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Evaluation of the antimicrobial susceptibility of *Staphylococcus aureus* to the K9CATH peptide by the resazurin microtiter method and the reference broth microdilution method

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ABSTRACT

The antimicrobial activity of the synthetic peptide K9CATH was determined by the resazurin microtitre method (RMM) and by the reference broth microdilution method (NCCLS) against a clinical mastitic isolate of *S. aureus* and a control strain of *S. aureus* ATCC. By both methods K9CATH inhibited both strains of *S. aureus*. The MIC for the field strain ranged between 4 and 8 µg/mL when determined visually and by absorbance, respectively, with both methods. For the control strain the MIC fell between 16 and 18 µg/mL by visual and absorbance interpretation, respectively, by both methods. Due to the color change from blue (not growth) to pink (growth) visual reading for MIC determination with the RMM showed to be easier, rapid, inexpensive and more sensitive for antimicrobial peptide screening than the reference method. This is the first time that the resazurin method is used to determine the MIC of antimicrobial peptides to *S. aureus*.

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1. Introduction

The increase of bacteria strains resistant to antibiotics has obligated the researchers to develop new therapeutic alternatives. The use of antimicrobial peptides (AMP's) has been evaluated for the last 20 years for its antimicrobial properties (Marr et al., 2006). The AMP's are essential components of the innate immune system conserved through evolution and are not toxic to the host (Raj and Dentino, 2002). Its antimicrobial spectrum includes gram positive and gram negative bacteria, fungi, parasites, viruses, and tumor cells (Gutiérrez y Orduz, 2003). The antimicrobial activity of these peptides is evaluated *in-vitro* using the reference broth microdilution method (BMM) described by the National Committee for Clinical Laboratory Standards (NCCLS, 2000), adapted for Wiegand et al., (2008). Specifically the synthetic K9CATH peptide has shown antimicrobial activity against gram positive, gram negative and yeast by the broth microdilution method (Sang et al., 2007). The BMM is used to determine the minimal inhibitory concentration (MIC) of an antimicrobial agent that will inhibit visible growth of bacteria and is performed in 96 well microtitre trays (Wiegand et al., 2008). An advantage of this method is that the reagents can be prepared in the laboratory and may be stored frozen or lyophilized. However 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 et al., 1996). In order to overcome this difficulty Alamar Biosciences, Inc., Sacramento, California developed the Alamar blue assay (MABA), a modification of the reference method that uses the conventional broth microdilution method and added a color indicator to enhance the detection of growth within the wells. Several researchers have used MABA to calculate the MIC of various antimicrobial drugs against gram positive and gram negative bacteria, as well as fungi and yeast and have demonstrated to be a useful tool easy to perform and read, and MIC's obtained were comparable with results obtained with the BMM. However a disadvantage of MABA is the economic cost resulting expensive for some low income laboratories. Therefore, a variant of the MABA, the resazurin microplate method (RMM) was developed, where resazurin dye is used as a colorimetric indicator making the test affordable to low income laboratories. This method is based in the reference method as well. The resazurin is an oxy-reduction indicator that has been employed to evaluate bacteria viability and contamination, as well as MIC indicator (Carter et al., 1998; Mann and Markham 1998; Smith and Townsend, 1999). 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.

Therefore, the objective of the present study was to evaluate the antimicrobial activity of the synthetic peptide K9CATH against a clinical bovine mastitic isolate of *Staphylococcus aureus* and a control strain (ATCC *S. aureus* MRSA), by using the colorimetric method RMM and the reference BMM.

2. Materials and methods

2.1. Bacteria strains

A strain of *Staphylococcus aureus* was isolated from a clinical case of mastitis from the dairy farm of the Veterinary school at the Autonomous University of Baja California. The strain was identified by the API staph identification system at the Microbiology Laboratory in the Veterinary School. The control strain used in the assays was *Staphylococcus aureus* (MRSA) ATCC 33591. Both strains were grown separately in blood agar at 37°C for 24 h. One isolate colony was picked from the plate, streaked onto a new blood agar plate and incubated as above. A suspension of growth for each strain was prepared in 5 mL of Mueller-Hinton II cation adjusted broth for the reference broth microdilution test and for the resazurin assay. The turbidities of these suspensions were adjusted to equal that of a 0.5 McFarland standard. For susceptibility testing with both assays, a 1:100 bacteria dilution was further done in Mueller-Hinton broth for a final concentration of 5×10^5 CFU/ml.

2.2. Antimicrobial peptide

The K9CATH antimicrobial peptide was donated by Dr. Melgarejo from Kansas State University. A vial of 10 mg of lyophilized synthetic antimicrobial peptide K9CATH (21 amino acids) was reconstituted in 1.5 mL of dionized water obtaining a final concentration of 6.6 µg/µL. This stock solution was stored at -70°C until used. For the assays a working solution was prepared four times more concentrated (512 µg/ml) to make twofold dilutions with the following concentrations: 128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml and 1 µg/ml.

2.3. Resazurin microplate method (RMM)

A sterile 96-well microtitre tray with lid was set up for each of the test bacteria ($n=2$) as follows: column 1-8, 100 μ l of Mueller-Hinton II cation adjusted broth plus 100 μ l of the antimicrobial peptide 4X (512 μ g/ml) only to column 1. Make twofold dilutions by transferring 100 μ l from column 1 to column 2 and continue to column 8 where the last 100 μ l were discarded. The final K9CATH concentrations were 128 μ g/ml, 64 μ g/ml, 32 μ g/ml, 16 μ g/ml, 8 μ g/ml, 4 μ g/ml, 2 μ g/ml and 1 μ g/ml. Then, 100 μ l of inoculum (5×10^5 CFU/ml) was added to all the wells. A viability control was included in column 10 containing 100 μ l of Mueller-Hinton broth plus 100 μ l of inoculum. Also, a sterility control containing only 100 μ l of Mueller-Hinton broth was included. Trays were covered and incubated for 16 h at 37°C. After incubation, 30 μ l of 0.01 % resazurin solution (w/v, prepared in sterile distilled water) were added to all the wells and incubated for 1 h. The MIC values were determined by two methods. First, visually by color change, with the highest dilution remaining blue indicating the MIC. Then by absorbance read at 650 nm using a Biorad Model 3550 universal microplate reader.

2.4. Broth microdilution assay (BMM)

This reference method was used to determine the MIC of the K9CATH peptide according to the NCCLS and adapted by Wiegand et al. (2008). The plate was set up identically as described for the resazurin assay, except that resazurin was not added. The MIC values were determined by two methods. First, visually with the highest dilution showing turbidity (bacteria growth) indicating the MIC. Then, by absorbance read at 650 nm using a Biorad Model 3550 universal microplate reader.

3. Results

The resazurin method was adapted to evaluate the *in vitro* susceptibility of *S. aureus* to the K9CATH peptide, and the broth microdilution method used as a reference. Incubation times for both methods were similar and the only difference was the addition of resazurin as color indicator for bacteria growth in the RMM. A total of 8 repetitions per method were made for each bacteria strain. Resazurin method depends on an easily recognized color change from blue (indicating inhibition of the test organism) to pink (no inhibition). By this method, visually determined MIC's for the field strain of *S. aureus* was 4 μ g/mL, while for *S. aureus* ATCC the MIC was 16 μ g/mL; when determined by absorbance MIC's were 8 μ g/mL and 18 μ g/mL, respectively. By the reference NCCLS method the MIC's determined visually and by absorbance were the same, 8 μ g/mL for the field strain of *S. aureus* and 18 μ g/mL for the *S. aureus* ATCC (Table 1).

Table 1

MIC's obtained of *S. aureus* to the antimicrobial peptide K9CATH with the reference NCCLS method and the Resazurin method (data shown in percentage).

	<i>S. aureus</i> field strain				<i>S. aureus</i> ATCC 33591			
	Resazurin		NCCLS		Resazurin		NCCLS	
	Visual	Absorbance	Visual	Absorbance	Visual	Absorbance	Visual	Absorbance
MIC μ g/mL								
4	100							
8		100	100	100				
16					100	87.5	87.5	87.5
32						12.5	12.5	12.5
Range μ g/mL	4-4	8-8	8-8	8-8	16-16	16-32	16-32	16-32
MIC average μ g/mL	4 \square	8	8	8 \square	16	18	18	18

4. Discussion

The antimicrobial activity of the K9CATH peptide was demonstrated by both methods against two different strains *S. aureus*. The higher MIC observed for the control strain of *S. aureus* (ATCC 33591) could be due to a higher

degree of virulence than the field strain, and therefore a higher concentration of the peptide was necessary to inhibit the bacteria.

Moreover, lower MIC's observed with the resazurin method could indicate that is more sensitive than the reference method. Mann and Markham (1998) also observed lower MIC's by the resazurin method when compared with the agar dilution assay for essential oils against a range of gram-positive and gram-negative bacteria. Also, a study performed to detect vancomycin and oxacillin resistant *S. aureus* showed the agreement between the MIC's obtained by the two colorimetric methods resazurin and nitrate reductase assay (NRA), with the reference broth microdilution method (Coban et al., 2006). In addition, the resazurin method has been utilized successfully to evaluate antimicrobial susceptibility and resistance for *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium smegmatis*, and proved to be sensitive and specific (Taneja and Tyagi, 2007; Rivoire et al., 2007; Campanerut et al., 2011; Palomino et al., 2002). Moreover, the colorimetric assay Alamar blue has been utilized in several studies to determine MIC's for several antimicrobial drugs against gram -positive, gram-negative, fungus and yeast, and have demonstrated to be effective, rapid, sensitive, and results correlated with those obtained with the broth microdilution method (NCCLS, 2000), agar dilution (Baker et al., 1994; Mann y Marham, 1998), BACTEC 460 (Collins y Franzblau, 1997), absolute concentration method (ACM) (Campanerut et al., 2011) and the nitrate reductase assay (NRA) (Cobban et al., 2006). Although the MABA is a reliable method involves an economic cost and the availability could be limited for some laboratorios in some countries.

Resazurin reduction results in an easily identified color change occurring at cell densities meaningful for MIC testing. Resazurin undergoes a color change when surrounding medium is reduced as a result of bacterial depletion of dissolved oxygen and acid production, and therefore it is necessary to asses reduction densities for each organism tested (Mann and Markhan, 1998). The resazurin microdilution method is a suitable alternative to the reference NCCLS method as demonstrated by similar MIC's obtained in this study with both methods, with the advantage of easier interpretation due to the color change. In addition, microdilution methods are affordable, rapid and easy to perform when compared to conventional methods. Moreover, for antimicrobial peptides susceptibility testing the microdilution methods is the best choice since small volumes of the peptides are required. While absorbance readings provided a useful comparison among MIC's obtained with both methods, plates can be more conveniently read visually during routine use of the resazurin method.

5. Conclusion

The K9CATH peptide inhibited the growth of both, the field strain and the reference strain of *Staphylococcus aureus*, and the MIC's obtained with both methods were very similar, although the resazurin method is easier to read and appears to be more sensitive than the reference method. 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 the K9CATH peptide to *S. aureus*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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