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Tachyplesin I Induce Drug Resistance in Bacteria *in vitro*

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Abstract: Tachyplesin I is a 17 amino acid cationic antibacterial peptide with a typical cyclic antiparallel β-sheet structure. It is not clear whether tachyplesin I can induce resistance in bacteria. Therefore, two selection procedures by gradually increasing the induction concentration of tachyplesin I were performed to determine whether tachyplesin I could induce resistance in *Escherichia coli* and *Staphylococcus aureus in vitro*. The results showed that tachyplesin I did not induce resistance in *Escherichia coli* ATCC25922, F41 and *Staphylococcus aureus* ATCC25923 in this study whereas high resistance to benzylpenicillin was induced using the same procedures in *Staphylococcus aureus* ATCC25923. Furthermore, no cross-resistance between tachyplesin I and benzylpenicillin was observed. It is suggested that tachyplesin I may be a safe antibacterial agent for future use in clinical practice.

Key words: Antimicrobial peptides, resistance, tachyplesin I, *Escherichia coli*, *Staphylococcus aureus*, China

INTRODUCTION

With the rapid emergence of antibiotic resistance, it is necessary to develop new alternative antibacterial agents (Falakaflaki *et al*., 2007; Karou *et al*., 2009; Habi and Daba, 2009). Antimicrobial Peptides (AMPs) are acknowledged to be promising candidates as novel alternative antibiotics (Zasloff, 2002; Brogden, 2005) due to their broad-spectrum antimicrobial activity and lack of acquired resistance in microbes. However, studies have indicated that some bacteria were resistant to certain AMPs. For example, most strains of *Lactobacillus casei* were found to be resistant to nisin (Breuer and Radler, 1996); Pexiganan, a Cationic Antibacterial Peptide (CAMP), the analogue of magainin could induce resistance in *Escherichia coli* and *Pseudomonas fluorescens* through successive selection methods in the laboratory (Perron *et al*., 2006); *Salmonella enterica* serovar typhimurium can rapidly acquire mutations which are resistant to the AMP of PR-39 with a stretching helical conformation (Pranting *et al*., 2008). Thus, microbial resistance to AMPs has become a crucial issue in the research and development of new AMPs.

Tachyplesin I, a CAMP, was originally isolated from hemocytes of the marine living fossil horseshoe crabs, *Tachypleus tridentatus* (Nakamura *et al*., 1988). It consists of 17 amino acid residues, two disulfide bonds and one unique arginine α-amide at the C terminal end and is characterized by a disulfide-stabilized β-sheet conformation, exhibiting potent and broad-spectrum activities against both gram-positive and gram-negative bacteria (Nakamura *et al*., 1988; Dai *et al*., 2008), fungi (Miyata *et al*., 1989), viruses (Nakashima *et al*., 1992), protozoa (Morvan *et al*., 1997) and cancer cells (Ouyang *et al*., 2002). Studies have indicated that tachyplesin I may kill bacteria not only by depolarization of the cytoplasmic membrane, preceded by permeabilization of the bacterial outer membrane for gram-negative bacteria but also by binding plasmid DNA and inhibiting the synthesis of macromolecules (Ohta *et al*., 1992; Imura *et al*., 2007; Dai *et al*., 2008). Because of its potency and relatively small size this peptide is a promising candidate as a novel alternative antibiotic in the pharmaceutical industry (Nakamura *et al*., 1988; Masuda *et al*., 1994; Hirakura *et al*., 2002), animal feed (Dai *et al*., 2009) and food industry.

To date, it is not clear whether tachyplesin I can induce resistance in bacteria. Tachyplesin I and other CAMPs can activate the aps system of *Staphylococcus epidermidis* (*S. epidermidis*). The aps system exclusively responds to CAMPs and controls the AMP resistance genes (*dlt* and *mprF*) on the surface of gram-positive

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bacteria (Li *et al*., 2007) which suggests that tachyplesin I may induce resistance in bacteria. Researchers speculated that if bacteria were continuously exposed to increasing concentrations of tachyplesin I, they may become resistant to tachyplesin I. Therefore, in this study, researchers determined whether tachyplesin I could induce resistance in *E. coli* and *S. aureus* using two selection procedures.

MATERIALS AND METHODS

Microorganisms, media and growth conditions: Gram-positive bacterium and gram-negative bacterium used in this study were provided by the Microbial Culture Collection Center of Guangdong (China). *E. coli* ATCC25922, *S. aureus* ATCC25923 and *E. coli* F41 were the original strains used in the study. Unless otherwise stated, the bacteria were cultured in nutrient broth media (1% peptone, 0.3% beef extract and 0.5% NaCl) and nutrient agar plates.

Antibacterial agents: The agents used in this study were the CAMP, tachyplesin I, synthesized by Shenzhen HYBIO (China) which had a purity >95%. Its amino acid sequence was as described (Nakamura *et al*., 1988):

The peptide was solubilized in phosphate buffered saline (pH 7.2) yielding a 10 mg mL^{-1} stock solution which was filter-sterilized before use. Benzylpenicillin sodium for injection (benzylpenicillin), 0.8 million units was provided by North China Pharmaceutical Group Corporation. Drug solutions were made fresh on the day of the assay or stored at -20°C for short periods.

MIC and MBC determination: The Minimum Inhibitory Concentration (MIC) was determined using a broth microdilution method for AMPs as described previously (Giacometti *et al*., 2000). Briefly, the strains were cultured in nutrient broth at 37°C with shaking at 200 r min^{-1} overnight. To obtain log phase growth microorganisms, 1 mL aliquots of the cultures were transferred to 100 mL of fresh nutrient broth and incubated for 2-3 h. The cells were diluted with 10 mM sodium phosphate, pH 7.4 buffer and resuspended in the same buffer. The cell concentrations were estimated by measuring the absorbance at 600 nm (Eppendorf BioPhotometer plus, Germany). The suspensions were diluted to 2×10^5-4×10^5 CFU mL^{-1}. The inoculum (100 μL) was added to each well of 96 well plates. The peptide samples diluted with fresh broth (100 μL) were added to each well and the plates were incubated at 37°C for 20 h. Cell growth was assessed by measuring the OD_{490} on a Spectralmax M_2 model microplate reader. The MIC was considered the lowest drug concentration that reduced growth by more than 50% compared with growth in the control well. The viable count in each well was determined by performing 10^3 dilutions and plating 10 μL of each dilution onto nutrient agar plates to obtain overnight cultures. The Minimum Bactericidal Concentration (MBC) was determined by plating out the contents of the wells that showed no visible bacterial growth onto nutrient agar plates and incubating at 37°C for 18 h. The MBC was considered to be the lowest concentration of each drug that prevented any residual colony formation. Experiments were performed in triplicate.

***In vitro* resistance study**

The first selection procedure: Bacteria (*E. coli* ATCC25922 and *S. aureus* ATCC25923) were cultured in nutrient broth overnight at 37°C. A 10 μL portion of the cell suspension was added to 2 mL nutrient broth with or without a final concentration of 1/2×MIC tachyplesin I. Following incubation for 20 h at 37°C with 200 r min^{-1} shaking if bacterial growth was observed, 10 μL of the culture was added to 2 mL of fresh nutrient broth lacking tachyplesin I for 3 successive passages under the same conditions. The regrown bacteria were then transferred into broth containing a higher concentration of tachyplesin I. The procedure was repeated with an increase in concentration of tachyplesin I at each step until no visible bacterial growth was observed.

Bacteria which were grown in the absence of tachyplesin I for the duration of the experiment acted as the control group. A sample of each induction generation was inoculated with 15% (v/v) glycerol and stored at -70°C. The MICs of tachyplesin I for each induced bacterial culture were determined at the same time.

The second selection procedure: This procedure was performed according to a previously reported method with some modifications (Ruiz *et al*., 2001). Researchers first prepared nutrient agar plates containing different concentrations of the drugs according to the MIC. The selected strain was initially grown in the nutrient agar medium with 1/2×MIC of tachyplesin I or benzylpenicillin at 37°C for 20 h. If bacterial growth was observed, one colony was both frozen and regrown on nutrient agar medium with 1×MIC drugs. This procedure was repeated 17 times with increasing concentrations of tachyplesin I or repeated 10 times with increasing benzylpenicillin concentrations until a final concentration of 640 μg mL^{-1} (= 64×MIC) tachyplesin I and 0.25 U mL^{-1} (= 20×MIC) benzylpenicillin was obtained. In addition, each strain was

grown in the absence of tachyplesin I or benzylpenicillin for the duration of the experiment which is referred to as the control selection experiment.

***In vitro* susceptibility testing:** Researchers assayed the resistance induced by measuring the MIC of tachyplesin I or benzylpenicillin for each selection strain. The MICs for the drugs used were determined according to the above method. Since Clinical Laboratory Standards Institute (CLSI) does not define standard breakpoints for isolates resistant to AMPs, researchers identified resistance using the MIC value of the inducible strain and initial strain according to a method previously described (Martinez and Baquero, 2000). The definition of bacterial resistance to AMPs was a significant increase in MIC for inducible strains compared to that of control strains.

Stability of resistance: To verify the stability of resistance, 10 μL of resistant strains were cultured in 2 mL nutrient broth medium without drug for 8 continuous passages each of 20 h and off-spring strains from each passage were stored in 15% (v/v) glycerol at -70°C. The MIC values for each passage strain were determined, respectively.

Determination of MICs for tachyplesin I in strains resistant to benzylpenicillin: The method for MIC determination was the same as earlier.

RESULTS AND DISCUSSION

MIC and MBC of tachyplesin I and benzylpenicillin: As shown in Table 1, tachyplesin I displayed similar antibacterial activity against gram-positive and gram-negative bacteria. In addition, benzylpenicillin also showed strong antibacterial activity against *S. aureus* ATCC25923. The MIC and MBC of benzylpenicillin for *S. aureus* ATCC25923 was 0.0125-0.025 and 0.05 U mL^{-1}, respectively.

***In vitro* studies of resistance development:** To explore the development of resistance to tachyplesin I *in vitro*, two selection procedures were performed. In the first procedure in order to investigate unadaptable resistance to tachyplesin I under laboratory conditions, researchers adopted non-successive induction methods. *E. coli* ATCC25922 and *S. aureus* ATCC25923 were cultured in an increasing concentration gradient of tachyplesin I in addition to three continuous passages in the absence of tachyplesin I nutrient broth. The bacteria were continuously cultured in a medium containing increasing concentrations of tachyplesin I (1/2×MIC, 2×MIC, 4×MIC, 5×MIC and so on).

Table 1: MIC and MBC of tachyplesin I (μg mL^{-1})

Organisms	MIC	MBC
Gram-positive bacterium		
S. epidermidis ATCC12228	10	20
Bacillus cereus AS1.196	20	40
Bacillus licheniformis AS1.269	5	20
S. aureus ATCC25923	10	20-40
S. aureus ATCC6538	10	20
Gram-negative bacterium		
E. coli ATCC25922	10	20-40
E. coli F41	5-10	20-40
E. coli K88	10	20
P. Aeruginosa ATCC27853	20	40

MIC was measured using broth microdilution method. It was considered the lowest drug concentration that reduced growth by >50% compared with growth in the control well. MIC and MBC were the average values obtained from three independent measurements

E. coli stopped growing when transferred into 9×MIC tachyplesin I broth whereas *S. aureus* stopped growing when transferred into 8×MIC tachyplesin I. During the process of resistance induction compared with the corresponding control induced strains, the growth of each successively induced strain was weaker. Furthermore, as would be expected less growth was observed with each successive transfer into higher concentrations of tachyplesin I. There was no significant difference in the MIC (10-20 μg mL^{-1}) between the control induced strains and the corresponding treatment induction strains in *E. coli* and *S. aureus*. These results showed that no inducible resistance to tachyplesin I in *E. coli* and *S. aureus* was produced. The induction procedure and the MIC of tachyplesin I can be shown in Table 2.

To explore the future use of tachyplesin I as an antibacterial agent in animal feed and in the clinic in the second selection procedure researchers adopted successive induction methods. The bacteria were successively transferred onto agar plates containing increasing concentrations (5, 10, 20, 30, 40, 60, 70, 80, 90, 100, 120, 140, 160, 200, 240, 320, 400, 480, 560 and 640 μg mL^{-1}) of tachyplesin I for twenty passages. During induction of resistance to tachyplesin I, greater colony numbers and better uniformity of morphology were observed in the control inducible strains compared with the treatment inducible strains. Fewer colonies were observed on the agar plates with each successive transfer into higher concentrations of tachyplesin I. A few visible colonies were seen on the nutrient agar plate containing 640 μg mL^{-1} tachyplesin I. As agar contains alginic acid, a major anionic component, this can bind to CAMPs and is probably the reason why such high concentrations (640 ug mL^{-1}) of tachyplesin I had to be used in the induction experiment. As shown in Table 3, no tachyplesin I resistant mutants in *E. coli* ATCC25922, *E. coli* F41 or *S. aureus* ATCC25923 were produced by this method.

Table 2: Induction of resistance to tachyplesin I

Organisms	Treatments	Concentration of tachyplesin I (μg mL^{-1})	MIC ranges of tachyplesin I (μg mL^{-1})	
			Before induction	After induction
E. coli	Control group	0	10	10-20
ATCC25922	Treatment group	5-90	10	10-20
S. aureu	Control group	0	10	10-20
ATCC25923	Treatment group	5-85	10	10-20

Inducible strains were successively cultured in nutrient broth supplemented with tachyplesin I (1/2×MIC, 1×MIC, 2×MIC, 4×MIC, 5×MIC, 6×MIC, 7×MIC, 8×MIC and 8.5×MIC) whereas control strains were successively cultured in unsupplemented medium under the same conditions. The method for MIC determination was the same as above. Each determination was made using three independent replicates from each line

Table 3: Induction of resistance to tachyplesin I

Organisms	Treatments	Concentration of tachyplesin I (μg mL^{-1})	MIC ranges of tachyplesin I (μg mL^{-1})	
			Before induction	After induction (20 successive passages)
E. coli	Control group	0	10	10-20
ATCC25922	Treatment group	5-640	10	10-20
E. coli F41	Control group	0	5-10	10-20
	Treatment group	5-640	5-10	10-20
S. aureus	Control group	0	10	10-20
ATCC25923	Treatment group	5-640	10	10-20

Inducible strains were cultured on nutrient agar plates supplemented with tachyplesin I whereas control strains were cultured in unsupplemented nutrient agar plates under the same conditions. The method for MIC determination was the same as above. Each determination was made using three independent replicates from each line

Table 4: Induction of resistance to benzylpenicillin*

Organisms	Treatments	Concentration* in agar medium (U mL^{-1})	MIC* ranges (U mL^{-1})	
			Before induction (original strain)	After induction (14 passages)
S. aureus	Control group	0	0.0125-0.025	0.05-0.1
ATCC25923	Treatment group	0.00625-0.25	0.0125-0.025	0.8-1.6

*Refers to benzylpenicillin. Inducible strains were cultured in nutrient agar supplemented with increasing concentrations of benzylpenicillin whereas control strains were cultured in unsupplemented nutrient agar under the same conditions. The method for MIC determination was the same as above. Each determination was made using three independent replicates from each line

Under the same conditions, the development of resistance to tachyplesin I in *S. aureus* ATCC25923 was compared to that of benzylpenicillin. After thirteen successive passages *in vitro* in the presence of increasing concentrations of benzylpenicillin (0.00625-0.25 U mL^{-1}), there was a notable increase in the MICs for *S. aureus* (16 or 64 fold increase compared with the corresponding control strain or original strain) (Table 4). The MICs of benzylpenicillin for each induced strain in *S. aureus* gradually increased (Fig. 1). The strain obtained in 0.25 U mL^{-1} benzylpenicillin was named QP.

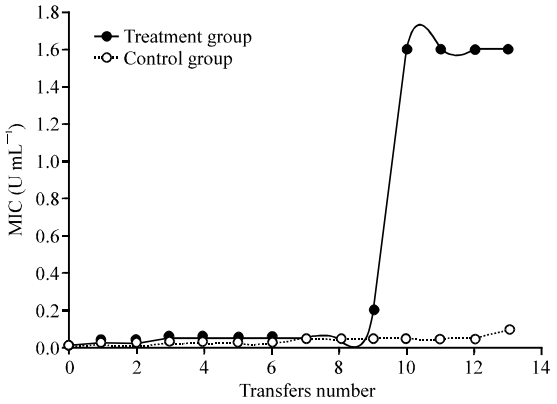

Fig. 1: Changes in MIC for the obtained strain of *S. aureus* ATCC25923 induced by benzylpenicillin. Inducible strains was successively transferred (13 passages) onto nutrient agar plates with increasing concentrations of benzylpenicillin. Whereas control induction strains were cultured in unsupplemented medium under the same conditions

Stability of resistance in QP: After eight continuous transfers in unsupplemented medium, the MIC of benzylpenicillin for the QP mutant gradually decreased. Following these eight passages, the MIC for QP was 0.0125 U mL^{-1} which was the same as the initial strain of *S. aureus* ATCC25923. This result showed that after eight transfers in unsupplemented medium, the change in MIC for each generation strain can be shown in Fig. 2.

Antibacterial activity of tachyplesin I in QP: The MIC of tachyplesin I in the QP mutant was 10 μg mL^{-1} which was the same as that of the original strain of *S. aureus* ATCC25923. This result indicated that a benzylpenicillin-resistant QP strain of *S. aureus* was also susceptible to tachyplesin I and no cross-resistance

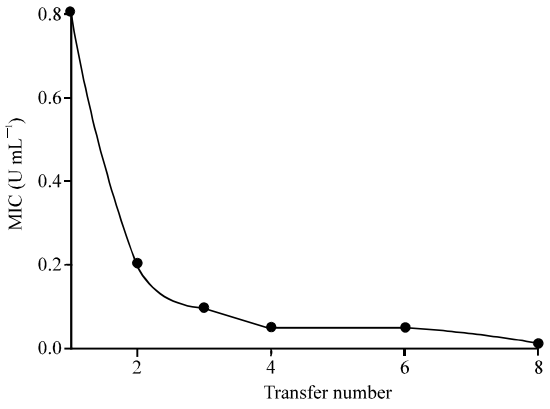

Fig. 2: Changes in MIC for induction strain QP after 8 continuous transfers in unsupplemented medium. MIC was measured using the broth microdilution method. Each determination was made using three independent replicates from each line

between tachyplesin I and benzylpenicillin was observed. The widespread use and misuse of antibiotics has led to bacterial resistance; similarly, the use of AMPs may result in similar problems. It has been proven that *S. aureus* is resistant to defensins and human AMPs and the mechanism of resistance in *S. aureus* is highly specific to special defensins, bacteriocin and CAMPs (Peschel *et al*., 1999).

It is unclear whether the widespread use of AMPs would result in a loss of sensitivity in the defense system to pathogenic microorganisms. Therefore, it is very important to study prospectively whether AMPs could induce resistance in microbes in order to avoid the results obtained for antibiotics.

It is known that microbial pathogens resistant to AMPs have two resistance mechanisms including constitutive mechanisms of resistance and inducible mechanisms of resistance. The constitutive mechanisms of resistance involve inherent properties of the pathogen which confer resistance and are normally expressed even without exposure to AMPs. The inducible mechanisms of resistance are induced by AMPs. These two resistance mechanisms are inter-coordinated to enhance the viability of microbes in all types of environments containing AMPs (Yeman and Yount, 2003). Prior to this study, the results showed that both *E. coil* and *S. aureus* were strongly inhibited and killed by tachyplesin I. According to these data in order to make clear the safety of tachyplesin I as an antibacterial agent which will most likely to be applied in animal feed, food and in the clinic in the future, both non-successive induction and successive induction by gradually increasing the induction concentration of tachyplesin I were performed to determine whether tachyplesin I could induce resistance in *E. coli* and *S. aureus in vitro*.

E. coli and *S. aureus* were chosen as the induced strains because these two bacteria are the most common intestinal tract pathogenic bacteria in animals and humans. In this case, the outcome of this study will be more valuable in guiding practice. At the same time, according to the literature, both gram-positive and gram-negative bacteria have their own unique response system to AMPs (Gunn and Miller, 1996; Moskowitz *et al*., 2004; Li *et al*., 2007).

Two inducible strategies (non-successive induction and successive induction) were adopted in this study. Non-successive induction is acknowledged to be a mature method for antibiotic induction experiments and in this way, many strains or mutants resistant to antibiotics have been successfully achieved. The aim of the first procedure was to induce an unadaptable resistant mutant. With the knowledge that tachyplesin I may be used as a feed additive in animal feed, researchers performed the second procedure using bacteria which were serially transferred onto nutrient agar plates with increasing concentration of drugs until a final concentration of 640 μg mL^{-1} tachyplesin I (20 serial passages) and 0.25 U mL^{-1} benzylpenicillin (13 serial passages) was obtained. The reason for the high concentration (640 ug mL^{-1}) of tachyplesin I used in the induction experiment was due to the presence of alginic acid, a major anionic component in the agar plates. Therefore, given the antimicrobial activity of CAMPs when the MIC determination or resistance induction of CAMPs for these bacteria were conducted using the agar plate method, agarose was used to replace agar as the support matrix. Both induction experiments showed that neither non-successive induction nor successive induction could induce resistance to tachyplesin I in *E. coli* ATCC25922, *E. coli* F41 or *S. aureus* ATCC25923 whereas there was a high level of resistance to benzylpenicillin in *S. aureus* ATCC25923 and tachyplesin I was able to kill the strains resistant to benzylpenicillin (QP). Moreover, these results also confirmed that the induction method researchers adopted was feasible, reasonable and compared with benzylpenicillin, resistance to tachyplesin I in bacteria was not easily induced. In addition if a control peptide used in the experiments e.g., LL37, a polymyxin or pexiganan, the experimental design may be fundamentally flawless given that its mechanism of resistance is same.

Most CAMPs did not induce resistant mutants after as many as 20 passages at a concentration close to the MIC in the laboratory (Hancock, 1997). Pexiganan is an

AMP of the analogue of magainin, it has a typical linear α-helix structure and its antibacterial mechanism involves disrupting the integrity of bacterial cell membranes (Gottler and Ramamoorthy, 2009). Pexiganan did not induce resistance in *P. aeruginosa* and *S. aureus* following 7-14 sequential passages in an agarose plate supplemented with a sub-inhibitory concentration. Moreover, following both chemical and UV light mutagenesis, both these strains were still sensitive to pexiganan. To date, no evidence of cross-resistance between pexiganan and any of the antibiotics in clinical use has been documented (Ge *et al*., 1999). However, through continued selection in the laboratory, 22/24 lineages of *E. coli* and *P. fluorescens* independently evolved heritable mechanisms of resistance to pexiganan when propagated in medium supplemented with increasing concentrations of this AMP for 600-700 generations (100 serial transfers) (Perron *et al*., 2006).

It is unclear whether there is a suitable method of ensuring that an AMP can induce resistance in bacteria and little is known about the relationship between the structure of AMPs and their ability to induce resistance in bacteria. Would a sub-inhibitory concentration of tachyplesin I used for a longer induction time induce resistance in bacteria? Further studies on this topic may be worthwhile.

The present study showed that it was difficult to induce resistance to tachyplesin I in bacteria which possibly have two reasons. On the one hand, the bacteria may not have exposed to tachyplesin I for a sufficient number of generations; on the other hand, the bacteria may not have enough time to develop resistance to tachyplesin I because of its multieffect of antibacterial mechanism on bacteria. Further studies are needed to elucidate the mechanism of action of tachyplesin I to determine why it could not induce resistance in bacteria.

CONCLUSION

In this study, tachyplesin I did not induce resistance in *E. coli* ATCC25922, *E. coli* F41 and *S. aureus* ATCC25923. It is suggested that tachyplesin I may be a safer antibacterial agent than other AMPs for future use in clinical practice.

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REFERENCES

Breuer, B. and F. Radler, 1996. Inducible resistance against nisin in Lactobacillus casei. Arch. Microbiol., 165: 114-118.

Brogden, A.K., 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria. Nat. Rev. Microbiol., 3: 238-250.

Dai, J.G., H.W. Xie, G. Jin, W. Wang, Y. Zhang and Y. Guo, 2009. Preliminary study on high-level expression of tandem-arranged tachyplesin-encoding gene in Bacillus subtilis Wb800 and its antibacterial activity. Mar. Biotechnol., 11: 109-117.

Dai, J.G., H.W. Xie, G. Jin, Y. Zhang, J.C. Zhu and Y. Guo, 2008. Preliminary study on antibacterial targets of tachyplesin. Prog. Biochem. Biophys., 35: 563-569.

Falakaflaki, B., R. Fallah, M.R. Jamshidi, F. Moezi and Z. Torabi, 2007. Comparison of nitrofurantoin and trimethoprim-sulphamethoxazole for long-term prophylaxis in children with recurrent urinary tract infections. Int. J. Pharmacol., 3: 179-182.

Ge, Y., D.L. MacDonald, K.J. Holroyd, C. Thornsberry, H. Wexler and M. Zasloff, 1999. *In vitro* antibacterial properties of pexiganan, an analog of magainin. Antimicrob. Agents Chemother., 43: 782-788.

Giacometti, A., O. Cirioni, F. Barchiesi, M. Del Prete, M. Fortuna, F. Caselli and G. Scalise, 2000. *In vitro* susceptibility tests for cationic peptides: Comparison of broth microdilution methods for bacteria that grow aerobically. Antimicrob. Agents Chemother., 44: 1694-1696.

Gottler, L. and A. Ramamoorthy, 2009. Structure, membrane orientation, mechanism and function of pexiganan-A highly potent antimicrobial peptide designed from magainin. Biochim. Biophys. Acta, 1788: 1680-1686.

Gunn, J.S. and S.I. Miller, 1996. PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in Salmonella typhimurium antimicrobial peptide resistance. J. Bacteriol., 178: 6857-6864.

Habi, S. and H. Daba, 2009. Plasmid incidence, antibiotic and metal resistance among enterobacteriaceae isolated from algerian streams. Pak. J. Biol. Sci., 12: 1474-1482.

Hancock, R.E.W., 1997. Peptide antibiotics. Lancet, 349: 418-422.

Hirakura, Y., S. Kobayashi and K. Matsuzaki, 2002. Specific interactions of the antimicrobial peptide cyclic β-sheet tachyplesin I with lipopolysaccharides. Biochim. Biophys. Acta, 1562: 32-36.

Imura, Y., M. Nishida, Y. Ogawa, Y. Takakura and K. Matsuzaki, 2007. Action mechanism of tachyplesin I and effects of PEGylation. Biochim. Biophys. Acta, 1768: 1160-1169.

Karou, S.D., D.P. Ilboudo, W.M.C. Nadembega, Y. Ameyapoh and D. Ouermi *et al*., 2009. Antibiotic resistance in urinary tract bacteria in ouagadougou. Pak. J. Biol. Sci., 12: 712-716.

Li, M., Y. Lai, A.E. Villaruz, D.J. Cha, D.E. Sturdevant and M. Otto, 2007. Gram-positive three-component antimicrobial peptide-sensing system. Proc. Natl. Acad. Sci., 104: 9469-9474.

Martinez, J.L. and F. Baquero, 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother., 44: 1771-1777.

Masuda, K., M. Ohta, M. Ito, S. Ohsuka, T. Kaneda and N. Kato, 1994. Bactericidal action of tachyplesin I against oral streptococci. Oral Microbiol. Immunol., 9: 77-80.

Miyata, T., F. Tokunaga, T. Yoneya, K. Yoshikawa and S. Iwanaga *et al*., 1989. Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II and polyphemusins I and II: Chemical structures and biological activity. J. Biochem., 106: 663-668.

Morvan, A., S. Iwanaga, M. Comps and E. Bachere, 1997. *In vitro* activity of the limulus antimicrobial peptide tachyplesin I on marine bivalve pathogens. J. Invertebr. Pathol., 69: 177-182.

Moskowitz, S.M., R.K. Ernst and S.I. Miller, 2004. PmrAB, a two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol., 186: 575-579.

Nakamura, T., H. Furunaka, T. Miyata, F. Tokunaga and T. Muta *et al*., 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*): Isolation and chemical structure. J. Biol. Chem., 263: 16709-16713.

Nakashima, H., M. Masuda, T. Murakami, Y. Koyanagi, A. Matsumoto, N. Fujii and N. Yamamoto, 1992. Anti-human immunodeficiency virus activity of a novel synthetic peptide, T22 ([Tyr-5,12, Lys-7]polyphemusin II): A possible inhibitor of virus-cell fusion. Antimicrob. Agents Chemother., 36: 1249-1255.

Ohta, M., H. Ito, K. Masuda, S. Tanaka, Y. Arakawa, R. Wacharotayankun and N. Kato, 1992. Mechanisms of antibacterial action of tachyplesins and polyphemusins, a group of antimicrobial peptides isolated from horseshoe crab hemocytes. Antimicrob. Agents Chemother., 36: 1460-1465.

Ouyang, G.L., Q.F. Li, X.X. Peng, Q.R. Liu and S.G. Hong, 2002. Effects of tachyplesin on proliferation and differentiation of human hepatocellular carcinoma SMMC-7721 cells. World J. Gastroenterol., 8: 1053-1058.

Perron, G.G., M. Zasloff and G. Bell, 2006. Experimental evolution of resistance to an antimicrobial peptide. Proc. R. Soc. B, 273: 251-256.

Peschel, A., M. Otto, R. Jack, H. Kalbacher, G. Jung and F. Gotz, 1999. Inactivation of the dlt Operon in Staphylococcus aureus confers sensitivity to defensins, protegrins and other antimicrobial peptides. J. Biol. Chem., 274: 8405-8410.

Pranting, M., A. Negrea, M. Rhen and D. Andersson, 2008. Mechanism and fitness costs of PR-39 resistance in *Salmonella enterica* serovar Typhimurium LT2. Antimicrob. Agents Chemother., 52: 2734-2741.

Ruiz, J., J. Sierra, M. De Anta and J. Vila, 2001. Characterization of sparfloxacin-resistant mutants of *Staphylococcus aureus* obtained *in vitro*. Int. J. Antimicrob. Agents, 18: 107-112.

Yeman, M.R. and N.Y. Yount, 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev., 55: 27-55.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature, 415: 389-395.