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Detection of midgut antigens of *Hyalomma anatolicum anatolicum* tick using SDS-PAGE and Western blot

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Summary

Ticks are important ectoparasites which are a considerable threat to human beings and to animals all over the world. Enormous economic losses annually occur in livestock production around the world as a result of their existance. One of the ways to control ticks and tick-borne diseases is to introduce resistance to these ectoparasites through immunization. For identification of the putative protective antigens, screening of large number of parasite antigens and their fractions are necessary. In this study, midguts of fed adult female *Hyalomma anatolicum anatolicum* were used to prepare antigen and to identify the midgut profile. Polypeptide profile was analysed by SDS-PAGE with 12.5% concentration under denaturated conditions and discontinuous buffer system. Humoral immunity and antigenic pattern were evaluated by Western blot. A total of 4 fractions were observed in the polypeptide profile. The molecular weight of the fractions were 97, 84, 66 and 55 kDa. The band with molecular weight of 66 kDa was dominant. Positive reaction with 84, 66 and 55 kDa bands were observed in immuno-blot of the midgut antigens.

Key words: *Hyalomma anatolicum anatolicum*, Midgut antigens, SDS-PAGE, Western blotting

Introduction

Ticks are important ectoparasites in public and animal health. They transmit many dangerous diseases to human and animals. Therefore, they are a significant threat to animals and human health (Aeschliman *et al*., 1990). *Hyalomma anatolicum anatolicum* is the most common tick vector of bovine and ovine tropical theileriosis and Crimean-Congo hemorrhagic fever in Iran (Hoshmand-Rad and Hawa, 1973; Hashemi-Fesharaki, 1986). The current methods for controlling of ticks are primarily based on the use of acaricides. Their use however, has had limited efficacy in the reduction of tick infestations and is often accompanied by serious drawbacks including selection of acaricide-resistant ticks and environmental contamination (De la Fuente and Kocan, 2003).

An alternative target and environment-friendly approach is to develop anti-ectoparasite vaccines (Trimnell *et al*., 2002). To achieve this goal, many surveys have so far been performed to detect protective antigens of ticks around the world (Das *et al*., 2000; Pipano *et al*., 2003). To date, only the midgut Bm 86 vaccine that affectcs *Boophilus microplus* feeding on cattle has successfully commercialized in Australia (Wiladsen *et al*., 1995). Since the immunity of midgut antigens of *H. anatolicum anatolicum* has been proved in Iran (Razmi *et al*., 2003), in this study the polypeptide profile of midgut antigens were identified by SDS-PAGE and Western blot.

Materials and Methods

Preparation of midgut antigens

Midgut of 100 fed adult female ticks

were separated by method described by Opdebeeck *et al*. (1988) using a stereo-microscope. Samples were mixed with PBS (pH = 7.2), sonicated in a 200 W ultrasonic disintegrator (Hielscher, Germany) on ice for 3 min, and haemogenized. The prepared suspention was centrifuged at 15000 g for 30 min at 4°C. The supernatant was then isolated as gut supernatant antigen (GSA) and stored at -20°C until used. The protein concentration of GSA sample was determined by Bradford method (1976).

Immunization procedure

Ten male rabbits were divided into two groups of five. Two and half ml of the prepared antigen was mixed thoroughly with equal volume of Freund's complete adjuvant (FCA). One ml of the adjuvanted antigen was inoculated subcutaneously to each rabbit in the test group on day zero. They were delivered two booster doses with the same amount of combined antigen with FCA and Freund's incomplete adjuvant (FIA) on days 14 and 28, respectively. The control rabbits were injected with saline in both adjuvants in a parallel inoculation regimen.

Sodium dodecyl sulphate polyacryl-amide gel electrophoresis (SDS-PAGE)

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for separation of proteins as described by Laemmli (1970) using a mini gel. The relevant solution was mixed, degassed by attaching the flask to a vacuum for 15 min, and poured into the mould as a separating gel (Biogene). The gel was allowed to set. The stacking gel was then poured on top of the running gel with the comb in place. Wide molecular weight marker (Sigma) was used to determine molecular weight of fractions. The gel was run in a Biogene electrophoresis apparatus for four hours in a gel running buffer (0.19 M glycine, 25 mM tris-base, 3.5 mM SDS). The gel was stained overnight in Coomassie blue (0.1% Coomassie G.250 (w/v) [Biogene] in 45% v/v methanol, 10% v/v acetic acid in dH_2O) followed by soaking in destaining solution (45% v/v methanol, 10% v/v acetic acid in dH_2O) with multiple changes for four hours on a gentle rocking platform. The gel was stored at 4°C in a solution containing 30% v/v methanol and 10% v/v glycerol.

Western blotting

For identification of antigenic subunits within parasite, samples prepared by running in SDS-PAGE were probed with sera obtained from immunized rabbits by immunoblotting as described by Towbin *et al*. (1979). Transfer of proteins from poly-acrylamide gel to nitrocellulose membrane took place at 220–250 mA using a mini-blotter (Hoeffer, USA). The nitrocellulose membrane was stained in Ponceau S (0.2% in 3% trichloroacetic acid, [Sigma, UK]) for 20–30 sec. The stain was removed in double distilled water (DDW). Molecular weight markers were marked. Non-specific binding sites on nitrocellulose membrane or strips were blocked with PBS plus 2% bovine serum albumin (BSA) for one hour at room temperature on a rocker platform. After washing of the nitrocellulose membrane, it was incubated in a dilution of 1/50 of sera from infected rabbits for 90 min at room temperature on a rocker platform. The washing was repeated then the nitrocellulose was incubated with the relevant alkaline phosphatase conjugates with appropriate conjugated sheep anti rabbit IgG (Biodesign) with PBS (1/5000) plus 1% BSA for 90 min at room temperature on a rocker platform. After washing the nitrocellulose membrane, it was developed at room temperature with agitation until the stain was suitably dark using 4-chloro-1-naphtol substrate as described by manufacturer instructions (Biogene). The nitrocellulose membranes were washed in distilled water and dried.

Results

The polypeptide profile was analysed by SDS-PAGE with 12.5% concentration under denaturated conditions and discontinuous buffer system. In electrophoresis, four bands with molecular weights of 97, 84, 66 and 55 kDa were observed. The 66 kDa band was noticeably dominant (Fig. 1). In Western blot of GSA protein bands with molecular weights of 84, 66 and 55 kDa had a positive

reaction (Fig. 2).

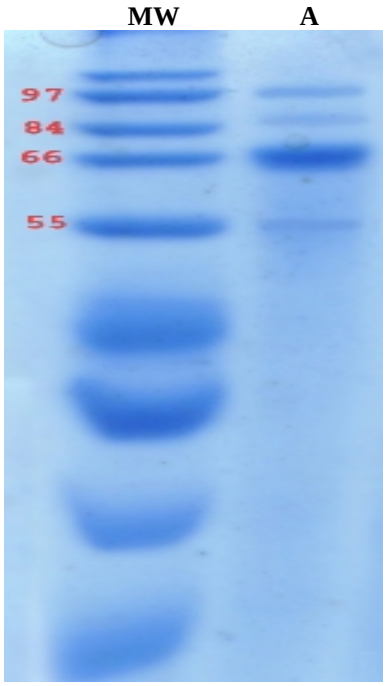

Fig. 1: The polypeptide profile of GSA was analysed by SDS-PAGE. Four bands with molecular weights of 97, 84, 66 and 55 kDa were observed. MW and A indicated molecular weight marker and GSA, respectively

Fig. 2: GSA Western blot. Three immuno-blot patterns with molecular wieghts of 84, 66 and 55 kDa were obtained from the GSA (the two columns are identical). Molecular sizes (in kDa) are indicated

Disccusion

Some experiments on immunization of rabbit (Ghosh *et al*., 1998) and cattle (Ghosh *et al*., 1999) with *H. anatolicum anatolicum* antigens have been reported. Antigen derived from midgut of *H. anatolicum anatolicum* provided protective immunity in rabbit (Razmi *et al*., 2005) and cattle (Razmi *et al*., 2003). It has been described that crossbred calves (*Bos indicus* × *Bos taurus*) were immunized with a fractionated midgut supernate antigen (GS-F Ag from *H. anatolicum anatolicum*) and that there was a significant decrease in the percentage larval engorgement and larval rejection of up to 34% on the immunized calves. Also a significant increase in the engorgement and preoviposition periods and a significant 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). Analyse of the polypeptide profile of dominant antigens is one of the primary and essential way for preparing an effective vaccine. In our experiment, we found four bands with molecular weights of 97, 84, 66 and 55 kDa with SDS-PAGE. The band with molecular weight of 66 kDa was dominant (Fig. 1). Also we found bands with molecular wieghts of 84, 66 and 55 kDa in Western blot (Fig. 2). Ghosh *et al*. (1998) reported bands of 97.4, 85, 66, 47.3, 42 and 31 kDa with SDS-PAGE. All of these bands were found in all of the life cycle of *H. anatolicum anatolicum*. Das *et al*. (2000) reported that SDS-PAGE and Western blot revealed three antigenic proteins of 100, 59.4 and 37 kDa responsible for induction of resistance in the host. Kumar *et al*. (2002) reported 26 discrete polypeptide bands with molecular weights ranging from 25 to 208 kDa with SDS-PAGE of gut supernatant antigen (GS Ag) derived from partly fed *H. anatolicum anatolicum* adult females. Of these, seven bands were of major polypeptides with molecular weights between 25.2 and 185.8 kDa. On immunoblotting with antisera raised in rabbits against gut supernatant, eight immunogenic polypeptides with molecular weights between 51.7 and 185.8 kDa were identified. Even those studied with the same

antigens, reported different results in the fractionating of proteins by SDS-PAGE. These differences may be due to different in preparing the antigenic solutions, chemical reagents of different quality and quantity or application procedures (Burgu *et al*., 2000).

In conclusion, we believe that gut-concealed antigen derived from *H. anatolicum anatolicum* can be strongly protective. Of course, it needs further studies to recognize which of these bands will be a good immunogen to be used for vaccination of animals.

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