

Preparation of antigens from midgut of *Hyalomma anatolicum anatolicum* and determining their immunoprotective effects

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ABSTRACT

Partially fed (4 -5 days) *H. a. anatolicum* (Iranian isolate) female adult ticks were used for preparation of antigens of midgut which are, the gut supernate antigen (GS), gut membrane antigen (GM), triton extracted gut membrane antigen (TX-GM) and sodium dodecyl sulphate treated Triton extracted gut membrane antigen (SDS-TX-GM). The New Zealand white rabbits were immunized with GS, GM, TX- GM and SDS-TX-GM antigens. The first inoculation on day 0 was administered after emulsification with Freund's complete adjuvant and second inoculation was given on day 14 with incomplete Freund's adjuvant and third inoculation was given on day 28 with incomplete Freund's adjuvant. Blood drawn was from the marginal ear vein on day 38. The humoral immune response was studied by Enzyme Linked Immunosorbent Assay (ELISA) and antibody titers in the sera of immunized rabbits on 10 days after the last booster dose of antigen was 1/32000. After electrophoresis of purified midgut antigens (GS, GM, TX-GM, SDS-TX-GM) 4µg of each were applied on a 10% acryl amide gel and blotted to nitrocellulose paper for reaction with its concern immune sera. By analysis of this Western blot, we got bands of 110, 95, 85, 66, 59, 49, 42 Kda for GSAg, 95, 85, 66, 49, 42 Kda for GM Ag, 66, 49 Kda for TX-GMAg and 66Kda for SDS-TX-GMAg responsible for induction of resistance in the host. The prepared antigens showed engorged nymphs ranged from 32-41%, 29-56%, 29-49% and 29-47% in response to GS, GM, TX-GM and SDS-TX-GM antigens respectively. Immunodominant antigenic proteins of 66 Kda were detected in the sera of rabbits immunized by all above antigens which can be candidate for single immunogen if in future studies show acceptable results. The immunized rabbits were challenged, 10 days after the last booster dose of antigens, with 700 larvae of *H. a. anatolicum* ticks by releasing them on one ear of rabbits and maximum protection in rabbits against *H. a. anatolicum* was induced with the one immunized with GSAg.

Keywords: *Hyalomma anatolicum anatolicum*, midgut, Western blot, ELISA

INTRODUCTION

Ticks and tick borne diseases constitute important constrains on the livestock industry, imparticular for

cattle in tropical and subtropical regions of the world. They cause immense economics losses directly by decreasing in milk and meat production and indirectly through transmission of various diseases like bovine tropical theileriosis, babesiosis

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and anaplasmosis (Opdebeeck *et al* 1988 a). Hard ticks (Acari: Ixodidae) are important obligatory blood feeding external parasites transmitting different viral, bacterial and protozoan agents to human and animals. There are six different genera of family ixodidae in Iran. The most prevalent species found in Iran are *Hyalomma*, *Rhipicephalus* spp. acting as vector of *Theileria annulata* and *Babesia* spp. in cattle and sheep. Theileriosis due to *Theileria annulata* is a disease causing drastic losses of cattle throughout a large area of Middle-East, South Asia and North Africa. It is an accepted fact that *Theileria annulata* infection is the worst parasitic disease of cattle in Iran. A vaccine has been produced since 1973 for pure-bred and cross-bred cattle which are highly at risk of the disease. The cost of using this virulent vaccine is two million dollars annually. Hooshmand-rad and Fesharki reported that *H. anatolicum anatolicum* is the prevalent tick and the main vector of sheep and bovine theileriosis in Iran (Hooshmand-rad *et al* 1973, Hashemi-Fesharki 1987). Direct impacts due to the ixodidae tick bite and their blood feeding from the host also is reported. Anemia, total loss of protein mass and defects to the hide products are also have got minor importance. Tick toxicosis and tick paralysis in human and animals are reported. There is at least one reported case of tick paralysis in human from Iran (Abdigoudarzi *et al* 2006). Lyme disease is a bacterial disease transmitted by *Ixodes* spp. in US and Europe. Suspected clinical cases of Lyme disease have been reported from Iran (Tabatabaie *et al* 2006). CCHF is a viral lethal disease transmitted by *Hyalomma* ticks and there were almost 45 cases of human disease from 1998 to 2004 from Iran.

Biological control of ticks by the use of predators, pathogens, sterile hybrid techniques and pheromones have had limited success (Nari 1990). Recently, recombinant vaccines against *Boophilus* ticks have become a new component of such integrated control strategies in Latin America

(mainly Cuba Gavac TM)(Rodrigues *et al* 1995) and also in Australia (Tick GARD) (Willadsen *et al* 1995), where *B. microplus* is the major tick species infesting cattle. But in Iran *Hyalomma anatolicum anatolicum* is the most common *Hyalomma* ticks and act as vector of economically important disease such as bovine tropical *Theileriosis*. The present study has been carried out to determine the protective efficiency of *Hyalomma anatolicum anatolicum* midgut derived antigens in rabbits challenged with it.

MATERIALS AND METHODS

Preparation of tick midgut antigens. *Hyalomma anatolicum anatolicum* was obtained from Parasitology Department of Razi research Institute. These partially fed adult ticks were used for preparation of antigen. The midgut diverticula's were dissected out and stored in cold PBS, pH 7.2 at -20 °C until use. This material was thawed and homogenized in membrane buffer, 10mM PBS buffer, pH 7.2, containing 5 mM EDTA. The material was centrifuged at 10,000 g for 30 min at 4° C. The supernate was ultra centrifuged at 100,000g for one hour at 4 °C. The supernate was called GSAg and pellet called GMAg. A part of GMAg was treated with 0.5% Tritonx-100 and used for homogenizing. The material was stirred for one hour and ultra centrifuged at 100,000g for one hour. The supernate was called Tx-GMAg. The pellet was further homogenized in membrane buffer and stirred with 0.01M SDS in membrane buffer for one hour and centrifuged at 100,000g for one hour. The supernate was called SDS-Tx-GMAg and pellet was discarded.

Immunization of rabbits. Protein contents of GSAg and GMAg was estimated by Lowry method and for other two antigens determined approximately same as above antigens. Ten New Zealand white rabbits, 3-4 months of age, all male weighed 1.5-2.0 Kg were obtained from Razi

animal house and divided into five groups. Groups I to IV were immunized with four types of antigens and the group V was kept as unimmunized control. First immunization dose was administered on day 0 after emulsification with Freund's complete adjuvant subcutaneously and intramuscularly, second dose was given on day 14 with the incomplete Freund's adjuvant same as before. The third dose was given on day 28 same as second dose and on day 38 the blood samples were collected from all rabbits and their sera were separated. The amount of antigen protein was 350µg in each inoculation.

Enzyme linked immunosorbant assay (ELISA).

Flat bottom 96-well ELISA plates were coated with four types of midgut antigens, 4µg of each antigen in different wells in 100 µl of coating buffer (0.1M carbonate-bicarbonate buffer, pH 9.6) were added to wells. The plates were incubated at 4 °C overnight and washed four times with PBS, pH 7.2, then each well were blocked by adding 200µl of 3 percent BSA in PBS, pH 7.2 and were incubated for one hour at 37 °C. After incubation, each plate was washed four times with PBS containing 0.5% Tween-20. The serum samples from animal of each group were diluted in PBS-T and 100 µl of it was added to wells. The plates were incubated at 37 °C in moist chamber for one hour and then washed four times with PBS-T. Then 100µl of diluted goat anti-rabbit IgG HRP conjugate were added to each well and plates were incubated at 37 °C for one hour and washed four times with PBS-T. Then 100µl of TMB substrate were added to each well and kept at room temperature for 15-20 min. and then reaction was stopped with H₂SO₄(1N) by adding 50µl to each well and OD was measured at 450 nm in ELISA reader (BioRad,USA).

Western blotting. Four types of antigens which were prepared, run by SDS polyacrylamide gel electrophoresis on 10% gel and transferred to nitrocellulose in an Bio-Rad semi-dry system according to the manufacturer's instructions, using

transfer buffer containing 0.025M Tris 0.04 M glycine and 20% (V/V) methanol. After blocking in 5% BSA in PBS (0.01 M, pH 7.2), the blots were developed using high titer animal sera in each group of antigens essentially as described for the ELISA. The peroxidase labeled secondary antibody was detected with chloronaphtol (5 mg/ml) in PBS (0.01 M pH 7.2) as substrate.

Challenge with ticks. There are laboratory bred *Hyalomma anatolicum anatolicum*(Bosher strain) and they are routinely grown up using 70 to 80 % relative humidity and 28 ° C for keeping eggs, starved larvae, engorged nymphs and adults. This tick has a two host strategy of life cycle. They are grown up at Reference Laboratory for ticks, Department of Parasitology, Razi Institute. All immunized and control rabbits were challenged 10 days after the last booster dose with 700 starved larvae as follow:

Fresh laid eggs from a female was weighed and divided as each pack was equal to 700 eggs and kept in different glass tubes. The hatched larvae were put in ear bag on different tested and control rabbits. The engorged nymphs were collected at the same time and have been counted for each rabbit.

RESULTS

Protein estimation of antigens.Protein of GS Ag and GMAg were determined by Lowry method and were 820 µg/ml and 830 µg/ml respectively. Protein content for other antigens (TX-GMAg & SDS-TX-GMAg) were approximately the same as above antigens.

Humoral immune response. Humoral immune response was determined by ELISA and it is shown in table 1. The antibody titer of GS Ag group reached to 1/32000 which showed higher titer than other antigen groups.

Western blotting. The appeared bands of proteins by running all antigens and their incubation with highest titer antibody from each group showed

on figs, 1, 2, 3 and 4 which on calculating their Rf value, the GSAg contains proteins with 110, 95, 85, 66, 59, 49 and 42 Kd molecular weights, GMAG contains proteins with 95,85,66,49 and 42 Kd molecular weights, TX-GMAG contains proteins with 66 and 49 Kd molecular weights and SDS-TX-GMAG contain protein 66 Kd molecular weight. Protein 66 Kd was seen for all the antigens which can be considered as nominated antigen.

Feeding and moulting performance of larvae to engorged nymphs. The results had shown that the engorged nymphs ranged from 45-59% in control group but in immunized groups it ranged from 32-41%, 29-56%, 29-49% and 29-47% in response to GS, GM, TX-GM and SDS-TX-GM antigens respectively. The Mean engorged weight was calculated for each group of engorged nymphs and compared with t student test. After comparison a significant ($p < 0.01$) reduction in the percentage of engorged nymphs was seen on rabbits immunized with GS antigen.

Table 1. Antibody titer of all antigens (Gs, GM, TX-GM, SDS-TX-GM) from immunized rabbits sera by ELISA.

Ab dil.	GSAg			GMAG	Tx-GMAG	SDS-Tx-GMAG
	8µg	6µg	4µg	4µg	4µg	4µg
1 : 25	1.905	1.883	1.793	1.739	1.512	1.552
1 : 50	1.839	1.881	1.741	1.796	1.500	1.502
1 : 100	1.883	1.779	1.673	1.763	1.476	1.500
1 : 200	1.834	1.765	1.644	1.755	1.435	1.489
1 : 400	1.828	1.732	1.607	1.714	1.401	1.449
1 : 800	1.812	1.699	1.603	1.622	1.340	1.421
1:1000	1.801	1.642	1.596	1.601	1.321	1.402
1:2000	1.765	1.604	1.523	1.578	1.300	1.382
1:4000	1.736	1.574	1.508	1.512	1.203	1.218
1:8000	1.641	1.521	1.432	1.469	1.143	1.109
1:16000	1.598	1.503	1.402	1.418	1.106	1.002
1:32000	1.476	1.458	1.308	1.214	0.945	0.932
1:100	0.321	0.301	0.289	0.299	0.256	0.264
Normal						
PBS	0.082	0.082	0.073	0.082	0.064	0.063

DISCUSSION

By preparing midgut antigens from *Hyalomma anatolicum anatolicum* ticks, our main purpose was whether determining immunological approach is feasible or not. (Allen & Humphreys 1979) was also obtained great resistance in guinea pigs against *D.andersoni* on immunization with extracts prepared from it.

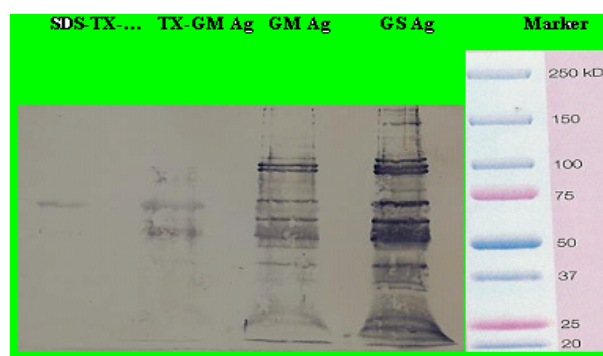


Figure.1 Western Blot of all antigens with GS Ag antibody.

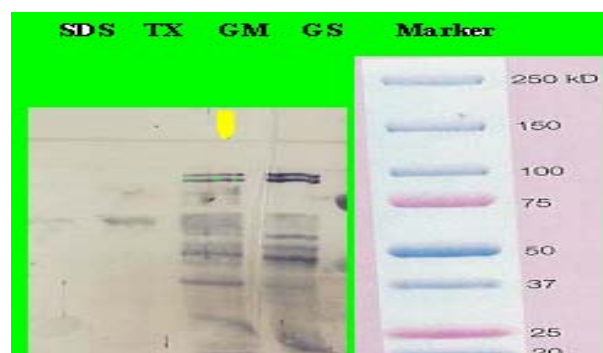


Figure2. Western blot of all antigens with GM Ag antibodies.

Attempts to artificially immunize potential hosts against ticks using crude antigens, whole tick extracts (McGowan *et al* 1981), reproductive organs and gut tissues (Allen & Humphreys 1979) and salivary glands (Wikel 1981, Brown & Askenase 1983) have already met. Salivary antigens also involved in the acquisition of resistance to *H. anatolicum anatolicum* an important vector of bovine tropical theileriosis (Gill *et al* 1986). A fractionated midgut supernate antigen (GS-F Ag) increase so much in the engorgement and preoviposition

periods and decrease in the engorged weight, egg mass weight and reproductive index were observed for adult female ticks when fed on the immunized calves (Banerjee *et al* 2003).



Figure3. Western blot of three antigens with SDS-TX-GM Ag.

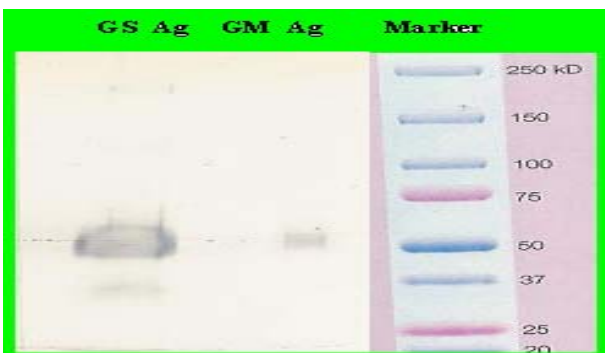


Figure4. Western blot two antigens with TX-GM Ag

One of the antigens with a molecular weight of 37 kDa isolated from larvae of *H.a.anatolicum* was found to have some adverse effect on development of *Theileria annulata* in the vector tick. This progress in the development of immunoprophylactic measures against *H. a. anatolicum* is also discussed by Gosh *et al* (1998). A significant reduction in percent engorgement and percent moulting of *H.a.anatolicum* larvae was observed when fed on the rabbits immunized with GS antigen. This indicates that there might be sharing of gut antigens between the larvae and adult stages of the ticks. If a protein can be identified which is common to all the development stages (larvae, nymph and adult) and protective in nature, it may be exploited in a better

way for control of ticks rather than aging stage specific proteins. A maximum level of protection was induced in response to GS Ag in terms of parameters of repletion of ticks. Opdebeeck *et al* (1988 a, b) reported significant antibody level against GS and GM antigen, derived from *B. microplus* tick in sera from the vaccinated cattle. There was significant correlation between the antibody and protection against *B.microplus* in immunized cattle (Opdebeeck *et al* 1988 b). The main antigen of two available commercial vaccines from Cuba and Australia is BM86 which showed good result for protection against *B. microplus* (Willadsen *et al* 1989) but this antigen did not show proper protection against *H.a.anatolicum* (Willadsen & Mckenna 1991). But our prepared antigens especially GS antigen showed protection for *H. a. anatolicum*. In our study also, there was a positive relation between the antibody titer and protection against ticks in the immunized rabbits. In present study, best protection was induced with the GS antigen in rabbits against *H.a.anatolicum* and by showing different proteins from each antigen on incubation with respective immunized rabbit sera, the protein complex in each antigen recognized and protein 66 Kd can be nominated as one of the effective immunogens. Certainly for other proteins, the efficacy may be enhanced further if we fractionate, isolate, and purify potential immunogens components.

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