IMMUNOPROTECTION INDUCED IN CALVES BY A SERINE PROTEINASE INHIBITOR FROM *BOOPHILUS MICROPLUS* UNFED LARVAE

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SUMMARY:The tick proteins have been studied to understand the host-parasite interaction and to find tools for tick control. A serine protease inhibitor from *Boophilus microplus* larvae was purified by affinity chromatography on trypsin-Sepharose and ion-exchange chromatography on a Resource Q column. It was preliminarily characterized as an active peak of approximately 18 kDA, and exhibited inhibition activity towards trypsin (Ki) 0.4 nM), elastase, (Ki 3.5 nM), and plasma kallikrein (Ki 35 nM). The inhibitor did not affect prothrombin time (PT) and thrombin time (TT), but it affected activated-partial-thromboplastin time (APTT) in a dose-dependent curve. A partial amino acid sequence indicated that the *B. microplus* protease inhibitor belongs to the BPTI-Kunitz-type inhibitor family. *Bos indicus*, nelore breed calves, previously sensitized with *B. microplus* larvae inhibitor and then challenged with adult ticks showed a 73.7% reduction of engorging adult female tick development and a 87.8% reduction in egg production when compared with control groups. Results suggest that this inhibitor can play a role in the feeding process of the tick and that development of antibodies against these proteins may impair the normal feeding and subsequent viability of the parasite.

KEY WORDS: *Boophilus microplus,* larvae, control, Serine proteinase inhibitor; tick; antigen; immunization.

INTRODUCTION

The tick, *Boophilus microplus,* is an important cattle parasite in South and Central America, Australia, Asia and Africa. Ticks in heavy infestation can cause death in cattle and is responsible for the transmission of diseases like anaplasmosis and babesiosis.

Tick control can be achieved by application of chemical products, but the development of resistance to many acaricides has created problems to this approach (ROULSTAN *et alii*., 1981). Cattle acquire a partial immunity to the ectoparasite due largely to an immediate hypersensitivity reaction to the tick after extensive natural exposure. However, this is still insufficient protection to prevent serious losses in cattle production (RODRIGUEZ, *et alii*., 1994). More recently, the first ectoparasite vaccine has been developed in Australia and commercially released under the name 'TickGARD'. The publication of the gene sequence enabled the development of a similar vaccine in Cuba (WILLADSEN *et alii*., 1995). The vaccine's active antigen, named Bm86 for the species of origin and the year in which it was first identified, is an 89 kDa glycoprotein with an extracellular location on the digestive cells of the tick gut (GOUGH and KEMP, 1993).

The identification and characterization of other protective antigens can be important in developing recombinant vaccines with long protection. It is possible that a vaccine containing more than one antigen may elicit a cooperative effect on protection (WILLADSEN, 1990). Following this idea, biochemical function of many molecules has been characterized to understand the complex interactions, which occur between parasites and their hosts.

Several proteinaceous components, including proteinase inhibitors, present in tick eggs and larvae have been described (WILLADSEN and McKENNA, 1983; WILLADSEN and RIDING, 1980), and some of this activity was connected with toxicity observed in guinea pigs (VERMEULEN *et alii*., 1988). The eggs and unfed larvae of the ectoparasite, *B. Microplus*, contain at least two proteolytic enzyme inhibitors that inhibit trypsin and/or chymotrypsin (VERMEULEN *et alii*., 1988).

B. microplus larvae have at least two forms of trypsin inhibitors, they were previously reported by WILLADSEN and RIDING (1980). They were described as doubled-headed inhibitors that are able to inhibit two enzyme molecules at the same time for example trypsin and chymotrypsin.

A serine protease inhibitor from *B. microplus* larvae was preliminarily characterized; the inhibitor was purified by affinity

chromatography on trypsin-Sepharose and ion-exchange chromatography on Resource Q column, showing an active peak, with 18 kDa. The purified protein inhibited trypsin (Ki 0.4 nM), elastase, (Ki 3.5 nM), and plasma kallikrein (Ki 35 nM). The inhibitor did not change prothrombin time (PT) and thrombin time (TT), but it affected activated-partial-thromboplastin time (APTT) in a dose-dependent function (Andreotti et al., in press). Moreover this inhibitor (BmTI-A) was purified and characterized as a member of the BPTI-Kunitz type serine protease inhibitor family. (TANAKA *et alii*., 1999)

The present work describes the effect of immune response of this larval serine proteinase inhibitor on growth and reproduction of the *B. microplus* larvae.

MATERIAL AND METHODS

Tick larvae production:

Boophilus larvae were obtained by culture of engorged female ticks in the lab. This tick strain were obtained from Dr Raul Henrique Kessler at EMBRAPA and were free of infection by *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale*.

Serine proteinase inhibitor purification:

Larvae ticks (1.13 g) were ground in a mortar with 5 mM tris-HCl buffer (pH 7.4) contain 20 mM NaCl (50 mL). The resulting homogenate was centrifuged at 10000x g for 30 min in a centrifuge (Hitachi model SCR20B). The resultant supernatant (42 mL) was filtered using 0.45 µM filter (Millipore). The filtrate (8 mL) was applied to trypsin-Sepharose column followed by washing with 0.05 M tris-HCl buffer (pH 8.0), and then elution bound inhibitors with 0.2 M KCl solution (pH 2.0). Fractions were immediately neutralized with 1 M tris-HCl buffer (pH 8.0). The fractions containing the inhibitory activity from several chromatographic runs were pooled and lyophilized. The concentrated material (1.8 mL) was desalted in a PD-10 column and eluted with distilled water. The partial purification was made according to (TANAKA *et alii*., 1999).

Animals:

Six *Bos indicus* nelore calves about 6 months old and around 150 kg. Animals were housed in pens during the development of essay. They were allocated into vaccinated and control groups each consisting of 3 calves. The blood was sampled weekly and the serum was frozen until tested.

ELISA:

Specific IgG antibodies to the larvae proteinase inhibitors were detected by an ELISA test. Microitration plates were coated with 1 µg/ml of larval proteinase inhibitor antigen in 20 mM carbonate buffer (pH 9.6) by incubation overnight at 4 C (Harlow and Lane, 1988). The plates were washed five times with 0.1% PBST. One hundred µl of bovine serum, diluted 1:100 in PBST, were added to each well and incubated for 45 minutes at 37°C. After washing 50 µl of rabbit anti-bovine IgG alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO), diluted 1:18.000 in PBST, were added to each well and plates were incubated for 30 minutes.

After washing plates with PBST, 50 µl of the substrate p-nitrophenyl phosphate (1.0 mM) were added to each well. Reactions were stopped after 15 minutes by addition of 100 µl of NaOH (0.2 M) to each well. Values were obtained using microplate reader at a wavelength of 405 nm (ARAUJO, *et alii*., 1998).

Experimental design:

The vaccination protocol involved two subcutaneous immunizations with 100 mg of the inhibitor and a saponin adjuvant with a period of 21 days. The control group received only the saponin with saline solution.

Three weeks after the second inoculation both groups received a challenge infestation with 20,000 larvae/animal. Engorged ticks were collected immediately after falling off, weighted and incubated at 28°C and 80% humidity until egg laying. Eggs were weighted and incubated to be evaluated in their hatchability. The hatchability was measured as the percent of larvae emerging from each egg clutch for each engorged tick.

RESULTS AND DISCUSSION

In this work the inhibitor was partially purified by affinity chromatography on a trypsin-Sepharose column.

The data displayed in the Figure 1A show that vaccination of calves resulted in a 73.5% reduction in the mean tick number. The mean weight of ticks collected from vaccinated calves was reduced in 84% (Figure 2B). These data show that vaccination produced an overall significant decrease in tick biomass.

The effect not only can be showed in engorged ticks number and biomass produced, but also in the time of peak production. Vaccinated group showed that production peaked two days later in the engorged period (Figures 1B and 2B). This effect of delayed tick development was reflected a three days of delay in the peak egg laying. (Figure 3B)

When the mean weight of ticks was compared of number between both groups, the data showed a reduction of the 41% tick biomass for the vaccinated calves.

The evaluation of the reproduction effect is showed in the first step with the evaluation of the eggs biomass production from laid eggs. The cumulative weight of eggs laid by engorged tick shows 9.6 gr. for the vaccinated group and 68.34 gr. for the control group. Figure 3A shows that the reduction of egg production in the vaccinated group was 86%. The effect in the individual tick when the mean of both groups were compared was about 50% of reduction in ticks for vaccinated calves.

The mean number of larvae that hatched from eggs of ticks collected from vaccinated calves was reduced by 24%.

Results of the serology study showed that vaccinated calves all had positive values reaction at maximum titer of 5,000 and this titer persisted until the last evaluation when the engorged period finishes. Antibody reactivity to the test antigen was not

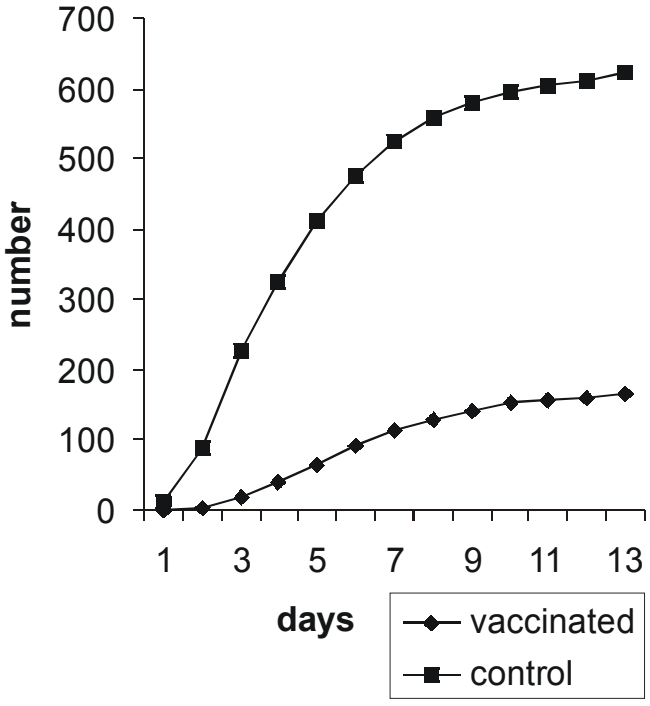

Fig. 1A – Number of engorged ticks collected in the engorging period after challenging with 20,000 larvae. The data show accumulation of the ticks during of engorging period. From the vaccinated group were collected 624 engorged ticks and 165 from the control group. The overall reduction of engorged ticks in the vaccinated group was of 73.5% when compared with control group.

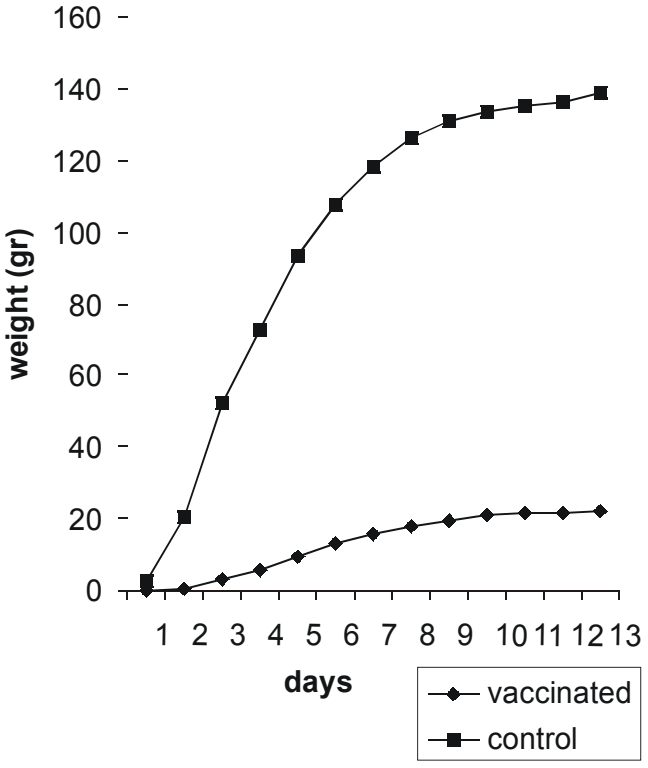

Fig. 2A. Accumulation of weight in amount of engorged ticks collected during engorging period. In this period the vaccinated group accumulated 9,6 gram and the control group 68,3. The vaccinated group showed a reduction of total weight of 84,1% when compared with control group.

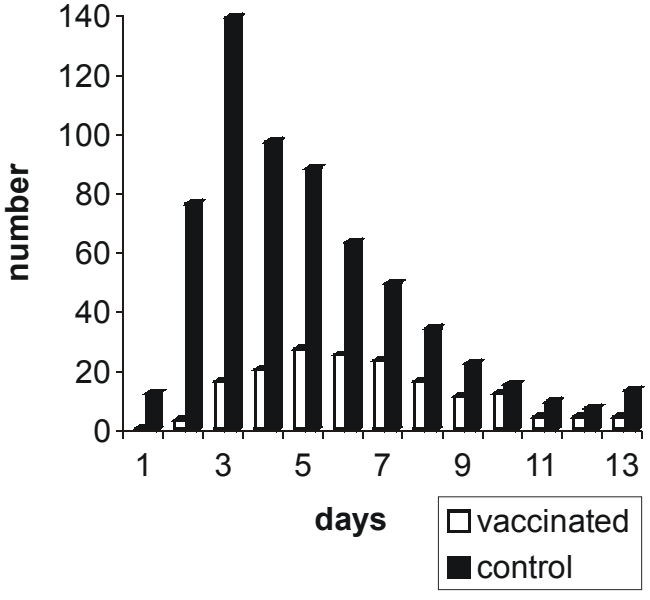

Fig. 1B – Shows the distribution of engorged tick number during engorging period. The peak was two days later in the vaccinated group.

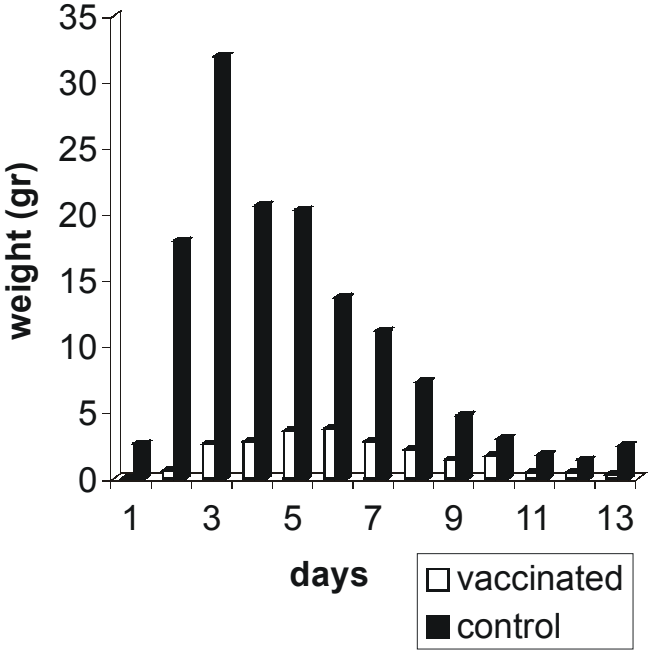

Fig. 2B – The distribution of engorged tick weight during engorging period. The vaccinated group showed the peak of production two days later than the control group.

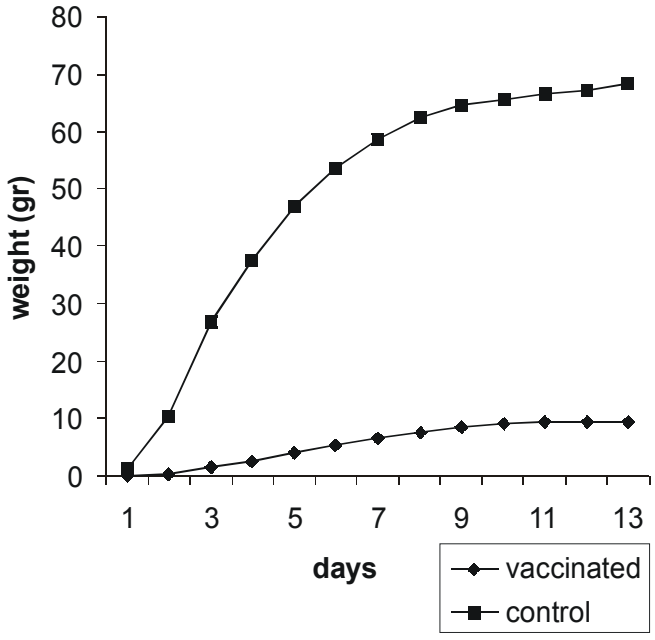

Fig. 3A – The cumulative weight of eggs laid by the engorged ticks shows a cumulative total of 9.6 grams for the vaccinated group and 68.34 grams for the control group. The reduction in the production of eggs in the vaccinated group was 86% when compared with control group.

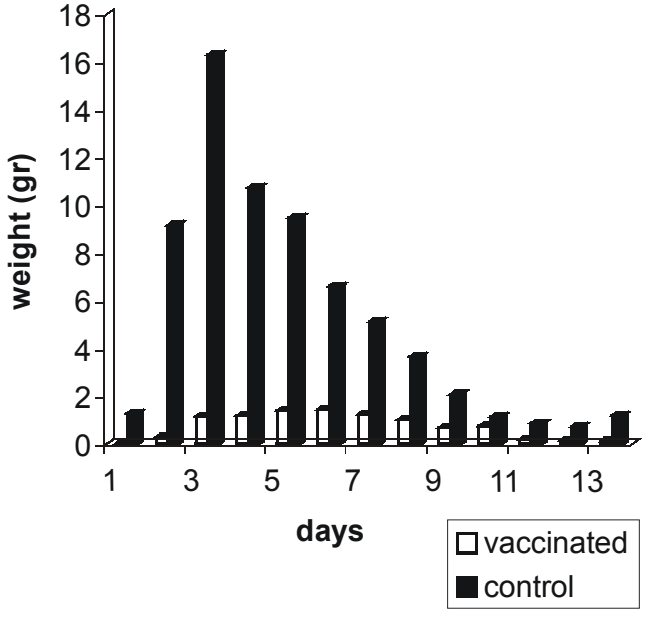

Fig. 3B – The distribution of egg weight during the egg laying period. The vaccinated groups showed the peak three days later than control group.

detected in the control groups. Those data suggest that bovine under natural conditions are not immune-stimulated by tick inhibitor when they are bitten by ticks. Apparently the amount of inhibitor introduced during a tick bite was not enough to stimulate the immune response.

In addition the characteristics of kallikrein inhibition in the site of attachment may play an important role by inhibition of bradykinin formation, the pain mediator, and edema forming consequently preventing the tendency to scratch which could lead to the removal of the parasite (BROSSARD, 1998).

The calves used in the essay did not lose weight or total plasma protein and they did not develop any skin reaction from the time of the first vaccination until the final collection of engorged ticks. Also no edema was observed in the animals, although the edema can has been shown to be a significant component in tick rejection reactions (RIBEIRO, 1989). Possibly the absence of edema might signify that the tick reaction was reduced sufficiently to not even produce edema.

It is clear that larvae need an efficient system to prevent blood clotting and the inflammation response during feeding. The hypersensitivity in the larval attachment site was found, so the inhibition of elastase-like enzyme may decrease this response. The double action of this inhibitor on both clotting and inflammatory enzymes might be an economical solution on the feeding process (TANAKA *et alii*., 1999). Not only did immunization of calves with inhibitor reduce the number and weight of the ticks which developed on vaccinated calves, but it also strongly reduced the reproductive capacity as demonstrated by a decreased biomass production of eggs and decreased hatchability.

Unfed larvae on the bovine skin died if they do not feed quickly by lose of water. In resistant animals they need frequent and short attachments causing stress to the tick state (KEMP *et alii*., 1976), and it is at this point in the life cycle that larvae are in their most vulnerable state. Also for the host, ticks have not yet begun to provoke any negative consequence related to taking large blood meals.

This work showed that immunoprotection was induced in calves by a serine proteinase inhibitor from *B. microplus* unfed larvae: not only the number and the weight of the ticks were reduced, but also their reproductive capacity.

As a pilot study this work raises several questions in this inhibitor immune protection studies: how much of protein need to inoculate to improve the immune response? what kind of adjuvant has the best antigen potentiality? how long and what kind of immunity it develops.

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