

Original Article

Immunization of Cattle with Tick Salivary Gland Extracts

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Abstract

Background: *Rhipicephalus (Boophilus) annulatus* tick is one of the most important ectoparasite of cattle. Recently, several laboratories in the world have been concentrated on immunizing cattle against tick using various types of tissue extracts of ticks. The aim of this study was to evaluate the effect of immunization of cattle with tick salivary gland extract on biological parameters of ticks and humoral immune responses of cattle.

Methods: Fourteen more dominant protein bands identified as immunogenic by Western-blot analysis were eluted from polyacrylamide gel. Test and control groups were injected three times with eluted proteins and sterile PBS (pH= 7.2) respectively with equivalent amount of adjuvant. After four weeks a tick challenge was performed. Finally, biological parameters of collected engorged female ticks were recorded and humoral immune responses to immunization measured by ELISA.

Results: The results indicated immunization of cattle resulted in reduction in mean tick counts, attachment, engorgement weights, feeding index, egg mass weight, hatchability and fertility index (respectively 63.1%, 62.6%, 30.2%, 36.4%, 40%, 78.7% and 13.3%) and increased duration of feeding, pre-oviposition and incubation period of eggs (respectively 8.6%, 45 and 31.34%). All changes were statistically significant ($P < 0.05$). Results showed an increase in antibody production of test group from the first week after immunization. The antibody level was boosted following tick infestation.

Conclusion: This investigation indicates that immunization of cattle with these antigens could induce a protective immune response against *Rh. (B.) annulatus* tick that would be expected to provide a safe non-chemical means of tick control.

Keywords: *Rhipicephalus (Boophilus) annulatus*, Immunization, Salivary gland, Humoral immune responses, Tick

Introduction

Rhipicephalus (Boophilus) annulatus tick is one of the most important ectoparasites of cattle that can occasionally complete its life cycle successfully on sheep, goats and wild ungulates such as antelopes. Surviving in Asia, Africa, America and Europe, this species of tick causes economic losses due to direct effects on the preferred hosts and by the pathogens they transmit (Murrell and Barker 2003, Estrada-peña et al. 2004, Rajput et al. 2006, Wikel 1999a, b). Therefore, tick control is a continuing global priority.

Traditional control methods such as using chemicals have had partially successful results, however, acaricide compounds are

expensive and have adverse effects such as a high incidence of resistance among tick populations, as well as harmful effects on vertebrate hosts, human beings and the environment. These increasing problems have stimulated researches on alternative methods of tick control in recent years (Mattewson et al. 1975, Walker 1994, Nari and Hansen 1999, FAO 2004, Rajput et al. 2006). These methods include biological control methods such as pasture spelling and artificial selection for tick-resistant cattle. Such steps can reduce tick burdens, but enhancement of host resistance through artificial immunization with a vaccine against ticks would constitute a

major advance in control process. Several attempts have been done to immunize actively cattle against ticks and it has been reported that protective host immune responses can damage female tick internal organs and interfere with tick's feeding and fertility behavior (Wikel 1999a, b, Willadsen and Jongejan 1999).

Performing various functions in the life cycle of Ixodid ticks especially in their feeding procedure, the salivary glands are the major route for pathogen transmission. To facilitate attachment and engorgement, ticks produce biologically active components with anti-haemostatic, anti-inflammatory and immunomodulatory effects.

“The initial secretion of the tick saliva contains a cement-like material, which helps the tick to establish a firm attachment with the host tissue” (Hager and Burgess 1980). Exposure to proteins secreted by ticks can provoke antigen-specific humoral and cellular responses in the mammalian hosts. Containing the natural antigens injected by the tick during infestation, salivary gland extracts might be an obvious choice to induce immunity against ticks (Willadsen 1980, Brossard and Fivaz 1982, Wikel 1996).

The purpose of this research was to determine the protein pattern of *Rh. (B.) annulatus* salivary glands by SDS-PAGE and to investigate the effects of immunizing cattle with immunogenic fractions of salivary gland extract of this tick against tick infestation. The quality of immune responses measured by ELISA and the efficacy of immunization was evaluated by analyzing feeding and fecundity parameters of ticks harvested from treatment group compared to the control groups.

Materials and Methods

Ticks

Engorged female *Rh. (B.) annulatus* ticks were collected from healthy cattle (free of any

transmissible pathogens), washed with 70% ethanol and then washed three times with sterile phosphate buffer saline (PBS) pH=7.2, after drying they were kept under a constant temperature of 28 °C, a relative humidity of 85% and a 12:12 (L: D) photo period.

Experimental animal

Eleven healthy Holstein calves (3–5 months old) were purchased from a tick-free area (Tehran Veterinary Medicine Faculty Research Institute) and kept in tick-proof pens.

Antigen preparation

Semi engorged adult female ticks fed for about 7–10 days on tick-bite free cattle, was collected to rear and maintain clean ticks. After washing with 70% ethanol and sterile PBS, ticks were opened along their dorsal surface as previously described (Brown et al. 1984). Then, salivary glands were removed, dissected free of other tissues, placed into PBS containing 1 mM phenyl methyl sulphonyl fluoride (PMSF) at 4 °C. Salivary glands were suspended in 200 µl PBS with PMSF, grinded by glass beads, sonicated (Dr Hielscher GmbH, Germany) for 30 minutes with 0.4 s cycles (40W) on ice and completely homogenized by freeze-defreeze alternatively. The mixture then were centrifuged at 12000×g for 15 minutes at 4 °C, supernatant were collected and stored at -20 °C until use. The protein concentration of salivary gland extract determined according to the method of Warburg (Hudson and Hay 1984, Tietz and Andresen 1986).

Protein Electrophoresis

Protein analysis by SDS-PAGE was carried out using a discontinuous gel system (Laemmli 1970) with an acrylamide concentration of 5% in stacking gel and 12% in the separation gel. A high molecular weight protein ladder (Fermentas, SMO661) was used as molecular weight markers.

Western blot analysis

The protein bands from polyacrylamide gel were electro blotted to a nitrocellulose paper (NCP) using a mini blotter (BioRad) according to Wang's method (Wang and Nuttall 1994). Non-specific reactive sites on the NCP blocked for 1 h in Tris-buffered saline with 0.5% tween 20 (TBS-T pH 8), containing 3% bovine serum albumin (HABtech) on a shaking plate at room temperature. The NCP was incubated for 1 h at 37 °C with bovine sera diluted 1:50 in PBS (pH= 7.2), The NCP was incubated for a further 1 h at 37 °C with sheep anti-bovine IgG (Serotech, conjugated with HRPO) diluted 1:2000 in PBS-tween, washed and reacted with Di amino benzidine (DAB) (Sigma) as a substrate. The NCP was washed 3 times between each step of the assay with PBS-tween. Finally, the immunogenic protein fractions were appeared on the NCP.

Protein extraction from polyacrylamide gel

After staining the polyacrylamide gel with Coomassie blue, the desired bands identified by western blotting were cut out with a scalpel blade, sliced to a minimum and put into a microcentrifuge tube. Gel slices were washed twice with distilled water and soaked in 1 mM 2-Mercaptoethanol for 15 min. Then, gel slices were incubated in elution buffer for 12 hours. After centrifuge, the supernatant was decanted and replaced by elution buffer and incubated for 4 hours. The two solutions were then pooled and added 5 volumes of -20 °C Methanol: Acetone solution to remove SDS and stain as well as precipitation of the protein. After 24 hours acetone solution were discarded and pellet were air dried on ice. To renaturation of protein with Guanidine HCl, the pellet was suspended in the minimum amount of buffer A, containing 100 mM KCl, 1mM DTT, 12.5 mM MgCl₂, 6 M Guanidine, 10% (v/v) Glycerol, 25 mM HEPES-KOH pH 7.6 and 0.1 mM EDTA. After 20 minutes incu-

bation at room temperature, several volumes of buffer B, composed of 1mM DTT, 0.1 mM KCl, 0.01% (v/v) IGEPAL CA-630, 12.5 mM MgCl₂, 10% (v/v) Glycerol, 25 mM HEPES-KOH pH 7.6 and 0.1 mM EDTA, were added and dialyzed at room temperature for 2–3 hours against buffer B. Protein concentration was measured by Warburg method. The protein solution was sterilized with a 0.45 µm filter (Schleicher and schuell, Germany) and stored at -20 °C (Hager and Burgess 1980, Hunkapiller et al. 1983, Kadonaga 2007).

Immunization

Cattle were divided into two groups (treatment and control). The treatment group were intradermally injected with a mixture of 100 µg eluted protein and equivalent amount of Freund's adjuvant (Biogen) every two weeks for a total three immunizations (first injection with complete Freund's adjuvant and two another with incomplete adjuvant). In a similar procedure the control group were injected with sterile PBS (pH= 7.2) and equivalent amount of complete and incomplete adjuvant as followed above.

Tick challenge

Four weeks after the last injection, each cattle were infested with about 500 *Rh. (B.) annulatus* tick larvae as described previously (Nikpay et al. 2012) on their right flank.

Tick parameters

Infested animals were checked daily and recovered ticks were collected, counted, weighed and isolated. Engorged ticks were collected twice a day, at 8:00 am and 4:00 pm. Each five engorged ticks were placed in a tube and were maintained in incubator with a constant condition as described earlier. The degree of protection was determined by measuring the usual parameters in ticks fed on immunized animals and comparing the results with those fed on control animals.

These parameters were including: mean of recovered tick counts, attachment rate, engorgement weight, length of feeding period, feeding index (engorgement weight(mg)÷feeding length (Day)), preoviposition period, egg mass weight, incubation period, hatchability rate and fertility index (egg mass weight(mg)÷engorgement weight (mg)).

Statistical analysis

The effect of treatment on level of tick fecundity and tick feeding parameters in two groups were analyzed by statistical methods. T-test and CHI square test was used to assess significant differences between immunized group means for each of variables ($P < 0.05$). All analyses were carried out using the SPSS program (version 16.0).

Humoral response

The levels of IgG antibodies produced against protein fractions of tick salivary gland extract injected to immunized cattle were monitored by ELISA according to the protocol described by Harlow (Harlow and Lane, 1988). Briefly, ELISA plates (Greiner) were coated with 5 µg per well of antigen in 20 mM carbonate buffer (pH= 9.6) by incubation overnight at 4 °C. They were washed three times and incubated for 1 h at 37 °C with 1% bovine serum albumin (HAB-tech) in PBS. Then, test sera diluted 1:200 in PBS, were incubated for 1 h at 37 °C. After this time, the plates were washed three times and 100 µl of 1:2000 sheep anti –bovine IgG conjugated with peroxidase were added to the individual wells. After incubation at 37 °C for 1 h the plates were washed. The color developed with 2, 2', Azino-bis 3-ethyl Benz- Thiazoline-6-Sulfonic acid (ABTS) as substrate. The optical density (OD) was determined at 405 nm (AWARENESS- STAT FAX 2100). Sera from immunized and control cattle were collected from week 0 (before the first injection) until week 18 and results were compared in two groups.

Results

Determination of protein pattern by SDS-PAGE

Coomassie blue staining of polyacrylamide gel visualized a broad protein profile in *Rh. (B.) annulatus* tick salivary gland extract, comprised of 32 fractions with molecular weight of >200, 160, 107, 103, 92, 82, 80, 78, 72, 66, 63, 56, 55, 50, 46, 40, 38, 37, 36, 33, 30, 29, 27, 25, 22, 19, 18, 17, 16, 15 kDa and two bands lesser than 15 kDa (Fig. 1).

Analysis of salivary gland antigens using bovine serum

Positive serum from cattle infested with about 10000 *Rh. (B.) annulatus* tick larvae, recognized approximately 22 proteins from female tick salivary gland extract including two bands more than 170 kDa, 160, 103, 92, 78, 72, 66, 63, 56, 55, 50, 40, 38, 36, 33, 29, 27, 26, 25, 19 kDa and a band less than 17 kDa . Sera from uninfested animals did not react with any of these antigens by this assay (Fig. 2).

From these immunogenic fractions, 14 bands, which were more dominant than others were (92, 78, 72, 66, 56, 55, 50, 40, 38, 36, 33, 27, 25 and 19 kDa), were dissected from polyacrylamide gel and used for immunization procedure.

Effects of immunization against tick challenge

Tick parameters

We used two groups of parameters to assess the effects of immunization on ticks feeding parameters and fecundity parameters.

Feeding parameters

Effects of immunization on feeding parameters are summarized in Table 1. Among immunized animals, the number, weight and feeding index of engorged female ticks were significantly reduced compared to the control group. The length of feeding period in

immunized group was longer than control group ($P < 0.05$).

Fecundity parameters

Female ticks recovered from immunized cattle passed longer period to lay egg after engorgement in comparison with ticks harvested from control cattle. The eggs were laid by ticks engorged on immunized cattle had longer incubation period than eggs produced by ticks fed on control cattle. Immunization with eluted antigens significantly reduced egg mass weight, hatchability rate of eggs and fertility index ($P < 0.05$) (Table 1).

Humoral responses

Cattle immunized with eluted fractions of salivary gland extract developed antibodies to these antigens. Humoral immune re-

sponses to immunization with tick salivary glands eluted proteins measured by ELISA. Results showed a steady increase in antibody production level of treatment group from the first week after immunization until the last injection. After fourth week, it reached a plateau during next four weeks. When a tick challenge was performed at week eighth, it rose sharply and peaked at week 12 and then this trend declined and gradually stabilized at week 18. In control group, there was a period of stability during the first eight weeks. After tick infestation, it surged dramatically and reached a peak at week 12. Then, it fell slightly to a constant level. Although both groups have had a significant increase in their antibody production level after 18 weeks, this level was boosted following tick infestation in treatment group (Fig. 3).

Table 1. Different parameters measured in female engorged ticks recovered from vaccinated and control groups and their change percentage

Variable	Group	Mean	Percent of changes
Number of collected ticks	Vaccinated	161	-63.1 ¹
	Control	436	
Attachment rate	Vaccinated	32.2	-55
	Control	87.2	
Engorgement weight (g)	Vaccinated	0.206	-30.2
	Control	0.295	
Length of feeding period (Day)	Vaccinated	25.250	8.6
	Control	23.511	
Preoviposition period (Day)	Vaccinated	2.906	45
	Control	2.011	
Egg mass weight (g)	Vaccinated	0.403	-40
	Control	0.665	
Incubation period (Day)	Vaccinated	25.406	31.34
	Control	19.345	
Feeding Index	Vaccinated	0.008	-36.4
	Control	0.013	
Fertility Index	Vaccinated	1.95	-13.3
	Control	2.25	
Hatchability rate	Vaccinated	747	-78.7
	Control	3504	

¹ Negative values indicates reduction in that value

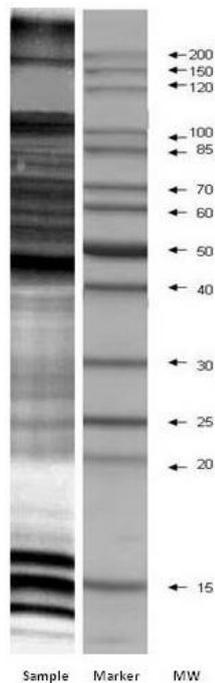


Fig. 1. SDS-PAGE analysis of *Rhipicephalus (Boophilus) annulatus* salivary gland extract. Sample: SDS-PAGE gel stained with coomassie blue. Marker: protein ladder. MW: molecular weight

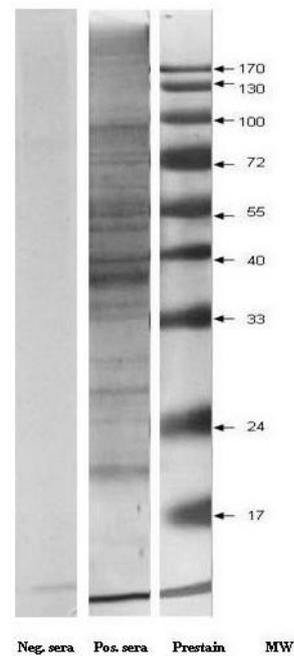


Fig. 2. Western blot analysis of *Rhipicephalus (Boophilus) annulatus* salivary gland extract. Neg sera: serum of uninfested cattle. Pos sera: serum of infested animals with tick larvae. Prestain: prestained protein ladder. MW: molecular weight

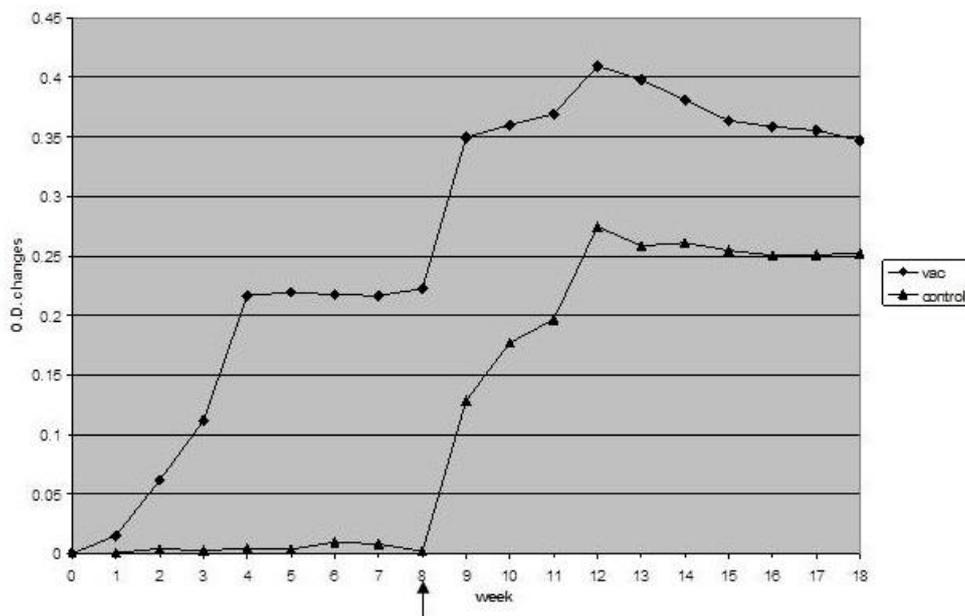


Fig. 3. Changes in antibody production to selected antigens in vaccinated animals \blacklozenge in comparison with control animals \blacktriangle during 18 weeks

\uparrow Indicates when tick challenge was carried out. Vac: vaccinated animals. Control: control group

Discussion

It is generally accepted that tick saliva plays an important role in pathogen transmission, suppression of host immune reactions, and successful engorgement. Therefore, biochemical and immunological characterization of tick saliva components introduced to the mammalian host during the feeding procedure is essential (Willadsen 1980, Brossard and Fivaz 1982, Wikel 1996, 1999a, b, 2006, Willadsen and Jongejan 1999).

Some mammalian hosts acquired resistance to tick bites after repeated experimental infestation with ticks (Brossard and Fivaz 1982, Dipeolu et al. 1992, Nikpay et al. 2008, Nikpay et al. 2012). According to these researches, although feeding behavior of ticks was affected after several experimental tick infestations, some components of tick saliva acting as host immune response modulator, suppressed host immune system. Therefore, this method could not reject ticks from host properly (Dipeolu et al. 1992, Cobon et al. 1996, Wikel 1999a, b, Willadsen and Jongejan 1999).

On the other hand, some researchers used various tissue extracts of ticks as a source of antigens for immunization of hosts. Since the salivary gland extracts contain the natural antigens injected by the arthropod during infestation, it appears to be an obvious choice to induce immunity against blood-sucking arthropods. It has been shown Immunization with salivary gland extracts of *Rh. (B.) annulatus* tick resulted in a clear reduction in the severity of clinical babesiosis. In addition, fewer *Babesia bigemina* positive ticks were collected from these cattle (Jittapalapong et al. 2004a, Jittapalapong et al. 2004b).

It is obvious that the importance of inhibiting tick feeding to prevent direct damage and pathogen transmission and reliability of using natural antigens to avoid repeated tick infestation justifies further investigations on natural salivary gland antigens of ticks.

Our results demonstrated that immunization of calves with eluted fractions of salivary gland extracts of *Rh. (B.) annulatus* ticks induced remarkable immunity expressed as an inhibition of feeding and fertility of the ticks with less direct trauma and damage and without the risk of disease transmission. As mentioned in results, immunization of cattle resulted in reduction in mean tick counts, attachment, engorgement weights, feeding index, egg mass weight, hatchability and fertility index (respectively 63.1%, 62.6%, 30.2%, 36.4%, 40%, 78.7% and 13.3%) and increased duration of feeding, preoviposition and incubation period of eggs (respectively 8.6%, 45 and 31.34%). Clearly, the host immunity affects the physiology of the tick in some permanent manner persisting even after the parasite detached. The shortening of the feeding time by the vaccine suggests that although the degree of immunity produced in this assay was relatively acceptable, the stronger immunization can probably inhibit feeding totally, which is highly desirable because it will prevent the transmission of tick-borne pathogens during feeding.

The humoral immune responses, measured by ELISA, showed cattle injected with tick salivary gland eluted proteins have expressed a constant increase in antibody production level from the first week of injection which were boosted after tick challenge. We had demonstrated tick infestation elevates the antibody level in infested cattle (Nikpay et al. 2008).

The results reported in this study confirmed that immunization against *Rh. (B.) annulatus* could be achieved under experimental conditions. Therefore, the immunogen fractions of the salivary gland extract would be an appropriate target to design an effective vaccine against blood-sucking parasites especially ticks. In addition, our pre-

vious studies indicated there are protein bands with the same molecular weight in different tissues of *Rh. (B.) annulatus* tick resulted in similar changes in antibody production level in cattle (Nikpay et al. 2008, Nikpay et al. 2012). Comparison of the results of SDS- PAGE and western blot analysis in this study and some previous studies reveals that there are several protein fractions having similar molecular weight and similar immunogenicity in different species of Ixodid ticks studied (Nabian et al. 2005, Razmi et al. 2005, Manzano-Román et al. 2007, Norouzi et al. 2007, Nikpay et al. 2008, Nikpay et al. 2012). Interestingly, some of these fractions were reported from the different tissues of ticks. Thus finding common crucial antigens in various tissues of different species of ticks would be our final goal in other studies.

Conclusion

Results of this investigation indicates that immunizing of cattle with these antigens could induce a protective immune response against *Rh. (B.) annulatus* tick infestation that would be expected to provide a safe non-chemical means of tick control. We would like to follow this study by characterization of each immunogenic fraction and finding common crucial antigens in other tick species to design a feasible and reliable vaccine against tick infestation

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