 5 × MIC resulted in more than 70% calcein leakage from cells, which is comparable to RRIKA at 10 × MIC. Vancomycin, as expected, had no effect on the bacterial cell membrane integrity even at high concentration (10 × MIC).

DNA binding properties

Since there was no obvious evidence that the antibacterial effect of AMPs was restricted to perforation of membranes, we explored the feasibility of other intracellular targets, such as DNA by assessing the DNA-binding properties of peptides (26, 27). As shown in figure 6, both peptides were able to bind the plasmid DNA and delay its electrophoretic migration into agarose gel, in a dose-dependent manner. At 8 μM concentration, of RRIKA peptide, a fraction of the plasmid DNA was still able to migrate into the gel as non-complexed DNA; however at 16 μM,

the majority of DNA remained in the gel's wells. At higher concentrations, complete retardation of DNA migration was exhibited, implying that the DNA was aggregated by peptide. Similar patterns of migration were observed with the RR peptide except that free DNA was still seen at 16 μM concentration. In contrast, magainin, a cationic antimicrobial peptide of amphibian skin, lacks this ability to bind DNA even at concentrations up to 80 μM.

Quantification of activity against biofilms

In order to evaluate the efficacy of RR and RRIKA against established biofilms, we measured the biofilm mass post-treatment by crystal violet staining biofilms formed by clinical isolates of *S. aureus* and *S. epidermidis*. As observed in figure 7a, both peptides significantly reduced *S. aureus* biofilms as compared to the antibiotics of choice. RRIKA and RR, at only 2 × MIC, dispersed more than 65% of mature biofilms ($p < 0.01$), while vancomycin and linezolid, at 16 × MIC, was only capable of reducing 40% of biofilm mass ($p < 0.01$). There was significant difference between peptide and antibiotics treated biofilms ($p < 0.01$).

With regards to *S. epidermidis*, both RR and RRIKA, at 32 and 64 × MIC, were able to reduce more than 50% of biofilm mass respectively ($p < 0.01$) (figure 7b). Vancomycin and linezolid, on the other hand, reduced only 9.5 and 10.7% of biofilm mass, respectively ($p < 0.01$) at concentrations equivalent to 256 × MIC. Also we found statistical significance between peptide and antibiotics treated biofilms ($p < 0.01$).

Hemolysis assay and cell toxicity

We assessed the release of hemoglobin from human erythrocytes exposed to different concentrations of peptides. As depicted in figure 8, even at concentrations as high as 300 μM, RRIKA and RR exhibited minimal hemolysis against RBCs (maximum of 10% hemolysis

observed). In contrast, melittin completely lysed RBCs (100% hemolysis) at a significantly lower concentration of 5 μM.

The cytotoxic effect of the peptides on HeLa cells was evaluated by the MTS assay. As depicted in figure 9, neither RRIKA nor RR was toxic to the mammalian cells tested up to concentrations of 32 μM and 64 μM, respectively. These values correlate to 8 and 4 × MIC for RRIKA and RR, respectively. The half maximal effective concentration (EC_{50}) of RRIKA and RR against macrophage cell lines was 64 and 128 μM respectively. On the other hand, melittin showed high toxicity even at bacteriostatic concentration with EC_{50} less than 5 μM.

DISCUSSION:

In this study, we evaluated the antimicrobial activity of two short synthetic peptides, and revealed their potential mechanisms of action. We observed that RRIKA and RR exhibited potent antibacterial activity against all tested *S. aureus* isolates. Moreover, our peptides demonstrated activity against multiple clinical isolates of MRSA, particularly MRSA USA300, a community-associated strain which is responsible for outbreaks of staphylococcal skin and soft-tissue infections (SSTI) in the United States (28). Similarly, the potent bactericidal activity was observed in other clinical MRSA isolates (USA100, USA200 and USA500) that are resistant to various antibiotic classes including macrolides, aminoglycosides, lincosamides, and fluoroquinolones. Additionally, we found that these peptides retained their activity against strains of *S. aureus* which have resistance to drugs of choice in treating MRSA infections, namely linezolid (MRSA NRS119) and vancomycin (VRSA strains). It is worthy to note that, the C terminus of the RR peptide was modified to contain three additional amino acids (isoleucine, lysine and alanine) which made the RRIKA peptide more amphipathic and hydrophobic and also increased its positive net charge. These modifications augmented the antibacterial activity of

RRIKA which exhibited MICs two to eight folds lower than those of RR. The enhanced efficacy of RRIKA may reveal a functional structure activity relationship and provide a template for future peptide synthesis.

Time-kill kinetics revealed a major advantage of the synthetic peptides over tested antibiotics. While RRIKA and RR eliminated MRSA within 1-2 hr after treatment, vancomycin required 24 hr to reduce the bacterial CFU by 3-$\log_{10}$. Linezolid, on the other hand, exhibited only a bacteriostatic effect.

Growth kinetic measurements of MRSA exposed to peptides clearly demonstrated that RRIKA and RR peptides exhibited kinetic behavior similar to membrane lytic peptides such as the lantibiotic nisin (24) and amphibian magainin (23) but different from non-lytic peptides such as fungal plectasin (29) and amphibian buforin II (23). These data suggest that our peptides act by disrupting the integrity of the bacterial membrane. To validate this hypothesis, we studied the effect of peptides on MRSA membranes using a calcein leakage assay. Similar to the well-known membrane damaging peptide, nisin, both RRIKA and RR permeabilized *S. aureus* cells in a concentration and time-dependent manner. These observations clearly validate that the mechanism of bacterial killing by our peptides is mediated by pore formation and disruption of the bacterial cell membrane, leading to leakage of cytoplasmic contents and ultimately cell lysis. However, recent evidence suggests that lysis of bacterial cells by pore formation is not the only mechanism of microbial killing of some AMPs and there are other possible intracellular targets, such as aggregation of DNA (26). To further explore this possibility, we examined the bacterial DNA-binding ability of the peptides by electrophoretic gel retardation. Our data indicated that both RRIKA and RR bind with bacterial DNA and alter its electrophoretic mobility in agarose gels. Although a detailed study on the interaction between peptides and nucleic acids *in vivo* is

392 needed, the current results lead us to suggest that RRIKA and RR might inhibit the bacterial
393 functions by binding to bacterial DNA after perturbing the cell membranes, resulting in an
394 augmented effect by two different mechanisms. While RRIKA and RR are not toxic to
395 mammalian cells at the levels used here, possible crossing of the peptides to the nuclear
396 membrane and binding to eukaryotic DNA must be examined further.

397 Major limitations with the use of AMPs for systemic applications are their possible inactivation
398 by serum or physiological concentrations of salts. Serum inactivate peptides either through
399 cleavage by proteases or through binding with proteins or lipids (30) We observed that RRIKA
400 but not RR retained its bactericidal activity, although at a slower rate, in the presence of 10%
401 FBS. Furthermore, both RRIKA and RR retain their antibacterial activity when tested in
402 increasing concentration of NaCl and $MgCl_2$; in contrast to many well studied AMPs (such as
403 LL-37, human β-defensin-1, gramicidins, bactenecins, and magainins) which showed
404 substantially reduced antibacterial activities under the same conditions (11). Previously, Turner
405 *et al*. reported that LL-37 and HNP-1 demonstrated a 12-fold and 100-fold increase in the MIC
406 of MRSA, respectively when 100 mM NaCl was added to the test medium (10). The ability to
407 resist the effects of salt and serum provide a selective advantage for our peptides for potential
408 therapeutics in physiological solutions.

409 After identifying that our peptides were capable of inhibiting bacterial growth alone, we wanted
410 to explore their ability to be used in combination with other antimicrobials such as lysostaphin.
411 Lysostaphin is a zinc metalloenzyme that specifically cleaves the abundant pentaglycine cross-
412 bridges of the staphylococcal cell wall. Several studies have reported the potential applications
413 of lysostaphin in the treatment of staphylococcal infections, however, *S. aureus* has developed
414 resistance against lysostaphin via different mechanisms (31). When we tested both RR and

RRIKA in combination with lysostaphin, we observed a synergistic relationship demonstrated by complete eradication of MRSA and VRSA within 20 minutes (at very low concentrations). The observed synergistic effect may be due the cleaving of the cell wall peptidoglycan by lysostaphin, which allows more access of peptides to the bacterial membrane. The synergistic relationship observed between our peptides and lysostaphin is important as it has the advantage of reducing the emergence of bacterial resistance to both agents while also minimizing drug-associated toxicity (by lowering the therapeutic dose needed for each antimicrobial agent to effectively treat MRSA infection).

One of the difficult challenges facing current antibiotics is bacterial biofilms, which are vital in the pathogenesis of staphylococcal infections. Biofilms hinder the penetration of antimicrobials to access bacteria, leading to failure of treatments (32). Our data showed that both RR and RRIKA are capable of disrupting *S. aureus* and *S. epidermidis* biofilms more efficiently than drugs of choice (vancomycin and linezolid). Most bacteria living in biofilms are either slow-growing or non-growing dormant cells that are difficult to treat with antibiotics that normally inhibit macromolecular synthesis in growing cells (33). However our peptides target mainly the microbial cell membrane, a characteristic that is not only in dividing organisms, but also in quiescent cells or stationary phase bacteria (34).

Cell toxicity is one of the major limitations in the development of antibacterials, particularly if the target of action is the cell membrane (7). We observed minimal hemolysis (less than 10%) with our peptides even with high concentrations (300 μM). On the other hand, 5 μM of melittin completely lysed human RBCs (100% hemolysis). Furthermore, RRIKA and RR were not toxic to HeLa cells at 8 and 4 × MIC, respectively.

437 In conclusion, our findings reveal the potent bactericidal action of peptides RRIKA and RR
438 against MRSA. The mechanism of action of the peptides is mainly due to bacterial lysis as a
439 consequence of bacterial membrane disruption and possibly by binding of the peptides to
440 bacterial DNA, which interferes with necessary cellular functions vital for microbial survival.
441 Such effects are extremely challenging for pathogens to overcome by developing resistance;
442 unlike current antibiotics, which usually inhibit metabolic pathways that can lead to bacterial
443 resistance (8). To date, issues such as poor pharmacokinetics have limited the potential systemic
444 applications of therapeutic AMPs (7, 8, 30). Therefore, AMPs which have advanced into
445 preclinical or clinical trials, are indicated for topical treatment of bacterial skin infections (7, 8,
446 30) such as Pexiganan (Access Pharmaceuticals, Inc.) for curing diabetic foot ulcers (35). *S.*
447 *aureus* is the most frequently isolated microorganism in diabetic foot infections, and those
448 caused by MRSA are associated with the worse outcomes and more frequent amputations (36-
449 41). Additionally, bacterial biofilms appear to play an important role in increasing the difficulty
450 of treating these ulcers (40). Furthermore, it has been proven recently that the clinical severity of
451 *S. aureus* skin infection is driven by the inflammatory response to the bacteria, rather than
452 bacterial burden (42-44). Taken together, the characteristics of the presented peptides with
453 combined bactericidal, anti-biofilm and anti-inflammatory effect may offer an effective way for
454 treating Staphylococcal skin infection. Therefore, these results support the potential for further
455 study and development of RRIKA and RR as topical therapeutics particularly in an era of
456 emerging drug resistance.

457 **ACKNOWLEDGEMENT**

458 Mohamed F. Mohamed and Maha I. Hamed are supported by a scholarship from the Egyptian
459 Cultural and Educational Bureau (ECEB) in Washington DC, USA. We would like to thank the

Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH Contract # HHSN272200700055C for providing *Staphylococcus aureus* strains used in this study. The authors wish to acknowledge Mr Haroon Mohammed for editing the manuscript.

REFERENCES

1. **Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, Johnson SK, Vandenesch F, Fridkin S, O'Boyle C, Danila RN, Lynfield R.** 2003. Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. JAMA : the journal of the American Medical Association **290:**2976-2984.
2. **Tsuji BT, Rybak MJ, Cheung CM, Amjad M, Kaatz GW.** 2007. Community- and health care-associated methicillin-resistant Staphylococcus aureus: a comparison of molecular epidemiology and antimicrobial activities of various agents. Diagnostic microbiology and infectious disease **58:**41-47.
3. **Stryjewski ME, Chambers HF.** 2008. Skin and soft-tissue infections caused by community-acquired methicillin-resistant Staphylococcus aureus. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **46 Suppl 5:**S368-377.
4. **Boucher HW, Corey GR.** 2008. Epidemiology of methicillin-resistant Staphylococcus aureus. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **46 Suppl 5:**S344-349.
5. **Chambers HF, Deleo FR.** 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nature reviews. Microbiology **7:**629-641.
6. **Boman HG.** 1995. Peptide antibiotics and their role in innate immunity. Annual review of immunology **13:**61-92.
7. **Zasloff M.** 2002. Antimicrobial peptides of multicellular organisms. Nature **415:**389-395.
8. **Hancock RE, Sahl HG.** 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature biotechnology **24:**1551-1557.
9. **Silva ON, Mulder KC, Barbosa AE, Otero-Gonzalez AJ, Lopez-Abarrategui C, Rezende TM, Dias SC, Franco OL.** 2011. Exploring the pharmacological potential of promiscuous host-defense peptides: from natural screenings to biotechnological applications. Frontiers in microbiology **2:**232.
10. **Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI.** 1998. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother **42:**2206-2214.
11. **Chu HL, Yu HY, Yip BS, Chih YH, Liang CW, Cheng HT, Cheng JW.** 2013. Boosting salt resistance of short antimicrobial peptides. Antimicrob Agents Chemother **57:**4050-4052.
12. **Lopes LB, Flynn C, Komalavilas P, Panitch A, Brophy CM, Seal BL.** 2009. Inhibition of HSP27 phosphorylation by a cell-permeant MAPKAP Kinase 2 inhibitor. Biochemical and biophysical research communications **382:**535-539.

13. **Lopes LB, Brophy CM, Flynn CR, Yi Z, Bowen BP, Smoke C, Seal B, Panitch A, Komalavilas P.** 2010. A novel cell permeant peptide inhibitor of MAPKAP kinase II inhibits intimal hyperplasia in a human saphenous vein organ culture model. Journal of vascular surgery **52:**1596-1607.
14. **Ward B, Seal BL, Brophy CM, Panitch A.** 2009. Design of a bioactive cell-penetrating peptide: when a transduction domain does more than transduce. Journal of peptide science : an official publication of the European Peptide Society **15:**668-674.
15. **CLSI.** 2007. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7. CLSI, Wayne, PA.
16. **Yan H, Hancock RE.** 2001. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob Agents Chemother **45:**1558-1560.
17. **White RL, Burgess DS, Manduru M, Bosso JA.** 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother **40:**1914-1918.
18. **Oliva B, Miller K, Caggiano N, O'Neill AJ, Cuny GD, Hoemann MZ, Hauske JR, Chopra I.** 2003. Biological Properties of Novel Antistaphylococcal Quinoline-Indole Agents. Antimicrobial Agents and Chemotherapy **47:**458-466.
19. **Xiong YQ, Mukhopadhyay K, Yeaman MR, Adler-Moore J, Bayer AS.** 2005. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of Staphylococcus aureus. Antimicrob Agents Chemother **49:**3114-3121.
20. **Yan J, Wang K, Dang W, Chen R, Xie J, Zhang B, Song J, Wang R.** 2013. Two hits are better than one: membrane-active and DNA binding-related double-action mechanism of NK-18, a novel antimicrobial peptide derived from mammalian NK-lysin. Antimicrob Agents Chemother **57:**220-228.
21. **Mosmann T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods **65:**55-63.
22. **Boman HG, Agerberth B, Boman A.** 1993. Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. Infection and immunity **61:**2978-2984.
23. **Park CB, Kim HS, Kim SC.** 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochemical and biophysical research communications **244:**253-257.
24. **Ruhr E, Sahl HG.** 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrobial agents and chemotherapy **27:**841-845.
25. **Koo SP, Bayer AS, Yeaman MR.** 2001. Diversity in antistaphylococcal mechanisms among membrane-targeting antimicrobial peptides. Infection and immunity **69:**4916-4922.
26. **Brogden KA.** 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Micro **3:**238-250.
27. **Park CB, Kim HS, Kim SC.** 1998. Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and Inhibiting Cellular Functions. Biochemical and biophysical research communications **244:**253-257.
28. **King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, Blumberg HM.** 2006. Emergence of community-acquired methicillin-resistant Staphylococcus aureus USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med **144:**309-317.
29. **Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK, Mygind PH, Raventós DS, Neve S, Ravn B, Bonvin AMJJ, De Maria L, Andersen AS, Gammelgaard LK, Sahl H-G, Kristensen H-H.** 2010. Plectasin, a Fungal Defensin, Targets the Bacterial Cell Wall Precursor Lipid II. Science **328:**1168-1172.

30. **Yeung AT, Gellatly SL, Hancock RE.** 2011. Multifunctional cationic host defence peptides and their clinical applications. Cellular and molecular life sciences : CMLS **68:**2161-2176.
31. **Kumar JK.** 2008. Lysostaphin: an antistaphylococcal agent. Applied microbiology and biotechnology **80:**555-561.
32. **Mah TF, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology **9:**34-39.
33. **Hurdle JG, O'Neill AJ, Chopra I, Lee RE.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nature reviews. Microbiology **9:**62-75.
34. **Yeaman MR, Yount NY.** 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacological reviews **55:**27-55.
35. **Lipsky BA, Holroyd KJ, Zasloff M.** 2008. Topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: a randomized, controlled, double-blinded, multicenter trial of pexiganan cream. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **47:**1537-1545.
36. **Dang CN, Prasad YD, Boulton AJ, Jude EB.** 2003. Methicillin-resistant Staphylococcus aureus in the diabetic foot clinic: a worsening problem. Diabetic medicine : a journal of the British Diabetic Association **20:**159-161.
37. **Lipsky BA, Stoutenburgh U.** 2005. Daptomycin for treating infected diabetic foot ulcers: evidence from a randomized, controlled trial comparing daptomycin with vancomycin or semi-synthetic penicillins for complicated skin and skin-structure infections. The Journal of antimicrobial chemotherapy **55:**240-245.
38. **Tentolouris N, Jude EB, Smirnof I, Knowles EA, Boulton AJ.** 1999. Methicillin-resistant Staphylococcus aureus: an increasing problem in a diabetic foot clinic. Diabetic medicine : a journal of the British Diabetic Association **16:**767-771.
39. **Fejfarova V, Jirkovska A, Skibova J, Petkov V.** 2002. [Pathogen resistance and other risk factors in the frequency of lower limb amputations in patients with the diabetic foot syndrome]. Vnitrni lekarstvi **48:**302-306.
40. **Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJ, Armstrong DG, Deery HG, Embil JM, Joseph WS, Karchmer AW, Pinzur MS, Senneville E, Infectious Diseases Society of A.** 2012. 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **54:**e132-173.
41. **Sotto A, Lina G, Richard JL, Combescure C, Bourg G, Vidal L, Jourdan N, Etienne J, Lavigne JP.** 2008. Virulence potential of Staphylococcus aureus strains isolated from diabetic foot ulcers: a new paradigm. Diabetes Care **31:**2318-2324.
42. **Montgomery CP, Daniels MD, Zhao F, Spellberg B, Chong AS, Daum RS.** 2013. Local inflammation exacerbates the severity of Staphylococcus aureus skin infection. PloS one **8:**e69508.
43. **Pirofski LA, Casadevall A.** 2008. The damage-response framework of microbial pathogenesis and infectious diseases. Advances in experimental medicine and biology **635:**135-146.
44. **Casadevall A, Pirofski LA.** 2003. The damage-response framework of microbial pathogenesis. Nature reviews. Microbiology **1:**17-24.

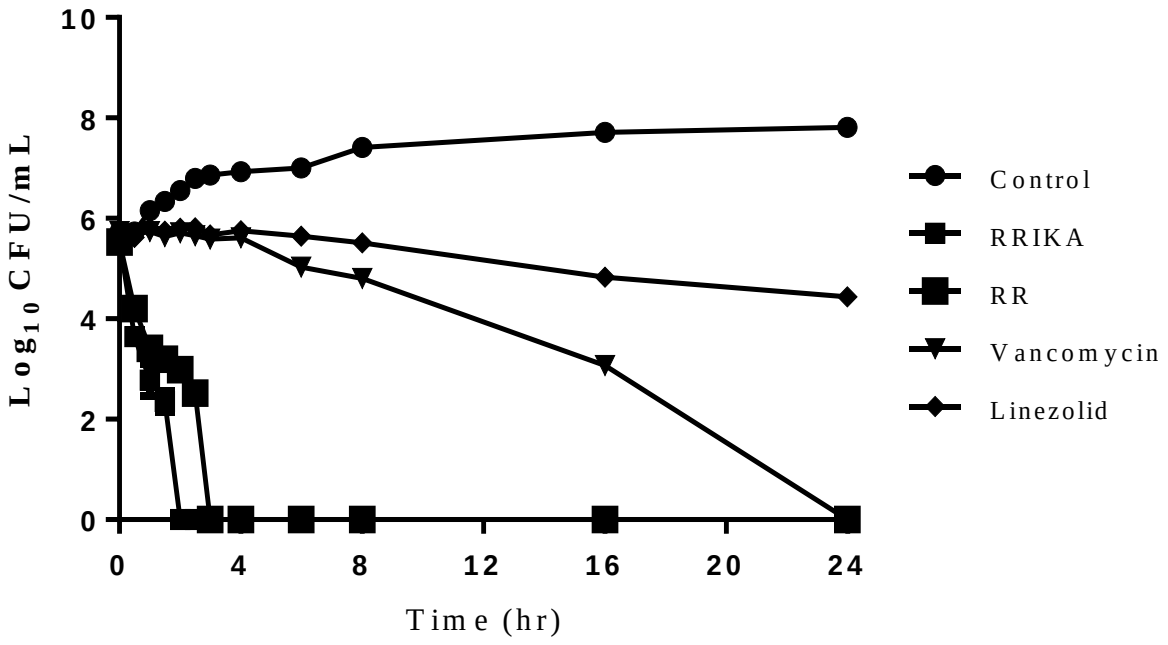

Figure 1: Bacterial killing kinetics of RRIKA, RR, vancomycin and linezolid at 5X MIC against MRSA USA300 in MHB (Mueller Hinton broth). Samples treated with peptide diluent (sterile water) were used as a control. The results are given as means ± SD (n = 3; data without error bars indicate that the SD is too small to be seen)

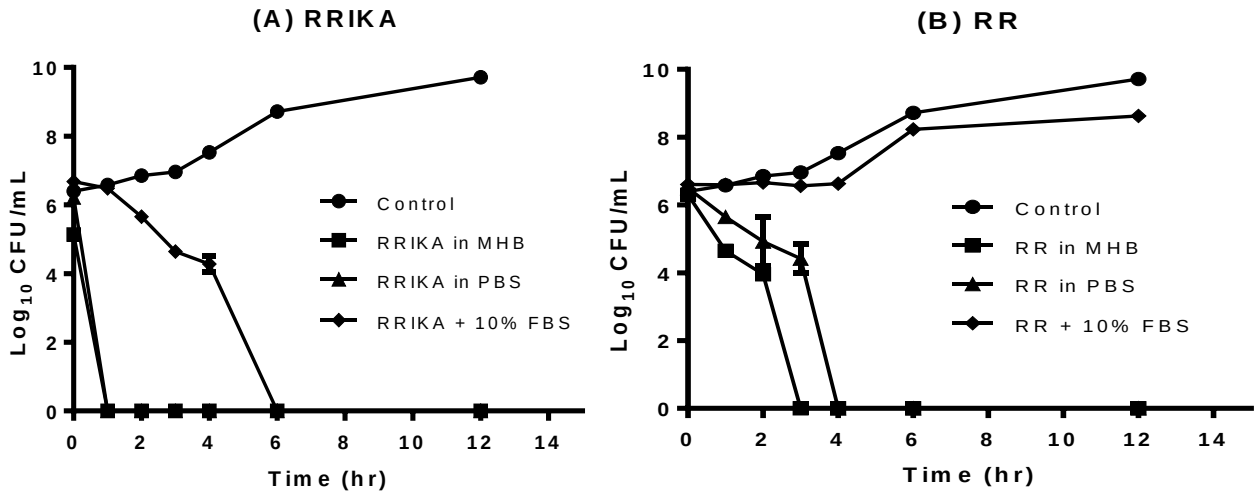

Figure 2: Effect of culture conditions on killing kinetics of RRIKA (A), RR (B) at 5X MIC against MRSA USA300. Abbreviations, MHB "Mueller Hinton broth", PBS "phosphate buffered saline" or MHB+10% FBS "fetal bovine serum". Untreated samples were used as a control was. The killing curves were identical (overlapping in the figure) for RRIKA in MHB and in PBS. Each experiment was done in triplicate, and the values represent means ± SD (data without error bars indicate that the SD is too small to be seen).

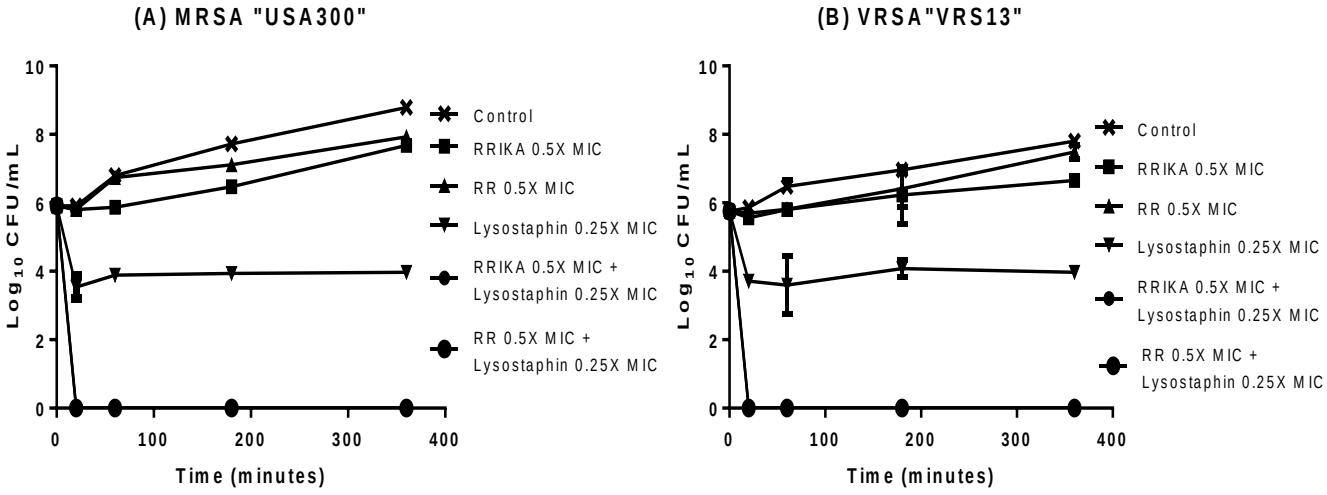

Figure 3: Synergistic killing of RRIKA and RR with lysostaphin by time kill assay. MRSA USA300 (A) and VRSA VRS13 (B) were incubated with 0.5X MIC of RRIKA or RR alone or in combination with 0.25X MIC of lysostaphin. Samples were obtained at different time points, then plated and counted. Control was untreated samples. The killing curves were identical (overlapping in the figure) for RRIKA and RR in combination with lysostaphin. The results are given as means ± SD (n = 3; data without error bars indicate that the SD is too small to be seen)

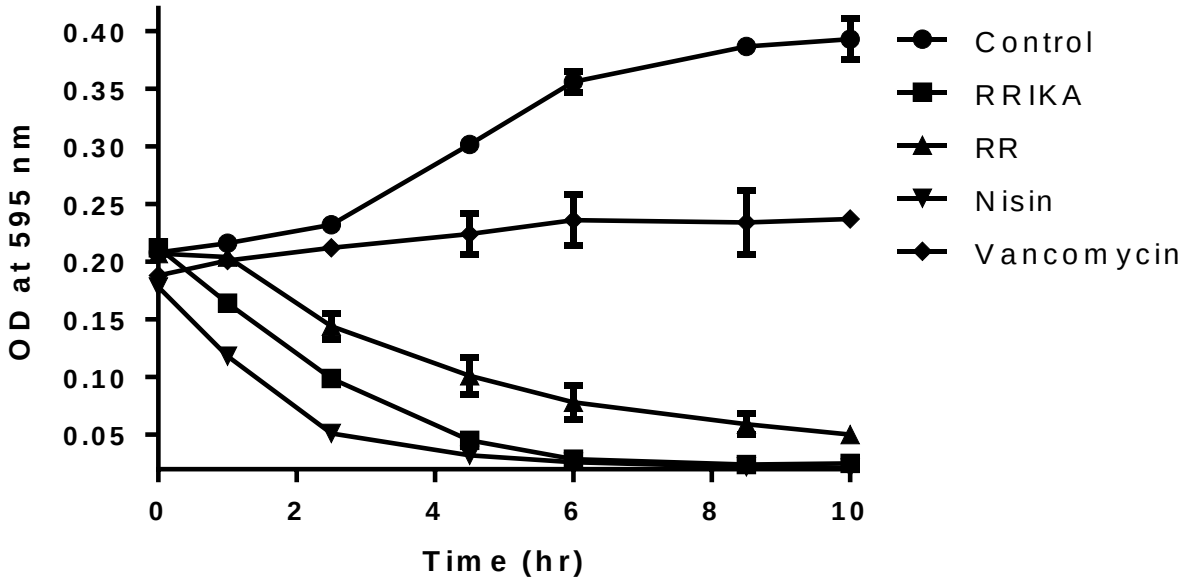

Figure 4: Killing kinetics of MRSA USA300 exposed to 4X MIC of RRIKA, RR, nisin, vancomycin and sterile water (control) as observed by measuring OD595 by microplate reader over time. Results are representative of two separate experiments, each was done in triplicate. Error bars represent standard deviation values (data without error bars indicate that the SD is too small to be seen)

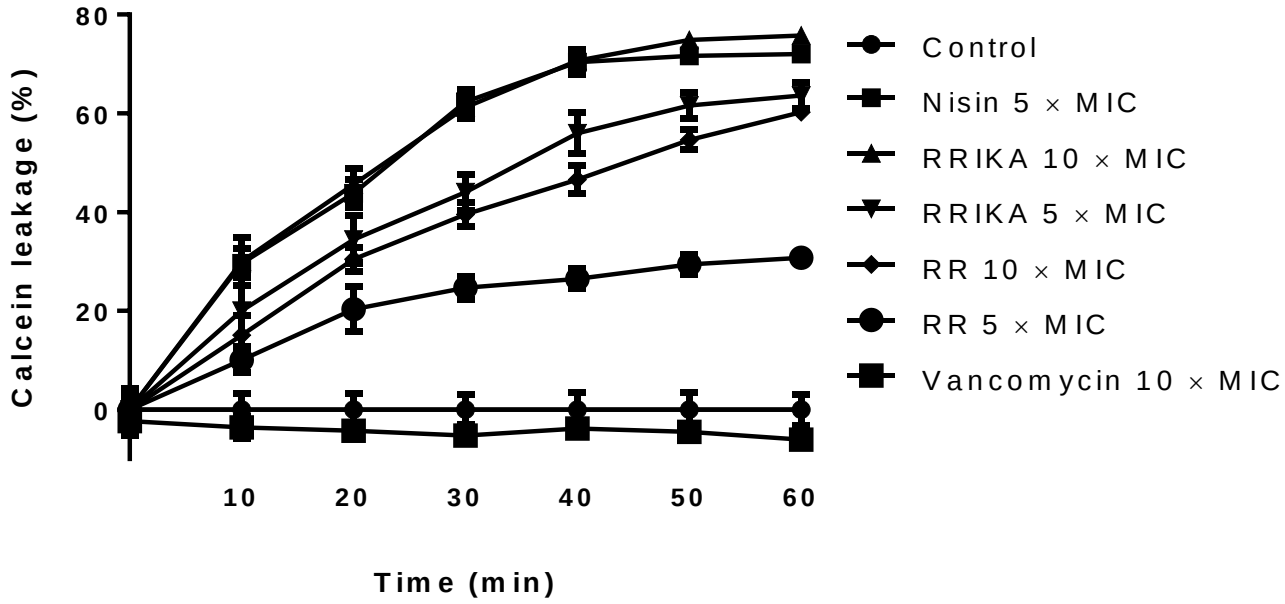

Figure 5: Permeabilization of the cytoplasmic membrane of MRSA USA300 as a function of peptide concentration, indicated by percent of calcein leakage for 60 min exposure. The results are given as means ± SD (n = 3; data without error bars indicate that the SD is too small to be seen).

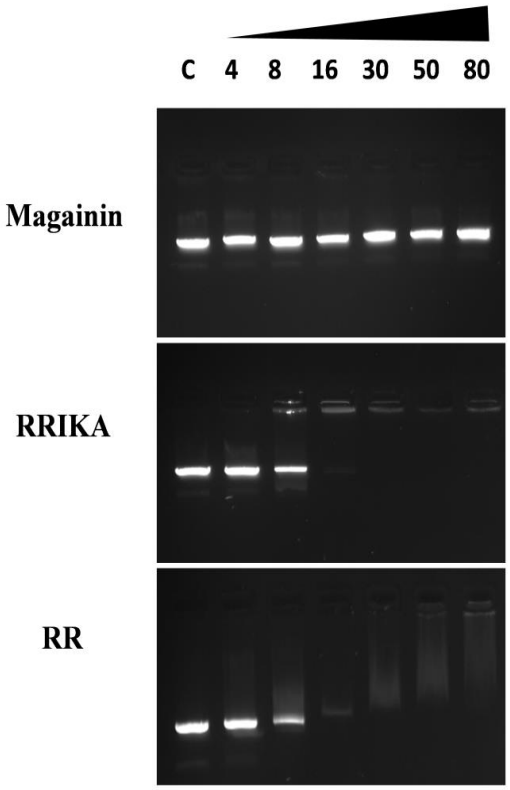

Figure 6: Interaction of peptides with plasmid DNA. Binding was assayed by measuring inhibition of migration by plasmid DNA. Different concentrations of peptides were incubated with 250 ng for 1 hr at room temperature prior to electrophoresis on a 1.0% agarose gel. The numbers above the lanes represent the concentration (in µM) of magainin, RRIKA or RR. Lane C, control consisting of plasmid DNA only.

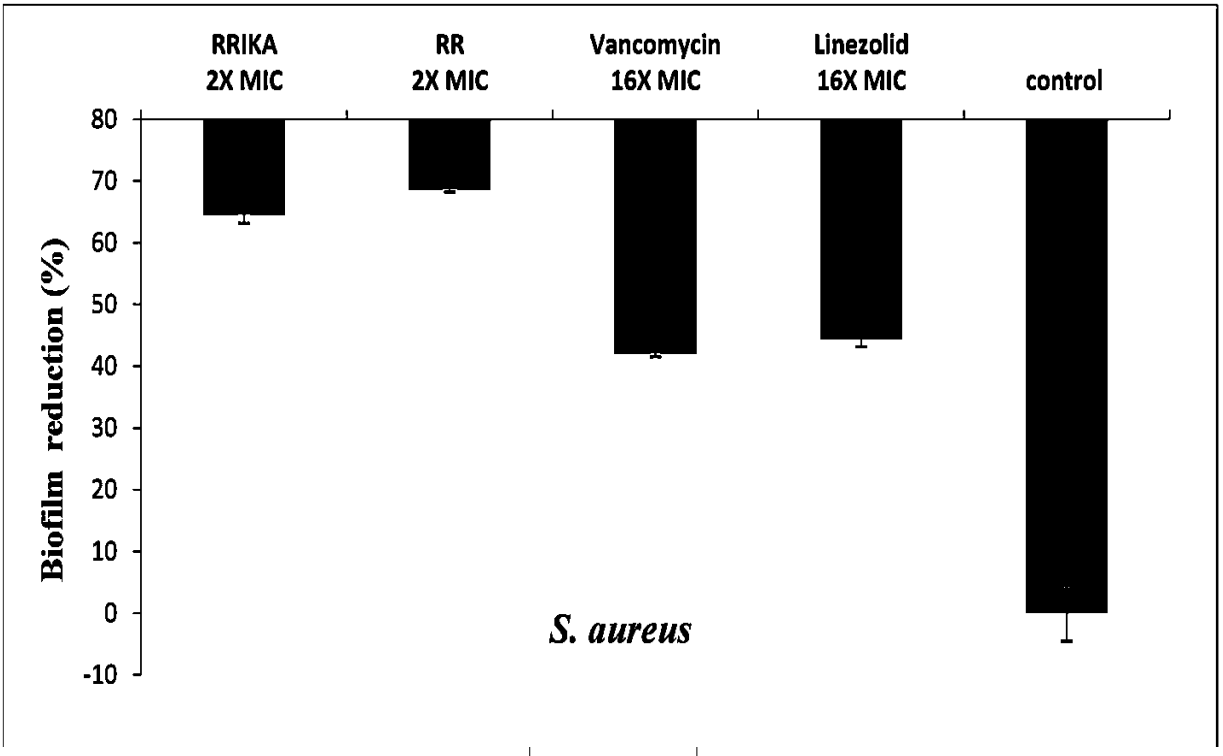

Fig. 7a

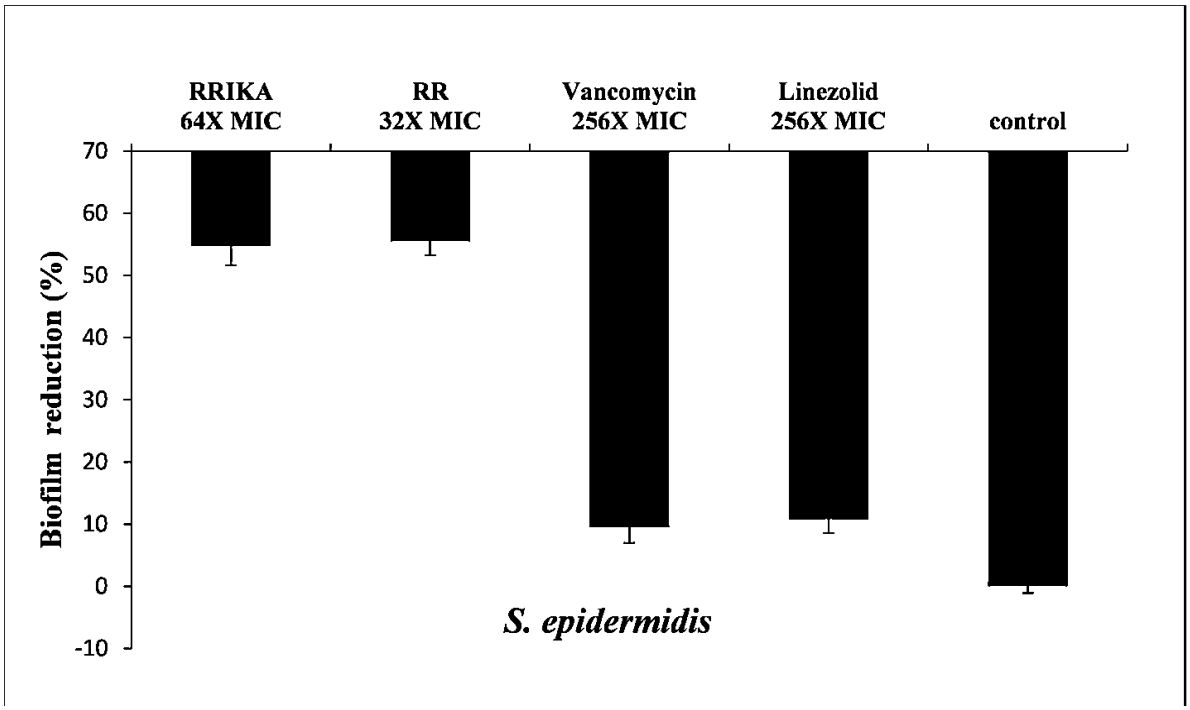

Fig. 7b

Figure 7: The effect of peptides (RRIKA & RR) and antibiotics (vancomycin & linezolid) on established biofilms of *S. aureus* (a) and *S. epidermidis* (b). The adherent biofilm stained by crystal violet, then the dye was extracted with ethanol, measured at 595 nm absorbance and presented as percentage of biofilm reduction compared to untreated wells "control". All experiments were done in triplicate for statistical significance. One asterisk (*) indicates statistically different than the positive control ($p < 0.01$). Two asterisks (**) indicates statistically different than the antibiotic treated wells ($p < 0.01$).

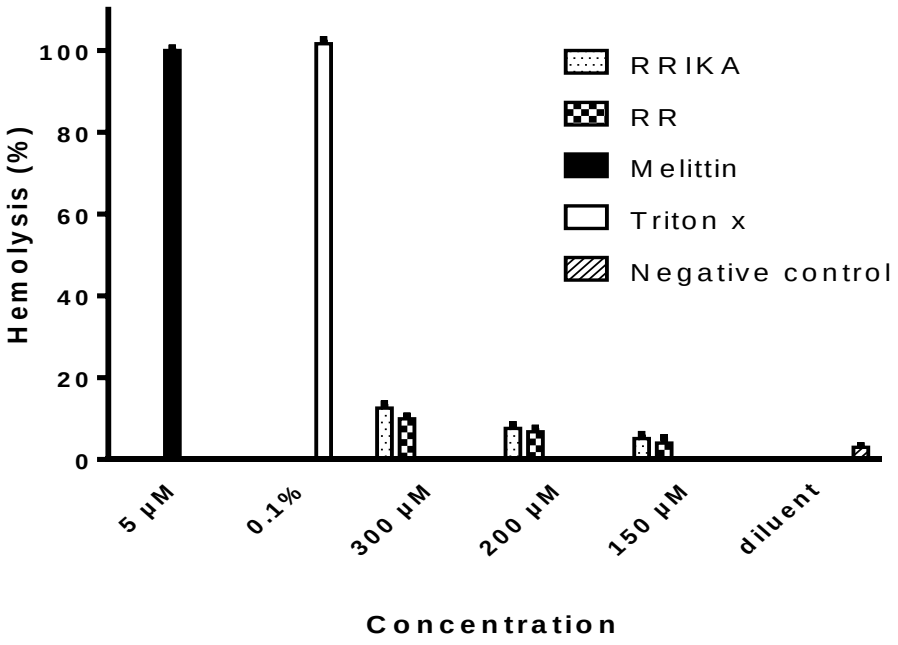

Figure 8: The release of hemoglobin in the supernatant of human erythrocytes after treatment with increasing amounts of RRIKA and RR was measured at 415 nm. Data collected after 1 hr of incubation are presented. Melittin (5 µM) and 0.1 % of triton X-100 served as positive controls. Phosphate buffered saline (PBS) served as a negative control.

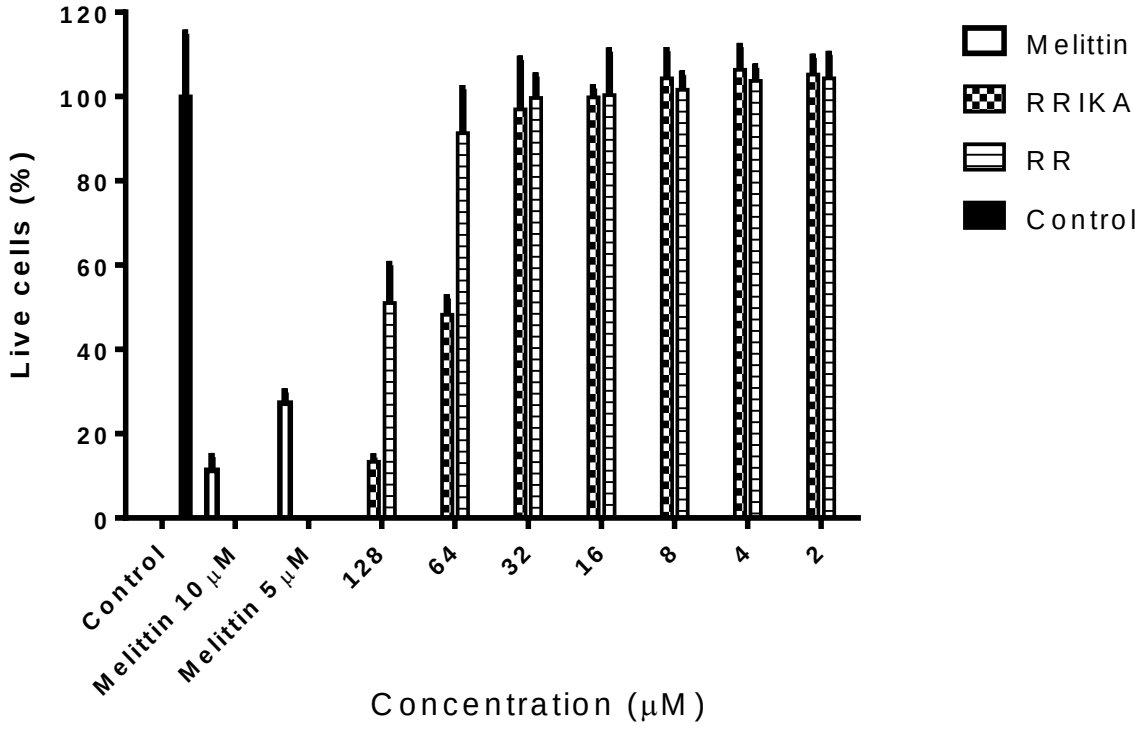

Figure 9: Cytotoxicity assay showing the percent mean absorbance at 490 nm after incubating HeLa cells with RRIKA and RR and melittin at different concentrations. Diluent was used as a control. Cell viability was measured by MTS assay. Results are expressed as means from three measurements ± standard deviation.

Table 1: Minimum inhibitory concentration (MIC) of peptides and antibiotics against clinical and drug-resistant *Staphylococci* strains

Strain Type	Strain ID	Origin	Phenotypic Properties	MIC(μM)					
				Peptide RRIKA	Peptide RR	Lin	Van	Nisin	Amp
Methicillin sensitive *Staphylococcus aureus* (MSSA)	ATCC 6538	-	Quality control and biofilm forming strain	2	16	2	0.5	1	≤0.25
	RN4220	United States	Resistant to mupirocin	4	8	1	0.5	nd	≤0.25
	NRS72	United Kingdom	Resistant to penicillin	4	32	4	0.5	2	>32
	NRS77	United Kingdom	-	4	16	2	0.5	2	≤0.25
	NRS846	-	-	4	16	4	0.5	2	≤0.25
	NRS860	-	-	4	16	1	0.5	2	≤0.25
Methicillin resistant *Staphylococcus aureus* (MRSA)	USA100	United States (Ohio)	Resistant to ciprofloxacin, clindamycin, erythromycin	2	16	4	0.5	nd	>32
	USA200	United States (North Carolina)	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, and methicillin	4	8	4	0.5	nd	>32
	USA300	United States (Mississippi)	Resistant to erythromycin, methicillin, and tetracycline	4	16	4	0.5	1	>32
	USA400	United States (North Dakota)	Resistant to methicillin and tetracycline	2	8	4	0.5	nd	>32
	USA500	United States	Resistant to ciprofloxacin,	4	8	4	1	nd	>32

		(Connecticut)	clindamycin, erythromycin, gentamicin, methicillin, tetracycline, and trimethoprim						
	USA700	United States (Louisiana)	Resistant to erythromycin and methicillin	2	8	4	1	nd	>32
	USA800	United States (Washington)	Resistant to methicillin	2	8	4	0.5	nd	>32
	USA1000	United States (Vermont)	Resistant to erythromycin and methicillin	4	8	4	0.5	nd	>32
	USA1100	United States (Alaska)	Resistant to methicillin	2	8	4	1	nd	>32
	NRS194	United States (North Dakota)	Resistant to methicillin	2	8	4	1	nd	>32
	NRS108	France	Resistant to gentamicin	4	8	4	0.5	nd	>32
	NRS119 (Lin^R)	United States (Massachusetts)	Resistant to linezolid	4	16	128	0.5	nd	>32
	ATCC 43300	United States (Kansas)	Resistant to methicillin	4	16	4	0.5	nd	>32
	ATCC *BAA-44*	Lisbon, Portugal	Multidrug-resistant strain	4	16	4	0.5	nd	>32
	NRS70	Japan	Resistant to erythromycin and spectinomycin	4	16	2	0.5	2	>32
	NRS71	United Kingdom	Resistant to tetracycline and methicillin	4	16	2	0.5	2	>32
	NRS100	United States	Resistant to tetracycline and methicillin	4	16	2	0.5	2	>32
	NRS123	United States (North Dakota)	Resistant to tetracycline and methicillin	4	16	2	0.5	1	>32
Vancomycin intermediate *Staphylococcus aureus*	NRS1	Japan	Resistant to aminoglycosides and tetracycline Glycopeptide-	2	16	2	8	nd	>32

(VISA)			intermediate *Staphylococcus aureus*						
	NRS19	United States (Illinois)	Glycopeptide-intermediate *Staphylococcus aureus*	4	8	2	4	nd	>32
	NRS37	France	Glycopeptide-intermediate *Staphylococcus aureus*	2	8	2	8	nd	>32
Vancomycin Resistant *Staphylococcus aureus* (VRSA)	VRS1	United States	Resistant to vancomycin	4	32	1	>128	1	>32
	VRS2	United States	Resistant to vancomycin	4	16	1	16	1	>32
	VRS3a	United States	Resistant to vancomycin	4	16	1	128	2	>32
	VRS4	United States	Resistant to vancomycin	4	32	2	128	nd	>32
	VRS5	United States	Resistant to vancomycin	4	32	2	128	nd	>32
	VRS6	United States	Resistant to vancomycin	4	32	1	>128	nd	2
	VRS7	United States	Resistant to vancomycin	4	16	2	128	nd	>32
	VRS8	United States	Resistant to vancomycin	4	16	1	>128	nd	32
	VRS9	United States	Resistant to vancomycin	4	16	1	>128	nd	>32
	VRS10	United States	Resistant to vancomycin	4	16	4	>128	nd	>32
	VRS11b	United States	Resistant to vancomycin	4	32	1	>128	nd	>32
	VRS12	United States	Resistant to vancomycin	4	32	2	>128	nd	>32
	VRS13	United States	Resistant to vancomycin	4	16	1	>128	1	16
S. epidermidis	NRS101	United States	Prototype biofilm producer, Resistant to methicillin and gentamicin	2	8	2	0.5	nd	>32

Lin: linezolid, Van: vancomycin, Amp: ampicillin, nd: not determined

Table 2: Amino acid sequence and physicochemical properties of peptides used in this study

Peptide	Amino acid sequence	Length	Molecular weight	Charge	% of amino acids that are hydrophobic
RR	WLRRIKAWLRR	10	1553.9	+ 5	54 %
RRIKA	WLRRIKAWLRRIKA	13	1866.3	+ 6	57 %
KAF	KAFAKLAARKA	11	1174.4	+ 4	63 %
FAK	FAKLAARLYRKA	12	1407.7	+ 4	58 %

Table 3: Fractional inhinitory concentration (FIC) index for the combination of peptides together or with lysostaphin

Compound	FIC index[a]					
	MSSA (RN4220)		MRSA (USA300)		VRSA (VRS13)	
	RRIKA	RR	RRIKA	RR	RRIKA	RR
RRIKA	-	0.75	-	0.75	-	0.75
RR	0.75	-	0.75	-	0.75	-
Lysostaphin	0.26	0.26	0.26	0.26	0.26	0.26

[a] The FIC index was determined in the presence of a fixed concentration of peptide, equivalent to ¼ ×MIC.