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Antibacterial activity of different types of snake venom from the Viperidae family against *Staphylococcus aureus*

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ABSTRACT. Toxins and venoms produced by living organisms have exhibited a variety of biological activities against microorganisms. In this study, we tested seven snake venoms from the family Viperidae for antibacterial activity and the activities of reversal of antibiotic resistance and inhibition of biofilm formation against 22 clinical isolates of *Staphylococcus aureus*. *Bothrops moojeni* venom exhibited anti staphylococcal activity with the lowest mean value of minimum inhibitory concentration (MIC). Moreover, reversal of antibiotic resistance was observed for combinations of *B. moojeni* venom (½ x MIC) and norfloxacin or ampicillin (both ½ x MIC) for 86.4% and 50% of the isolates, respectively. *B. moojeni* venom alone at ½ MIC inhibited 90% of biofilm formation, whereas in combination with ciprofloxacin, both at ½ MIC, a reduction on the NorA efflux pump activity was observed. The detection of *in vitro* mutants colonies of *S. aureus* resistant to *B. moojeni* venom was low and they did not survive. A phospholipase A2 was purified from the venom of *B. moojeni* and displayed anti-staphylococcal activity when tested alone or in combination with ciprofloxacin. The results presented here will contribute to the search for new antimicrobial agents against resistant *S. aureus*.

Keywords: *Bothrops moojeni*, bacteria, antibiotic-resistance, NorA efflux pump, biofilm.

Atividade antibacteriana de diferentes tipos de veneno de serpentes da família Viperidae contra *Staphylococcus aureus*

RESUMO. Toxinas e venenos exibem uma variedade de atividades biológicas contra micro-organismos. Neste estudo, investigou-se a atividade de sete venenos de serpentes, da família Viperidae, sobre o crescimento de *Staphylococcus aureus*, na reversão fenotípica da resistência a antibióticos e inibição de formação de biofilme contra 22 isolados clínicos de *S. aureus*. O veneno de *Bothrops moojeni* apresentou a menor média de concentração inibitória mínima (CIM). Além disso, observou-se reversão da resistência a antibióticos para combinações do veneno de *B. moojeni* (½ x CIM) e norfloxacina ou ampicilina (ambos ½ x CIM) para 86,4% e 50% dos isolados, respectivamente. O veneno de *B. moojeni* na concentração de ½ CIM inibiu 90% de formação de biofilme, enquanto ele em combinação com ciprofloxacina, ambos na concentração de ½ CIM, diminuiu a atividade da bomba de efluxo NorA. A detecção *in vitro* de colônias mutantes de *S. aureus* resistente ao veneno de *B. moojeni* foi baixa e eles não sobreviveram. Uma fosfolipase A2 purificada a partir do veneno de *B. moojeni* exibiu atividade antibacteriana quando testada sozinha ou em combinação com ciprofloxacina. Os dados obtidos poderão contribuir para a pesquisa de novos agentes antimicrobianos contra *S. aureus*.

Palavras-chave: *Bothrops moojeni*, bactéria, resistência, antibiótico, bomba de efluxo NorA, biofilme.

Introduction

Since the late 1940s, bacterial resistance to antibiotics has drastically increased worldwide. It negatively affects the treatment of all infectious diseases, and is a major cause of mortality and morbidity, significantly increasing the cost of health care (Martinez et al., 2009). *S. aureus* can be responsible for both local and generalized infections, and is naturally susceptible to nearly all antibiotics that have been developed (Chambers & DeLeo, 2009). The emergence of strains of *S. aureus* resistant to penicillin, methicillin, vancomycin and linezolid was reported soon after their clinical use (North & Christie, 1946; Labischinski, Ehlert, & Berger-Bächi, 1998; Tsiodras, et al., 2001). Multiple drug resistant *S. aureus* is one the most common nosocomial

pathogens worldwide and is of great concern to the global health community. The NorA efflux pump is one of the major contributors to the resistance of *S. aureus*, promoting extrusion of chemically unrelated compounds, such as ethidium bromide, quaternary amine compounds, chloramphenicol and fluoroquinolones from the cell (Costa, Viveiros, Amaral, & Couto, 2013). Moreover, *S. aureus* has the ability to form biofilm, which prevents antibiotics from accessing bacterial cells, thereby contributing to its success as a human pathogen (McCarthy et al., 2015).

As multidrug resistance is an increasing problem, it highlights the urgent need for new antibiotics and treatment strategies. The discovery of chemically diverse and relatively non-toxic antimicrobials from different natural sources shows the promise that natural products have as a source of new antimicrobial drugs (Lima et al., 2005; Abreu, McBain, & Simões, 2012). A promising strategy to restore antibiotic effectiveness against pathogens has been the use of a combination of two or more antibiotics or of antibiotics and natural products, and the identification of efflux pump and biofilm inhibitors (Braga et al., 2005; Credito, Lin, & Appelbaum, 2007; Nascimento, Brandão, Oliveira, Fortes, & Chartone-Souza, 2007).

Snake venom, a complex mixture of proteins and peptides with potential biological activity, could lead to the development of new drugs with therapeutic significance (Ferreira et al., 2011; Vyas, Brahmbhatt, Bhatt, & Parmar, 2013). Snake venoms of the family Viperidae in particular are an important source of peptides, but they remain underexplored (Ferreira et al. 2011). Studies have already demonstrated that snake venoms of *Bothrops jararaca* (Ciscotto et al., 2009), *B. leucurus* (Nunes et al. 2011) *B. marajoensis* (Costa-Torres et al., 2010), *Bothrops alternatus* (Bustillo et al., 2008) *Crotalus adamanteus* (Samy, et al., 2014), *C. durissuscumanensis* (Vargas et al., 2012) and *Porthidium nasutum* (Vargas et al., 2013) have antibacterial activity.

The aim of this study was to evaluate the potential antibacterial activity of crude snake venom from snake species of the genera *Bothrops*, *Bothropoides* and *Rhinocerophis* of the family Viperidae on *S. aureus* isolates. We also investigated the antibacterial activity of *Bothrops moojeni* venom, its interaction with antibiotics, as well as its inhibitory effect on biofilm formation and the NorA efflux pump.

Material and methods

Bacterial strains

Antibacterial activity and synergistic effects were evaluated against 22 clinical isolates of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) (Braga et al., 2005), and *S. aureus* strain RN 7044 carrying the plasmid pWBG32 which encodes the NorA efflux pump (Pillai, Pillai, Shankel, & Mitscher, 2001). In addition, *S. aureus* ATCC 25923 was included as a negative control. The bacteria were cultured in Müller-Hinton (MHB; Difco Laboratories, Detroit, Michigan) or Luria-Bertani (LB; Difco Laboratories, Detroit, Michigan) broths at 37º C for 24 hours.

Venoms and antibiotics

Venoms from *Bothropoides erythromelas*, *Bothropoides jararaca*, *Bothropoides neuwiedi*, *Bothrops atrox*, *Bothrops jararacussu*, *Bothrops moojeni* and *Rhinocerophis alternatus* were kindly donated by the Serpentarium of *Fundação Ezequiel Dias*, which is a recipient of the authorization n° 117521 from the brazilian institute of environment and renewable natural resources (IBAMA) to work with the wild fauna under the category 20.45 (scientific animal husbandry of the wild fauna for research purposes). Initially, crude venom was dissolved in ammonium acetate buffer (0.2 M, pH 8) in order to make stock solutions of 20 mg mL^{-1} that were centrifuged at 10,000 x g for 10 minuntes at 4º C. The supernatant was aliquoted and then stored at -20° C until use.

The following antibiotics were used in this study: amoxicillin/clavulanate (Glaxo Smith Kline, Brentford, Middlesex, UK), and ampicillin, ciprofloxacin, levofloxacin, norfloxacin, and ofloxacin (Sigma Chemical Co. St. Louis, MO, USA). Beta-lactams were chosen for having an effect on a wide range of infectious agents, whereas fluoroquinolones are widely used against multi-resistant cocci infections.

Fractionation of *Bothrops moojeni* venom

Crude venom of *B. moojeni* was dissolved in 0.2 M ammonium acetate pH 8 at a concentration of 50 mg mL^{-1} and centrifuged for 10 min at 10,000 g at 4° C. The supernatant was removed and subjected to gel filtration. Fractionation was performed on an Akta Purifier System using the chromatographic column Sephacryl S-100 XK 16/60, both from GE Healthcare (Uppsala, Sweden), with 0.2 M ammonium acetate pH 8 as elution buffer in a 1 mL min^{-1} flow. Aliquots of 2 mL were collected, pooled into six major fractions according to their elution time, lyophilized and resuspended in ultrapure

water. The fraction with antibacterial activity was subjected to high performance liquid chromatography, using a µRPC C2/C18 ST 4.6/100 reverse phase column (GE Healthcare, Uppsala, Sweden), previously equilibrated with 0.1% trifluoroacetic acid (TFA). Elution of the unbound sample was carried out for 2 column volumes with 0.1 % TFA (buffer A). The bound sample was eluted under a linear gradient from 0 to 80% acetonitrile (buffer B) added to buffer A at a flow rate of 0.7 ml min^{-1}.

Amino acid sequencing

Intact protein (20 μg) was solubilized in acetonitrile/water solution (1:1) and submitted to Edman degradation using a Shimadzu PPSQ-21A automated protein sequencer. The resulting sequence was compared with the sequences of other related proteins in the SWISS-PROT/TREMBL data bases using the programs FASTA 3 (http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=fasta) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast). Later, sequences were aligned using the program Mafft (http://mafft.cbrc.jp/alignment/software/).

Determination of the minimum inhibitory concentrations (MIC)

Determination of MIC was performed by the broth dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2016) and using MHB with an inoculum of approximately 10^5 colony-forming units per milliliter (CFU mL^{-1}). The MHB was supplemented with serial antibiotic concentrations ranging from 0.0612 to 1,024 μg mL^{-1} and venoms at concentrations from 2 to 1,024. To evaluate the effect of venoms in combination with antibiotics, increasing concentrations (with a 2-fold step, i.e., 0.0612, 0.125, …, 1024 μg mL^{-1}) of these antibiotics were added to MHB containing venom at 1/2 × MIC. MICs were interpreted as the lowest concentration of antibiotics or venoms that inhibited visible growth after 24 hours of incubation at 37° C.

To evaluate the effect of *B. moojeni* venom as a resistance-modifying agent, crude venom in combination with antibiotics in different concentrations (½, ¼, ⅛ x MIC) were used. Cultures that contained neither venom nor antibiotics were included as controls; all tests were carried out in duplicate. The MIC was defined as the lowest concentration that completely suppressed visible growth after 24 hours of incubation at 37º C. The bactericidal concentration was the lowest concentration at which bacteria failed to grow in MHB and after plating onto Muller-Hinton agar (Smith-Palmer, Stewart, & Fyfe, 1998).

Survival curves

Growth curves for *S. aureus* RN 7044 were determined by the use of the dilution tube method with 1×10^5 CFU mL^{-1} as standard inoculum in the presence of *B. moojeni* venom at concentrations of ½ and 1 x MIC, in MBH and in combination with ½ x MIC of ciprofloxacin. A tube containing only MHB was inoculated and included as a control. Tubes were incubated at 37º C for 24 hours. At different times (3, 6, 9 12, 24, 36 hours), the optical density (OD) of each culture was read at 600 nm using a NanoDrop Spectrophotometer (NanoDrop Technologies) for CFU mL^{-1} determination. Bacterial density (CFU mL^{-1}) was then determined.

Monitoring of ethidium bromide efflux

S. aureus RN 7044 was cultured with aeration in MHB at 37° C up to OD600 = 1.8. The culture was further incubated for 30 min at 37° C in the absence or presence of *B. moojeni* venom after which 20 µg mL^{-1} ethidium bromide were added and the cells were again incubated for 30 min at 37° C. The cells were then collected by centrifugation (5,000*g*) and washed twice with 20 mmol L^{-1} HEPES–NaOH (pH 7) buffer, followed by resuspension in the same buffer at a final OD269 = 0.1. Fluorescence of ethidium bromide was measured using a Varian fluorescence reader (Cary Eclipse Fluorescence Spectrophotometer, Palo Alto, CA, USA) with excitation at 269 nm and emission at 600 nm for 1 hour. Readings were taken with the fluorescence reader set at high sensitivity every minute for the first 5 min, then every 5 min until the end of 90 min.

Biofilm inhibition assays

The effect of *B. moojeni* venom on the biofilm formed by *S. aureus* RN7044 was tested according to Pimenta, Martino, Bouder, Hulen, & Bligh (2003) with modifications. Briefly, *S. aureus* was grown in LB medium at 37° C for 24 hours. Afterwards, 0.1 mL of culture containing approximately 10^5 CFU mL^{-1} were transferred to a polystyrene 96-well microplate containing either LB medium or LB medium supplemented with the *B. moojeni* venom at the concentrations of ½, ¼, ⅛ x MIC, and then incubated at 37° C for 24 hours. Following the incubation period, the suspension cultures were discarded, the plate was washed three times with distilled water and the biofilms were stained with 0.1% crystal violet for 30 minutes at room

temperature. Extra dye was then removed by five washes with distilled water. The dye retained by the cells of the biofilm was dissolved with 120 µL of 1% (w/v) sodium dodecyl sulfate. The results were recorded as absorbance at 595 nm to quantify total biofilm mass.

Detection of mutants resistant to *Bothrops moojeni* venom

Spontaneous mutants of *S. aureus* RN 7044 resistant to *B. moojeni* venom were obtained as described previously (Szybalsky & Bryson 1952). Twenty milliliters of culture in MBH was grown for 24 hours at 37° C, centrifuged at 3,000 x g for 20 min at 4° C and resuspended in 10^{-1} of the initial volume in saline (0.9% NaCl). After this, 0.1 mL was spread onto a gradient plate supplemented with venom at concentrations of 2, 5, and 10 x MIC and incubated at 37° C. The results were read at 24, 36, 72 and 96 hours. Mutant colonies were considered those that had grown beyond the edge of confluent growth.

Determination of the fractional inhibitory concentration

The fractional inhibitory concentration (FIC) index is frequently used to assess the drug interactions (Mackay et al. 2000). The indices were calculated as follows: FIC of drug A = MIC drug A + venom/ MIC drug A alone. The interpretation was made as follows: synergy (≤0.5), indifference (>0.5 to 4), and antagonism (>4).

Statistical analysis

Mann-Whitney U test at R platform was used to determine significant differences between venom´s MIC, with 5% of significance.

Results and discussion

In this study, the susceptibility of *S. aureus* to seven snake venoms from the family Viperidae (*Bothropoides erythromelas, B. jararaca, B. neuwiedi, B. atrox, B. jararacussu, B. moojeni* and *Rhinocerophis alternatus*) was determined. MICs of the crude snake venoms are shown in Table 1. Although all the venoms came from snakes of the same family, great variation in MIC (from 2 to >1,024 μg mL^{-1}) was observed against *S. aureus*. Moreover, no significant difference ($p>0.05$) was observed among the MICs of different venoms, except for the *Bothropoides erythromelas* and *B. jararaca* venoms. However, the mean MIC of *B. moojeni* venom was the lowest, being chosen for further studies.

For all venoms tested, MICs were greater than 1,024 μg mL^{-1} for ten out of the 22 *S. aureus* isolates, whereas the remaining isolates had MICs >1,024 μg mL^{-1} against at least one of the venoms. *B. jararaca* showed weak antibacterial activity against all clinical isolates and the reference strain (ATCC 25923) with MIC >1,024 μg mL^{-1}. Similar results were obtained with *B. erythromelas* venom. In contrast, *S. aureus* RN 7044 was more sensitive to venoms of *B. atrox* and *B. neuwiedi*. Moreover, our data revealed that the type ATCC strain was less sensitive than the *S. aureus* isolates. Interestingly, minimum bactericidal concentrations (MBCs) correlated with the MICs for all venoms tested.

There are several reports in the literature on the antibacterial activity of snake venom against gram-positive and gram-negative bacteria (Lu et al., 2002; Stábeli et al., 2004; Klein et al., 2015), including *Bacillus subtilis*, *Sarcina* spp., *Escherichia coli* and *S. aureus*. A previous study with *B. marajoensis* venom (also of the family Viperidae), revealed that it was able to inhibit the growth of *P. aeruginosa*, *S. aureus* and *Candida albicans*, thereby demonstrating an antifungal effect is also present in some venoms (Costa-Torres et al., 2010).

The MICs of six antibiotics belonging to two classes are shown in Table 2. Overall, the isolates were resistant to three of the six antibiotics tested. The isolates exhibited resistance to β-lactams, with the highest frequency of resistance to ampicillin (100%) and amoxicillin/clavulanate (63.6%). The increase in the frequency of isolates of *S. aureus* resistant to β-lactam antibiotics has been reported in the literature since the beginning of its clinical use in 1940 (Livermore, 2000). Alzolibani et al. (2012) found 96.7% ampicillin resistance in clinical isolates of *S. aureus*, which agrees with the data obtained in our study.

Among the quinolones of clinical use, the isolates were resistant to only ofloxacin (45.4%). It should be noted that all the isolates were susceptible to ciprofloxacin, levofloxacin and norfloxacin. Similarly, Kowalski et al. (2003) suggest that *S. aureus* had increased susceptibility to fourth generation fluoroquinolones. In contrast, other studies revealed high frequency of resistance to ciprofloxacin (Alzolibani et al., 2012; Flamm et al., 2012; Kwak et al., 2013). A possible explanation for this discrepancy could be that the isolates tested in this study were collected in the late 70s and early 80s prior to the introduction of fluoroquinolones to clinical use.

Table 1. Minimum inhibitory concentration (MIC) of snake venom from the Viperidae family against clinical isolates and strains of *Staphylococcus aureus*

Isolate/Strain	MIC ($\mu g\ mL^{-1}$)						
	Bothropoides erythromelas	*Bothropoides jararaca*	*Bothropoides neuwiedi*	*Bothrops atrox*	*Bothrops jararacussu*	*Bothrops moojeni*	*Rhinocerophis alternatus*
RN 7044	32	512	2	2	8	4	4
ATCC 25923	>1,024	>1,024	64	64	64	128	64
7	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
8	>1,024	>1,024	64	64	64	8	64
10	>1,024	>1,024	64	64	64	>1,024	64
12	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
17	512	>1,024	64	64	64	2	64
22	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
23	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
25	>1,024	>1,024	64	64	64	>1,024	64
29	>1,024	>1,024	>1,024	>1,024	>1,024	2	>1,024
30	>1,024	>1,024	>1,024	>1,024	>1,024	4	>1,024
31	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
34	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
37	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
40	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
41	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
42	>1,024	>1,024	64	64	64	8	64
44	>1,024	>1,024	>1,024	>1,024	>1,024	128	>1,024
48	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024
49	>1,024	>1,024	64	64	>1,024	256	64
50	>1,024	>1,024	64	64	64	128	64
52	512	>1,024	64	64	64	16	64
54	>1,024	>1,024	64	64	>1,024	8	64

Table 2. Minimum inhibitory concentration (MIC) of antibiotics alone, and in combination with ½ x MIC of *Bothrops moojeni*.

Isolate/Strain	Amoxicillin/ Clavulanate		Ampicillin		Ciprofloxacin		Levofloxacin		Norfloxacin		Ofloxacin	
	MIC	MIC[a]	MIC	MIC[a]	MIC	MIC[a]	MIC	MIC[a]	MIC	MIC[a]	MIC	MIC[a]
RN 7044	4	2	0.5	0.25	128	64	16	16	256	128	64	64
ATCC 25923	0.0612	0.0612	0.5	0.25	1	0.5	0.5	0.25	0.5	0.25	0.5	0.25
7⋆	128	128	256	128	0.0612	0.0612	0.125	0.125	0.5	0.25	0.125	0.125
8	0.5	0.5	0.5	0.25	2	1	1	0.5	1	0.5	0.5	0.5
10	128	128	128	128	0.25	0.25	0.125	0.125	0.5	0.5	0.125	0.125
12	128	128	128	128	0.0612	0.0612	0.0612	0.0612	0.5	0.25	0.125	0.125
17	128	128	16	8	1	0.5	0.5	0.5	0.5	0.25	0.25	0.25
22	128	128	256	128	0.0612	0.0612	0.5	0.5	0.5	0.25	0.25	0.25
23⋆+	1,024	1,024	512	256	0.125	0.0612	0.125	0.125	0.5	0.25	0.25	0.25
25	0.5	0.5	0,5	0.25	2	1	0.5	0.5	2	1	8	4
29	64	64	128	128	0.25	0.25	0.0612	0.0612	0.5	0.25	0.0612	0.0612
30	0.5	0.5	2	2	0.5	0.25	0.5	0.25	2	1	0.5	0.5
31	128	128	64	64	0.125	0.125	0.125	0.125	0.5	0.25	0.125	0.125
34	256	256	128	128	0.0612	0.0612	0.0612	0.0612	0.5	0.25	8	4
37⋆+	256	256	64	64	0,5	0,25	0.0612	0.0612	1	0.5	16	8
40⋆+	256	128	256	128	0.125	0.125	0.125	0.125	4	2	4	**2**
41	512	512	256	256	0.0612	0.0612	0.0612	0.0612	1	0.5	8	4
42	0.5	0.25	0.5	0.25	1	1	0.25	0.25	4	2	4	4
44⋆	256	256	256	128	0.0612	0.0612	0.0612	0.0612	1	0.5	16	16
48	128	128	256	256	0.0612	0.0612	0.0612	0.0612	1	1	8	8
49	0.5	0.25	0.0612	0.0612	1	0.5	0.125	0.125	2	1	8	4
50	0.5	0.25	0.5	0.25	1	0.5	0.5	0.25	4	2	0.25	0.25
52	0.5	0.5	0.125	0.125	2	1	0.125	0.125	4	2	0.25	0.25
54⋆	0.5	0.25	1	0.5	2	1	1	0.5	4	2	4	**2**

Critical point for determination of drugs resistance in μg/mL for *S. aureus* according to CLSI (2016): Amoxicillin-Clavulanate ≥ 8; Ampicillin ≥ 0.25; Ciprofloxacin ≥ 4; Levofloxacin ≥ 4; Norfloxacin ≥ 16; Ofloxacin ≥ 4.

⋆β-lactamase positivos; + Methicillin-resistant *S. aureus* (MRSA).

[a] Antibiotic concentration in combination with *B. moojeni* venom that prevented the development of turbidity (i.e., growth).

Results highlighted in bold refers to reversal of resistance.

The capacity of *B. moojeni* venom to enhance the activity of the tested antibiotics was also investigated. The MICs of antibiotics and *B. moojeni* venom individually and in combination are shown in Table 2. A synergic effect (FIC index of 0.5) was observed between *B. moojeni* venom and all antibiotics investigated. The combinations of the *B. moojeni* venom with, ampicillin, amoxicillin/clavulanate, ciprofloxacin, levofloxacin, norfloxacinin and ofloxacin achieved synergy of 50%, 22.7%, 45.5%, 18.2%, 86.4% and 31.8%, respectively, in the *S. aureus* clinical isolates. Moreover, no antagonistic action was found. These results suggest that *B. moojeni* venom increased the antibacterial activity of these antibiotics against *S. aureus*. It should be noted that five of the six β-lactamase positive isolates were

inhibited by the combination of ampicillin and *B. moojeni* venom. Additionally, the reduction of the MIC of ofloxacin, when in combination with *B. moojeni* venom, for the isolate 40 (β-lactamase positive, methicillin-resistant *S. aureus*-MRSA), and the isolate 54 (β-lactamase positive) led to the reversal of ofloxacin resistance in these isolates.

Previous studies have reported antimicrobial peptides in various animal venoms, which are traditionally associated with defense mechanisms, such as antibacterial activity (Jenssen, Hamill, & Hancock, 2006; Wang, Li, & Wang, 2009). Moreover, other studies have shown that the fluoroquinolones, erythromycin and rifampicin had their effects enhanced by antimicrobial peptides, demonstrating synergistic action (Ulvatne, Karoliussen, Stiberg, Rekdal, & Svendsen, 2001; Fehri, Wróblewski, & Blanchard, 2007).

Among the fractions of *B. moojeni* venom obtained by gel filtration chromatography, the fraction BmooIV (Figure 1A) was the only one that exhibited antibacterial activity when tested against *S. aureus* RN 7044 (Figure 2A). The antibacterial activity of BmooIV was detected in the amount of 1.14 mg mL^{-1} of protein. To investigate whether BmooIV also had the ability to enhance the susceptibility of *S. aureus* RN 7044 to ciprofloxacin it was tested in combination with this antibiotic, both in ½ x MIC concentration. The result indicated the reversal of phenotypic resistance to ciprofloxacin of *S. aureus* RN 7044

The fraction BmooIV was lyophilized and subjected to a high-performance liquid chromatography (HPLC) on the μRPC C2/C18 reverse phase column (Figure 1B). The fraction obtained from reverse phase named Bmoo-SII presented antibacterial activity when tested against *S. aureus* RN 7044. It also enhanced the susceptibility of *S. aureus* RN 7044 to ciprofloxacin, when tested in combination, both at ½ x MIC concentration (Figure 2B).

The comparison of 50 amino acids residues from Bmoo-SII placed it in the phospholipase A_2 superfamily with high identity to Myotoxin II from *B. moojeni* and basic phospholipase A_2 from *B. moojeni* and *B. asper* (Figure 3). Silveira et al. (2013) characterized the phospholipase A_2 (PLA_2) from *B. moojeni*. PLA_2 was first purified and characterized from cobra venom and later from rattlesnake venom. They are small, secreted proteins of 14–18 kDa that usually contain 6 to 8 disulfide bonds. Queiroz et al. (2011) compared their results with other studies (Soares et al. 1998; Soares et al., 2000; Borja-Oliveira et al., 2007; Calgarotto, et al., 2008; Santos-Filho et al., 2008), which reported isoforms of PLA_2 myotoxin of *B. moojeni* with molecular weight ranging between 13,400 Da to 16,500 Da. Thus, we were able to infer from the amino acids residues obtained in our analysis that Bmoo-SII has a molecular weight within this range.

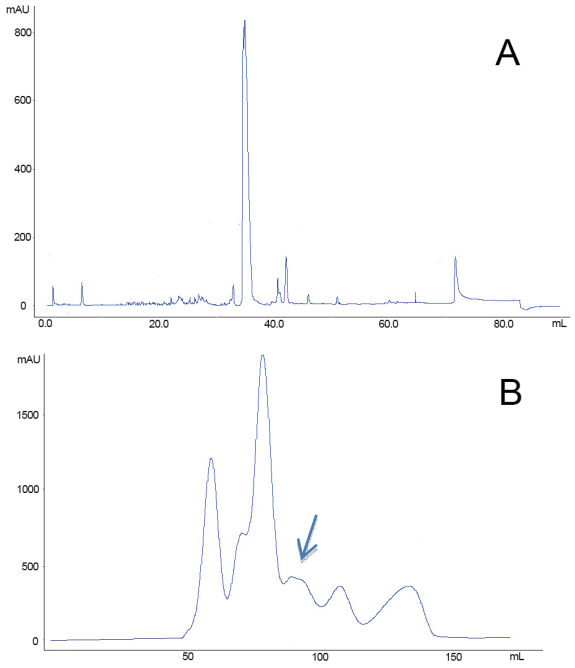

Figure 1. A. Chromatographic profile of *Bothrops moojeni* venom separation in gel filtration Sephacryl-S100 XK 16/60 column. The pointer indicates the fraction named BmooIV. B Chromatographic profile of BmooIV in μRPC C2-C18 the reverse phase column. The highest peak is Bmoo-SII.

PLA_2 plays important roles in cellular signaling and metabolism. It also participates in the first line of antimicrobial defense (Nevalainen, Graham, & Scott, 2008; Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011). The bactericidal action of PLA_2 depends on whether the bacteria are gram-positive or gram-negative. In general, PLA_2 hydrolyses the phospholipid membrane of the bacteria cell causing death to both gram-positive as gram-negative bacteria (Nevalainen et al., 2008). In vitro studies by Grönroos, Laine, Janssen, Egmond, & Nevalainen, 2001, showed that PLA_2s from groups IIA and V were found to kill both methicillin-resistant staphylococci and vancomycin-resistant enterococci. Snake venom from the family Viperidae possesses PLA_2 from group IIA (Dennis et al., 2011). The efficiency of PLA_2 against antibiotic-resistant bacteria is an important property that holds promise for biotechnological applications.

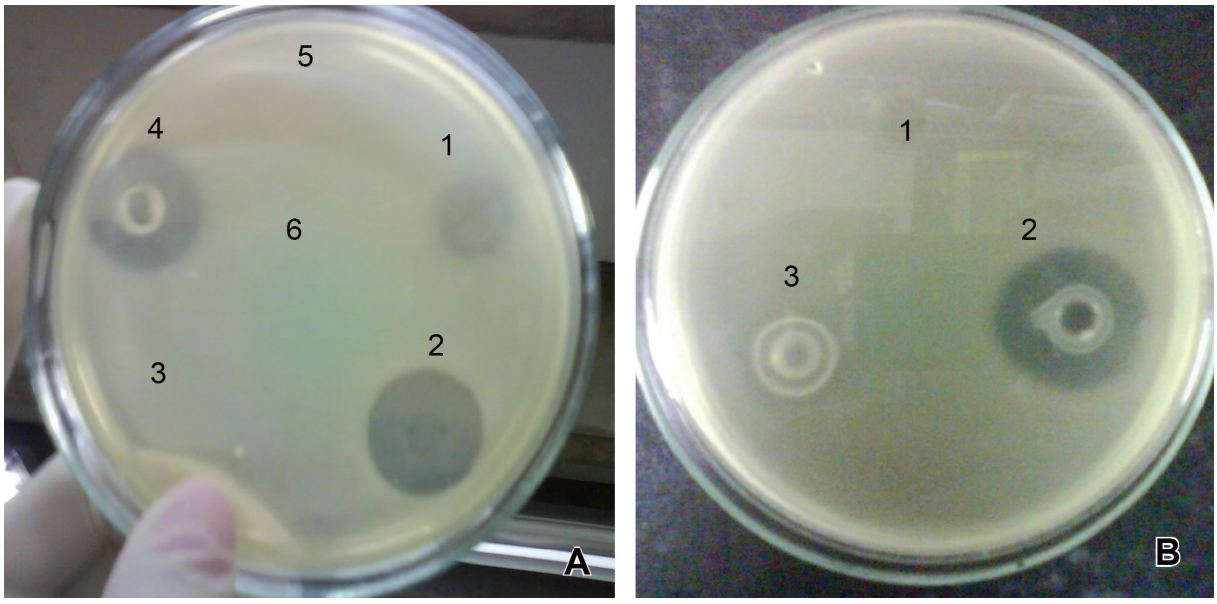

Figure 2. A. Activity of BmooIV against *Staphylococcus aureus* RN 7044. 1- BmooIV (1.14 mg mL^{-1} – 1 x MIC); 2- *Bothrops moojeni* crude venom (1 x MIC); 3- BmooIV (0.57 mg mL^{-1} – ½ x MIC); 4- BmooIV (0.57 mg mL^{-1} – ½ x MIC) in combination with ciprofloxacin (64 μg mL^{-1} – ½ x MIC); 5- Ciprofloxacin (64 μg mL^{-1} – ½ x MIC); and 6- Elution buffer 0.2 M ammonium acetate pH 8. B. Antibacterial activity of Bmoo-SII against *S. aureus* RN 7044. 1- Elution buffer 0.2 M ammonium acetate pH 8; 2- Bmoo-SII (50 μL – ½ x MIC) in combination with ciprofloxacin (64 μg mL^{-1} – ½ x MIC); and 3- Bmoo-SII (100 μL – 1 x MIC).

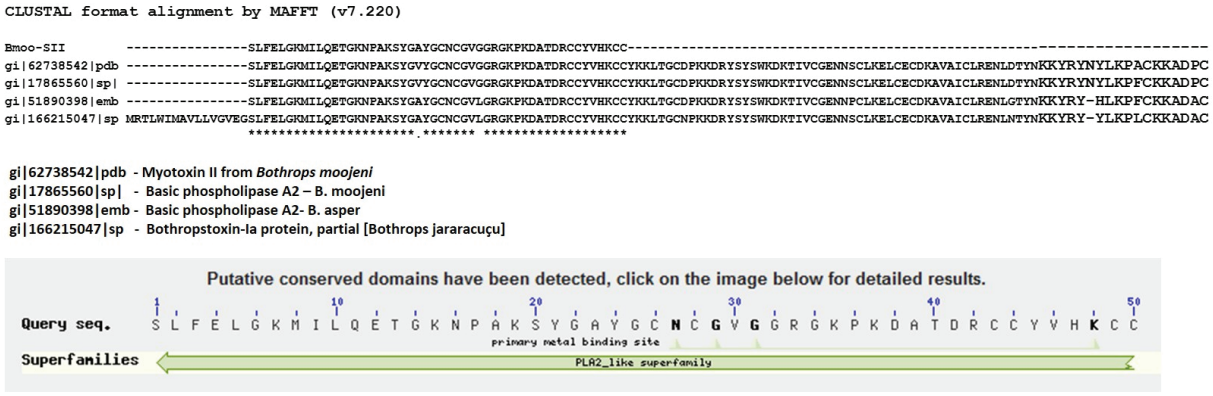

Figure 3. Alignment of Bmoo-SII with other phospholipase A_2-like sequences.

Survival kinetics were evaluated for *S. aureus* RN7044, for which synergistic activity had been observed (Figure 4). A bactericidal profile was observed at sub-inhibitory concentrations of ciprofloxacin (½ x MIC) and *B. moojeni* venom (½ x MIC) and of *B. moojeni* crude venom alone (1 x MIC). It should be noted that *B. moojeni* venom or ciprofloxacin, when tested individually in sub-MIC concentrations, allowed bacterial growth similar to that of the control, although there was a longer lag phase in these concentrations.

The possible inhibitory action to the efflux pump of *S. aureus* RN 7044 by *B. moojeni* venom was evaluated by the loss of fluorescence (Figure 5). In the control culture, a greater reduction in the fluorescence between 10 and 20 minutes due to the higher extrusion of ethidium bromide by the NorA efflux pump was observed. Cultures exposed to *B. moojeni* venom (½ x MIC) presented similar results to the control. However, when *B. moojeni* venom was used in combination with ciprofloxacin (both ½ x MIC) a slower descent in fluorescence was noticed, indicating a reduction in pump activity. Although the crude venom and ciprofloxacin (both ½ x MIC) by themselves were not effective antibacterials, they can reverse the resistance by blocking the NorA efflux pump. Previous studies have identified products from natural sources as inhibitors of the *S. aureus* NorA efflux pump, which co-administered with fluoroquinolone can potentiate its antibacterial activity (Marquez et al., 2005).

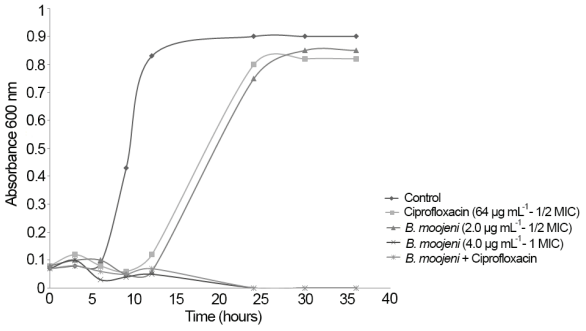

Figure 4. Survival curves of *Staphylococcus aureus* RN 7044 alone (control), in the presence of ciprofloxacin (½ MIC) alone, in the presence of *Bothrops moojeni* venom (½ and 1 x MIC) alone, and in the presence of the combination of the two (½ MIC).

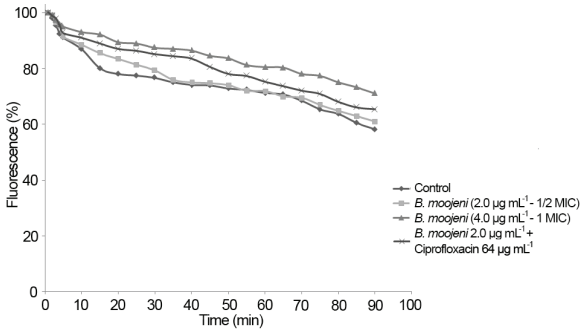

Figure 5. Measurement of active efflux of ethidium bromide in *Staphylococcus aureus* RN 7044 with excitation at 269 nm and emission at 600 nm.

To date, most studies of the activity of snake venom on bacteria have focused on their bactericidal and bacteriostatic effects. In this study, we evaluated the influence of *B. moojeni* venom as a therapeutic agent for biofilm formation by *S. aureus* RN 7044. When tested with ½ x MIC, *B. moojeni* venom was able to inhibit 90% of biofilm formation, without affecting bacterial growth. Recently, Klein et al. (2015) demonstrated for the first time that a lectin purified from the venom of *Bothrops jararacussu* disrupts staphylococcal biofilms. These findings are of interest because nosocomial infections involving the formation of biofilm caused by *S. aureus* are often difficult to treat with antibiotics.

Considering the relevance of resistant mutants in the search for potential antimicrobial agents against *S. aureus*, we investigated the occurrence of mutants in a gradient plate. At concentrations of 2, 5 and 10 x MIC of *B. moojeni* venom, a small number of mutant colonies was detected after 48 hours of incubation (Figure. 6). The relatively low frequency of the spontaneous emergence of mutants resistant to *B. moojeni* in the population of *S. aureus* RN 7044, as well as their inability to grow after consecutive subcultures on non-selective medium, is relevant since the loss of viability of the mutant *S. aureus* and the inhibitory power of *B. moojeni* venom can be explored further with the aim of possible biotechnological application.

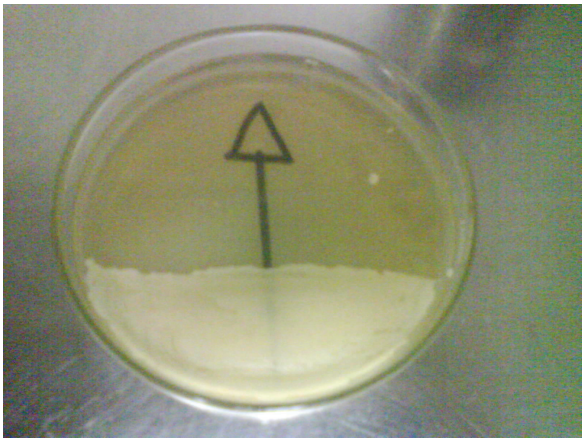

Figure 6. Mutants on Müller-Hinton agar gradient plate with *Bothrops moojeni* venom (10 x MIC) after 48 hours of incubation at 37º C.

The results show that *B. moojeni* venom and its bioactive constituent (phospholipase A_2) possess strong antimicrobial activity against *S. aureus* and its biofilm formation. The fact that *B. moojeni* venom displayed greater potency than other venoms was not investigated, but may be due to the presence of the enzyme PLA_2. *B. moojeni* venom enhanced antibiotic susceptibility, mostly in combination with norfloxacin. The effects of *B. moojeni* venom on the biofilm formation, efflux pump, and occurrence of mutants of *S. aureus* all promise to be useful in the search for antibacterial agents against drug resistant *S. aureus*.

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