Structural analysis and determination of the expression of *Microplusin* from *Rhipicephalus microplus* during a challenge with pathogenic bacteria

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Resumen

En México, la garrapata *Rhipicaphalus microplus* es la especie de mayor impacto económico y sanitario, debido a su gran capacidad expoliativa y vectorial para transmitir agentes infecciosos al ganado bovino como *Babesia* spp. y *Anaplasma marginale*. En este sentido, *R. microplus*, posee una amplia variedad de mecanismos inmunológicos de protección, incluyendo la producción de péptidos antimicrobianos (PAMs). Al momento, existen escasos reportes del papel del sistema inmune de *R. microplus*, ante la exposición con bacterias patógenas, ni mucho menos en la interacción patógeno-vector. Por lo anterior, este trabajo pretende caracterizar algunos de los mecanismos inmunológicos de *R. microplus* asociados a la expresión de PAMs, durante el reto con *Staphylococcus saprophyticus* y *Anaplasma marginale*, así como el análisis estructural de la *microplusina*. Nuestros resultados muestran la expresión diferencial de *microplusina* en presencia de hemolinfa de garrapatas infectadas con *S. saprophyticus*, y hemolinfa de garrapatas infectadas con *A. marginale*. Así mismo, se demostró la expresión basal del péptido antimicrobiano en hemolinfa de garrapatas sin infección. Adicionalmente, se encontró que la *microplusina* presenta una estructura molecular con 6 residuos de cisteínas conservados, similar al que contienen los PAMs pertenecientes a la familia de las defensinas. Estos hallazgos, corroboran la idea de la alta plasticidad que presenta la respuesta inmune de los artrópodos, dependiente del patógeno. El avance en el conocimiento de la biología de *R. microplus*, contribuye al descubrimiento de blancos, para el desarrollo de nuevos fármacos y/o vacunas para el control integrado de plagas.

Palabras Claves: *Rhipicephalus microplus,* Péptidos antimicrobianos, *Microplusin*a, *Anaplasma marginale*

Abstract

In Mexico, the *Rhipicaphalus microplus* tick is the species with the most significant economic and health impact due to its great predatory and vectorial capacity to transmit infectious agents to cattle, such as *Babesia* spp. and *Anaplasma marginale*. In this regard, *R. microplus* has various immunological protection mechanisms, including the production of AMPs. At the moment, there are few reports on the role of the immune system of *R. microplus* to the pathogenic bacteria exposure, much less information exists about the pathogen-vector interaction. Therefore, this work aims to characterize some immunological mechanisms of *R. microplus* associated with the expression of AMPs during the challenge with *Staphylococcus saprophyticus* and *A. marginale*, as well as the structural analysis of *microplusin*. Our results show the differential expression of *microplusin* in the presence of hemolymph from ticks infected with *S. saprophyticus* and hemolymph from ticks infected with *A. marginale*. Likewise, the *microplusin* basal expression in the ticks´ hemolymph without

infection was demonstrated. Additionally, it was found that *microplusin* has a molecular structure with six conserved cysteine residues, similar to that contained in AMPs belonging to the defensin family. These findings corroborate the idea of the high plasticity that the immune response of arthropods presents, depending on the pathogen. Advances in knowledge of the biology of *R. microplus* contribute to the discovery of targets for developing new drugs and/or vaccines for integrated pest control.

Key Words: *Rhipicephalus microplus*, Antimicrobial peptides, *microplusin*, *Anaplasma marginale*

Introduction

Parasitic diseases are a global problem that limits veterinary health and the productive performance of livestock. In relation, ticks represent the most important group of pathogens that cause diseases in cattle. Specifically, *Rhipicephalus microplus* is the hematophagous ectoparasite considered one of the leading causes that limit livestock production in the tropics and subtropics due to the direct and indirect damages it causes. Among these affectations, its great plundering and vectorial capacity stand out in transmitting various infectious agents such as *Babesia* spp. and *Anaplasma marginale* [1,2]. Different control strategies have been developed, where chemical acaricides are the most used tools for tick control [3]. However, the inappropriate use of these components has selected resistant populations to these compounds [4]. As a result, the importance of searching for molecular and immunological targets for developing more efficient vaccines, drugs, and/or control strategies has been highlighted. In this regard, vector control has been proposed based on acaropathogenic organisms, such as *Staphylococcus saprophyticus* bacteria, which cause yellow beak disease in *R. microplus.* This infection produces a yellowish exudate in females that blocks the genital pore, preventing oviposition and causing the death of the tick [5].

On the other hand, the tick immune system has a wide variety of protective mechanisms, where hemolymph is the circulatory fluid initiating the orchestrated protective immune response analogously to the blood, and the lymphoid system takes place by hemocytes [6]. These cells can respond through various mechanisms such as phagocytosis, production of reactive oxygen species, nodulation, and encapsulation, among others [7]. However, several pathogens manage to evade the mechanisms of the immune system and colonize the tick´s organs. Additionally, it is known that the immune system of arthropods has a remarkable plasticity that is reflected in the type of response it generates; it is even pathogen-dependent and can present significant differences between Gram-positive and Gram-negative bacteria [8].

In ticks, AMPs are genetically encoded polypeptides synthesized on ribosomes that are part of the innate immune response [9]. These molecules are synthesized in the fat body and released into the hemolymph in response to microorganisms' invasion. They can recognize surface membrane components such as lipopolysaccharides (LPS) and peptidoglycan (PGN) generated by Gram-positive and Gram-negative bacteria. Structurally the AMPs are short sequences (30-60 amino acids), and the vast majority have a positive charge, and classify into three groups: a) Linear peptides that form α-helices without cysteine residues; b) Cyclic peptides with cysteine residues; and c) Peptides with an excessive representation in amino acid residues [10–12]. Within these groups, defensins are the most studied AMPs in invertebrates and show a classic conserved pattern of Cys-1-Cys-4, Cys-2-Cys-5, and Cys-3-Cys-6 cysteine residues, linked together by three disulfide bridges that stabilize the molecule and maintain the tertiary structure [13]. Likewise, the mechanism of action of AMPs is based on the formation of pores in the membrane of the pathogen due to the insertion of peptides by electrostatic interactions, generating an osmotic shock that culminates in cell lysis [14]. In relation, it has been shown that *microplusin* is constitutively expressed in fatty body, ovaries, and eggs, and its mechanism of action is based on the blockage of copper ions necessary for the respiratory metabolism of microorganisms, thus inhibiting the growth of Gram-positive bacteria.

In contrast, the tick immune response mediated by the AMPs in the presence of their pathogens, or those they transmit remains unclear. Therefore, the main objective of this work was to perform a structural analysis of the *R. microplus microplusin*. As well as to evaluate its relative expression during infection with *S. saprophyticus* and *A. marginale* within the pathogen-vector interaction, to propose new molecular and immunological therapeutic targets, resulting in the development of new drugs and/or vaccines or control methods that contribute to integrated pest control.

Materials y Methods

Bioinformatic analyses

In order to identify the *microplusin* mRNA sequence of *R. microplus*, a selective search was performed by BLASTn analyses in three databases including GeneBank, SwissProt, and KEGG. Additionally, for alignment and phylogenetic analysis, we selected the AMPs mRNA sequences of ticks species with the highest similarity percentages (>95%) to *R. microplus microplusin* sequence.

Tridimensional structure analysis of microplusin

The prediction of the 3D structure of the complete sequence of the *microplusin* of *R. microplus* and the cysteine identification was carried out in the SwissModel program and VMD 1.9.3. The stereochemical and energetic validation of the 3D structure was performed using different tools of the ExPASy and Proteomics Tools servers.

Sequence alignment of cysteine residues

In order to compare the ticks *microplusin* sequences with *Drosophila melanogaster* antimicrobial peptides [13], an alignment analysis was performed with the MUSCLE program. The AMPs sequences of *D. melanogaster* have conserved cysteine regions widely studied.

Prediction of the physical-chemical parameters of microplusin

The physical-chemical parameters were determined using an *in silico* analysis from the amino acid sequence of the *microplusin* in the Protparam and ProtScale programs. The parameters evaluated included molecular weight, theoretical isoelectric point, amino acid composition, and hydrophobicity.

Phylogenetic Analysis

The phylogenetic analysis was carried out in the MEGA 7 software using the ticks *microplusin* sequences aligned with the MUSCLE program. The analysis included a sequence of *Streptomyces* spp as the orthologous taxa (outgroup). Finally, the phylogenetic tree was reconstructed by the Maximum Likelihood and Jones-Taylor-Thornton method and a bootstrap value of 500.

Infection of R. microplus ticks with S. saprophyticus and A. marginale

The *R. microplus* strain INIFAP-Media Joya CA was expanded by infesting pathogen-free cattle under controlled conditions, with 1 g of eggs equivalent to 20,000 tick larvae. Twenty-one days post-infestation engorged female ticks were collected and washed with an Antibiotic-Antimycotic mixture (Gibco™ Thermo Fisher Scientific). Cleaned ticks were infected by immersion in SIGMA-ALDRICH® soy trypticasein selective liquid medium containing $1x10^8$ *S. saprophyticus* bacteria. The presence of the bacterium was confirmed by end-point PCR of the 16S rRNA of *S. saprophyticus* and by evaluating the characteristic signs of infection of the yellow beak in ticks. Internal controls were engorged ticks without infection but treated under the same conditions in the presence of a selective medium. For the infection with *A. marginale,* eggs from the ticks obtained in the expansion were used to infest a bovine positive for infection with *A. marginale* in the same way as previously mentioned. Verifying the infection in the hemolymph tick was carried out by amplifying by end-point PCR the *Anaplasma* sp *msp5* gene.

Hemolymph extraction

Hemolymph extraction from all experimental and control groups was performed by subcuticular puncture of engorged females with a scalpel blade, as described by [15]. The extracted hemolymph was centrifuged at 500 x g, resuspended in anticoagulant citrate buffer at 4ºC (0.098 mM NaOH, 0.14M NaCl, 0.017 M EDTA, 0.04 M citric acid pH 4.5), and stored at -20°C.

Specific oligonucleotide design
Specific oligonucleotides were designed in the NCBI PRIMER-BLAST program based on the *R. microplus microplusin* gene sequences obtained:
FMp:5'-TTCGACTTCGGCTCATCACC-3' and RMp: 5'-AACGGACATCGCTTGTTCCA-3'. The specificity of the designed oligonucleotides was performed using a BLASTn analysis in the NCBI, SwissProt, and KEEG databases.

Determination of the presence of the microplusin gene in the R. microplus genome by end-point PCR assays
The presence of the *microplusin* gene sequence in the tick genome was determined by conventional end-point PCR assays under the parameters indicated by the manufacturer (PCR Master mix, Promega®). Previously, the culture and expansion of an embryonic cell line of *R. microplus* was carried out in 50 mL and MEM-L-15 medium supplemented with 20% fetal bovine serum. DNA extraction was performed from confluent embryonic cells using the phenol-chloroform-isoamyl alcohol technique, as indicated in the technical data sheet. The DNA obtained was resuspended in nuclease-free water and stored at -70°C until use. The PCR products obtained were visualized in 1.5% agarose gels, documented in a Photo Doc-It Viresa equipment and analyzed with the OMAX TopView 3.7 software.

Housekeeping gene validation
As a constitutive control, the phospholipid-hydroperoxidase glutathione peroxidase (*phgpx*) gene was used, with the oligonucleotides: Fw-phgpx GTTTGCAGACACCTCAGCGTGCC, and Rv-phgpx GCTAGACTGCACAAGCAATACGGG [16]. The constitutive gene was validated by RT-PCR, with the Super Script ® III One-Step RT-PCR System with Platinum® Taq High Fidelity DNA Polymerase Kit, according to the manufacturer´s specifications.

Determination of relative expression levels of microplusin by RT-PCR
The total RNA extraction from the hemolymph of uninfected ticks (CTR), infected with *S. saprophyticus* and *A. marginale* was carried out using the conventional TRIzol technique, according to the manufacturer's instructions. Finally, the RNA obtained was resuspended in nuclease-free water and stored at -70ºC until use. The integrity of the samples was evaluated by electrophoresis in 2% agarose gels, and for all reactions equimolar concentrations of RNA from the different conditions were used. The relative expression of the *microplusin* gene in ticks infected with different pathogenic bacteria was performed by Super Scritp® III One-Step RT-PCR System Kit according to the technical sheet. The oligonucleotides used for each reaction were those previously described for *microplusin* and *phgpx* genes. The PCR amplicons were visualized on a 2% agarose gel and documented on a Photo Doc-It Viresa equipment. Densitometric analysis of the gel were performed in the ImageJ program to quantify the intensity and determine the relative differential expression for each antimicrobial peptide, normalized with the constitutive *phgpx* gene.

Results

Bioinformatic analyses
The search in the different databases and the *in silico* analyses revealed several *microplusin* sequences for *R. microplus* with high levels of identity (>95%) with sequences of other tick species.

Tridimensional structure analysis of microplusin
The structural analysis of the *microplusin* sequence and the stereochemical and energetic validation of the 3D structure (Z-score, Local Quality Estimate, and Comparison PDB structures) showed values within the parameters established for the quality of the estimated model. Based on the conserved structure of AMPs reported in insects, the structural prediction shows highly conserved regions, different from the classical pattern Cys-1-Cys-4, Cys-2-Cys-5, and Cys-3-Cys-6 (Fig.1) [13].

Sequence alignment of cysteine residues
The alignment showed high similarity in the cysteine location of *R. microplus* and the *R. zambeziensis, R. appendiculatus* and *R. pullcelus* ticks. Interestingly, all ticks sequences have similarity to only two out six cysteines from *D. melanogaster*, which have a

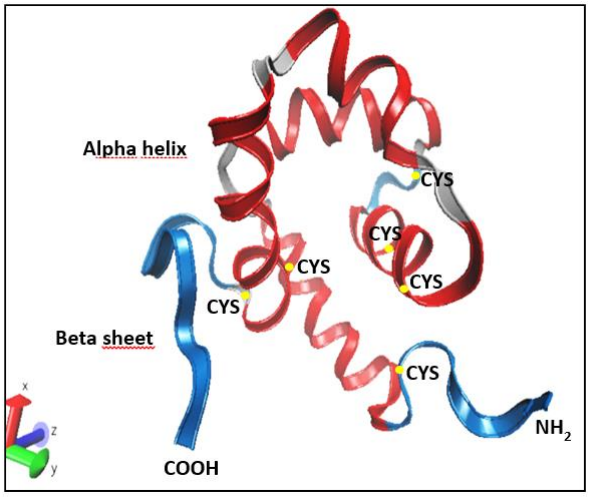

Figure 1. Prediction of molecular structure of *R. microplus microplusin*. The model shows the presence of β-sheet and α-helix, with 6 cysteine residues conserved in the defensin family, showing a differential arrangement. Swiss-Model and VMD programs 1.9.3

spatial arrangement of Cys-1-Cys-4, Cys-2-Cys-5, and Cys-3, Cys-6 characteristic of most insect AMPs (Fig. 2).

Prediction of physical-chemical structural parameters of microplusin

The evaluation of the physical-chemical properties predicted a molecular weight (MW) of 12 kDa; an isoelectric point (IEP) of 5.34, and 110 amino acids (aa). Regarding hydrophobicity, the results predicted that the molecule has an amino acids distribution with a net negative charge while the first amino acids have hydrophobic properties.

Phylogenetic Analysis

The phylogenetic tree revealed that the *microplusin* of *R. microplus* organizes as a monophyletic group that included *R. pulchellus, R. appendiculatus,* and *R. zambeziensis*. Interestingly, sequences from *Ixodes scapulars* and *Ixodes ricinus* groups in a different clade (Fig. 3).

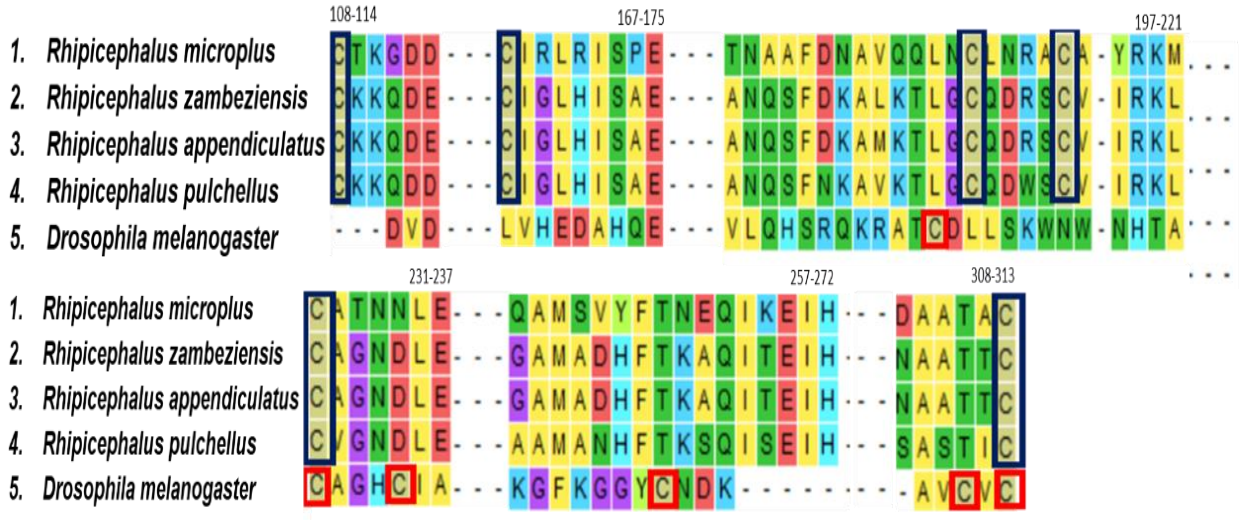

Figure 2. Alignment analysis of the cysteine residues of *microplusin* of several species of ticks and *D. melanogaster*. The sequences of *R. zambeziensis, R. appendiculatus,* and *R. pulchellus* aligned with the *microplusin* of *R. microplus.* The red box indicates the Cys-1-Cys-4, Cys-2-Cys-5, and Cys-3-Cys-6 location present in most insect AMPs. The dark blue box shows the different location of the cysteines.

Determination of the presence of the microplusin gene in the R. microplus genome

The results of the PCR showed an amplicon of approximately 250 bp, in the three different DNA extractions performed (Fig. 4).

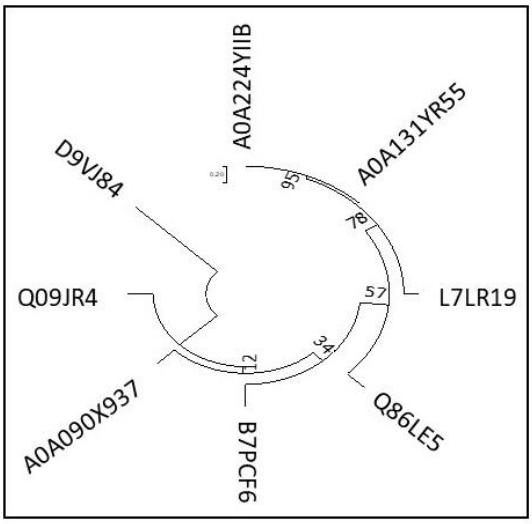

Figure 3. Phylogenetic tree of the *microplusin* sequence of *R. microplus* reconstructed with the Maximum Likelihood analysis and the Jones-Taylor-Thornton evolutionary method. The numbers near the nodes show the replicates of the bootstrap. The accessing number corresponding to A0A224YIIB *R. zambeziensis;* A0A131YR55 *R. appendiculatus;* L7LR19 *R. pulchellus*; Q86LE5 *R. microplus*; B7PCF6 *I. scapularis*; A0A090X937 *I. ricinus*; Q09JR4 *Argas monolakensis*; D9VJ84 *Streptomyces* spp.

Housekeeping gene validation

The relative expression of the *phgpx* gene in the *R. microplus* genome was validated using end-point RT-PCR assays. The results showed the presence of an amplicon of approximately 500 bp (Fig. 5, upper gel). The molecular weight of the amplicons of the *phgpx* gene was the reference for subsequent tests.

Determination of relative expression levels of microplusin by RT-PCR

The expression of *microplusin* in the hemolymph of ticks infected with the pathogenic bacteria *A. marginale* and *S. saprophyticus*, and without infection (control), showed an amplicon of 250 bp, with a relative differential expression in each case (Fig. 5).

It should be noted that this expression increased in the condition where there is no infection, in contrast to ticks infected with *A. marginale*, where a decrease in the relative expression of the messenger was observed (Fig. 5, lane C). Likewise, in the treatments with *S. saprophyticus*, a low relative expression was observed compared to hemolymph without infection (control), however, the expression was higher than that observed in the infections with *A. marginale* (Fig. 5, lane B). The densitometry analyzes of the amplicons were normalized with the constitutive gene *phgpx*. The relative expression in the hemolymph of uninfected ticks (control) was of 102.08% (Fig. 5 panel b).

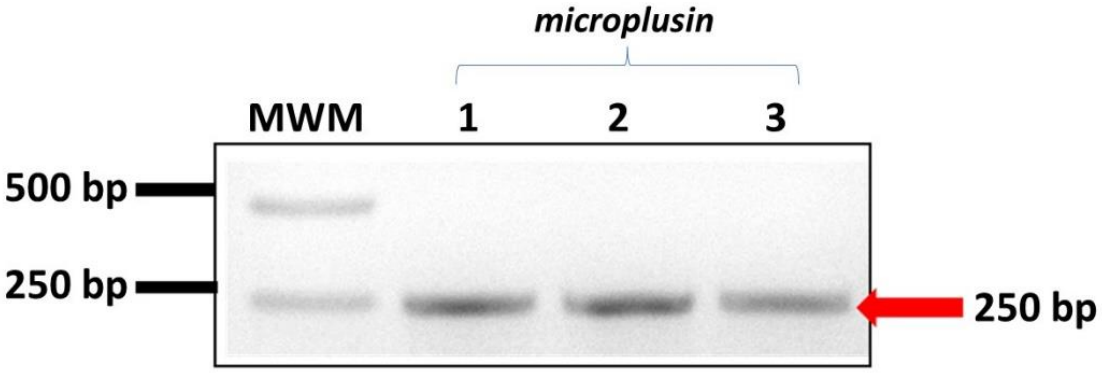

Figure 4. PCR amplification of the *microplusin* gene in the *R. microplus* genome. Three amplicons of approximately 250 bp were observed. Lanes 1, 2 and 3 correspond to genomic DNA extracted from embryonic cells of *R. microplus* from three different samples. MWM: 1 Kb molecular weight marker.

In contrast, in the hemolymph of ticks infected with *S. saprophyticus*, a 1.2-fold decrease was observed. The relative expression percentage remains at 85.475% (Fig. 5, panel b). Finally, in hemolymph from ticks infected with *A. marginale*, a 3.2-fold decrease in relative expression percentage was observed, this is 32.648% lower than the controls (Fig. 5, panel b). Additionally, the plot profile results reveal a marked variation in amplicon intensity in hemolymph from ticks infected with *S. saprophyticus* and *A. marginale (*Fig. 5, panel b).

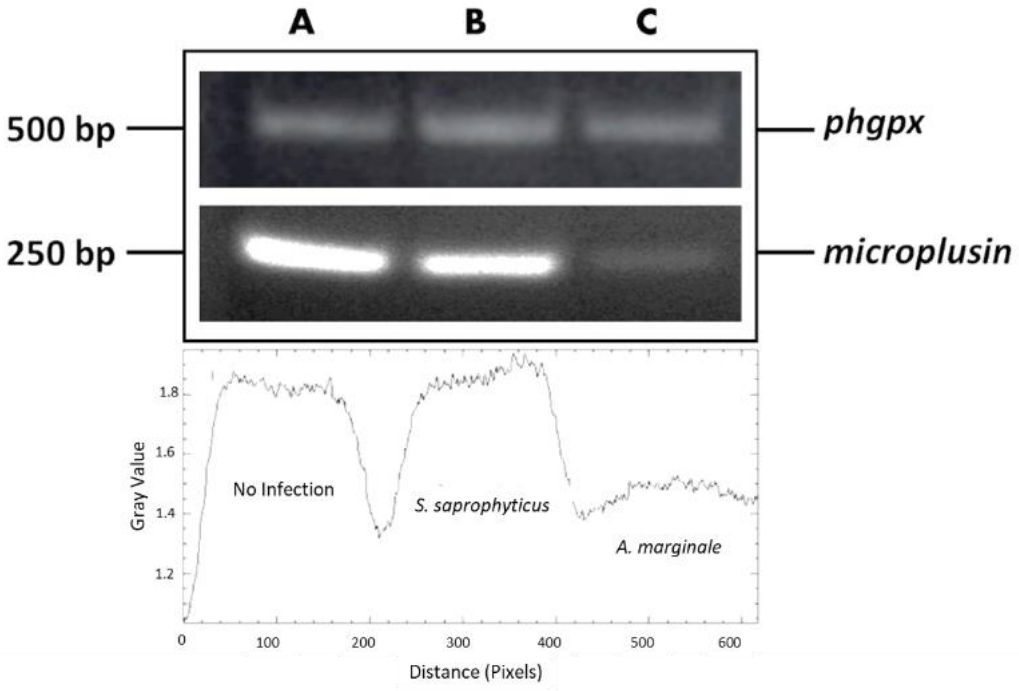

Figure 5. Determination of the relative expression of the *R. microplus microplusin* gene in hemolymph from ticks infected with *S. saprophyticus* and *A. marginale* by RT-PCR assays. Upper gel: lanes A, B and C, expression of the constitutive gene *phgpx* (control). Bottom gel: lane A: relative expression in hemolymph from ticks without infection (control); lane B: relative expression in hemolymph of ticks infected with *S. saprophyticus*; lane C: relative expression in hemolymph of ticks infected with *A. marginale.* All amplicons of *microplusin* have a molecular weight of 215 bp, while the amplicons of 500 bp correspond to the control (*phgpx*). Bottom panel: plot profile showing the intensity variation of the bands corresponding to hemolymph from ticks without infection, hemolymph from ticks infected with *S. saprophyticus*, and hemolymph from ticks infected with *A. marginale* (plot obtained with ImageJ).

Discussion

Bioinformatic analyses are an interesting tool for studying infectious diseases and the pathogens that cause them. Thus, the search for new vaccination targets using these tools has contributed to advances in reverse vaccinology. On the other hand, in recent years, veterinary sciences have shown a trend toward the development of new forms of vector control. In this regard, studying the molecular and immunological mechanisms in the vector-pathogen interaction has gained significant interest in elucidating the associated mechanisms. Therefore, this work focused on the structural and functional characterization of a type of AMPs known as *microplusin*, which is part of the immune response of the cattle tick *R. microplus*.

We observed that *microplusin* had a different arrangement of cysteine residues present in the AMPs of insects that have been widely studied and characterized [13]. Additionally, the predicted physical-chemical (negative charge) characteristics of *microplusin* are not like those of other AMPs (positive charge). Therefore, this negative charge arrangement could suggest a change in the mechanism of disruption of the membrane integrity of microorganisms. On the other hand, the analysis of the relative expression of

microplusin in response to infection by Gram-positive and negative bacteria represents a great advance in the biology of *R. microplus*, suggesting the role of these AMPs in response to infections with differential mechanisms. These infections include a pathogenic bacterium for the tick itself, *S. saprophyticus*, and *A. marginale*, a bacterium that *R. microplus* transmits to cattle.

Interestingly, both bacteria can mediate a differential immune response mediated by AMPs. Our results corroborate the idea of the high plasticity of the arthropod's immune response that, depending on the pathogen, the immune response varies. Finally, our results contribute to advancing in the knowledge and elucidation of vector-pathogen interactions, opening the possibility of searching for new targets based on tick immunosuppression, which contributes to the development of strategies to achieve effective biological control.

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