Short Communication

Evaluation of antimicrobial peptide K9CATH in a murine model of mastitis

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Abstract

In the present study the effect of antimicrobial peptide K9CATH was evaluated in a murine model of mastitis by *Staphylococcus aureus*. Mastitis was induced with a clinical isolate of *S. aureus* in 20 female Balb/c mice at 7 to 10 days of lactation. Ten mice (treatment group) received daily intramammary infusions of 32 µg/50 µL K9CATH peptide, for 3 consecutive days, and 10 other mice (control group) received only 50 µL of saline solution. Results showed a significant reduction ($P<0.01$) in *S. aureus* CFU counts in the treatment group compared to the control group. These data suggest that the K9CATH peptide could be a potential candidate for the treatment of bovine mastitis.

Keywords: murine mastitis, K9CATH, antimicrobial peptide, *Staphylococcus aureus*

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Introduction

Bovine mastitis, described as inflammation of the mammary gland, is the most damaging infectious disease that affects dairy cattle worldwide, causing economic losses estimated at $35 million per year (Wellenberg et al., 2002). *Staphylococcus aureus* has been found to be responsible for up to 30% of clinical mastitis cases and is also the most difficult to cure. Treatment is restricted to the use of a broad range of antibiotics such as tetracycline, penicillin, and pirlimycin usually administered through intramammary infusion. However, low cure rates (lower than 15%) (Schmelcher et al., 2012) and the emergence of antibiotic-resistant bacteria warrant the need for novel antimicrobials for the treatment of *S. aureus* bovine mastitis, and antimicrobial peptides (AMPs) are considered attractive control agents.

AMPs have been studied for more than two decades and their antimicrobial properties emerge nowadays as a solid alternative to common antibiotics (Steinstraesser et al., 2011). They are essential components of the innate immune system conserved through evolution, therefore resistance development to these compounds is considered unlikely. Their antimicrobial spectrum includes gram-positive and gram-negative bacteria, fungus, parasites, virus and tumor cells (Ge et al., 1999). Several AMPs have shown antimicrobial activity against mastitis causing pathogens, *in vivo* and *in vitro*. The antimicrobial peptides Lacticin 3147, Nisin, and Nisin Z have been used in intramammary infusion in cows with clinical and subclinical mastitis by *S. aureus* (Twomey et al., 2000; Cao et al., 2007; Wu et al., 2007; Kim et al., 2010). Also, other AMPs, such as the rabbit neutrophil defensins NP-1 and NP-5, the neutrophil derived human bactericidal/permeability-increasing protein (BPI), bovine cathelicins (BMAP-27, BMAP-28, Bac5, Indolicin), and the bovine β-lactoglobulin, have shown antimicrobial activity against gram-positive and gram-negative isolates from mastitic cows in *in vitro* experiments (Cullor et al., 1991; Chockalingam et al., 2007; Tomasinsig et al., 2010; Chaneton et al., 2011). Furthermore, an experiment conducted by Kim et al. (2010) showed the antimicrobial effect of the peptide Lacticin NK34 against 70% of the *S. aureus* strains *in vitro*, and in a mouse infection model with *S. aureus* 80% survival rate. The mouse model of bovine mastitis was developed in the early 1970s and is a suitable and relatively inexpensive analysis as a prelude to a cow study (Chandler, 1970). Numerous studies have been carried out using this model to test the efficacy of various antimicrobial agents against *S. aureus*-induced mastitis (Chandler, 1971; Craven et al., 1982; Anderson and Craven, 1984; Bramley and Foster, 1990; Brouillette et al., 2004; Brouillette and Malouin, 2005; Schmelcher, 2012). In particular, the antimicrobial peptide Canine Cathelicidin (K9CATH), derived from canine neutrophil granules, has shown antimicrobial activity *in vitro* against gram-positive and gram-negative bacteria, yeast (Sang et al., 2007), and field *S. aureus* (Barreras-Serrano et al., 2014). Also, mice infected with *M. tuberculosis* H37Rv treated with K9CATH showed a significant decrease in colony-forming units (CFU) and pneumonic area (Tamayo-Sosa et al., 2012).

The objective of the current study was to evaluate the effect of the antimicrobial peptide K9CATH in a murine model of mastitis primarily by its effect on bacteria cell number in the gland.

Materials and Methods

Preparation of bacterial inoculum: A field strain of *S. aureus* was isolated from milk from a cow with clinical mastitis, in brain-heart infusion broth (BHB) (Becton, Dickindon, USA) for 24 h at 37°C. To verify its purity, the bacteria were plated in blood agar, following enzymatic tests (catalase and oxidase) and the biochemical commercial test API-Staph (API Staph, bioMérieux, USA).

For the preparation of inoculum, the bacteria were diluted in physiological saline solution and concentration was determined by absorbance at 625 nm. An absorbance of 0.8 corresponded to a bacteria concentration of $1x10^5$ CFU/50 µL used in the experiment. The inoculum concentration was verified by its seeding in agar and colony counting at 24 h.

Preparation of antimicrobial peptide K9CATH: The synthetic peptide K9CATH was synthesized as 38 mer by the Biochemical Research Service Laboratory, Structural Biology Center, University of Kansas, Lawrence, KS 66047. The lyophilized peptide was weighed and diluted in 500 µL of sterile deionized water to obtain a final concentration of 7.2 µg/ µL (stock solution) and was stored at -70°C. From this stock solution dilutions were made to obtain a concentration of 32 µg/mL of physiological saline to be used for the treatment of mice. K9CATH is an antimicrobial molecule protected under USPTO Patent No. US 7,985,832 B2. The concentration of the K9CATH peptide used in this study was determined based on the minimum inhibitory concentration (MIC) obtained for this peptide in an *in vitro* experiment (Barreras-Serrano et al., 2014), and also for the non-cytotoxic effect in human type II alveolar pneumocytes (Tamayo-Sosa et al., 2012; V.M. Del Villar-Perez, unpublished observations).

It is important to consider that the concentration used for *in vivo* experiments had to be higher than that obtained *in vitro* to ensure optimal diffusion of the peptide into the targeted infected tissue to kill the bacteria, but also to be non-cytotoxic for the tissue (Tamayo-Sosa et al., 2012).

Mouse model of S. aureus mastitis: Animal work was performed in accordance with Mexican National Regulations on Animal Care and Experimentation (NOM-062-ZOO-1999). Twenty female Balb/c lactating mice were infected with *S. aureus* by intramammary infusion as described by Chandler (1970). Briefly, before bacterial inoculation of the mammary glands, dams between days 7 and 10 of lactation were separated from their pups for 4 h. After that, the pups were allowed to nurse for 1 h for depletion of milk from the mammary glands. The experimental mice were anesthetized with a mixture of tiletamine/zolazepam (Zoletil® 100, Virbac, Mexico) at 100 mg/kg of weight, intramuscularly. Using a stereoscope, the left and right fourth abdominal

mammary glands (L4 and R4, respectively) were injected with 50 µL of a suspension containing $1x10^5$ CFU of *S. aureus* through the teat canal using a 33-gauge blunt needle (Hamilton, Kent, UK). The mice were placed in cages for recovery.

Therapeutic effect of peptide K9CATH: After 24 h of bacterial inoculation, the mice were randomly distributed into two groups of 10 mice each. One group (treatment group) received 50 µL containing 32 µg of K9CATH by intramammary infusion in each teat. The other group (control group) received only 50 µL of physiological saline solution by intramammary infusion in each teat. The treatments were repeated every 24 h for 3 days. For the application of treatments, the mice were anesthetized as previously described.

Determination of colony forming units (CFU) in mammary glands: The animals were euthanized 24 h after the last treatment following the protocol described by NOM-033-ZOO-1995. The mammary glands were aseptically dissected, weighed, and homogenized with a polytron (Kinematica, Luzern, Switzerland) in Falcon tubes with 1 mL sterile physiological saline (100 mg/mL). Serial dilutions of 1:10 (10^{-1}, 10^{-2}, 10^{-3}) and plating on tryptic soy agar (TSA), in duplicate, were performed to determine intramammary bacterial concentrations. For this determination the L4 mammary glands were used.

Statistical analysis: Bacterial $\log_{10}$ CFU counts obtained from each experimental group were compared for statistical significance by using the non-parametric Kruskall-Wallis test. When a significant ($P<0.05$) difference was observed between treatments, Dunn's multiple comparison test was applied. Analysis was performed with the statistical program SAS (SAS version 9.3, SAS Institute, Inc., Cary, NC).

Results and Discussion

The mammary glands of the mice treated with the K9CATH peptide at a concentration of 32 µg/mL showed a significant decrease in CFU of *S. aureus* ($P<0.05$), compared with the mammary glands of the mice in the control group, which only received saline solution (Figure 1). The treated animals showed an average of $1.842x10^3$ CFU/mL while the control group showed $122.498x10^3$ CFU/mL.

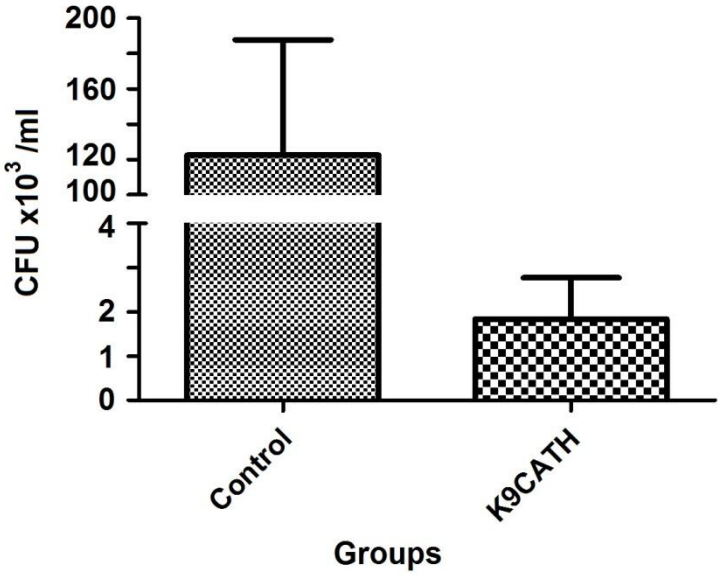

Figure 1 CFU counts in the mammary gland tissues collected from mice from the control and treatment (K9CATH) groups showing significant ($P<0.05$) difference. Also, the variability in the CFU count per group given by the standard error is displayed.

The decrease in CFU found in this study is consistent with a previous study in which K9CATH inhibited *S. aureus* in an *in vitro* assay at a concentration of only 5.6 µg/mL (Barreras-Serrano et al., 2014), and with the results obtained by Tamayo-Sosa et al. (2012), in which K9CATH was effective against the intracellular bacteria *M. tuberculosis* H37Rv in mice with pulmonary tuberculosis also at 32 µg/mL, as well as *in vitro* at 10.66 µg/mL.

Although the concentration of the K9CATH peptide used in this experiment was higher than that used in *in vitro* experiments against *S. aureus*, this concentration has been shown to be effective against intracellular bacteria in *in vivo* studies, and also non-cytotoxic to host cells (alveolar pneumocytes and erythrocytes) (Tamayo-Sosa et al., 2012; Sang et al., 2007), therefore safe for its use in *in vivo* experiments. Similar antimicrobial effectiveness has been reported of the use of several antimicrobial peptides both in *in vitro* assays against pathogens isolated from mastitic cows (such as the NP-1 and NP-5, BPI, bovine cathelicidins, and β-lactoglobulin), as well as in the treatment of infected cows by intramammary infusion. For instance, the peptides Nisin and Nisin Z have shown to be effective when administered by intramammary infusion in cows with clinical and subclinical mastitis, respectively, caused by several pathogens, including *S. aureus* (Cao et al., 2007; Wu et al., 2007). Likewise, the peptide Lacticin 3147 was effective when infused in the mammary gland in cows

infected with *S. aureus*. Furthermore, mice infected with *S. aureus* treated with the antibiotic peptide NK34 had 80% survival rate, higher than those that received distilled water (Kim et al., 2010).

The capacity to survive intracellularly has been proposed as a factor contributing to the persistence of *S. aureus* in the bovine mammary gland. It is known that less than 15% of cases of mastitis caused by *S. aureus* respond to conventional antibiotics, and this is mostly due to its poor diffusion into the mammary tissue (Craven and Anderson, 1984; Yancey et al., 1991). However, the results obtained in this study showed that the peptide K9CATH at 32 µg/mL concentration significantly decreased the CFU in mice inoculated with *S. aureus*, therefore reaching optimal levels into the gland. These results are similar to those found by Tamayo-Sosa et al. (2012), in which mice infected with the intracellular bacteria *M. tuberculosis* H37Rv treated with the K9CATH peptide showed a significant decrease in CFU and pneumonic area.

Furthermore, antimicrobial compound efficacy evaluated *in vitro* against *S. aureus* does not necessarily apply to intramammary infections (IMI) since important *in vivo* interactions with the host cells are not accounted for (Schmelcher et al., 2012). Thus, screening of new potential antimicrobial compounds adequate for IMI needs to be performed in a test system that is better suited than *in vitro* methods. The mouse mastitis model has been increasingly used to study ruminant mastitis due to similarities between mice and cows (Brouillette et al., 2004; Brouillette et al., 2005; Notebaert and Meyer, 2006). According to the significant decrease in CFU of *S. aureus* in this study, the mouse model could be a reliable tool for the evaluation of other antimicrobial peptides with potential for the treatment of bovine mastitis.

Conclusion

This study showed the effectiveness of the peptide K9CATH to decrease CFU in the mammary glands of mice infected with *S. aureus*. Further studies are suggested to elucidate the efficacy of K9CATH against other mastitis causing pathogens.

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บทคัดย่อ

การประเมิน สารต้านจุลชีพเปปไทด์ K9CATH ในแบบจำลองการเกิดโรคเต้านมอักเสบในหนู

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การศึกษาครั้งนี้ได้ประเมินผลของสารต้านจุลชีพเปปไทด์ K9CATH ในแบบจำลองการเกิดโรคเต้านมอักเสบในหนู โดย Staphylococcus aureus โดยกระตุ้นให้เกิดโรคเต้านมอักเสบเกิดจากเชื้อ S. aureus ในหนูเพศเมีย พันธุ์ Balb / c จำนวน 20 ตัว ในวันที่ 7 ถึง 10 ของการให้นม โดยหนูในกลุ่มที่ได้รับการรักษา ได้รับเปปไทด์ K9CATH จำนวน 32 ไมโครกรัม / 50 ไมโครลิตร ฉีดเข้าทางเต้านม เป็นเวลา 3 วันติดต่อกัน ในขณะที่หนูในกลุ่มควบคุมจำนวน 10 ตัว ได้รับน้ำเกลือ 50 ไมโครลิตร ผลการทดลองแสดงให้เห็นว่าในกลุ่มที่ได้รับการรักษาพบเชื้อ S. aureus ลดลงอย่างมีนัยสำคัญ (P <0.01) เมื่อเทียบกับกลุ่มควบคุม ข้อมูลนี้ชี้ให้เห็นว่าเปปไทด์ K9CATH มีศักยภาพในการรักษาโรคเต้านมอักเสบในวัว

คำสำคัญ: เต้านมอักเสบในหนู K9CATH สารต้านจุลชีพเปปไทด์ *Staphylococcus aureus*

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