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Effect of Bioactive Peptide, KR-12-a5, on Growth of Clinical Methicillin-resistant *Staphylococcus aureus* Isolates

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Abstract

Staphylococcus aureus is one of the most frequent infectious agents causing hospitals and community associated infections. Developing the treatment for methicillin resistant *S. aureus* (MRSA) is a challenging arena for effective therapeutics. Application of antimicrobial peptides is an alternative option to control MRSA infection. The potential of the bioactive peptide against MRSA was previously revealed; however, the study on the effect of KR-12-a5 on the various clinical MRSA growth was not elucidated. This study aimed to investigate the effect of bioactive peptide KR-12-a5 on the growth of MRSA isolated from patients. Evaluation of KR-12-a5 peptide using antimicrobial susceptibility test was performed. Antimicrobial activity assay showed that the KR-12-a5 at 2-64 µM could inhibit the growth of *S. aureus* ATCC 29213 in the range of 55.0±0.8%-81.0±6.2%. In case of KR-12-a5 treatment at 2, 4, and 8 µM, growth of all twenty clinical MRSA isolates could be inhibited less than 90% in the range of 0.0-75.9±1.6%, 0.0-73.0±4.2%, and 0.0-77.1±1.7%, respectively. Whereas the treatment of those at 16, 32, and 64 µM, growth of clinical MRSA could be significantly inhibited more than 90% ($p < 0.05$) in the range of 94.3±6.7%-100.4±0.4% in 3 isolates (15%), 97.3±0.3%-99.5±0.2% in 4 isolates (20%), and 91.6±0.8%-99.0±0.1% in 9 isolates (45%), respectively. These findings indicated that the KR-12-a5 effectively controls growth of both *S. aureus* ATCC 29213 and clinical MRSA isolates.

Keywords: *Staphylococcus aureus*, MRSA, KR-12-a5, Antimicrobial peptide

1. Introduction

Staphylococcus aureus is one of the most frequent infectious causes of hospitals and communities-associated infection. The bacteria are located on the skin and mucous membranes, mostly in the nose area. Methicillin resistant *S. aureus* (MRSA) strains are divided into hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) (Cerini et al., 2023; Otto, 2012). Developing the treatment for MRSA is challenging due to its antimicrobial resistance. Antimicrobial peptides (AMPs) are considered an alternative used to treat MRSA infection, generally made up of 10-50 amino acid residues. Antimicrobial peptides from microbes and animals are active against multidrug-resistant Gram-positive and Gram-negative bacteria especially the inhibition of biofilm formation. LL-37 is a synthetic antimicrobial peptide reveals the potential for antimicrobial activity *in vitro* against bacteria, fungi, viruses, and parasites. There are also shortening peptide lines and amino acid changes to increase the effectiveness of pathogen inhibition (Xhindoli et al., 2016). Consequently, the α-helical wheel diagram of KR-12 developed from LL-37, was altered the hydrophobicity and net positive charge of the peptides to obtain the effective short AMPs having antimicrobial activities without mammalian cell toxicity (Jacob, Park, Bang, & Shin, 2013). KR-12 was designed in a series of analogs and synthesized as KR-12-a (1-8). The antimicrobial activity of KR-12 and its analogs were determined against Gram-positive (*Bacillus subtilis*, *Staphylococcus epidermidis*, *S. aureus*), and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium). Studies on KR-12-a5 revealed a minimal inhibitory concentration (MIC) of 4 µM against *S. aureus* KCTC 1621 (Jacob et al., 2013) and higher (2- to 4-fold) antimicrobial activity against MRSA

than LL-37 (Kim, Rajasekaran, & Shin, 2017). The activity of KR-12-a5 surpasses that of LL-37 against multidrug-resistant bacteria and demonstrates synergistic effects with antibiotics against multidrug-resistant *Pseudomonas aeruginosa* (MDRPA). Additionally, KR-12-a5 exhibits lack of cytotoxicity for mammalian cells and resistance to physiological salts, as reported by Kim et al. (2017). Consequently, KR-12-a5 emerges as a promising candidate for the development of antimicrobial agents. Even though the potential of the bioactive peptide KR-12-a5 against MRSA was previously reported (Kim et al., 2017); however, there is limited report on the effect of KR-12-a5 on growth of various MRSA isolated from patients. This study aimed to determine the effect of bioactive peptide KR-12-a5 on growth of clinical MRSA.

2. Materials and Methods

2.1 Peptide synthesis

The antimicrobial peptide KR-12-a5 (KRIVKLILKWLR-NH_2) was synthesized through standard solid-phase methods using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Synpeptide, China). The synthesized peptide was subsequently purified and identified utilizing HPLC and Electrospray Ionization Mass Spectrometry (ESI-MS) (Synpeptide, 2015). The purity of peptide was more than 95.5% as ascertained by HPLC. The resulting lyophilized peptide was dissolved in Millipore-purified water, yielding a stock solution with a concentration of 10 mM, and was stored at -20°C for further use.

2.2 Bacterial strains

S. aureus ATCC 29213 was used as a standard strain and the twenty clinical methicillin resistant *S. aureus* isolates in this experiment were obtained from the stock culture in a private hospital in Bangkok and Department of Microbiology, Faculty of Public Health, Mahidol University. Clinical specimens, including sputum, pus, urine, catheter tips, rectal swabs, and blood cultures, were collected during 2021-2022 and processed using standard microbiological techniques and MRSA identification was confirmed through antimicrobial susceptibility testing.

2.3 Bacterial culture

Bacteria were cultured individually on tryptic soy agar (TSA) at 37°C for 18-24 h. A single colony was picked up and inoculated into tryptic soy broth (TSB) and incubated at 37°C in 200 rpm shaker for 2-6 h. The turbidity of bacterial culture was adjusted to standardize the inoculum density for an antimicrobial susceptibility test using a $BaSO_4$ 0.5 McFarland standard (CLSI, 2018).

2.4 Evaluation of antimicrobial activity of KR-12-a5 peptide using antimicrobial susceptibility test

In the laboratory experiment, an evaluation of the antimicrobial susceptibility of KR-12-a5 was conducted. To ensure standardized conditions, the bacterial suspension used in this evaluation was adjusted to an approximate concentration of 1 x 10^8 CFU/ml. This adjustment was achieved by matching the turbidity of the bacterial suspension to that of a 0.5 McFarland standard, following the guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) document of 2018. Subsequently, the adjusted bacterial suspension was further diluted to a 1:100 ratio using a 1% peptone solution. The antimicrobial evaluation of KR-12-a5 peptide was conducted utilizing a growth rate curve analysis. This experiment took place in a controlled environment within a sterile 96-well microtiter plate. Growth rate curves allowed for a comprehensive assessment of the antibacterial activity of the peptide.

The KR-12-a5 stock peptide was two-fold diluted (4-128 µM) in 1% peptone. The 50 µl of stock peptide (2-64 µM: final concentration) and the 50 µl of diluted bacteria (~ 5x10^5 CFU/ml: final concentration) were inoculated to each well of microtiter plate (CLSI, 2018; Kim et al., 2017). The mixtures were incubated at 37°C and measured by microtiter plate reader at 600 nm every h for 18 h. The 100 µl of peptone served as a control. The 50 µl of vancomycin (2 mg/ml) combined with 50 µl of diluted bacteria (~5x10^5 CFU/ml), and 50 µl of TSB mixed with 50 µl of diluted bacteria (~5x10^5 CFU/ml) used as the positive control and negative control, respectively. To assess the inhibition percentages of KR-12-a5 and vancomycin, all experiments were done in triplicate, and the following formula was employed:

$$\% \text{ inhibition} = [(OD_{\text{negative control}} - OD_{\text{treatment}})/OD_{\text{negative control}}] \times 100\%$$

2.5 Statistical analysis

Each experiment was performed triplicate and the results were expressed as mean±SD. Analysis of variance (one-way ANOVA; assume α at 0.05) was used to analyze statistical significance of differences between the experiment groups and control groups in antimicrobial susceptibility test. The analysis was performed by using SPSS statistics 18.

3. Results and Discussion

Antimicrobial activity on growth of *S. aureus* ATCC 29213 and twenty clinical MRSA isolates was evaluated using antimicrobial susceptibility test. Bacterial culture was tested with three different treatments for 18 h incubation in 96-well plate. 1) six concentrations of KR-12-a5 (2 μM, 4 μM, 8 μM, 16 μM, 32 μM, and 64 μM), 2) TSB (negative control), and 3) antibiotic (positive control). The antimicrobial activity of all six concentrations of KR-12-a5 on *S. aureus* ATCC 29213 growth was observed within the incubation period spanning 8 to 18 h in the course of the study (Figure 1A). The results indicated that all six concentrations of KR-12-a5 exhibited significant antimicrobial effects on the growth of *S. aureus* ATCC 29213. At 18 h of experiment, those treated with all six concentrations of KR-12-a5 revealed significant differences compared to negative control (p-value < 0.01) and positive control (p-value < 0.01). The experiment depicted in Table 1 and Figure 2 suggested that KR-12-a5 peptide has an effect in controlling the growth of *S. aureus* ATCC 29213. The growth of those could be inhibited by KR-12-a5 at different concentrations. At concentrations of 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, and 64 μM, the KR-12-a5 peptide demonstrated inhibitory effects on *S. aureus* ATCC 29213 growth, yielding inhibitions of 56.6±0.9%, 55.7±1.5%, 55.8±0.9%, 55.0±0.8%, 57.0±2.4%, and 81.0±6.2%, respectively. These results were consistent with antimicrobial activity against *S. aureus* KCTC 1621 with the MIC of 4 μM (Jacob et al., 2013; Kim et al., 2017).

In this investigation, twenty samples that were isolated from the various clinical samples were employed, including sputum 7 (35.0%), pus 5 (25.0%), urine 5 (25.0%), catheter tip 1 (5.0%), rectal swab 1 (5.0%), and haemoculture 1 (5.0%) isolates (Table 2). The growth of twenty clinical MRSA isolates was assessed to determine the antimicrobial action of KR-12-a5 at concentrations ranging from 2 μM to 64 μM (Figure 1B-1U and Table 1). During the time period 3-18 h, growth of all clinical MRSA isolates exhibited significant difference of least one pair (p-value < 0.05). At 18 h for the treatment of 2 μM KR-12-a5, the growth of twenty clinical MRSA isolates could be inhibited less than 90% in the range of 0.0±0.0% to 75.9±1.6%. For the treatment of 4 μM KR-12-a5, the growth of the twenty isolates were inhibited in the range of 0.0±0.0% to 73.0±4.2%. For the treatment of 8 μM KR-12-a5, the growth of the twenty isolates were inhibited in the range of 0.0±0.0% to 77.1±1.7%. For the treatment of 16 μM KR-12-a5, growth of three clinical MRSA isolates (15%) including isolate 8, isolate 13, and isolate 16, were inhibited more than 90% within 18 h in the range of 94.3±6.7% to 100.4±0.4%, whereas the growth of other 17 clinical MRSA isolates were inhibited less than 90% in the range of 28.1±9.4% to 79.9±3.3%. For the treatment of 32 μM KR-12-a5, growth of four clinical MRSA isolates (20%) (isolate 2, isolate 8, isolate 13, and isolate 16) were inhibited more than 90% in the range of 97.3±0.3% to 99.5±0.2%, whereas the growth of other 16 clinical MRSA isolates were inhibited less than 90% in the range of 20.1±7.8% to 88.8±1.6%. For the treatment at 64 μM, growth of nine clinical MRSA isolates (45%) (isolate 1, isolate 2, isolate 3, isolate 4, isolate 7, isolate 8, isolate 9, isolate 13, and isolate 16) were inhibited more than 90% in the range of 91.6±0.8% to 99.0±0.1%, whereas the growth of other 11 clinical MRSA isolates were inhibited less than 90% in the range of 66.4±5.6% to 85.4±0.8% (Table 1 and Figure 2).

The percent inhibition resulting from the 64 μM KR-12-a5 treatment was also compared at 6, 12, and 18 h, as illustrated in Figure 2. The growth of *S. aureus* ATCC 29213 was inhibited for 96.6±1.0%, 94.1±4.4%, and 81.0±6.2%, respectively, and those of twenty clinical MRSA isolates were inhibited in the range of 68.4±6.0% to 100.4±0.7%, 83.4±0.9% to 98.4±0.6%, and 66.4±5.6% to 99.0±0.1%, respectively. Despite the apparent inhibitions observed during the early stages of growth in both *S. aureus* ATCC 29213 and twenty clinical MRSA

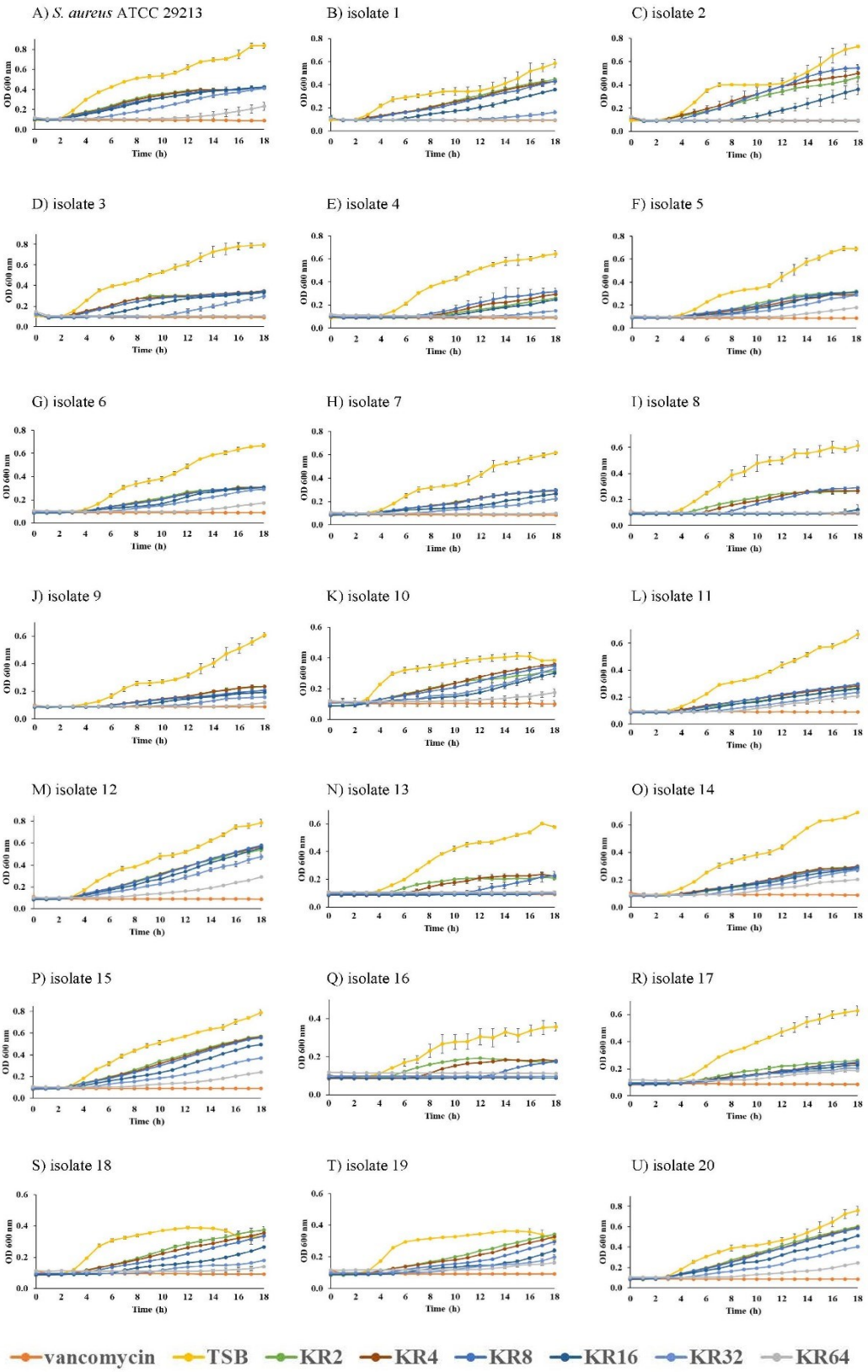


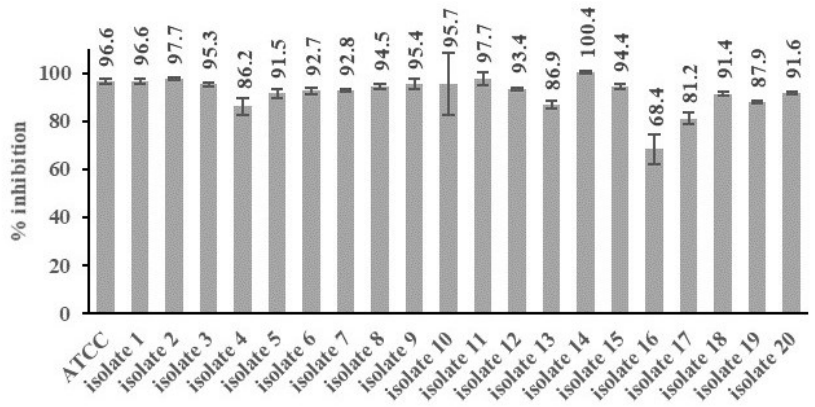
Figure 1. Antimicrobial activity of 2 - 64 µM KR-12-a5 on growth of *S. aureus* ATCC 29213 A) and clinical MRSA strains, B) - U) isolate 1-20 comparison to positive control (vancomycin) and negative control (TSB). All experiments were done in triplicate.

Table 1. Inhibitory effect and percent inhibition of KR-12-a5 on growth of *S. aureus* ATCC 29213 and clinical MRSA isolate 1-20 at 18 h of treatment.

Stains	Concentrations (μM)					
	2	**4**	**8**	**16**	**32**	**64**
S. aureus ATCC 29213	+ 56.6±0.9%	+ 55.7±1.5%	+ 55.8±0.9%	+ 55.0±0.8%	+ 57.0±2.4%	+ 81.0±6.2%
Isolate 1	+ 27.7±6.0%	+ 31.1±9.3%	+ 30.9±3.0%	+ 46.2±1.3%	+ 85.6±2.5%	++ 98.7±0.4%
Isolate 2	+ 41.6±4.1%	+ 35.9±12.7%	+ 29.2±3.2%	+ 57.4±7.4%	++ 99.5±0.2%	++ 99.0±0.1%
Isolate 3	+ 64.1±1.6%	+ 63.2±0.2%	+ 64.3±0.7%	+ 65.5±1.5%	+ 70.7±3.2%	++ 98.0±0.2%
Isolate 4	+ 69.3±2.6%	+ 63.2±3.1%	+ 58.9±7.9%	+ 71.4±2.2%	+ 88.8±1.6%	++ 98.1±0.3%
Isolate 5	+ 61.1±0.8%	+ 65.9±1.1%	+ 62.6±1.2%	+ 62.7±2.0%	+ 66.5±2.3%	+ 84.5±1.2%
Isolate 6	+ 62.5±0.5%	+ 62.1±0.7%	+ 62.4±1.3%	+ 62.2±1.0%	+ 64.7±1.0%	+ 85.4±0.8%
Isolate 7	+ 60.9±2.0%	+ 60.8±2.0%	+ 60.0±1.5%	+ 65.8±1.9%	+ 74.2±3.1%	++ 97.8±1.3%
Isolate 8	+ 66.3±1.0%	+ 66.3±2.2%	+ 61.8±1.0%	++ 94.3±6.7%	++ 98.9±0.4%	++ 97.5±0.0%
Isolate 9	+ 71.8±2.8%	+ 72.0±0.6%	+ 77.1±1.7%	+ 79.9±3.3%	+ 86.5±0.8%	++ 94.3±2.2%
Isolate 10	+ 25.6±14.5%	+ 9.5±4.3%	+ 12.4±3.9%	+ 29.7±12.0%	+ 20.1±7.8%	+ 75.0±11.2%
Isolate 11	+ 68.4±1.6%	+ 66.0±0.6%	+ 64.0±2.3%	+ 70.0±1.1%	+ 74.5±0.6%	+ 79.2±2.2%
Isolate 12	+ 35.6±4.3%	+ 31.0±2.8%	+ 29.6±3.5%	+ 33.1±3.5%	+ 44.6±2.1%	+ 70.9±1.3%
Isolate 13	+ 75.9±1.6%	+ 73.0±4.2%	+ 72.3±6.8%	++ 97.2±2.6%	++ 99.2±0.3%	++ 97.0±0.2%
Isolate 14	+ 65.7±1.1%	+ 65.2±1.6%	+ 66.3±2.4%	+ 68.0±2.6%	+ 69.6±3.1%	+ 81.2±1.0%
Isolate 15	+ 31.1±3.4%	+ 32.1±2.7%	+ 33.0±3.4%	+ 42.0±1.6%	+ 59.7±2.5%	+ 78.2±0.8%
Isolate 16	+ 68.9±2.8%	+ 66.8±2.1%	+ 67.8±2.2%	++ 100.4±0.4%	++ 97.3±0.3%	++ 91.6±0.8%
Isolate 17	+ 67.5±2.1%	+ 71.3±4.3%	+ 70.1±3.3%	+ 73.9±0.8%	+ 78.0±2.7%	+ 81.3±2.3%
Isolate 18	- 0.0±0.0%	- 0.0±0.0%	- 0.0±0.0%	+ 28.1±9.4%	+ 63.5±5.1%	+ 80.6±5.1%
Isolate 19	- 0.0±0.0%	- 0.0±0.0%	+ 3.9±12.6%	+ 29.2±16.4%	+ 50.2±9.9%	+ 66.4±5.6%
Isolate 20	+ 22.6±5.6%	+ 24.8±3.6%	+ 25.5±3.0%	+ 36.3±3.8%	+ 52.4±3.8%	+ 76.2±0.2%

- means no inhibition; + means % inhibition less than 90% and ++ means % inhibition more than 90%

A)



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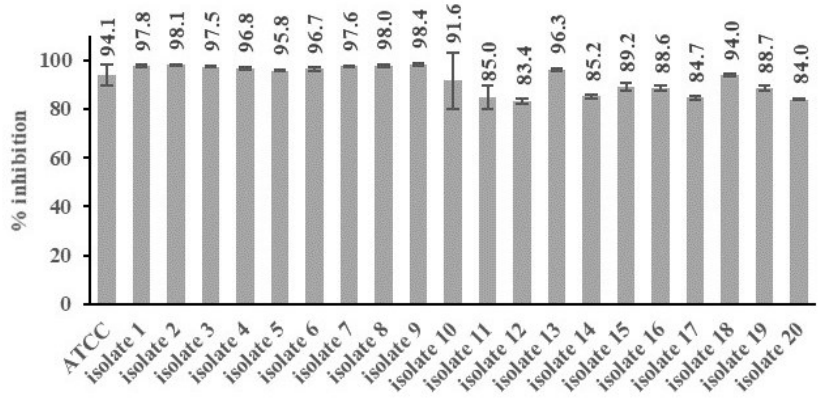

C)

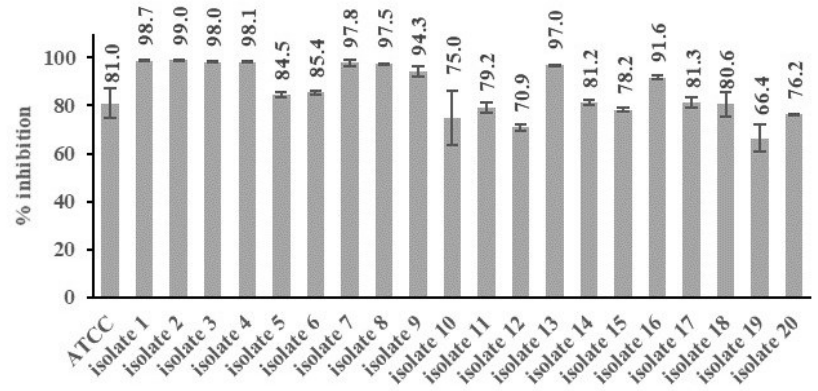

Figure 2. Percent inhibition of 64 µM KR-12-a5 on growth of *S. aureus* ATCC 29213 and clinical MRSA isolate 1-20 at A) 6 h, B) 12 h, and C) 18 h of treatment. All experiments were done in triplicate.

Table 2. Source of clinical MRSA in stock culture

Isolates	Specimen	Diseases in patient
1	Sputum	Acute respiratory failure
2	Pus	Acute bronchitis
3	Sputum	Cancer
4	Pus	Pneumonia
5	Urine	Cancer
6	Pus	Cancer
7	Catheter tip	Cancer
8	Rectal swab	Cancer
9	Pus	Pneumonia
10	Sputum	Liver cirrhosis
11	Pus	Osteomyelitis
12	Sputum	Cancer
13	Urine	Cancer
14	Urine	Cancer
15	Urine	Cancer
16	Sputum	Cancer
17	Urine	Cancer
18	Sputum	Osteomyelitis
19	Hemoculture	Cellulitis
20	Sputum	Cancer

isolates, the antibacterial activity at 6 and 12 h of treatment exhibited a consistent trend, aligning with the observations made at 18 h.

The growth anomalies were observed in some isolates. From the unexpected growth pattern especially early decline phase in isolate 10, isolate 18, and isolate 19, the growth of those isolates was in the death phase after 16 h. Despite all of them being MRSA and having received similar KR-12-a5 treatment, the divergent outcomes could be attributed to several factors, including variations in the source of clinical MRSA, the presence of distinct drug-resistant strains, and disparities in antimicrobial susceptibility patterns. These multifaceted elements contribute to the heterogeneous response to treatment, emphasizing the complexity of MRSA infections and highlighting the need for comprehensive research to elucidate the underlying mechanisms governing treatment efficacy in diverse clinical scenarios.

The effect of antimicrobial peptide OP-145 on growth of ten clinical MRSA isolates has been determined (Ming & Huang, 2017). The OP-145 showed significant antibacterial activity against nine clinical MRSA strains (90%, p-value < 0.05). Previous research on the effect of KR-12-a5 on growth of MRSA isolated from patients is very limited. The present study showed that treatment with KR-12-a5 at concentrations ranging from 4 μM to 8

μM for 18 h resulted in bacterial inhibition for a subset of MRSA isolates. Specifically, out of the total isolates tested, twelve isolates (60%) showed bacterial inhibition in the range of 60.8±2.0% to 73.0±4.2%, while eleven isolates (55%) exhibited inhibition in the range of 60.0±1.5% to 77.1±1.7%. These findings suggested that the treatment with KR-12-a5 at concentrations between 4 μM and 8 μM were successful in inhibiting the growth of MRSA isolates. Furthermore, the results from our study showed that the antimicrobial activity of KR-12-a5 was consistent with three MRSA strains, specifically CCARM 3089, CCARM 3090, and CCARM 3095 with the MIC in the range of 4 μM to 8 μM (Jacob et al., 2013; Kim et al., 2017).

Besides, KR-12-a5 exhibited an HC_{50} value of 96 μM and did not cause any hemolytic activity at MIC 4 μM (Jacob et al., 2013). Previous study revealed that KR-12-a5 is a non-cytotoxic agent with potent antimicrobial and antibiofilm activity against pathogens; moreover, KR-12-a5 might serve as a potential therapeutic agent and play key effects in the treatment of infections (Caiaffa et al., 2017; Li et al., 2019). Limitations in this study were the lack of MIC determination of each MRSA isolate and the presence of early death phase in some isolates.

4. Conclusions

Antimicrobial activity assay demonstrated that the KR-12-a5 at concentrations ranging from 2 μM to 64 μM inhibited the growth of *S. aureus* ATCC 29213 at 18 h with the growth inhibition percentages ranged from 55.0±0.8% to 81.0±6.2%. The antimicrobial activity of KR-12-a5 against twenty clinical MRSA isolates was determined. In this study, the KR-12-a5 inhibited growth of all tested clinical MRSA isolates less than 90% at the concentrations of 2 μM, 4 μM, and 8 μM in ranged of 0.0±0.0%-75.9±1.6%, 0.0±0.0%-73.0±4.2%, and 0.0±0.0%-77.1±1.7%, respectively. Nevertheless, KR-12-a5 at the concentration of 16 μM, 32 μM, and 64 μM significantly inhibited growth of clinical MRSA isolates more than 90% ($p < 0.05$) in 3 isolates (15%), 4 isolates (20%), and 9 isolates (45%) in ranged of 94.3±6.7%-100.4±0.4%, 97.3±0.3%-99.5±0.2% and 91.6±0.8%-99.0±0.1%, respectively. These findings indicate that bioactive peptide KR-12-a5 effectively inhibits growth of both *S. aureus* ATCC 29213 and clinical MRSA isolates. The review of KR-12-a5 peptide on this research is rarely carried out, especially when MRSA that is isolated from patients.

This pioneering research delved into MRSA strains collected from patients in Thailand. Remarkably, despite uniform treatment, diverse outcomes emerged, highlighting the distinct characteristics of the studied MRSA variants. This study provides valuable data for the practical application of the investigated peptide, offering insights into its potential as a therapeutic or preservative agent. For further studies, the determination of MIC and MBC is pivotal in evaluating the safety and efficacy of antimicrobial peptide. The findings contribute to the growing body of knowledge surrounding antimicrobial peptides, paving the way for their judicious utilization in various fields.

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Conflict of Interest

No conflict of interest.

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Ethical Approval

This research was reviewed and approved according to the Standard Operating Procedures of Ethical Review Committee for Human Research, Faculty of Public Health, Mahidol University, to consider to be complied with a "Research with Exemption" category (Protocol number 40/2564).

Publication Ethic

The submitted manuscript has not been previously published or is under review for publication by another print or online journal or source.

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