

Research Article

Evaluation of the Antimicrobial Activity of the K9CATH Peptide (38 Amino Acids) Against a Mastitis Isolated Strain of *Staphylococcus aureus* by the Resazurin microtiter Method

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Abstract: The antimicrobial activity of the synthetic peptide K9CATH was determined by the Resazurin microtiter Method (RMM) against a strain of *S. aureus* isolated from a case of mastitis. To the antibiogram this bacteria strain showed to be resistant to Ampicillin, Erythromycin, Cefepime, Dicloxaciline and Penicillin (10 U), while the MIC obtained for the K9CATH was 5.66 µg/mL. Unlike the reference broth method, visual reading for MIC determination with the RMM showed to be easier, rapid, inexpensive and more sensitive for antimicrobial peptide screening, based in a color change from blue (not growth) to pink (growth). This is the first time that the resazurin method is used to determine the MIC of the 38 aa's K9CATH peptide against a mastitic isolate of *S. aureus*.

Keywords: AMPs and mastitis, K9CATH peptide, resazurin assay, *Staphylococcus aureus*

INTRODUCTION

Mastitis is the inflammation of the mammary gland and is the disease with the highest incidence in milk production worldwide (Bradley, 2002). Annual worldwide losses are estimated at 35 billion U.S. dollars. Mastitis is treated with antibiotics such as tetracycline and penicillin (Deluyker *et al.*, 2005), however the indiscriminate use of antibiotics is favoring the emergence of bacteria resistance and the elimination of antibiotics in milk is undesirable (Erskine and Barlett, 1996). One of the main challenges to overcome for the milk industry is to reduce the use of antibiotics and therefore the development of new antimicrobial therapies to treat mastitis is necessary.

The use of Antimicrobial Peptides (AMP's) could be an alternative to conventional antibiotics because of its wide spectrum (Gutiérrez and Orduz, 2003). Specifically the synthetic K9CATH peptide has shown antimicrobial activity against gram positive, gram negative and yeast by the Broth Microdilution Method (BMM) (Sang *et al.*, 2007).

Although the BMM is easy to perform a disadvantage could be the MIC interpretation due to inoculum sedimentation or very scant or transparent growth that occurs with some species of bacteria (Baker and Tenover, 1996). For this reason the Microplate Alamar Blue Assay (MABA) was developed by Alamar Biosciences, Inc., Sacramento, California and is based on the conventional broth microdilution method but

with a color indicator. However MABA could be unaffordable to low income laboratories and for this reason the Resazurin microtiter Method (RMM) was developed, where resazurin dye is used as a colorimetric indicator and is based in the reference method as well. Resazurin is an oxy-reduction indicator that has been employed as MIC indicator (Mann and Markham, 1998). The validity of the RMM to predict MIC is due to the correlation of visible color change (from blue to pink) with bacterial densities in the microplate wells. In the present study the antimicrobial activity of the peptide K9CATH (38 aa's) was evaluated against a bovine mastitic isolate of *Staphylococcus aureus* by the resazurin microtiter method.

MATERIALS AND METHODS

Bacteria isolation and identification: A 50 mL milk sample was aseptically collected in a sterile falcon tube from a first lactation cow diagnosed with mastitis by the California Mastitis Test (CMT). Milk sample was stripped onto MacConkey and blood agar and incubated for 24 h at 37°C. Grown colonies were subjected to a gram stain to identify morphology and also the catalase and potassium hydroxide tests were performed. Then, bacteria were grown in Brain Heart Infusion (BHI) media (Becton, Dickinson, USA) for 24 h at 37°C and identified by the API staph identification system (API® staph BioMerieux). An antibiogram was used to

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determine bacteria susceptibility or resistance to conventional antibiotics by a commercial multi-disks kit (Biorad).

For the resazurin assay, after bacteria was grown in BHI media a suspension was prepared in saline solution with a turbidity equal to that of the 0.5 McFarland standard. A 1:100 bacteria dilution was further done in BIH media for a final concentration of 5×10^5 CFU/mL.

Antimicrobial peptide K9 CATH: The K9CATH antimicrobial peptide was donated by Dr. Melgarejo from Kansas State University. A vial of 2 mg of lyophilized synthetic antimicrobial peptide K9CATH (38 amino acids) was reconstituted in 500 μ L of sterile dionized water obtaining a final concentration of 4 μ g/ μ L. This stock solution was stored at -70°C until used. For the assay a 4x working solution was prepared (512 μ g/mL) to make twofold dilutions with the following concentrations: 128, 64, 32, 16, 8, 4, 2 and 1 μ g/mL.

Resazurin Microplate Method (RMM): Minimal inhibitory concentration for the K9CATH was determined following the protocol described by Franzblau *et al.* (1998) with a minor modification. Briefly, 200 μ L of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 2 to 11 received 100 μ L of BHI media. One hundred microliters of 4x antimicrobial peptide solution were added to wells B3 to B5 and B8 to B10 and by using a multichannel pipette 100 μ L were transferred from column 3 to 5 and from column 8 to 10 down the rows until G to make twofold dilutions and from last dilution 100 μ L of excess medium was discarded. One hundred microliters of *S. aureus* inoculum was added to all the wells, except wells B6 and B7 that served as drug-free (inoculum-only) controls. The plates were incubated at 37°C for 24 h. Thirty microliters of freshly prepared resazurin at 0.01% diluted in BHI media was added to all the wells and reincubated at 37°C for 6 h and the colors of all wells were recorded. A blue color in the well was interpreted as no growth and a pink color was scored as growth. The MIC was defined as the lowest drug concentration which prevented a color change from blue to pink.

RESULTS AND DISCUSSION

Bacteria isolated from milk was determined to be *Staphylococcus aureus* according to presence of clusters of cocci gram positive, catalase positive, potassium hydroxide and by biochemical identification. For the antibiogram this strain of *S. aureus* showed to be susceptible to 5 of the 12 antibiotics indicated for the treatment of gram positive bacteria, while resistance was shown to Ampicillin (10 μ g), Erythromycin (15

μ g), Cefepime (30 μ g), Dicloxaciline (1 μ g) and Penicillin (10 U). However the antimicrobial peptide K9CATH inhibited *S. aureus* growth in vitro at 5.66 μ g/mL, a concentration lower than those of the antibiotics in the antibiogram.

The MIC obtained with this method are similar to those obtained with the peptide WBC14 (water buffalo cathelicidin 14) from water buffalo with antimicrobial activity against *Streptococcus dysgalactiae* (12.5 μ M), *Klebsiella pneumoniae* (25 μ M), *Corynebacterium species* (12.5 μ M) and *Staphylococcus aureus* (25 μ M), all isolated from mastitis clinical cases (Tamayo *et al.*, 2010). Furthermore, Sang *et al.* (2007) reported that the 38 aa's K9CATH peptide have shown antimicrobial activity against Gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella enteritidis* and *Neisseria gonorrhoeae*) and yeast (*Candida albicans*). Although in both cases the antimicrobial activity of these peptides has been evaluated *in-vitro* using the reference broth microdilution method. Furthermore, antimicrobial susceptibility and resistance for *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium smegmatis* has been evaluated successfully with the RMM and proved to be sensitive and specific (Taneja and Tyagi, 2007; Rivoire *et al.*, 2007; Campanerut *et al.*, 2011; Palomino *et al.*, 2002).

The resazurin method follows the principle of the reference broth method and differs only in the addition of resazurin and depends on the observation of a color change from blue (indicating inhibition of the test organism) to pink (no inhibition). This color change appears when surrounding medium is reduced as a result of bacterial depletion of dissolved oxygen and acid production and therefore it is necessary to assess reduction densities for each organism tested (Mann and Markham, 1998).

CONCLUSION

The K9CATH peptide (38 aa's) antimicrobial activity against a field strain of *S. aureus* was effectively determined by the resazurin method. The resazurin method could be an alternative to the reference NCCLS method for its easy interpretation due to the color indicator. Further studies are necessary to validate the resazurin assay and determine if the MIC's are in concordance with those of the reference method for this peptide.

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