

Short communication

Comparative IgG recognition of tick extracts by sera of experimentally infested bovines

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Abstract

Enzyme-linked immunosorbent assay (ELISA) and Western blot were used to investigate the pattern of antibody responses of six bovines infested twelve times with *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) (six heavy infestations followed by six light infestations) against salivary gland, gut and larvae extracts. During heavy infestations, bovine IgG levels were shown to be higher, and a decrease in the number and weight of ticks that completed the parasitic cycle was observed. The pattern changed starting from the seventh infestation, showing a decrease in IgG levels. An initial increase followed by a significant decrease in the proportion of ticks that completed the parasitic cycle was also observed from the seventh infestation. The number of molecules recognized by Western blot was higher from sera collected following heavy infestations than after light infestations, although a great variation in the profiles detected could be seen when the bovines were compared. These results indicate that IgG responses to different tick antigens may not be generally associated with bovine resistance, and that infestation levels modulate the magnitude of humoral responses and possibly the immune mechanisms in the natural acquisition of tick resistance.

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1. Introduction

The tick *Rhipicephalus (Boophilus) microplus* is a hematophagous ectoparasite of bovines, and constitutes a major health concern as a debilitating parasite and

vector of a great variety of diseases, such as bovine babesiosis and anaplasmosis (Ribeiro et al., 2007).

Currently, the control strategies against tick infestation are almost exclusively focused on synthetic chemicals applied during its parasitic phase. Control of ticks by vaccination offers a number of advantages, like cost-effectiveness, reduced environmental contamination, and the prevention of the selection of drug-resistant ticks that result from repeated acaricide application (Barros and Evans, 1989; Gomes et al., 1999; Willadsen, 2006). But the development of an immunological control strategy depends on the identification of tick immunogenic

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molecules able to induce a host's protective immune response. Concerning potential antigens, two kinds of molecules are described: (i) "exposed" antigens, which are naturally in contact with the host immune system during tick infestation, such as those secreted in the saliva during attachment and feeding on a host; (ii) and "concealed" antigens, which are not naturally exposed (or recognized by) to the host's immune system (Nuttall et al., 2006; De la Fuente and Kocan, 2006). In addition, development of vaccines using multiple antigens that could target a broad range of tick species and further prevent or reduce transmission of pathogens would become a great advancement in veterinary sanitation (Da Silva Vaz et al., 2004; De la Fuente et al., 2007).

Cattle acquire resistance to a variety of tick species with repeated exposure (Wikel and Whelen, 1986). The host's immune response causes premature detachment, reduced engorgement size, increased mortality, decreased fecundity and diminished hatching of ticks (Barriga et al., 1993). Therefore, the analysis of the immune responses developed by infested bovines may become of great importance in the identification of the characteristics of this resistance. Here we reported the analysis of the IgG response of bovines repeatedly infested with *R. microplus* and the development of resistance by hosts under heavy and light infestations.

2. Materials and methods

2.1. Infestation

Six male *Bos taurus taurus* Hereford calves of about 6 months of age were purchased from an area free of *R. microplus*. Heavy infestations were performed with each calf being infested once a month for 6 months with 18,000 *R. microplus* larvae along the back. Light infestation ensued, with each calf being infested once a month for more 6 months with 800 *R. microplus* larvae. The ticks used were of the Bagé strain (Barriga et al., 1995), and the proportion of females was considered as 50% (Da Silva et al., 2007).

2.2. Antigen preparation

Antigens were obtained from twelve-day-old larvae, partially and fully engorged female ticks according to Da Silva Vaz et al. (1994). Briefly, the dorsal surface was dissected and gut and salivary glands were separated and washed in PBS. The tissues were sonicated and solubilized in a medium containing

0.5% sodium deoxycolate, 0.1% pepstatin A, 0.1% leupeptin and 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in 10 mM tris buffer (pH 8.2). The material was centrifuged and the supernatants were stored at -70°C . The protein concentration was measured according to Bradford (1976).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Microtitration plates were coated with 3 μg per well of antigen in 20 mM carbonate buffer (pH 9.6) by incubation overnight (Harlow and Lane, 1988). Plates were blocked with 5% nonfat dry cow milk-PBS (blotto), and incubated with sera diluted 1:50. Rabbit anti-bovine IgG-peroxidase conjugate (diluted 1:6000 in blotto 5%) was then used, and chromogen and substrate were added (3.4 mg *o*-phenylenediamine, 5 μl H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0). The reaction was stopped with 12.5% H_2SO_4 and the optical density (OD) was determined at 492 nm. Sera from the six bovines collected after each infestation were tested six times against *R. microplus* tissue on three different microplates.

2.4. Western blot

For the Western blot analysis, tissue extracts were separated by SDS-PAGE 10% gel electrophoresis under denaturing conditions and transferred to nitrocellulose in 12 mM carbonate buffer pH 9.9 (Dunn, 1986). The strips were blocked with 5% blotto and the test sera were diluted 1/50 in 5% blotto and incubated overnight. Membranes were incubated with rabbit anti-bovine IgG antibody conjugated to alkaline phosphatase (Sigma) diluted 1/10,000 in blotto and stained with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT). All Western blot analyses were performed using the pre-immune bovine sera or incubations with secondary antibody alone as negative controls.

2.5. Statistical analyses

The variance analysis was used to compare the percentile of tick recovery, after transformation in logarithmic scale, in function of the successive infestations. The Tukey's test was used to reveal the significance order (Statistix[®].v8.0, 2003. Tallahassee, FL: Analytical Software). For the comparison of the mean weights of detached engorged females the test used was of Kruskal–Wallis. The values were considered different to the level of $P < 0.05$.

3. Results

3.1. Total numbers and mean weights of detached female ticks during infestations

The mean percentiles of detached adult female ticks throughout infestations are shown in Fig. 1 (Table 1, available online as additional information, shows the total numbers of detached female ticks after each infestation in the six bovines). The percentile of recovered ticks after infestation 7 was higher, which was significantly different from most of the other infestations, except for infestations 1, 8 and 9 ($P < 0.05$). Infestation 12 presented the smallest recovery of ticks, significantly different from the recovery rate observed for infestations 1–9.

The mean weights of detached females (Fig. 2) produced during infestation 6 were significantly lower than those of all other infestations. Also, the values obtained in infestation 1 decreased following the remaining heavy infestations, but they were shown to be similar of those from infestations 7 and 8, within the beginning of light infestations.

3.2. Variations in the IgG levels and profiles of antigen recognition during infestations and between bovines

ELISA was used to compare the IgG levels developed against *R. microplus* salivary gland, gut and larvae protein extracts of sera from six bovines submitted to heavy and light infestations (Fig. 3). Fig. 3(A) shows that the means of salivary gland antigens recognition presented a major increase in infestation 2, after which a decrease was seen. A major increase in IgG levels against gut antigens (Fig. 3B) could be detected in the sera collected after infestations 3 and 6, which then decreased and stabilized until the last infestation. Similarly, anti-larvae IgG levels (Fig. 3C) presented a peak in sera from infestation 3,

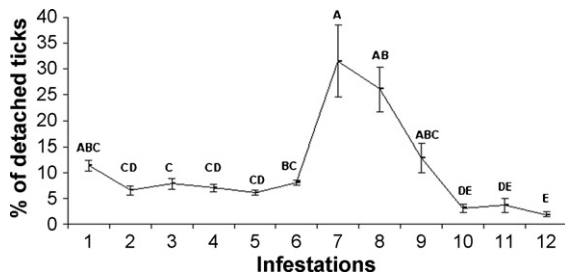


Fig. 1. Mean (\pm S.E.) percentiles of number of detached adult female ticks of 6 bovines infested successively for 12 months with *R. microplus* larvae (infestations from 1 to 6 with 18,000 larvae and from 7 to 12 with 800 larvae).

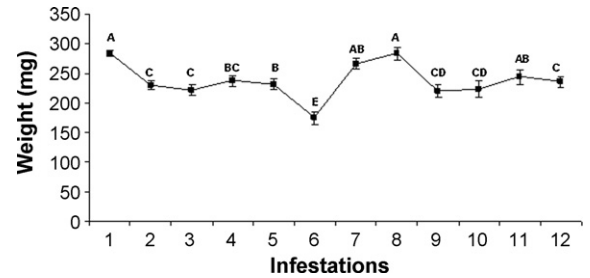


Fig. 2. Mean (\pm S.E.) of individual weight of detached engorged females (mg) of 6 bovines infested successively for 12 months.

after which a decrease occurred, with stabilization in infestations 9–12.

The antigen recognition profiles of salivary gland, gut and larvae protein extracts by sera of the infested bovines are shown in Fig. 4. A considerable variation was observed between individuals. Fig. 4(A) shows that greater numbers of salivary antigens were recognized by the sera from infestations 2 to 8, though this recognition diminished from infestations 10 to 12. Bands of 130 kDa, 97.6 kDa and 86 kDa were recognized by all bovine sera in all infestations. Two bands of 20 kDa and 35 kDa were detected in sera from bovines 1, 5 and 6 in the last infestations.

The recognition of gut antigens was shown to be greater from infestations 2 to 8, as compared to sera collected in the last infestations (Fig. 4B). A 103.6-kDa antigen was recognized by bovines 1 and 5, mainly in the sera from the last infestations, and bovine 6 recognized this antigen only in infestation 12. All bovines recognized a 125.6-kDa antigen in the first infestations. Bovine 1 generated a profile with the highest number of recognized molecules, some of which were recognized exclusively by this individual, such as antigens of 85.4, 69.8, 25 and 19.3 kDa.

The recognition profiles against the larvae extract (Fig. 4C) showed a considerable number of antigens recognized in the sera collected from infestations 2 to 6, which decreased in the following infestations. Low-molecular mass antigens presented higher reactivity mainly against the sera from the first infestations: 33.6 kDa and 25.1 kDa were detected by sera from infestations 2 to 6 of bovines 1, 3 and 5; bovine 2 recognized a 25.1-kDa antigen only in the serum from infestation 4; bovines 2 and 6 recognized a 15.5 kDa in sera obtained after infestations 2 to 6.

4. Discussion

The main purpose of the current investigation was to evaluate the profiles of humoral antigenic recognition

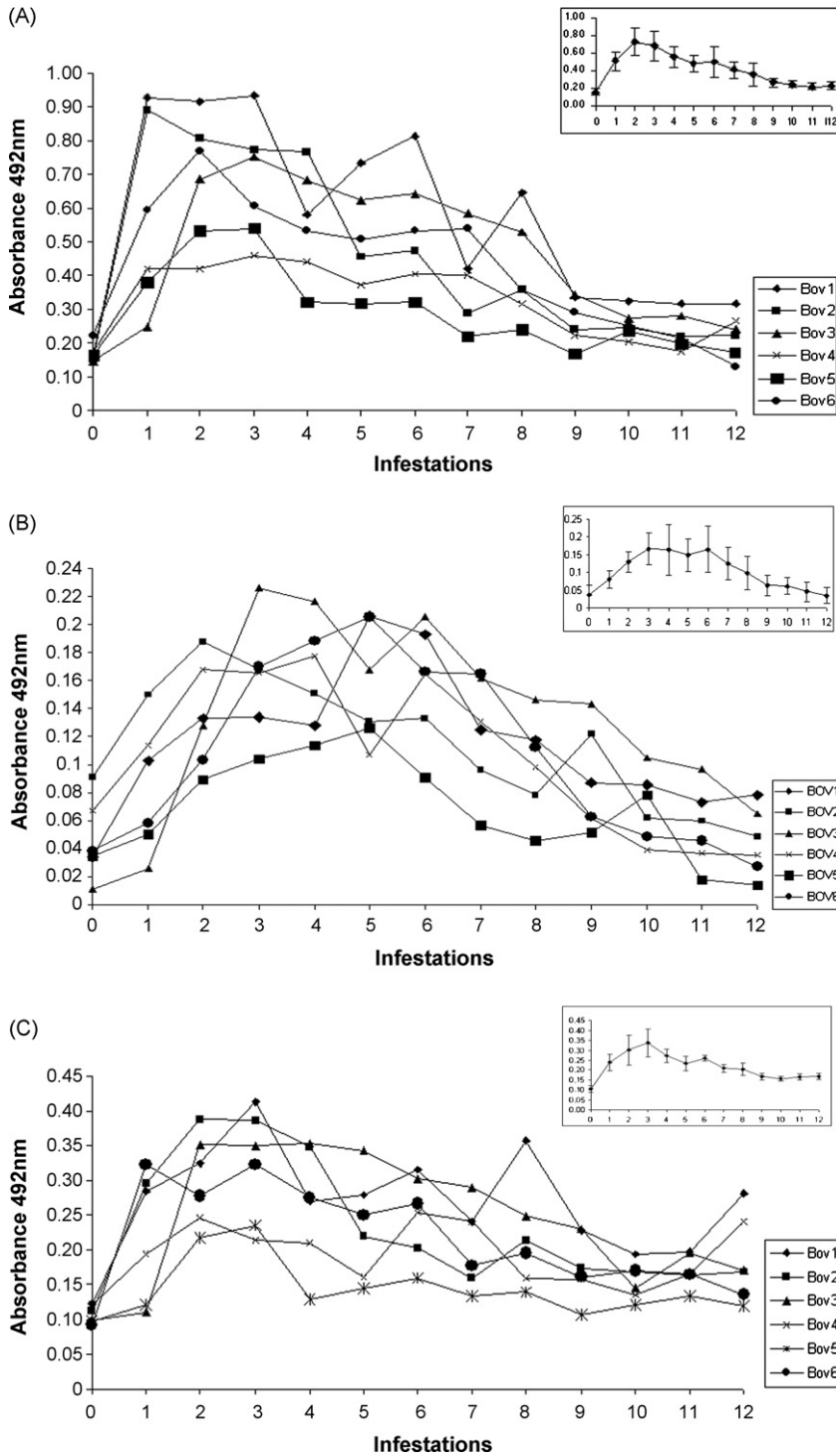


Fig. 3. IgG levels of infested bovine to *R. microplus* antigens measured by ELISA. Extract of salivary gland (A), gut (B) and larvae (C) were used as antigens. The upper box represents the means (\pm S.E.) of the sera for six infested bovine.

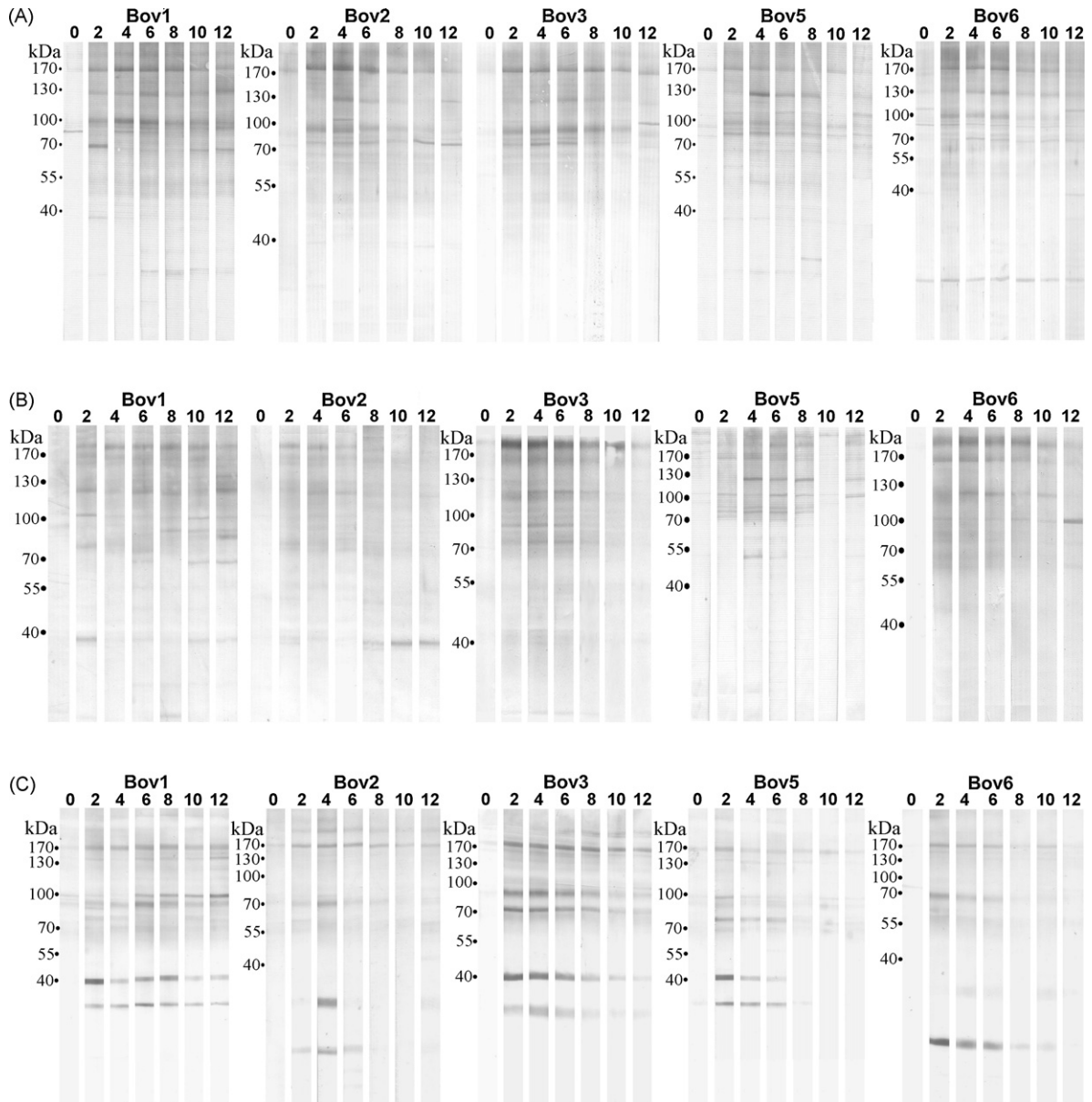


Fig. 4. Western blot analyses of *R. microplus* antigens recognized by pre-infestation (0) and post-infestation (2–12) sera collected after experimental infestations. Extracts from (A) salivary glands, (B) gut, and (C) larvae were used as antigen.

from salivary gland, larvae and gut extracts of *R. microplus*, as a result of repeated exposure under high and low tick densities. The reactivity degrees detected by ELISA and the profiles of molecules recognized by Western blot observed indicate the presence of individual variations between bovines, suggesting that humoral immune responses against different molecules may account, if related, for the resistance to tick infestations. End-point titration assays (Fig. 3A–C) determined that the animals presented higher levels of IgG against

different antigens of *R. microplus* during heavy infestations as compared to light infestations, suggesting that the variations in the IgG levels and profiles of antigenic recognition may be modulated by tick infestation levels. These results are in agreement with observations from sheep naturally exposed to the tick *Ixodes ricinus*, as the IgG responses to salivary gland extract varied between seasons, which were higher in spring (heavy infestation period) than in autumn of 1999 (low infestation period) (Ogden et al., 2002a).

Heavy infestations seem to have caused a decrease in the number and the mean weight of ticks that completed the parasitic cycle (Figs. 1 and 2), and induced a higher level of IgG response against all the extracts analyzed. Furthermore, infestation 6 showed a significant decrease in the mean weight of recovered ticks, in comparison to all other infestations. This reduction is consistent, at least partially, if taken as a consequence of the high levels of anti-tick IgG developed. However, this effect was shown to be comparatively ineffective against the first light infestations, showing an increase in the tick recovery index and in mean weight of these ticks (infestations 7 and 8). This pattern was more similar to that seen in infestation 1, when the bovines presumably did not possess any anti-tick adaptative immune response, as compared to the patterns of any other infestation. The variation in the patterns of resistance may indicate that the immune response developed against the tick after the six first heavy infestations were not protective against the first light infestations.

On the other hand, the last infestations (10, 11 and 12) showed a significant decrease in the proportion of ticks that completed the parasitic cycle, presenting simultaneously very low levels of anti-tick IgG, although the mean weight of the recovered ticks did not reach the values obtained in infestation 6. These results indicate two important points. First, light and heavy consecutive infestations present differences in the expression of resistance. Similarly, sheep infested with *I. ricinus* exhibit density-dependent intraspecific facilitation at different infestation levels (Ogden et al., 2002b). As the lowest number of recovered ticks was obtained after infestation 10, the second important point is that a different protective immune response was developed against less intense infestations, with a different contribution of the IgG response. Ogden et al. (2002a) observed similar results in sheep naturally exposed to *I. ricinus*, as IgG levels against salivary gland extract vary inversely with resistance in the low infestation period. It is well established that *R. microplus* infestation levels are intensively influenced by seasonal factors (Brum et al., 1987; Furlong, 1993). Therefore, it may be assumed that cattle herds also alter levels and/or mechanisms of anti-tick immune responses during the year in response to different seasonal tick densities, as seen on the experimentally infested bovines.

Higher anti-tick IgG levels and a major number of tick molecules were detected by bovine sera when the salivary gland extract was analyzed by ELISA and Western blot. In Western blot, a greater number of salivary gland antigens was recognized by the sera collected after the first infestations of all bovines,

corroborating the higher IgG levels detected by ELISA. The profiles of the sera from the last infestations predominantly showed a lower number of bands, in comparison to the higher infestation sera, but it could also be seen that new molecules were recognized. For example, two bands, of 20 kDa and 35 kDa, were detected from sera of bovines 1, 5 and 6 after the last infestations. A 20-kDa salivary protein was identified by Brown et al. (1984) from *Amblyomma americanum*, which was thought to be a component of tick cement and was immunogenic in guinea pigs and elicited protective immunity in vaccine/challenge experiments (Brown and Askenase, 1986). A larval *R. microplus* 19.1-kDa protein, possibly the same described by Willadsen and Riding (1979) as 18.5 kDa, was shown to be recognized by infested cattle, and induced immediate-type hypersensitivity responses (Pruett et al., 2006). Another 36-kDa salivary protein, identified from *Dermacentor andersoni*, has been shown to inhibit T lymphocyte proliferation (Bergman et al., 1995).

Using gut extract (Fig. 4) as the antigenic source, the responses of the bovines showed that the heavy molecules, of 125.6 kDa and 103.6 kDa, were recognized by most of the bovines and in almost all infestations. These antigens are components most likely shared with the salivary glands, although some controversy may arise concerning the possible regurgitation of gut contents (Kemp et al., 1982; Brown, 1988). The low mass larvae antigens, of 15.5 kDa, 25.1 kDa and 33.6 kDa, were detected with more intensity in the sera from the first infestations, in most bovines. Future identification and cloning of these antigens may contribute to the development of an anti-tick vaccine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetpar.2008.08.016](https://doi.org/10.1016/j.vetpar.2008.08.016)

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