


Immunization schedules using Bm86 protein on reproductive parameters of *Rhipicephalus sanguineus* fed on a rabbit model



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Abstract:

Vaccination with the Bm86 antigen is a scarcely control alternative for *Rhipicephalus sanguineus*; immunization with this antigen could be a helpful tool to prevent infestations without generating resistance to ixodicides. This study aimed to compare the effects of two immunization schedules with the Bm86 protein on the biological parameters of *R. sanguineus*. Three groups of eight rabbits were formed: group one was inoculated with three doses of Bm86 antigen, group two received two doses, and group three (control group) was inoculated with physiological saline solution (PSS). The parameters and biological cycle of the ticks fed on the animals of each group were compared. The results showed that experimental groups one and two exhibited significant decreases ($P \leq 0.05$) in the parameters

of larval weight, adult tick weight, egg mass weight, and hatching rate compared to the control group. Likewise, significant increases in the lengths of preoviposition, oviposition, incubation, hatching, larva-nymph molting, and feeding periods of adult ticks were detected in the experimental groups.

Keywords: Ticks, Reproductive parameters, *In vivo* infestation, Biological cycle.

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Introduction

In Mexico, *Rhipicephalus sanguineus sensu lato* is the primary transmitter of diseases, such as human monocytic ehrlichiosis and Rocky Mountain spotted fever, making this tick the second most important vector of disease in this country^(1,2,3). This tick has a cosmopolitan distribution⁽⁴⁾ and a life cycle with an average length of 60 to 123 d^(5,6).

Chemical ixodicides are the most common option for tick control⁽⁷⁾. However, the biggest problem associated with their use is the generation of resistance in ticks^(8,9). It is for this reason that the constant search for new control methods, both chemical and alternative, is essential.

Immunological control methods base their operation on the induction of an immune response in tick hosts against tick-specific antigens⁽¹⁰⁾, which can reduce the reproductive efficiency of ticks, decreasing the size of the tick population in the environment and therefore the parasite load of the hosts⁽¹¹⁾.

The Bm86 protein is a key antigen in the development of immune control methods against ticks. Bm86 is a glycoprotein with a molecular weight of 89 kDa, consisting of approximately 650 amino acids (aa)⁽¹²⁻¹⁵⁾.

The vaccine efficiency of immunogens against the Bm86 protein ranges from 35 to 89 %^(16,17), depending on factors such as host and tick species, as well as the immunization protocols used. In most of these studies, vaccine efficiency is determined by the decrease in parasite load^(18,19,20). The objective of this study was to compare the effect of two immunization schemes with the Bm86 protein on the biological parameters of *R. sanguineus*.

Material and methods

The study was conducted at the “La Posta-INIFAP” Research Station located at kilometer 22.5 of the Veracruz-Córdoba highway in the municipality of Medellín, Veracruz, Mexico. To carry out this work, approval 01/21 from the Bioethics Committee of the Faculty of Veterinary Medicine and Animal Sciences of the Universidad Veracruzana, which verified that this research was conducted in accordance with the provisions of NOM-033-ZOO-1995, subsection 6.1.b⁽²¹⁾.

Handling and feeding of experimental animals

A total of 24 six-month-old rabbits (*Oryctolagus cuniculus*) of the New Zealand breed, irrespective of sex, were used as hosts. The disposal of the animals at the conclusion of this research was carried out in accordance with the provisions of NOM-033-ZOO-1995⁽²¹⁾.

Vaccination with the Bm86 antigen

Three groups of eight animals each were established and assigned to different treatments with a commercial vaccine based on the Bm86 protein, designed to induce immunity in cattle against the tick *Rhipicephalus microplus* in Mexico. Experimental group 1 received three 1 mL doses of the commercial anti-Bm86 vaccine through subcutaneous injection in the left scapular region at 7-d intervals between each dose; experimental group 2 received two 2 mL doses of an anti-Bm86 vaccine through subcutaneous injection in the left scapular region at 14-d intervals between each dose. The control group was administered a single dose of 2 mL of physiological saline solution (PSS) through subcutaneous injection in the left scapular region on day 0 of the experiment.

Artificial infestation

Artificial infestations were carried out in rabbits every 30 d, always in the morning, using controlled feeding chambers 60 mm in diameter and 40 mm in height, following the methodology proposed by Barradas *et al*⁽²²⁾. The ticks used in the infestation process came from an experimental colony of *R. sanguineus*. To establish this colony, adult females of *R. sanguineus* with a minimum length of 8 mm were selected; these were obtained from veterinary clinics in the Veracruz-Boca del Río metropolitan area from naturally infested animals that had no history of previous treatments for the control of ticks in order not to modify the reproductive parameters of the ticks due to factors external to the experiment. Each animal was infested with 500-800 14-d-old larvae; after their feeding period, the surviving larvae were retrieved, quantified, and grouped into batches of 100 larvae in Eppendorf vials with cotton plugs. These batches were housed in an incubator under

controlled conditions of temperature ($28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) and humidity ($80 \pm 10\%$)⁽²³⁾. The following stages of the life cycle were housed with the same humidity and temperature parameters.

Before their feeding period, the nymphs were kept for 14 d. They were later used to re-infest rabbits, with approximately 200 nymphs per animal. The fed nymphs were collected and grouped into batches of 50 individuals. Subsequently, the number of days of the molting process to their adult stage was recorded.

Adult stage

Adult ticks were kept for 14 d. The rabbits were then infested with 10 male and 40 female ticks. Adult females that completed their feeding process were collected daily and housed individually in Eppendorf vials with cotton plugs.

Measured parameters

The live weight of the larvae was considered as the weight of each batch of 100 larvae that had completed their feeding process. The measurement was performed on the same day that each batch was collected.

The live weight of the nymphs was considered as the weight of each batch of 50 nymphs that had finished their feeding process. The measurement was performed on the same day that each batch was collected.

Adult live weight was defined as the individual weight of each adult female after her feeding period. This measurement was performed on the same day that each female was collected.

The weight of the egg mass was measured on the day each female completed her oviposition process.

The percentage of oviposited weight was calculated as the proportion of the adult female's weight equivalent to the weight of the egg mass of each collected adult female.

The weight-loss rate was calculated as the percentage of weight lost by each female from her date of collection to the end of her oviposition process.

The number of eggs was determined by counting the total number of eggs produced by each tick using a stereoscopic microscope.

The viable larvae per tick were determined by counting the larvae that hatched from each egg mass using a stereoscopic microscope.

The hatching rate was calculated as the quotient of the number of larvae produced by each tick divided by the number of eggs produced by each tick, multiplied by one hundred.

The preoviposition period was considered as the number of days elapsed from the collection of adult females to the beginning of the oviposition period.

The oviposition period was considered as the number of days elapsed from the beginning of laying to the day on which the absence of oviposition in comparison to the previous day's review was confirmed.

The incubation period was considered as the number of days elapsed from the start of oviposition to the observation of the first live, hatched larva.

The hatching period was considered as the number of days elapsed from the first live, hatched larva to the hatching of all eggs or the absence of viable unhatched eggs.

The larval feeding period was considered as the number of days elapsed between the date of infestation of a batch of larvae and the date on which they completed their engorgement process and detached from their host.

The larva-nymph molting period was determined as the number of days elapsed from the end of the larval feeding period to the day there was at least one larva that had successfully completed its ecdysis process to become a live nymph.

The nymphal feeding period was determined as the number of days elapsed from the date of infestation of each batch of nymphs to the time when the nymphs completed their engorgement process and detached from the host.

The nymph-adult molting period was determined as the period from the date of collection of each batch of nymphs to the date there was at least one tick that had completed its molt from nymphal stage to adult stage.

The feeding period of adult ticks was considered as the number of days from infestation to the time the tick spontaneously detached from the host after completing its feeding.

Statistical analysis

Data from the three groups were compared using a one-way ANOVA, and Tukey's test was employed to assess these differences. All analyses were performed using the Minitab 17 ® statistical program.

Results and discussion

Larval weight

The larval weight was 40.50 ± 14.53 mg for ticks fed on group one, 33.31 ± 9.22 mg (Table 1) for group two, and 36.30 ± 11.85 mg for group three ($P=0.000$); these differences suggest that ticks fed on rabbits subjected to two doses of Bm86 vaccine reached a lower post-feeding weight than ticks fed on group one and the control.

Table 1: Comparison of biological parameters in ticks fed on rabbits immunized against the Bm86 protein

Parameter	n	G1	G2	G3
Live weight of larvae, mg	351	40.50 ± 14.53^a	33.31 ± 9.22^b	36.30 ± 11.85^a
Live weight of nymphs, mg	67	99.80 ± 69.40^a	81.10 ± 70.00^a	98.30 ± 85.70^a
Live weight of adults, mg	305	119.79 ± 37.06^b	131.99 ± 46.54^a	144.70 ± 51.97^a
Egg mass weight, mg	305	77.91 ± 26.20^b	80.46 ± 31.47^b	95.03 ± 40.07^a
Oviposited weight, %	305	66.72 ± 15.8^a	61.72 ± 14.79^a	66.30 ± 17.83^a
Weight loss rate, %	305	75.51 ± 6.74^a	73.65 ± 7.02^a	74.29 ± 5.50^a
Number of eggs, n	234	$1,419 \pm 326.05^a$	$1,165 \pm 868.65^a$	$1,299 \pm 661.77^a$
Larvae /tick, n	234	940 ± 202^a	908 ± 702.13^a	916 ± 611.80^a
Hatching rate, %	234	71.4 ± 20.78^b	64.23 ± 25.57^b	73.97 ± 30.99^a

Data are presented as the mean \pm standard deviation.

G1= immunized with three doses; G2= immunized with two doses; G3= control group (not immunized).

^{ab} Different letters indicate significant differences ($P \leq 0.05$).

Nymphs weight

The weight of the nymphs after their feeding period was 99.80 ± 69.40 mg in group one, 81.10 ± 70.00 mg in group two, and 98.3 ± 85.70 mg in group three (Table 1) ($P=0.689$). This suggests that this parameter is not affected by the immunization against Bm86 present in the blood of which the ticks fed; even so, it can be seen that group two was where ticks obtained a lower average weight compared to the other groups. The absence of statistically significant differences could also be due to the relatively small number used to compare this parameter ($n= 67$).

Adults weight

The mean weight of adult female ticks for group one, group two, and the control group was 119.79 ± 37.06 mg, 131.99 ± 46.54 mg, and 144.70 ± 51.97 mg, respectively (Table 1) ($P=0.000$). In a study carried out in 2010⁽¹⁶⁾, Pérez *et al.* reported similar results, with the average weight of adult ticks fed on immunized dogs being 115 mg, slightly lower than that

in the non-immunized group, which was 126 mg. Accordingly, Bechara *et al*⁽²⁴⁾ identified a decrease in the weight of *R. sanguineus* females fed on hamsters and guinea pigs immunized with the Bm86 protein compared to ticks fed on non-immunized animals. The results indicate that the ticks fed on group one had a lower weight compared to those in groups two and the control group; this can be explained by immunization against Bm86 affecting the intestinal epithelium of ticks, compromising their ability to obtain nutrients from the blood on which they fed, which would be reflected in a significant weight reduction compared to the control group⁽¹⁶⁾.

Egg mass weight

The average weight of the egg mass was 77.91 ± 26.20 mg in group one, 80.46 ± 31.47 mg in group two, and 95.03 ± 40.07 mg in group three (Table 1) ($P=0.00$). Pérez *et al*⁽¹⁶⁾ obtained a similar mean: 71 mg in the control group, whereas in the immunized group, it was 55 mg. Likewise, Rodríguez *et al*⁽²⁵⁾ reported that the egg mass weight in the control group was 64.93 mg, and in the immunized group, was 62.06 mg, the latter being slightly lower. The results show that ticks belonging to groups one and two (fed on vaccinated hosts) were affected in terms of the size of the egg masses they were able to oviposit; this could be explained by the presence of the Bm86 protein in cells of the ovarian epithelium of ticks. Alternatively, this decrease in egg mass weight could not be a direct effect of anti-Bm86 immunization, but a side effect of the reduction in body weight of engorged adult female ticks, since their ability to oviposit is related to their body weight.

Percentage of oviposited weight

The percentage of oviposited weight was 66.72 ± 15.86 % in group one, 61.72 ± 14.79 % in group two, and 66.30 ± 17.83 % in group three (Table 1) ($P=0.095$). These results contrast with those reported by Pérez *et al*⁽¹⁶⁾, who detected a significant decrease in this parameter from 55 to 46 %. Since the differences detected between the three groups are not statistically significant, it can be assumed that the immunoglobulins present in the blood of vaccinated hosts are not effective in reducing the percentage of oviposited weight; this suggests that the decrease in the reproductive capacities of *R. sanguineus* associated with immunization with Bm86 protein is not an effect associated with the decline in body weight of females.

Weight loss rate

The mean weight loss rate was 75.5 ± 6.74 % in group one, 73.65 ± 7.02 % in group two, and 74.29 ± 5.50 % in group three (Table 1) ($P=0.115$). The differences among the three groups were not significant for this parameter; this shows that anti-Bm86 immunization is not able to modify the weight loss of *R. sanguineus* females.

Number of eggs

The mean number of eggs oviposited per tick was $1,452.41 \pm 326.05$ eggs in group one, $1,318.57 \pm 868.65$ eggs in group two, and $1,411.83 \pm 661.77$ eggs in group three (Table 1) ($P=0.413$). Although differences can be identified, the lack of statistical significance indicates that this parameter is similar in the three groups. Therefore, the results suggest that, under the experimental conditions of the present study, anti-Bm86 immunization does not significantly affect the number of eggs produced by *R. sanguineus* females.

Ticks in group one had a mean of 894.80 ± 292.65 live larvae (Table 1), those in group two had 948.69 ± 702.13 live larvae, and those in group three had $1,053.55 \pm 611.80$ ($P=0.109$). Since the differences among the three groups for this parameter were not statistically significant, it can be assumed that the number of larvae is similar across groups regardless of anti-Bm86 immunization present in the hosts of each group.

Hatching rate

The mean hatching rate was 63.34 ± 20.78 % in group one (Table 1), 62.44 ± 25.56 % in group two, and 74.02 ± 30.99 % in group three ($P=0.006$). In a similar study⁽²⁵⁾, the control group had an average of 86 %, whereas the immunized group had 94.00 %. It was possible to identify those ticks in group three (fed on unvaccinated hosts) achieved significantly higher hatching rates than those in groups one and two; this demonstrates a significant impact on the reproductive capacity of *R. sanguineus* females that were fed on hosts immunized with the Bm86 protein.

Length of the biological cycle

Preoviposition

The preoviposition period had a mean of 3.8 ± 1.16 d in group one, 4.16 ± 0.90 d in group two, and 3.68 ± 0.98 d in group three ($P=0.011$) (Table 2). There was an increase in the preoviposition period of ticks in group two (immunization with two doses) compared to ticks in the control group; this could indicate a delay in oogenesis in ticks fed on immunized animals. These results contrast with those reported by Bechara *et al*⁽²⁴⁾, who did not observe significant differences in this parameter between females fed on immunized hamsters and guinea pigs compared to non-immunized animals; this could be due to differences in the experimental host used.

Table 2: Life cycle length in ticks fed on rabbits immunized against the Bm86 protein

Parameter	n	G1	G2	G3
Preoviposition	305	3.82 ± 1.16 ^{ab}	4.16 ± 0.90 ^a	3.68 ± 0.98 ^b
Oviposition	305	3.95 ± 1.15 ^a	3.62 ± 1.12 ^{ab}	3.56 ± 0.78 ^b
Incubation	305	26.06 ± 4.09 ^b	28.21 ± 2.70 ^a	26.89 ± 4.33 ^b
Hatching	305	5.22 ± 2.40 ^b	6.17 ± 2.00 ^a	6.36 ± 2.05 ^a
Feeding period of larvae	351	3.92 ± 0.99 ^a	3.68 ± 0.81 ^a	3.79 ± 0.87 ^a
Larval-nymph molting	351	4.99 ± 1.30 ^b	6.11 ± 4.03 ^a	4.95 ± 1.07 ^b
Feeding period of nymphs	67	4.96 ± 1.09 ^a	5.37 ± 1.11 ^a	4.96 ± 1.07 ^a
Nymph-adult molting	67	15.59 ± 0.79 ^a	15.32 ± 0.88 ^a	15.73 ± 1.37 ^a
Feeding period of adults	305	8.77 ± 1.11 ^b	9.04 ± 1.06 ^a	8.63 ± 1.03 ^b
Total length of the cycle		77.27	81.67	78.55

Data are presented as mean ± standard deviation.

G1= immunized with three doses; G2= immunized with two doses; G3= control group (not immunized).

^{ab} Different letters indicate significant differences ($P \leq 0.05$).

Oviposition

The oviposition period lasted 3.95 ± 1.15 d in group one, 3.62 ± 1.12 d in group two, and 3.56 ± 0.78 d in group three ($P=0.010$) (Table 2). While the variation between the groups was slight, there was a statistically significant difference between groups one and three. This result suggests that ticks fed on hosts immunized with Bm86 (group one) had a slightly longer oviposition process compared to ticks with limited exposure (group three).

Incubation

The mean incubation period was 26.06 ± 4.09 d, 28.21 ± 2.70 d, and 26.89 ± 4.33 d in groups one, two, and three (Table 2), respectively ($P=0.001$). An increase in the length of the incubation period of group two compared to group three was observed; this could be attributed to a delay in embryonic development caused by ticks consuming blood from animals immunized against the Bm86 protein.

Hatching

The hatching period had a mean of 5.22 ± 2.40 d in group one, 6.17 ± 2.00 d in group two, and 6.36 ± 2.05 d in group three (Table 2) ($P=0.000$). The reduction in hatching time observed in group one, compared to group three (control), could be due to greater fragility of the shells of group one eggs, possibly resulting from exposure of gravid females to animals immunized against Bm86.

Larval feeding

The feeding period of the larvae was 3.92 ± 0.99 d in group one (Table 2), 3.68 ± 0.81 d in group two, and 3.79 ± 0.87 d in group three ($P=0.106$). It is assumed that feeding on animals immunized with Bm86 does not affect the time required for larvae to complete their feeding process.

Larva-nymph molting

The molting period from larval stage to nymphal stage lasted 4.99 ± 1.30 d in group one, 6.11 ± 4.03 d in group two, and 4.95 ± 1.07 d in group three (Table 2) ($P=0.000$). There was an increase in the time required for the larvae in group two to complete their molting compared to those in group three; this delay could be due to a nutritional deficit caused by anti-Bm86 antibodies acting on villus cells, thereby affecting nutrient use during feeding.

Feeding nymphs

The feeding period of the nymphs had a mean of 4.96 ± 1.09 d in group one, 5.37 ± 1.11 d in group two, and 4.96 ± 1.07 d in group three ($P=0.388$), suggesting that immunization with Bm86 does not significantly affect the length of the feeding period in the nymphal stage.

Nymph-adult molting

The molting period from nymphal stage to adult stage lasted 15.59 ± 0.79 d in group one, 15.32 ± 0.88 d in group two, and 15.73 ± 1.37 d in group three ($P=0.421$), which could indicate that this parameter is highly stable, and that the nymphs of *R. sanguineus* did not present delays in their development.

Adult feeding

The feeding period of adult ticks was 8.77 ± 1.11 d in group one, 9.04 ± 1.06 d in group two, and 8.63 ± 1.03 d in group three (Table 2) ($P=0.043$). The females in group two had a more extended feeding period than those in the control group. This could be due to a nutritional deficit associated with the consumption of blood from animals immunized with the Bm86 protein⁽²⁶⁾. The results of the present study coincide with those reported by Bechara *et al*⁽²⁴⁾, who observed an increase in the length of the feeding period in adult females of *R. sanguineus* fed on immunized hamsters and guinea pigs compared to those fed on non-immunized animals.

Conclusions and implications

Immunization with the Bm86 protein can decrease some reproductive parameters of the tick *Rhipicephalus sanguineus* at least under controlled laboratory conditions and parasitizing an alternative host. This suggests the possibility of using immunization with Bm86 as a tool to control other species of hard ticks besides *R. microplus*, which, so far, represents the only target species for the application of this antigen. However, additional studies are required to quantitatively evaluate the increase in serum titers of anti-Bm86 antibodies in vaccinated hosts, as well as to identify which tick species are really susceptible to the effect of these anti-Bm86 vaccine antibodies or, alternatively, to search for new antigenic targets that allow conferring protection against different tick species of importance in veterinary medicine.

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