Original Article

Analysis of Immunogenic Relevant Proteins in *Rhipicephalus* (*Boophilus*) *annulatus* Tick

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Abstract

Background: Considering the importance of ticks as a main group transmitting pathogen organisms, this study designed to recognize immunogenic proteins in different tissues of *Rhipicephalus* (*Boophilus*) *annulatus* tick and to find out if there are common proteins in these tissues.

Methods: Seven cattle were experimentally infested with about 10000 *R. annulatus* larvae and their humoral immune response to extracts of salivary gland and ovary of adult ticks and larval extracts during infestation were determined by ELISA and Western blot analysis. Measurements of serum antibodies level recorded weekly, from week 0 to week 9.

Results: Using Western blot analysis, 15 fractions from soluble antigens extracted from salivary gland and larvae, and 14 fractions in the larval extracts were recognized. These findings illustrate the recognition of common antigens with molecular weight of 170, 117, 100, 70, 37, 33 and 30 kDa from different antigens by resistant cattle sera.

Conclusion: Common antigens are present in different tissues of *Rhipicephalus* (*Boophilus*) *annulatus*, which can be used as a target in immunization against ticks.

Keywords: *Rhipicephalus* (*Boophilus*) *annulatus*, Salivary gland, Ovary, Larva, Immunoblot, ELISA

Introduction

Cattle develop resistance to ticks following natural infestation (Reik 1962, Dipeolu et al. 1992, Cruz et al. 2008) and vaccination (Opdebeek et al. 1989, Willadsen et al. 1988, 1999, 2006, Wikel 1999a, b, Ogden et al. 2002, Trimnel et al. 2005, Leal et al. 2006, Perez 2010).

It has been shown that infested animals with *Boophilus microplus* induce an immune response and acquire a partial resistance to subsequent infestations, in which the internal organs of the ectoparasite become damaged, leading to an increase of feeding time, a decrease in the numbers of engorged females, less uptake of blood meal, a decrease in the number and viability of eggs (Wikel 1988). There is a general view, however, that a vaccine with only one antigen will not be sufficient to generate an effective immune response able to control the proliferation of the ticks under field conditions (Willadsen 1990).

Salivary gland, gut, embryo, and larval extracts of various ticks have been studied as possible target for vaccine. However, these tissues have been examined independently (Wikel 1984, Wiladsen et al. 1988, Wong and Opdebeek 1989).

We examined the cattle immune response to salivary gland, ovary, and larval extracts of *R. annulatus* tick at the same time with the aim of finding common antigens, which in vaccination protocol could be advantageous upon stage, or organ specific antigens. The tick *R. annulatus* is an important ectoparasite

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of cattle that present in West Africa, Central Africa, Asia, certain parts of Southern Sudan and Europe.

In this study, for the first time, salivary gland, ovary, and larval extracts of this species have been studied at the same time as possible target antigens to determine the cattle immune response to them.

Materials and Methods

Tick infestation

Seven healthy 3-5 months old Holstein cattle being negative sera for tick infestation were provided from Tehran Veterinary Medicine Faculty Research Institute (Amin abad). They were housed in tick-proof pens independently.

Each cattle was infested with about 10000 *R. annulatus* larvae as described by Brown (1988). Briefly, tubes containing larvae fastened to a shaved flank of cattle with adhesive tape. Then, the surface of the infestation area was covered with a piece of cloth.

Antigen preparation

Partly engorged female ticks were washed with 70% ethanol and then washed three times with sterile distilled water. After drying, they were maintained at 28^{o} C and 85% relative humidity. The antigens used in the ELISA were extracts of salivary glands, ovaries, and larvae. Salivary glands and ovaries were dissected with fine-tipped forceps in cold PBS, rinsed in fresh PBS and then each of these organs and tick larvae were homogenized in a glass tissue grinder separately in PBS containing 1 mM phenyl methyl sulphonyl fluoride at 4^{o} C following sonicated for 30 minnutes on ice with 40 W. The homogenates centrifuged at 20000 g for 30 min at 4^{o} C, the supernatant were stored at -70^{o} C. The proteins of the extracts were determined using the method of Warburg (Tietz 1986, Hudson and Hay 1994).

Sera collection

Positive and negative references sera collected from infested and uninfested cattle were used to standardize the ELISA test. The positive sera were obtained by infesting seven three months-old calves with 10,000 of *R. annulatus* larvae. Weekly for 12 consecutive weeks, blood was collected by jugular vein and allowed to clot for 2 hour at room temperature and then centrifuged at 800 g for 15 min and extracted serum aliquoted and kept at -20^{o} C.

ELISA

ELISA plates were coated with 4 µg per well of three antigens in 20 mM carbonate buffer (pH= 9.6) by incubation overnight at 4^{o} C (Harlow and Lane 1988). Having been washed three times, the plates were incubated for 1 hour at 37^{o} C with 5% skim milk-PBS. Then, test sera diluted 1/200 were incubated at 37^{o} C. After 1 hour, the plates were washed three times and 100 µl of 1/2000 diluted sheep anti –bovine IgG conjugated with peroxidase were added to the individual wells. After incubation at 37^{o} C for 1 hour, the plates were washed again and the enzyme substrate was added. The substrate was prepared by dissolving 62 mg of (2, 2, Azino-bis 3-ethyl benz- thiazoline-6-sulfonic acid) in 50 ml of distilled water and the optical density (OD) was determined at 405 nm. Sera from cattle were tested against anti *R. annulatus* antibodies at 1-week intervals from week 0 (the week of infestation) to week 9.

SDS-PAGE electrophoresis of different tissues

Tick extract preparations (20 µg/lane) were resolved by sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) using a discontinuous gel system in reducing condition (Laemmli 1970) with an acrylamide concentration of 5% in stacking gel and 12% in

the separation gel. A high molecular weight protein ladder (Fermentas) was used as molecular weight markers. Finally, the gel was stained with Coomassie brilliant blue.

Western blot analysis

Soluble proteins extracted from salivary glands, ovaries and larvae of *R. annulatus* (20 µg/lane) were resolved by SDS-PAGE using a discontinuous gel system as described previously (Laemmli 1970). The antigens separated were transferred to nitrocellulose paper (NCP) (Wang and Nuttal 1994), and non- specific reactive sites on the NCP were blocked for 1h in Tris-buffered saline with 0.5% tween 20 (TBS-T pH8), containing 3% bovine skim milk on a shaking plate at room temperature. The NCP was incubated for 1 h at 37^{o} C with bovine sera diluted 1/50 in PBS (pH= 7.2). The NCP was incubated for a further 1 h at 37^{o}C with sheep anti- bovine IgG (conjugated with HRPO) diluted 1/2000 in PBS-tween20. After 1 hour, the paper was washed and reacted with Di amino benzidine (DAB) (Sigma) as a substrate. The NCP was washed three times between each step of the assay with PBS-Tween.

Results

Characterization of humoral immune response

The experimental infestation of seven cattle with approximately 10000 larvae of *R. annulatus* was performed to characterize the humoral immune response to tick extracts. Furthermore, approximately 2500±252 ticks were collected per animal between Days 21 and 26.

Figure 1 shows changes of antibody levels measured by ELISA in the sera of the experimentally infested animals against salivary glands, ovaries, and larvae antigens. All extracts showed positive reactions with all sera tested starting from the first week of infestation. A steady increase of the antibody level was observed during the experiment from week 0 to week 9, however, a slight decrease antibody production occurred in the week 9 of infestation.

SDS-PAGE electrophoresis of different tissues

Coomassie brilliant blue stain differentiates a vast profile of proteins in tick's antigens. In salivary gland, ovary and larval extracts 22, 18, and 25 fractions were more dominant respectively. Protein bands ranging in molecular weight from 18 to more than 200 KDa .The bands >200, 170, 117, 100, 93, 70, 55, 50, 44, 37, 30 and 27 kDa were common in all extracts (See in Fig. 2).

Western blot analysis of salivary, ovary antigens and larva extracts

In order to analyze the peptides reacting with the bovine antibodies, Western blot assays were carried out using the sera with higher ELISA OD that belonged to 21st day after infestation. The profile of three different antigens recognized by cattle positive sera are presented in Fig.3. Serum from experimentally infested cattle recognized approximately 15 fractions from soluble antigens extracted from salivary gland and larvae. Challenged cattle also recognized 14 protein bands in the ovary extract.

The negative control sera recognized no band. These findings illustrate the recognition of common proteins with molecular weight of 170, 117, 100, 70, 37, 33 and 30 kDa from different antigens by sera of infested cattle.

Discussion

It is extremely important to have defined immunogenic molecules in order to dissect the events involved in acquisition and expression of tick resistance. Some studies clearly show that a number of tick tissues can be used to induce artificial resistance to tick feeding, an approach that holds significant

promise as an alternative method for tick control (Wickel et al. 1988, Turni et al. 2004, Leal et al. 2006, Willadsen 2006, Cruz et al. 2008). Antibodies reactive with tick extracts have been described by several researchers (Willadsen 1980, Wikel 1984). Immunoelectrophoresis, immunofluorescence, ELISA (Wikel and Osburn 1982, Brossard and Wikel 1997, Cruz et al. 2008, Perez 2010, Kopp et al. 2010) have been used to examine serological responses against ticks. Many investigators have used the Western blot technique to identify relevant antigens of several tick species using sera collected from different animal hosts (Whelen et al. 1986, Brown 1988, Trimnel 2005, Cruz et al. 2008). Salivary glands, gut and larval extracts have been tested as immunogens to control tick infestation (Opdebeeck et al. 1989, Barriga et al. 1991). Da Silva Vaz et al. (1994) also indicated that *Rhipicephalus* (*Boophilus*) *microplus* infested cattle develop antibodies to components of tick salivary gland, gut, embryo and larvae during the first infestation.

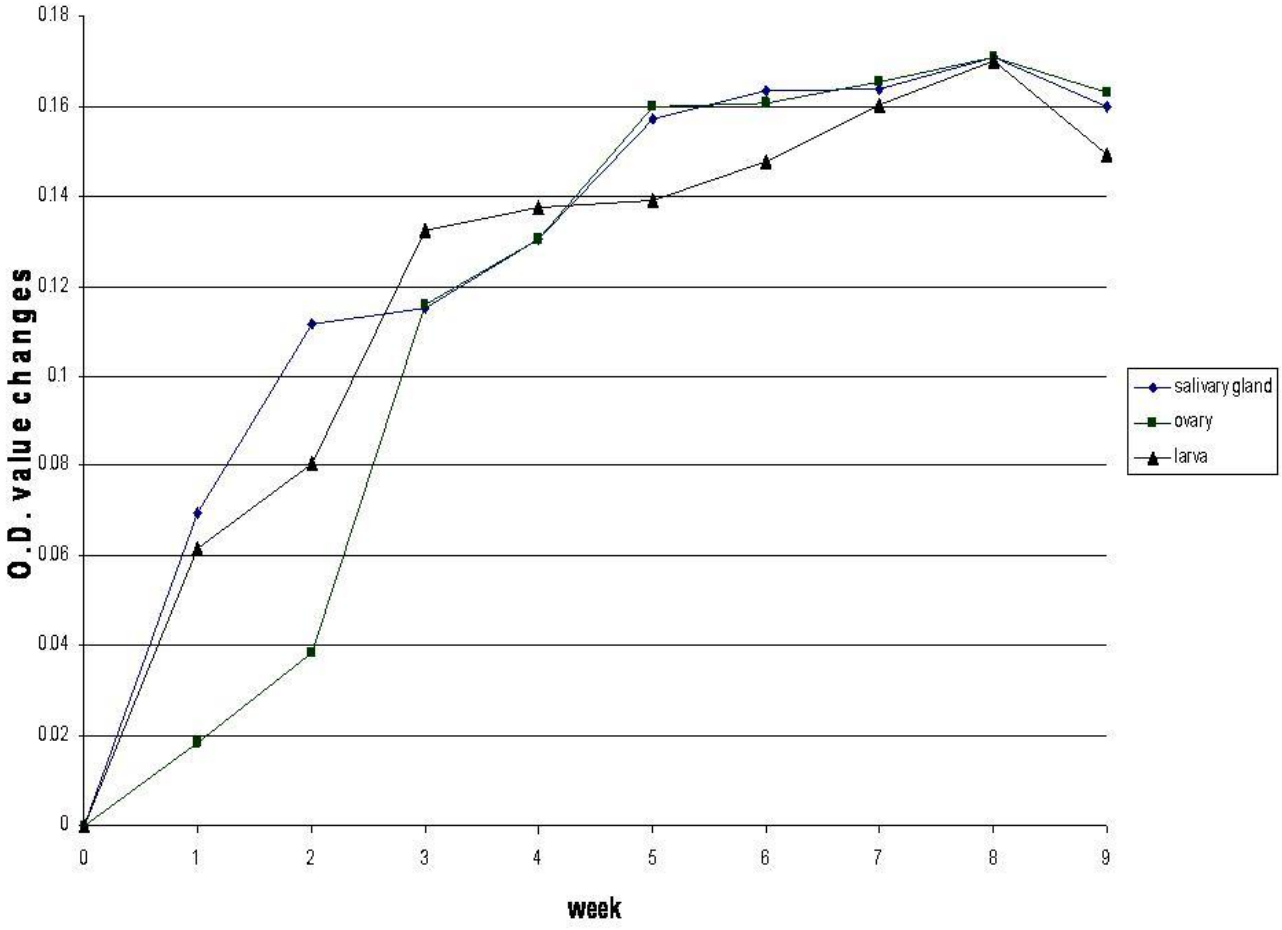

Fig. 1. The comparison of serum antibody response changes (OD value changes) in seven calves infested with *R. annulatus* using different tissues antigens. Results expressed as mean of seven infested bovine sera

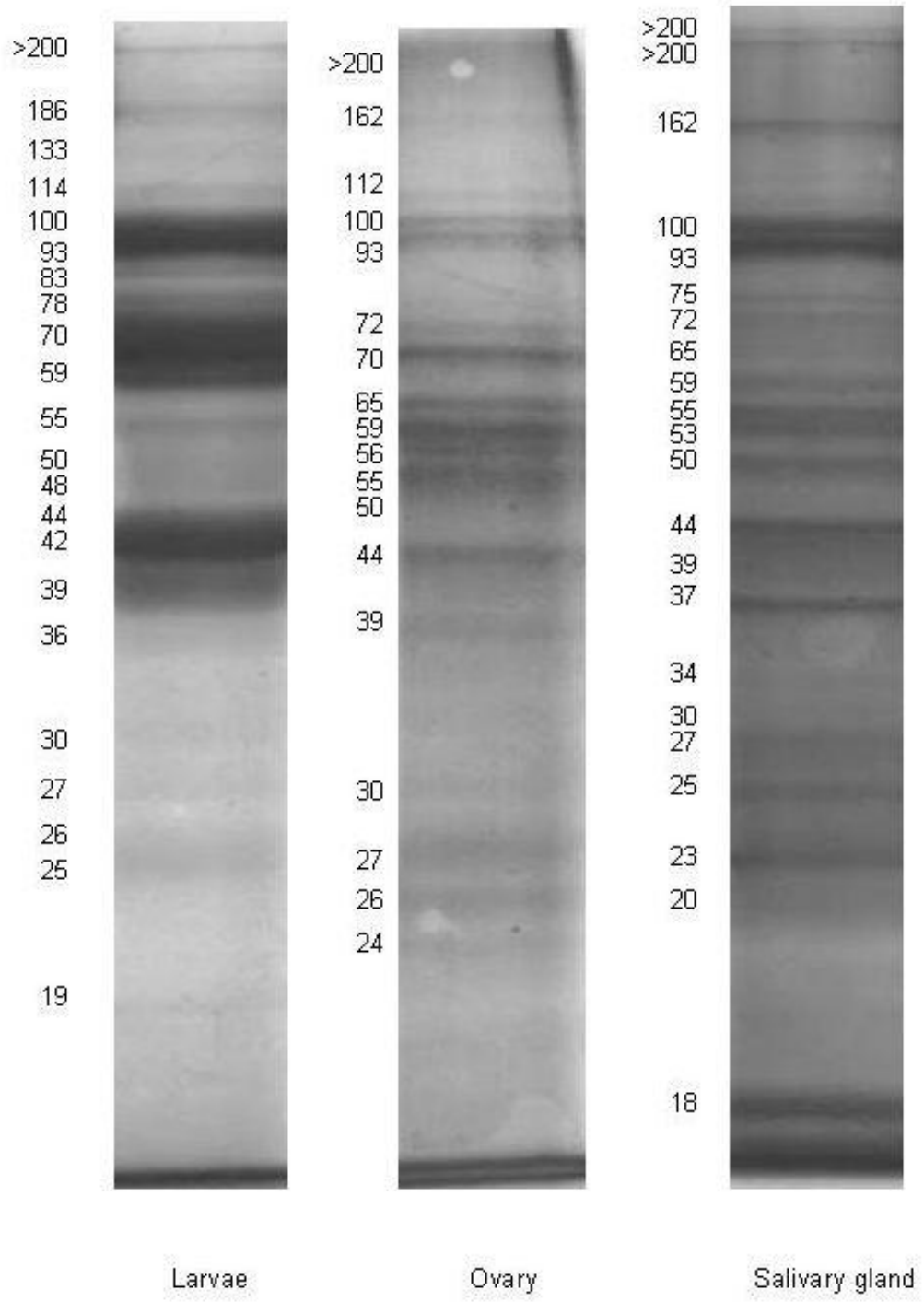

Fig. 2. SDS-PAGE analysis of proteins derived from salivary glands, ovary and larvae of *Rhipicephalus* (*Boophilus*) *annulatus*. The numbers in left side of each column indicate the molecular weight (kD) of each fraction

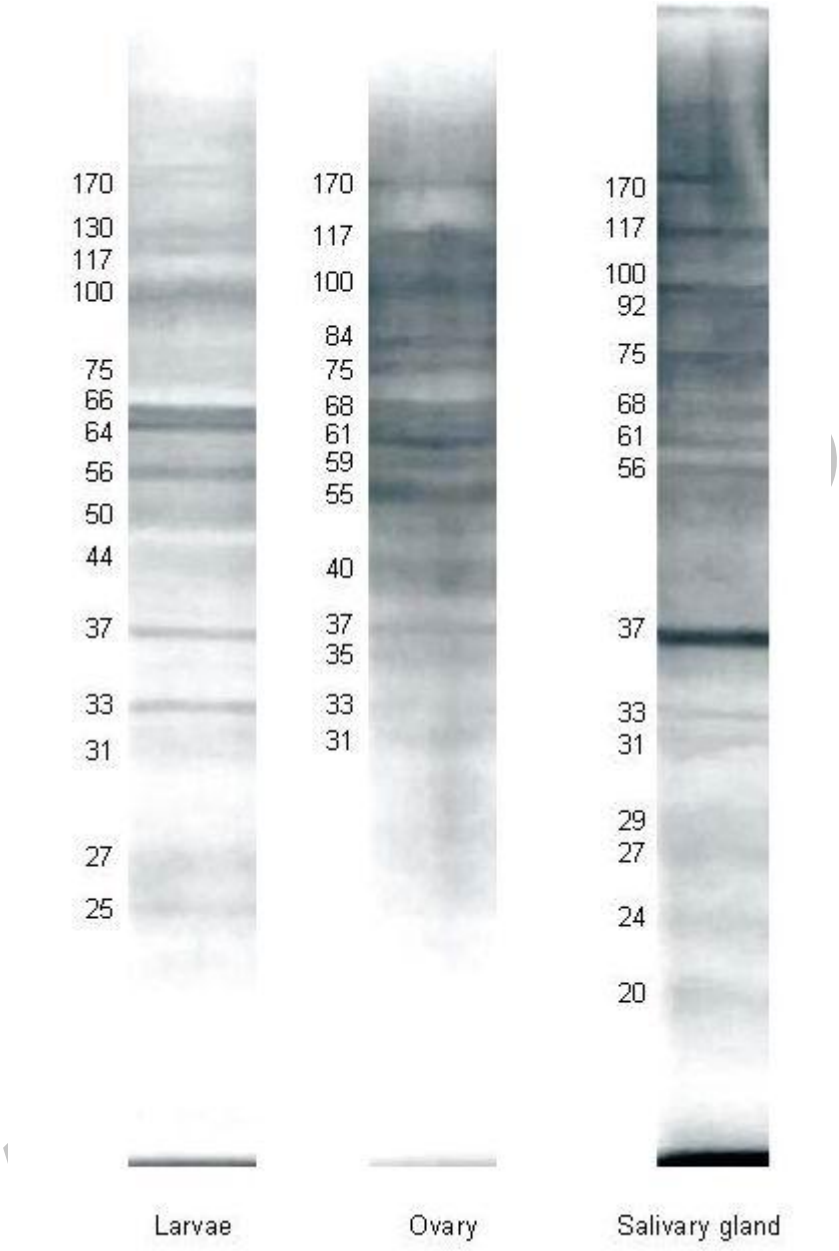

Fig. 3. Western blot analysis of *R. annulatus* antigens by tick infested bovine sera (positive serum). The numbers in left side of each column indicate the molecular weight of each fraction

In the present study, ELISA was used to compare the antibody levels developed against *R. annulatus* salivary gland, ovary and larvae protein extracts of sera from cattle submitted to experimental infestation. The results of this part of our study showed the impact of approximately high-density tick infestation on humoral immune responses induced in experimentally infested cattle suggesting the antibody level increased during the period of infestation and as mentioned in Fig. 1, a significant increase was observed in third week after infestation that the greatest amount of ticks were harvested at this time. These results are in agreement with our previous study and some other researchers (Oegden et al.

2002, Cruz et al. 2008, Nikpay et al. 2008, Perez 2010). Using Western blot analysis, some basic information have provided about antigens extracted from *R. annulatus* tick recognized by sera collected from cattle infested with this species. The recognized fractions could be used as target for subsequent antigen characterization studies, and points out some probable antigenic sites in specific tick tissues. ELISA and Western blot data indicate that *R. annulatus* infested cattle develop antibodies to components of tick salivary glands, ovaries and larvae during the infestation.

In our experiment, all extracts showed the same absorbance value changes. One possible explanation of the similarities in the reactivity of different tissues is that the bovine is inoculated with various tissue antigens during the tick feeding process, or that there are common antigens in different tissues of *R. annulatus* and according to these results it seemed to be a correct assumption. These findings further illustrate the presence of common crucial antigens, which are believed to be 170, 117, 100, 70, 37, 33 and 30 kDa depending upon the assay system employed because they were recognized in three extracts by anti tick sera. Electerophoretic comparison of salivary gland extracts of *R. annulatus* specimens demonstrated some similar proteins with salivary gland extracts of Hyalomma specimens (Nabian et al. 2005). Also it was shown that some proteins of salivary gland of *R. annulatus* tick changed in different temperatures but protein bands of 29, 36, 55 and 97 were constant at all temperatures (Nabian et al. 2005).

For the first time in the current study salivary glands, ovary and larvae extracts have been used as antigenic sources at the same time and it was showed that ovaries could be a source of immunogenic proteins such as the other tissues of tick studied previously. It should be mentioned that there was not any similar study about *R. annulatus* tick and this investigation was conducted for the first time in Iran to characterize the immunogenic antigens in this species.

The findings of the present study establish the presence of common antigens between ovary, larvae, and salivary glands of *R. annulatus* ticks. Further studies will be aimed at the isolation of common proteins and testing for antigenicity and immunogenicity followed by purification and subsequent in vitro expression for potential vaccine development.

Vaccination of hosts with antigenic tissues and secretions of ticks may provide a new method to control ticks. Extracts of whole tick body homogenates and various organs especially have been tested as to their ability to induce tick resistance in the host (McGowan et al. 1980). The significance of these observations is with regard to the induction of host immunity to ticks and the development of tick vaccines using common proteins.

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