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Immunogenic potential of *Rhipicephalus (Boophilus) microplus* aquaporin 1 against *Rhipicephalus sanguineus* in domestic dogs

Potencial imunogênico da aquaporina 1 de *Rhipicephalus* (*Boophilus*) *microplus* contra o carrapato *Rhipicephalus sanguineus* em cães domésticos

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Abstract

This study evaluated a recombinant aquaporin 1 protein of *Rhipicephalus (Boophilus) microplus* (*Rm*AQP1) as antigen in a vaccine against *R. sanguineus*. Five dogs were immunized with *Rm*AQP1 (10 µg) + adjuvant (Montanide) (G1), and five were inoculated with adjuvant only (G2), three times. Twenty-one days after the last immunization, animals of both groups were challenged with *R. sanguineus* larvae, nymphs and adults, and their biotic potential was compared. Blood samples were collected before each immunization and every 28 days after the last immunization for 10 weeks. Serum antibody titers (IgG) were assessed by ELISA. We observed that: engorgement period of adult females from G1 was 12% shorter than G2; larvae from G1 had 8.7% longer engorgement period than G2 and weighed 7.2% less; nymphs from G1 had 4.5% shorter engorgement period than G2 and weighed 3.6% less; although the antibody titers increased following the second immunization, they rapidly decreased after the third immunization. Results indicated low immunoprotection of *Rm*AQP1 against adult *R. sanguineus* ticks, and possible efficacy on larvae and nymphs fed on immunized dogs. Further studies should be performed for a full evaluation of the immunoprotection of *Rm*AQP1 against *R. sanguineus* infestations in dogs.

Keywords: *Rhipicephalus sanguineus*, aquaporin, *Rm*AQP1, immunity, domestic dog, ticks.

Resumo

Este estudo avaliou a proteína recombinante (aquaporina) do carrapato *Rhipicephalus (Boophilus) microplus* como antígeno em vacina contra *Rhipicephalus sanguineus*. Cinco cães foram imunizados com *Rm*AQP1 (10 µg) + adjuvante (G1) e cinco foram inoculados apenas com adjuvante (G2), três vezes. 21 dias após a última imunização todos os animais foram desafiados com larvas, ninfas e adultos de *R. sanguineus,* e potencial biótico dos carrapatos foi comparado. Amostras de sangue foram coletadas antes de cada imunização e a cada 28 dias após a última imunização, durante 10 semanas. Títulos de anticorpos dos soros dos cães foram avaliados por ELISA. Resultados: o período de ingurgitamento das fêmeas do G1 foi 12% mais curto que o período de ingurgitamento de G2; o período de ingurgitamento das larvas do G1 8,7% foi mais longo e o peso 7,2% menor que no caso de G2; o período de ingurgitamento das ninfas do G1 4,5% foi mais curto e peso 3,6% menor que no caso do G2; aumento dos títulos de anticorpos do G1 após a segunda imunização e declínio após a terceira imunização. Os resultados indicaram baixo potencial de imunoproteção de *Rm*AQP1 contra *R. sanguineus* adultos, e possível eficácia contra larvas e ninfas, na dose testada. Sugere-se desenvolver novos estudos para melhor avaliação da eficácia de *Rm*AQP1 contra *R. sanguineus* em cães.

Palavras-chave: *Rhipicephalus sanguineus*, aquaporina, *Rm*AQP1, imunidade, cão doméstico, carrapatos.

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Introduction

Ticks are obligate blood-sucking ectoparasites from the phylum Arthropoda, class Arachnida, subclass Acari and order Ixodida (KRANTZ & WALTER, 2009). Three families of ticks have been described so far: Ixodidae (hard ticks), Argasidae (soft ticks) and the most recently identified family Nuttalliellidae (GUGLIELMONE et al., 2010). The genus *Rhipicephalus* belongs to the Ixodidae family and includes about 84 tick species, almost all originating from the Afrotropical region, among which are included the brown dog tick, *R. sanguineus*, and the cattle tick, *R. (B.) microplus* (APANASKEVICH et al., 2013; GUGLIELMONE et al., 2010; HORAK et al., 2013). *R. sanguineus* can be found parasitizing different domestic and wild animals, including humans, despite having the domestic dog as its main host (DANTAS-TORRES et al., 2006; GUGLIELMONE et al., 2003; SZABÓ et al., 2008; RODRIGUEZ-VIVAS et al., 2016).

Bearing in mind that an average 95% of the ticks are on the environment and only 5% on the host, an integrated control strategy for eliminating tick population of both animal and environment is required (DANTAS-TORRES, 2008). Acaricides are widely used to eliminate and prevent reinfestation for a certain period; however, the excessive use of acaricides has contributed to the development of resistant tick populations as well as environmental and animal products contamination (KUNZ & KEMP, 1994; DE LA FUENTE et al., 2015). A sustainable option for tick control is the combination of chemicals with vaccines (GHOSH et al., 2007; GUERRERO et al., 2012; SPRONG et al., 2014). Thus, as part of a *R. (B.) microplus* genome study aiming the discovery of transcripts that produce antigens for an effective vaccine against the cattle tick, studies focused on the genome (GUERRERO et al., 2010), transcriptome (GUERRERO et al., 2005) and proteome (RACHINSKY et al., 2007, 2008) of *R. (B.) microplus* identified genes and gene coding regions that encode proteins with essential functions in ticks (BELLGARD et al., 2012). One of these gene coding regions was found to encode proteins whose amino acids were significantly similar to aquaporins.

Aquaporins, also called "water channels", allow the regulation of water transport through the highly hydrophobic lipid bilayer of cell membranes (GUERRERO et al., 2014). Members of the aquaporin family are observed from mammal individuals (ROJEK et al., 2008) to bacteria (FU et al., 2000), being common in certain types of cells such as erythrocytes (DENKER et al., 1988). Two constrictions on the aquaporin structure act as filters, and the selectivity to water, glycerol, urea, and other small molecules is determined by the size and charge of the constricting pore (BEITZ et al., 2006; GUERRERO et al., 2014).

Due to the fact that cattle ticks ingest large volumes of blood in relation to their size and weight, they are obliged to concentrate blood components and have effective water transport mechanisms for their digestion (MEGAW, 1974). Thus, because the ticks' aquaporins are fundamental in their physiology, they seem to be good candidates for antigens in a vaccine against ticks (GUERRERO et al., 2014).

In this context, a cDNA that encodes an aquaporin of *R.* (*B.*) *microplus* was expressed as a recombinant protein in *Pichia pastoris* and the amino acid sequence of the cloned aquaporin protein fragment is representend on Figure 1. The recombinant protein, named *Rm*AQP1, was tested as an anti-cattle tick vaccine in cattle and provided 76% and 73% efficiency in two experiments, being considered a promising antigen in vaccines against infestation with *R. (B.) microplus* in cattle (GUERRERO et al., 2014).

Considering that the brown dog tick and the cattle tick belong to the same genus (*Rhipicephalus*) as shown by molecular phylogeny studies (BEATI & KEIRANS, 2001; MURRELL & BARKER, 2003; BARROS-BATTESTI et al., 2006), the present study investigated the immunogenic potential of the recombinant aquaporin protein of *R. (B.) microplus*, *Rm*AQP1, against different instars of *R. sanguineus* (aquaporin sequences unkown until now) infesting domestic dogs.

Materials and Methods

Ticks

Rhipicephalus sanguineus ticks were obtained from colonies maintained at the Immunopathology Laboratory of the Department of Veterinary Pathology, School of Agricultural and Veterinary Sciences -FCAV, UNESP, Campus of Jaboticabal.

For colony maintenance, the various instars of *R. sanguineus* (larvae, nymphs and adults) were fed on rabbits that were provided by the Central Animal Facility of UNESP, Botucatu. The rabbits received food and water "ad libitum" and were kept in individual cages. The ticks were released on the hosts inside specially designed plastic chambers, affixed with synthetic glue (Brascoplast®) to the rabbits' shaved dorsum. The rabbits had no prior contact with ticks.

To facilitate the metamorphosis of engorged instars as well as their maintenance, once detached from the host, the ticks were placed in clear plastic tubes with a perforated lid, to allow adequate aeration, and placed in an incubator (CD347 model, FANEM) at 27°C, 80% humidity and 12 hours photoperiod.

Hosts and experimental groups

Male and female mongrel dogs (n = 10), 1 to 2 years of age, were used as hosts in the infestation challenge with *R. sanguineus* larvae, nymphs and adults. The dogs were kept in individual boxes at the Experimental Kennel of the Department of Veterinary Pathology, FCAV-UNESP, where there is strict control of parasites, including ticks. The animals were vaccinated against parvovirus,

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNN
GLLFINTTIASIAAKEEGVSLEKREAEAEFMKIENLLIRQLINEFLGTMILITIGDSIMAIIIAG
DNESLAACVGPLGWGVAIYVAVQISGGVSSHLNPAVTLAQASVRKFPIAKVPLYFAAQYLG
GFVGAALVFATYKDAIEHFDQGIRQVTGEKATAGIFATYPRPHVSTLTCFIDQVIATGIMM
VCVEAIGDTRNFGGIPPHIHPICLGLMIMAIIFSFAYNCMCPAAASFLEQKLISEEDLNSAVD
HHHHHH*

Figure 1. Amino acid sequence of the cloned aquaporin protein fragment produced by expression of *Pichia pastoris*. Amino acids of *R. (B.) microplus* protein are underlined. Additional amino acids not originating from the tick are not underlined. Image adapted from Guerrero & Pérez-de-Leon (2014).

canine distemper, leptospirosis, hepatitis, parainfluenza and rabies, as well as dewormed and received appropriate food and water "ad libitum".

The dogs were distributed into two groups with five animals each: G1-immunized with *Rm*AQP1 (10 µg/mL) plus adjuvant (Montanide ISA61VG, SEPPIC, Paris) and G2-control, inoculated only with the adjuvant. All experimental procedures were performed in accordance with the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Experimentation, and approved by the Ethics Committee on Animal Use of FCAV-UNESP, campus of Jaboticabal (Protocol 010221/13, 04/06/2013).

Anti-tick vaccine (R. (B.) microplus recombinant aquaporin)

The *Rm*AQP1 development is detailed elsewhere (GUERRERO et al., 2014). The vaccine was kindly provided by Dr. Felix D. Guerrero and Dr. Adalberto Pérez de León (USDA-ARS Knipling-Bushland US Livestock Insects Research Laboratory, Kerrville, Texas, USA) as part of a scientific cooperation agreement signed by UNESP and USDA-ARS.

Blood collection

Blood samples (3 mL) were collected from all 10 animals using vials with no anticoagulant immediately before each vaccination (days 0, 21, 42) and every 28 days after the last immunization during 10 weeks (days 70, 98, 126, 154). Subsequently, the blood samples were centrifuged at 3,400 rpm for 15 minutes at 25°C and the serum was collected and stored at -20°C until further analysis by ELISA.

Immunization and challenge infestation

The experiments were developed at the Department of Veterinary Pathology, School of Agricultural and Veterinary Sciences -FCAV, UNESP, Campus of Jaboticabal, São Paulo, Brazil.

Following the methods described by Andreotti (2006), G1 animals were immunized intramuscularly with 1 mL of a solution containing 10 µg of recombinant protein (*Rm*AQP1) plus adjuvant (Montanide ISA61VG, Seppic, Paris, France) at the beginning of weeks 1, 4 and 7. The animals from G2 received, by the same route and at the same days, an equal volume of adjuvant.

Twenty-one days after the last immunization, each animal from G1 and G2 was challenged with 20 *R. sanguineus* adult tick couples, 100 *R. sanguineus* larvae and 100 *R. sanguineus* nymphs from the Jaboticabal strain. Each tick instar was released in a separate feeding chamber (BECHARA et al., 1995) that was affixed with glue (Brascoplast®) to the dogs' shaved dorsum.

The feeding chambers were opened daily. Engorged females were individually weighed and placed separately in plastic containers in an incubator at 27°C, 80% humidity and 12 hours photoperiod, until complete oviposition. Detached larvae and nymphs were individually counted but weighed and stored into plastic vials in daily batches. Feeding period was assumed as the time elapsed from tick liberation on the host until its detachment, partially or fully engorged; pre-oviposition was the number of days from detachment to the beginning of oviposition; each egg mass was weighed and incubated separately in the BOD under the same conditions previously described; the larval hatching rate for each female offspring was the mean value of visual evaluation performed by three different persons, according to Szabó & Bechara (1995); finally, molting rates were the proportion of recovered engorged ticks that molted.

Biological parameters of the ticks under laboratory conditions

The effect of the aquaporin antigen on the biotic potential of the different stages of *R. sanguineus* was assessed by the differences between immunized and control groups regarding: recovery rates of engorged females (% RecA), larvae (% RecL) and nymphs (% RecN); weights of engorged female (EFW), larvae (ELW) and nymphs (ENW); egg mass weight (EMW); engorgement periods of females (FEP), larvae (LEP) and nymphs (NEP); female pre-oviposition period (POP); larval hatchability (% LH), larvae to nymph molting rate (% MoltN) and nymph to adult molting rate (% MoltA). EMW was determined 15 days after tick detachment as there was no significant increase in this parameter after this period (BECHARA et al., 1994).

Indirect Enzyme-Linked Immunosorbent Assay - ELISA

Briefly, microtiter plates were coated with antigen (100 µL/well of a solution of 10 µg antigen/mL in coating buffer) and incubated overnight in a humid chamber at 4°C. The wells were washed three times with PBS + 0.05% Tween-20 (PBST) and blocked with 5% skimmed milk in PBST and incubated for 2 hours at 37°C in a humidified chamber. After further washing with PBST, 100 µL of the serum to be tested was added per well, diluted 1:50, following serial dilutions 1:2 from A to H, with subsequent incubation of 1 hour and 30 minutes at 37°C in a humidified chamber. Further washing was performed, as previously described, and 100 µL of anti-dog IgG conjugated to alkaline phosphatase was added to each well diluted 1:10,000. After 1 hour and 30 minutes incubation followed by washing, 100 µL of substrate for alkaline phosphatase, P-nitrophenylphosphate, diluted in diethanolamine buffer (1 mg/mL) was added to each well. After 30 minutes incubation at room temperature, the optical density measurements were performed on a MRX ELISA High Performance reader™ (DYNEX Technologies, Chantilly, VA, USA) at 405 nm. Optimal dogs' serum dilution was determined after preliminary testing with 1:50, 1:100, 1:200 and 1:400 dilutions.

Results

Biological parameters of adult R. sanguineus ticks after challenge infestation

Biological parameters of engorged females obtained after challenge infestation are shown in Table 1. Only the FEP was statistically different, with the immunized group having 12% shorter engorgement period than the control group.

Biological parameters of R. sanguineus larvae after challenge infestation

Biological parameters of engorged larvae obtained after challenge infestation are shown in Table 2.

Larvae were statistically different in the immunized and control groups regarding their engorgement periods and engorged weights. Although the larvae of the immunized group presented 8.7% longer engorgement period than the control group, they weighed 7.2% less.

Biological parameters of R. sanguineus nymphs after challenge infestation

Biological parameters of engorged nymphs obtained after challenge infestation are shown in Table 3.

The nymphs were statistically different in the immunized and control groups regarding their engorgement periods and engorged weights. Nymphs of the immunized group had 4.5% shorter engorgement period and weighed 3.6% less than the control group.

Table 1. Biological parameters of engorged females after challenge infestation.

PARAMETERS	CONTROL	IMMUNIZED
% RecA	52.8 ± 6.86 [a]	49.00 ± 16.11 [a]
FEP (days)	9.11 ± 0.14 [a]	7.98 ± 0.15 [b]
EFW (mg)	145.40 ± 4.05 [a]	146.40 ± 4.07 [a]
POP (days)	5.57 ± 0.26 [a]	5.89 ± 0.23 [a]
EMW (mg)	65.08 ± 4.58 [a]	74.85 ± 3.96 [a]
% LH	87.07 ± 2.69 [a]	87.47 ± 1.68 [a]

% RecA, engorged females recovery rate; FEP, female engorgement period; EFW, engorged female weight; POP, pre-oviposition period; EMW, egg mass weight; % LH, larval hatchability. Means in the same line followed by the same letter do not differ statistically by Mann-Whitney test ($p<0.05$).

Table 2. Biological parameters of engorged larvae after challenge infestation.

PARAMETERS	CONTROL	IMMUNIZED
% RecL	40.40 ± 4.34 [a]	30.75 ± 7.28 [a]
LEP (days)	5.01 ± 0.006 [a]	5.45 ± 0.10 [b]
ELW (mg)	0.2512 ± 0.0028 [a]	0.2234 ± 0.004 [b]
% MoltN	91.76 ± 5.03 [a]	91.27 ± 3.17 [a]

% RecL, larvae recovery rate; LEP, larvae engorgement period; ELW, engorged larvae weight; % MoltN, larvae to nymph molting rate. Means in the same line followed by the same letter do not differ statistically by Mann-Whitney test ($p<0.05$).

Table 3. Biological parameters of nymphs after challenge infestation.

PARAMETERS	CONTROL	IMMUNIZED
% RecN	26.80 ± 5.38 [a]	27.75 ± 9.94 [a]
NEP (days)	6.90 ± 0.10 [a]	6.59 ± 0.08 [b]
ENW (mg)	3.86 ± 0.03 [a]	3.72 ± 0.04 [b]
% MoltA	15.60 ± 3.69 [a]	33.53 ± 8.64 [a]

% RecN, nymphs recovery rate; NEP, nymphs engorgement period; ENW, engorged nymphs weight; % MoltA, nymph to adult molting rate. Means in the same line followed by the same letter do not differ statistically by Mann-Whitney test ($p<0.05$).

Indirect Enzyme-Linked Immunosorbent Assay - ELISA

The ELISA showed that the animals of G1 had an increase in anti-*Rm*AQP1 antibody titers post first and second immunization, but these titers began to decrease after the third immunization (Figure 2).

Individual analysis of each animal showed that two dogs of G1 presented longer antibody titers post-second immunization (Figure 3); however, the titers began to decline soon after the third immunization and, like the other animals, decreased until the last analyzed time.

Discussion

Advances in the biology of ticks may favor vaccines development due to the opening of new opportunities to identify candidates for antigens (GHOSH et al., 2007). The development of anti-tick

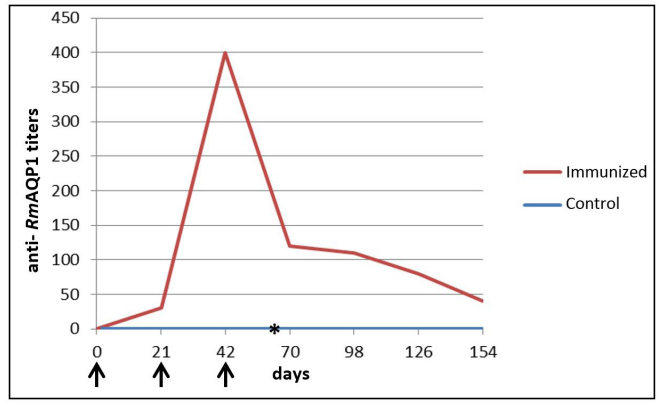

Figure 2. Anti- *Rm*AQP1 antibody titers present in domestic dogs' serum in different post-immunization times measured by ELISA. Results represent the last serum dilution with a mean optical density greater than three times the mean of the negative control group. ↑ – Represents the days that dogs from G1 were immunized with *Rm*AQP1. *- Represents day 63, date of the challenge infestation with *Rhipicephalus sanguineus*.

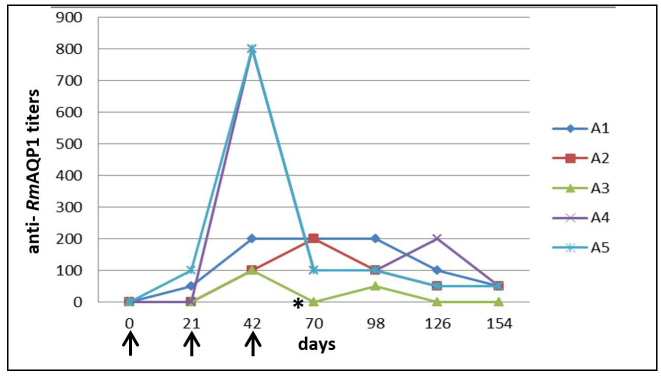

Figure 3. Individual analysis of anti- *Rm*AQP1 antibody titers of dogs immunized with recombinant aquaporin protein at different times after immunization by indirect ELISA. Results represent the last serum dilution with a mean optical density greater than three times the mean of the negative control group. ↑ – Represents the days that dogs from G1 were immunized with *Rm*AQP1. *- Represents day 63, date of the challenge infestation with *Rhipicephalus sanguineus*.

vaccine is one of the most promising alternatives to chemical control of ectoparasites with the advantage of being target specific, of easy management and low cost. Moreover, it does not jeopardize human health nor offer environmental risk (WIKEL, 1996). Finally, with tick infestation reduction, a vaccine would consequently help to lower pathogens transmission by ectoparasites (DE LA FUENTE et al., 2015).

Until now, the only commercially available anti-tick vaccine, a Bm86 recombinant protein named Gavac, is triggered against-*Rhipicephalus (B.) microplus* on bovines (DE LA FUENTE et al., 2007). However, sequence variations in the Bm86 locus in ticks, among other factors such as cattle genetic variations, could affect the effectiveness of Bm86-containing vaccines (GARCIA-GARCIA et al., 1999). Consequently, the development of integrated tick control strategies, including vaccines and acaricides, is important and could lead to the sustainable control of ticks and tick borne diseases (DE LA FUENTE et al., 2015).

A more recent study described the ATAQ, a homologous Bm86 protein with primary and secondary structures similarities (AGUIRRE et al., 2016), as a promising antigen in the development of an anti-tick vaccine. Other types of immunogens that may be effective against ticks are the synthetic peptides, the 64P cementum protein, subolesin/akirin, ferritin 2, P0 protein, SILK antigen and aquaporins (PATARROYO et al., 2002; TRIMNELL et al., 2005; ALMAZÁN et al., 2003; DE LA FUENTE et al., 2011; HAJDUSEK et al., 2010; RODRÍGUEZ-MALLON et al., 2012; MERINO et al., 2013; BELLGARD et al., 2012; GUERRERO et al., 2014).

Accordingly, the present study evaluated a recombinant aquaporin 1 protein of *R. (B.) microplus* as an antigen in a vaccine against the tick *R. sanguineus,* in domestic dogs. The evaluation included: 1) comparisons between the biotic potential of ticks fed on immunized and control animals and; 2) determination of immunized dogs' serum antibody titers (IgG) by ELISA test.

Among the biological parameters analyzed in this study, only the engorgement period of adult female ticks was statistically different between immunized and control groups, with the immunized group presenting 12% shorter FEP than the control group. Thus, the results suggest low protective potential of the *Rm*AQP1 antigen against adult *R. sanguineus*, in the dose used. Different results were observed by Guerrero et al. (2014) in a study conducted in Campo Grande, MS, Brazil, using the *Rm*AQP1 antigen against *R. (B.) microplus* on cattle. In that study, authors observed a marked decrease in ticks recovery rate from immunized animals, which was equivalent to only 29% of what was recovered in the control group. The results showed efficacy of 76% and 73% of the vaccine in two trials regardless of the effects on production and hatchability of eggs being insignificant (GUERRERO et al., 2014). The differences between the two studies with the *Rm*AQP1 can be due to numerous factors, including: a) in the present study, *Rm*AQP1 was used against *R. sanguineus* in dogs while in the study conducted by Guerrero et al. (2014) this protein was used in cattle against *R. (B.) microplus*; and b) the protein dose used in this study may have been insufficient to induce immunity in dogs.

The engorgement period and engorged weight of larvae and nymphs of G1 and G2 was significantly different. Although larvae of the immunized group presented an 8.7% longer engorgement period than the control group, they weighed 7.2% less. Moreover, the nymphs of the immunized group showed 4.5% shorter engorgement period and weighed 3.6% less than the control group. These results suggest a possible effect, though discreet, of the *Rm*AQP1 on larvae and nymphs of *R. sanguineus* in the dose tested.

To perform the ELISA, the optimum concentration of sera dilution was established by testing sera at 1:50, 1:100, 1:200 and 1:400, with the 1:50 dilution being chosen. The concentration of the tested antigen (10 µg *Rm*AQP1/mL), in turn, was determined by its suppliers.

In the immunized group, the analysis of the antibody titers mean, represented by the last serum dilution that presented a mean optical density three times greater than the mean presented by the negative control group, revealed that although the animals showed an increase in antibody titers post-second immunization, these titers quickly declined after the third immunization. Moreover, individual analysis of the immunized dogs showed that only two of the five animals vaccinated with *Rm*AQP1 showed considerable increase in antibody titers post-second immunization, which decreased after the third immunization. These results point to a low immunogenicity of the antigen in the dose used. In contrast, different results were observed in the ELISA test from the study conducted by Guerrero et al. (2014) where the cattle immunized with *Rm*AQP1 showed greater increase in antibody titers, and these remained high post third immunization.

Thus, the low immunogenicity of the *Rm*AQP1 in the present study could possibly be related to the fact that here an aquaporin protein of *R. (B.) microplus* was used against *R. sanguineus* while in the work of Guerrero et al. (2014) this protein was used against *R. (B.) microplus* itself. Furthermore, in the study conducted by Guerrero et al. (2014) it was administered to each animal 2 mL containing 100 µg of the antigen + Montanide, however, to adjust the antigen dose for a dog´s size, in the present study it was administered to each animal 1 mL containing 10 µg of *Rm*AQP1, which may have been a low dose to induce effective combat against adult ticks. Nonetheless, significant results were observed in larvae and nymphs, indicating possible effectiveness of the antigen against these instars.

Finally, we suggest that further studies should be developed using a higher dose of *Rm*AQP1 for a better evaluation of its immunogenic potential against *R. sanguineus* in dogs.

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