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Immunization of rabbits against *Hyalomma anatolicum anatolicum* using larval and nymphal extracts

Moshaveri-nia, A.[1]; Naiminik, M. H.[1]; Razmi, G. R.[2*] and Hashemitabar, G. R.[2]

[1]Graduated from School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

[*]**Correspondence:** G. R. Razmi, Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: razmi@ferdowsi.um.ac.ir

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Summary

The protective capacity of the crude extracts from larval and nymphal stages of *Hyalomma anatolicum anatolicum* was examined in the New Zealand white rabbits. The rabbits were randomly divided into 3 groups of five animals. The rabbits in groups 1 and 2 were immunized with crude extracts of larval and nymphal antigens, respectively and group 3 was served as control. Following challenge of each rabbit with 2000 larvae of *H. a. anatolicum*, engorged nymphs were collected, weighed and then cultured in an incubator. A significant decrease in weight of engorged nymphs was only observed in group 1 that immunized with crude extracts of tick's larvae ($P<0.05$). Polypeptide profile of the larval and nymphal extracts was analysed by SDS-PAGE, and antigenic pattern with serum of immunized rabbits was evaluated by Dot and Western blot test. The molecular weight of the fraction of larval extract after SDS-PAGE showed six polypeptide bands as follows: 97, 84, 66, 55, 45 and 36 kDa, and in nymphal extract 13 polypeptide bands of 205, 116, 97, 84, 66, 55, 45, 36, 29, 24 and 20 kDa and two bands between 116 and 205 kDa were found. In Western blotting, positive reaction was only observed with sera of group 1 in the bands with 97, 84 and 66 kDa. It seems that the larval extract of *H. a. anatolicum* can be used as a source of biological material for isolation of protective antigen.

Key words: *Hyalomma a. anatolicum*, Immunization, Larval antigen, Nymphal antigen, Rabbit

Introduction

Hyalomma anatolicum anatolicum is the most common tick vector of bovine and ovine tropical theileriosis in Iran (Hooshmand-Rad and Hawa, 1973; Hashemi-Fesharaki, 1986) which causes considerable production losses in the livestock industry. Without effective tick control, it would be virtually impossible to raise livestock production. Although, a practical method for controlling of ticks is the use of acaricides, increasing in the acaricide-resistant ticks show the importance of research on alternative methods of tick control. Immunization of host against ticks is a promising alternative to the expensive and laborious acaricide treatment. To date, only the midgut Bm86 vaccine that affects *Boophilus microplus* feeding on cattle has successfully passed all tests and has been commercialized in Australia (Willadsen *et al*., 1995) and Cuba (De la Fuente *et al*., 1998). Acquired immunity against the tick *H. a. anatolicum* has been studied previously in rabbits (Manohar and Banerjee, 1992) and cattle (Sran *et al*., 1996; Sangwan *et al*., 1998; Ghosh *et al*., 1999). The present study was conducted to study the effect of immunization in rabbits using larval and nymphal extracts of *H. a. anatolicum* against experimental challenge infestation with ticks.

Materials and Methods

Ticks

Laboratory colonies of *H. a. anatolicum* were used in the experiment. These colonies were fed on ears of rabbits. In the

laboratory, all stages of ticks were kept at 28°C in a biological oxygen demand incubator in cotton-plugged glass tubes inside a desiccator over a 10% solution of potassium hydroxide to maintain the required relative humidity of 85%.

Experimental animal

The New Zealand white rabbits of both sexes at the age of 4-5 months were procured from the branch of Razi Institute in Mashhad.

Antigen preparation

The larvae and engorged nymph ticks were surface-sterilized by washing in 30% hydrogen peroxide followed by rinses in 70% alcohol and in phosphate buffered saline (PBS, 0.01 M, pH = 7.3 composed of NaCl, K_2HPO_4, KH_2PO_4, 100 IU/ml penicillin, 100 µg/ml streptomycin and distilled water). Then, the extracted antigens were prepared by thoroughly disrupting larvae and nymph ticks in PBS with sterile pestle and mortar after addition of sterilized glass beads. The suspensions were filtered through a Buchner funnel (pore size, 25-50 µm) to remove the tick cuticles. The sample was homogenized by ultrasonic disintegrator (Soniprep-150, MSE England) using standard probe at 200 W for 3 min, while cooling on ice. The protein concentration of the extracted antigens was determined by the method of Lowry *et al*. (1951).

Immunization

Rabbits were randomly divided into three groups of five animals. The rabbits were inoculated subcutaneously in 3 divided doses on day 0, 14 and 28. The rabbits in all groups received antigen in Freund's complete adjuvant (FCA) on day zero. The antigen was mixed with equal amount of Freund's incomplete adjuvant (FIA) for inoculation on days 14 and 28. Two and half ml of the prepared antigen was mixed thoroughly with equal volume of FCA, FIA or PBS and then 1 ml of the antigen was inoculated subcutaneously to each rabbit. The control rabbits were injected with both PBS and adjuvant.

Infestation challenge

Two weeks after the third immunization, each rabbit of the test and control groups were challenged with 2000 active unfed larvae. After 2 weeks, engorged nymph ticks were collected, weighed and then placed individually in glass tubes in an incubator at 28°C with a relative humidity of 85%. Biological parameters, including engorgement period, weight of engorged nymph and moulting length were determined for each group. Data were analysed by Student's t-test.

Serum preparation

The sera of the control and two test groups were prepared from fresh blood clotted in refrigerator for 1 h. The serum was separated from the red blood cells by centrifuging at 6000 rpm for 10 min and aliquoted and stored at -20°C.

SDS-PAGE and Western blot analysis

SDS-PAGE was carried out as described by Laemmli (1970) using a mini gel. Samples prepared by running in SDS-PAGE were probed with sera obtained from rabbits in the test and control groups by immunoblotting as described by Towbin *et al*. (1979).

Results

The results of this experiment are shown in Table 1. Immunization with larval antigen significantly affected the weight of engorged *H. a. anatolicum* nymph ($P<0.05$).

The SDS-PAGE of larval extract showed

Table 1: Results from *H. a. anatolicum* infestation of immunized and control rabbits (mean ± SD)

Biological parameters of tick	Larval tick extract[a] (group 1)	Nymphal tick extract[b] (group 2)	Control
Engorgement period (days)	14 ± 1.9	14 ± 1.9	14 ± 1.9
Weight of engorged nymph (mg)	15.18 ± 10.79[*]	35.4 ± 5.5	34.15 ± 0.5
Moulting length (days)	17 ± 0.4	16 ± 0.7	16 ± 0.7

[a] each dose contained 6 mg protein in 1 ml of PBS. [b] each dose contained 8 mg protein in 1 ml of PBS. [*]Significantly different from control group by Student's t-test ($P<0.05$)

six polypeptides of 97, 84, 66, 55, 45 and 36 kDa and nymphal extract showed thirteen polypeptides of 205, 116, 97, 84, 66, 55, 45, 36, 29, 24 and 20 kDa and two bands between 116 and 205 kDa (Fig. 1). In Western blotting, only sera of group 1 (larval extract) showed 97, 84 and 66 kDa bands.

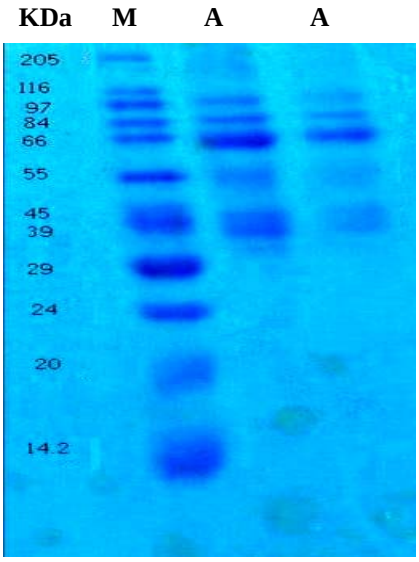

Fig. 1: Polypeptide pattern of larval antigen after staining with Coomassie blue. M = Molecular weight marker; A = Larval antigen

Discussion

The principle of vaccination against ectoparasites using concealed antigens is now well established (Willadsen and Kemp, 1988). The concept has led to commercial development of a vaccine against the tick *Boophilus microplus*, known as TickGARD (Willadsen *et al*., 1995). This work stimulates the further development of vaccines against other tick species. Immunization trial conducted in the present study using larval and nymphal antigens of *H. a. anatolicum* were based on the assumption that ticks feeding on appropriately immunized hosts might ingest antibodies, specific for target antigen in midgut of tick, which produces deleterious effect on the feeding and reproductive performance of the tick (Allen and Humphreys, 1979; Johnston *et al*., 1986). In the pervious studies, a significant protection against adults of *H. a. anatolicum* following immunization of rabbits and calves with antigen derived from midgut was observed (Razmi *et al*., 2003, 2005). In three-host tick such as *H. a. anatolicum*, one antigen alone cannot induce the cross-protection among different stages of tick development. Therefore, it is very important to identify the protective antigens in all stages of tick development. In the present study, when the protective efficacy of the larval and nymphal extract of *H. a. anatolicum* were compared, only the larval extract of *H. a. anatolicum* showed significant protection against ticks challenge in immunized rabbits. This may be due to the presence of higher immunoprotective antigen concentrations in larval extract in comparison to nymphal extract. Allen and Humphreys (1979), Opdebeeck *et al*. (1989) and Varma *et al*. (1990) showed that the antigens from partially fed and unfed ticks can be protective in various host animals. Agbede and Kemp (1986) also reported that partial feeding of ticks increases the gut cells to maximum as the source of concealed antigens. Following Western blot examination, the sera of group 1 that were immunized with larval extract showed 66, 84 and 97 kDa antigens. In similar studies, Norouzi (2004) and Parmar *et al*. (1996) also detected 66 and 84 kDa antigens such as immunodominant proteins in the midgut and salivary glands extracts of *H. a. anatolicum*. Based on the above-mentioned results, a 66 kDa protein in larval extract can be a common immunoprotective antigen in all stages of *H. a. anatolicum*, but the identified protein must be tested for its potential protective effect. Although, it has been suggested that future vaccines should be cross-protective, however, very limited information are available on the identification of candidate antigenic molecules from *H. a. anatolicum*. In some studies, high protective effect against tick infestation was reported by using purified 39 kDa larval antigen (Ghosh *et al*., 1999), 37 kDa larval antigen (Das *et al*., 2000, 2005) and 34 kDa larval glycoprotein (Singh and Ghosh, 2003) of *H. a. anatolicum*. These studies demonstrated that the larvae of *H. a. anatolicum* are an important source of biological material for isolation of protective antigens. The results of the present study also confirmed the protective effect of larval

extract. However, in order to develop a suitable immunoprotective measure against *H. a. anatolicum*, improvement in methods for isolation and purification of the immunogenic antigens from larval extract is necessary.

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