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Finding Immunologically Related Bm86 Molecules in *Hyalomma Anatolicum Anatolicum* by Monoclonal Antibody against Bm86

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Abstract

Ticks are widely distributed around the world and they are greatly important for human and livestock as a vector of serious infectious diseases. Lyme disease, Crimean Congo fever as well as Theileriosis and Babesiosis are amid major diseases transmitted by ticks to both human and animals respectively. Despite great success in introduction of Bm86 based vaccines (Gavac™ and TickGARD) for tick control, study on different tick species such as *Hyalomma anatolicum anatolicum* which is the prevalent tick species in IRAN, showed moderate success. Therefore, the need to find Bm86 immunologically related molecules are mostly desired. In this study we have focused on finding immunologically similar structure to Bm86 molecules in midgut of our regional predominant tick species *Hyalomma anatolicum anatolicum* by development of monoclonal antibody against Bm86. Initially, Bm86 molecule purified from Gavac™ vaccine and monoclonal antibody was developed by standard protocol and then this monoclonal antibody was used in different immunological methods like Immunofluorescent test and Western blot analysis to find similar structure in *Hyalomma anatolicum anatolicum* midgut epithelial cell.

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Our study leads to development of a hybridoma clone which we called 4E11 and it is reacted with 37 and 40 kDa molecules in midgut epithelial cells of *Hyalomma anatolicum anatolicum*.

Keywords: Tick control; *Hyalomma anatolicum anatolicum*; Bm86; Monoclonal antibody.

1. Introduction

Tick and tick-borne diseases still remains on top list of major livestock problems in tropical and subtropical countries. Traditional tick control methods mainly based on use of chemical-acaricides which leads to environmental pollution, poor animal productivity, emergence of resistant tick strains [1,2], contamination of milk and meat products with drug residues [3]. Recent approaches include exploiting the immunological techniques by means of vaccine against tick [4]. Studies revealed beneficial role of usage of vaccine in tick control programs by reducing the use of chemical acaricides, diminish the cost of animal production, lower environmental contamination and decrease the risk of chemical residues in animal-derived products [5,6]. According to this approach, two Bm86-based vaccine named TickGARD PLUS (Intervet Australia Pty. Ltd., 91-105 Harpin Street, Bendigo East, Victoria) and Gavac™ (Heber Biotec S.A., Havana, Cuba) were commercialized in the 1990s in Australia and Cuba, respectively [7]. Bm86, is a 89,000 Mr membrane bound glycoprotein which located at extra-cellular surface of tick gut microvilli of digest cells [8] and it is also a well-known concealed antigen which in contrast to exposed antigen, they are normally hidden from the host immune response. One of advantages of application of concealed antigen is, their low natural selection pressure on these antigens, and emergence of resistance is not of major concern. There is also another advantage of this concealed antigen over exposed antigen and that is, "the anti-tick effect can take place over a longer period of time compared to exposed antigens. This effect may even extend beyond the mere feeding period into the inactive stages where digestion and molting/egg laying takes place" [9, 10].

Bm86 was originally derived from *Rhipicephalus (Boophilus) microplus* tick species. "This molecule is arguably the most economically important ectoparasite of livestock in tropical and subtropical regions of the world" [11], with significant rate of protection (between 55 and 100 % control of R. microplus ticks) [12]. Despite initial success in use of Bm86 based vaccines, the development of anti-tick vaccines faces a number of unique obstacles due to complex interactions between tick and vertebrate hosts. Increases in efficacy are most likely to come through the discovery of additional, effective vaccine antigens and the use of a tick vaccine as the single, standalone control technology is likely to require more efficacious vaccines than those currently available vaccines [9]. The problem will appear when prevalent tick species is somehow different from originally Bm86 derived tick species, i.e. *Rhipicephalus (Boophilus) microplus*. For example, one of tick species which prevalent in the southern Mediterranean area and also in eastern Africa (Sudan, Somalia and Ethiopia) and central Asia [13] is *Hyalomma anatolicum anatolicum* which is also the predominant tick species of Iran [14]. Study on effectiveness of Bm86 containing vaccines against *Hyalomma anatolicum anatolicum* displayed its relative effectiveness (an overall 50% reduction in the total weight of nymphs engorging on vaccinated calves) [15]. The potential for use of Bm86 antigen or its homologues in vaccination against other tick species has also been studied [15]. According to that study 95% of reduction in number of engorged nymphs and 55% reduction in weight of surviving *Hyalomma dromedarii* ticks were observed. In *B. decoloratus*

reduction of 70% in reproductive capacity was observed. Study on other *Boophilus* species like *Boophilus annulatus* showed more than 99.9% protection efficacy which support application of Bm86-containing vaccines for the control of Boophilus spp. infestations [16]. Therefore, it is highly desirable for the ideal anti-tick vaccine is the ability to elicit a protective immune response across more than one tick species or genus [17].

Our study was conducted to find immunologically similar structures or homologue of Bm86 molecule in *Hyalomma anatolicum anatolicum* by exploiting anti-Bm86 monoclonal antibody as a tool. Previous studies on application of monoclonal antibody to find Bm86 homologues also showed promising results. For example, in one study they used a Bd86 derived synthetic peptide, BD86-3, to raise a series of mouse monoclonal antibodies. One of these mAbs, named 12.1, recognized Bm86 homologues in immuno-histochemical analyses in four out of five tick species including *R. (B.) microplus, Rhipicephalus (Boophilus) decoloratus, Hyalomma anatolicum anatolicum and Rhipicephalus appendiculatus* [18]. In another study monoclonal antibodies (mAb) were produced against midgut membrane (GM) antigens of *Boophilus microplus* [19]. Eighteen monoclonal antibodies were developed, among them QU13 was the prototype. Antigens selectively precipitated from reaction of this monoclonal antibody with Triton X100 precipitated gut membrane (TXGM) showed 62% and 59% protection in two different animal studies. When results were compared with protection promoted by TXGM precipitated antigen alone it was showed that TXGM precipitate had 97% and 94% protection rate in two different animal studies, which mean higher protection. The prominent feature of our study is extraction of Bm86 from commercial vaccine (Gavac™) and making monoclonal antibody against this molecule. Then we use this developed monoclonal antibody to find homologues structure in *Hyalomma anatolicum anatolicum* by common immunological methods.

2. Materials and Methods

2.1. Bm86 containing vaccine

Gavac™ (Heber Biotec S.A., Havana, Cuba) vaccines were kindly provided by the company to Razi vaccine and serum research institute (RVSRI) of Iran.

2.2. Extraction of Bm86 from Gavac™ vaccine

Gavac™ vaccine composition include 60%

Aqueous Phase (Phosphate buffer saline + recombinant Bm86 (API), 40% Oily Phase (90% Mineral Oil and 10% Emulsifier M888VG) based on manufacturer claim. To separate aqueous phase from oily phase of vaccine, 5 ml of vaccine was mixed with 3 ml of Para-formaldehyde or chloroform (Merck) and vortexed for 10-15 minutes to completely liquefy the oil phase of vaccine. The suspension stored at refrigerator for 15-20 minutes to initiate phase separation. Then, suspension was centrifuged at 4500 rpm, 4°C for 60 minutes to complete phase separation. In case of incomplete separation of two phases, further centrifugation for another 1 hour was employed. The water phase was separated carefully from the oil phase and transferred into a new tube.

2.3. Trichloroacetic acid (TCA) precipitation of Bm86

The abovementioned separated aqueous phase of vaccine was precipitated by trichloroacetic acid (TCA) method [20]. The resulting pellet was washed with ethanol-ether (1:1 V/V) to remove TCA. A small portion of precipitate was dissolved in sterile distilled water used for Lowry protein determination method to quantify Bm86 as protein for ELISA development , SDS-PAGE sample load , etc., and the remaining precipitated Bm86 either dissolved in sterile PBS, which used for final immunization of mice, or in carbonate buffer to develop indirect ELISA to screen antibody rise in Balb/c mice serum.

2.4. Immunization protocol

Five female Balb/c mice were provided by laboratory animal department of Razi vaccine and serum Research Institute. Mice were immunized by Gavac™ vaccine (3 injection of 100 □l of vaccine in multiple sites subcutaneously with two weeks interval). The last injection was done by injection of 10 □l of TCA precipitated Bm86 via intra-peritoneal injection.

2.5. Indirect ELISA

To assess antibody increase in immunized Balb/c mice as well as to screen monoclonal antibody against Bm86, an indirect ELISA test was developed using checkerboard technique [21] to find optimized concentration of antigen, antibody and secondary antibody conjugate. Based on findings of checkerboard, 1 □g/ml TCA precipitated Bm86 was dissolved in carbonate buffer (1.59g Na_2CO_3 + 2.93 g Na_2HCO_3 up to 1 liter of double distille water) and coated in Nunc Maxisorp microtiter plate overnight at 4^oC. Microtiter wells then were washed 3 times by 300 □l of wash solution which contains Phosphate Buffer Saline (PBS) + 0.05% Tween 20. This stage proceeds by blocking of wells by 100 □l/well of 1% BSA + PBS for 30 minutes at room temperature to prevent nonspecific binding. Microtiter plates then were dried and kept at refrigerators until use. To test mouse serums 50 □l of 1:100 diluted serums (in wash solution) was added into the wells and incubated at room temperature for 1 hour. Wells were washed three times by 300 □l/well of wash solution. Then 100 □l diluted rabbit anti-mouse whole serum HRP conjugate (Sigma) (1:2000 in PBS-Tween 20 + 1% BSA solution) was added to each well. Plates incubated at room temperature for 1 hour followed by three times washing by wash solution. Tetra Methyl Benzidine (TMB, Pishtaz Teb Diagnostics) was added to each well (100 □l per well) as chromogen and plates were incubate at room temperature in darkness for 15 minutes. Finally the reaction was stopped by adding 100 □l of stop solution (1 N Hcl) to each well and the color intensity of wells were read by ELISA Reader (Biotek) at 450 nm with 630 nm reference filters.

2.6. Fusion protocol and limit dilution

The method described by [22] was used which briefly described. Four days after last intra-peritoneal injection, an intravenous injection via tail vein was carried out and after 24 hours the fusion protocol was run. Prior to fusion protocol, NS-1 myeloma cells (mouse myeloma, Reference no. 82102301 provided by cell and gene bank of Razi vaccine and serum research institute (RVSRI) of IRAN were cultured and passaged in Iscove's modified Dulbecco's medium containing 10% FCS, 2 mM L-Glutamine and 10 mg/ml of gentamicin sulfate at 37^oC+ 5% CO2. The cells have been kept in exponential growth phase and retain at this phase for fusion protocol. The

fusion protocol was done briefly by removal of spleen from immunized mice aseptically and their attached tissues and fats were removed. Single cell suspension was prepared using two needles (one for keeping the spleen and another for scrapping the cells) and finally pressing the pieces against metal sieve by barrel of 2 ml syringe. Spleen red blood cells were removed by treatment of cell suspension by 85% ammonium chloride. Spleen cells were washed serum free Iscove's medium in order to remove ammonium chloride and then counted. Myeloma cells were also counted using 4% aqueous Trypan blue suspension and two cell types (spleen and NS-1 myeloma cells) were mixed in 10:1 ratio of spleen to NS-1 myeloma cells. The cell mixture were washed by fetal calf serum (FCS) free medium and its pellet were mixed gently by 0.3 ml of warm Poly Ethylene Glycol (PEG) MW 1450) slowly within 1 minutes drop by drop. Then 15 mL of Iscove's media without FCS was added slowly over a period of 90 seconds and cells were incubated at room temperature for 10 minutes. Then, cells were centrifuged at 400 g for 10 minutes and re-suspended in 30 ml of complete culture media containing OPI Media supplement (Sigma) and Hypoxanthine-Aminopterin-Thymidine (HAT) to adjusted cell concentration to 2.5-3 $\times 10^6$ cell /ml. Resultant cell suspension, were dispensed in five 96 wells microtiter tissue culture sterile plates by 10 ml pipette in a manner of one drop/well. After 24 hours incubation at 37°C + 5% CO2 another drop of complete culture media containing OPI + HAT was added into each well. After 5 days 50% of each well supernatant media were removed and replaced with 1 drop of fresh complete media containing HAT + OPI. After 10 days microscopically positive wells supernatant were screened for presence of monoclonal antibody.

The emerged clones were screened for the presence of antibody by the formerly described developed indirect ELISA. The eleven positive clones were used for the limit dilution.

2.7. Tick midgut sample preparation

Engorged females of *Hyalomma anatolicum anatolicum* were kindly provided by insect rearing department of Razi vaccine Serum Research institute. Engorged tick female were blood fed on ear bag on rabbit ear was aseptically dissected and their midgut were transferred into PBS buffer containing 1% anti-protease cocktail (complete Sigma cocktail buffer). Host red blood cells were separated from the midgut tissue by several consecutive steps of cold PBS washing followed by low speed centrifugation (3 minutes 800 rpm 4°C). The final pellet was re-suspended in the complete buffer and checked microscopically for the presence of host red blood cells. Large midgut tissue was gently smashed aseptically to create single cell suspension. Single cells which prepared mechanically are used both for preparation of Immunofluorescent slide and also for the SDS-PAGE and Western blot analysis.

2.8. Immunofluorescence study

Single cells of midgut epithelial cell were used for the immunofluorescence study. Cell suspension adjusted to 10^4-10^5 cells/ml by sterile PBS. Twenty five □l of cell suspension was dispense on 12 spot teflon-coated multi-spot microscope slides (Hendley-Essex Multiple Microscopic Slide, UK), and checked by inverted microscope to ensure enough number of cells were in each spot. Slides were left under the laminar hood to be air dried. Slides were labeled and kept at -20 °C in a plastic slide box until use. To run the test, slides were immersed in

pre-cold acetone (Merck) for 10 minutes. Then they were left at room temperature to dry. To each spot of slide, 20 μl of blocking buffer (PBS +2% BSA) was added and slides kept in humid chamber at room temperature for 30 minutes. Slides were washed three times by PBS each time for 5 minutes and then 20 μl of ELISA positive hybrid cell supernatant was added and again slides incubated in humid chamber at room temperature for 1 hour. Wash step was repeated as mentioned before and then 20 μl of diluted rabbit anti-mouse FITC conjugate was added to each spot of slide and they were incubated again in humid chamber for 30 minutes at room temperature. Slides were washed as before and watched under immunofluorescent microscope.

2.9. SDS-PAGE and Western Blot Analysis

SDS-PAGE was accomplished by method of [23], denaturing method in which 3% of stacking gel and 12.5% of resolving gel were used. The Western blot analysis was performed by the method of Towbin and his colleagues [24]. The primary antibody was our monoclonal antibody and the secondary antibody was anti-mouse HRP conjugate (Sigma).

3. Results

Gavac vaccine was shown in figure 1. These vaccines are kindly provided by (Heber Biotec S.A., Havana, Cuba) to Razi vaccine and serum research institute.

Figure 1: Gavac™ vaccine

The predominant tick species in Iran is *Hyalomma anatolicum anatolicum* (Figure 2a). This tick species can be found in all Iran provinces and also it is one of the important tick species in Middle-east region. Single cell suspension (Figure 2b) was prepared by abovementioned protocol and these cells were used both for preparation of immunofluorescent slides and SDS-PAGE followed by Western blot analysis.

Figure 2a: *Hyalomma anatolicum anatolicum* engorged female tick.

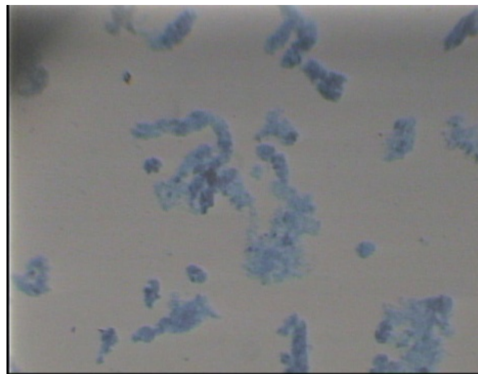

Figure 2b: Single cell suspension prepared from midgut of *Hyalomma anatolicum anatolicum* (Trypan blue staining, 4 X)

To develop ELISA test for screening of both immunized laboratory animals and hybridoma clones, the most common method is checkerboard method (Figure 3 a) in which serial dilution of antigen was prepared in microtiter plates from column 1 to 11, column 12 was used as antigen control and after overnight incubation and blocking, serial dilution of antibody was prepared from A-G row and H row was used as antibody control. Finally after addition of conjugate, chromogen, stop and reading the microplate, a color gradient was developed from A1 to H12. Those wells with high OD and low background both in antigen control (column 12) and antibody control (row G) was selected and their corresponding antigen concentration and antibody titer were used for further optimization of our ELISA. (Figure3b) Hybridoma clone was developed after 1 week and appeared at the corner of wells which subsequently limit diluted to ensure single clone isolation.

Figure 3a: The ELISA checkerboard

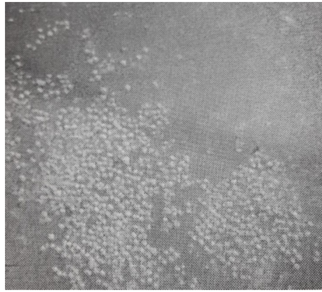

Figure 3b: Hybridoma clone

Midgut single cell which prepared earlier was used for SDS-PAGE test and visualization of SDS_PAGE electrophoresis products was made by Commassie brilliant blue R250 staining (Figure 4a). Major bands of 208, 97, and 47 were appeared. Western blotting displayed bands of 37 and 40 kDa as predominant bands (Figure4b, test run in duplicate) which means some bands may not appear as prominent bands or show very tiny bands in Commassie brilliant blue staining and need more sensitive staining like silver nitrate staining to overcome the problem.

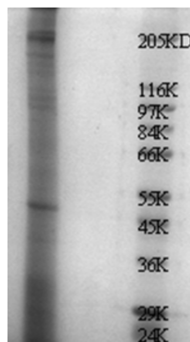

Figure 4 a: Midgut Single cell SDS-PAGE showed major bands of 208, 97 and 47 kDa

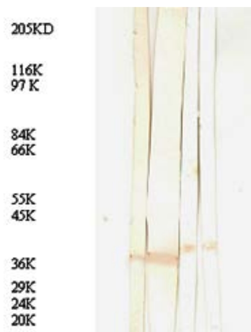

Figure 4b: Western blotting showed 37 and 40 kDa with 4E11 monoclonal antibody (duplicate test).

Tick midgut single cells were also used for indirect immunofluorescent test (IFA) (Figure 5). Immunofluorescent microscope was showed two patterns of diffuse and spot like appearance (Figure 5a & 5 b) which may coincide with finding of two bands in our Western blotting test.

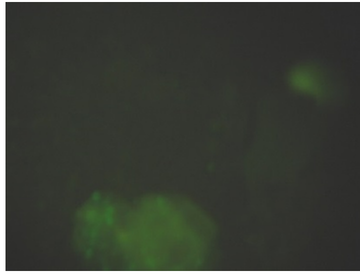

Figure 5a

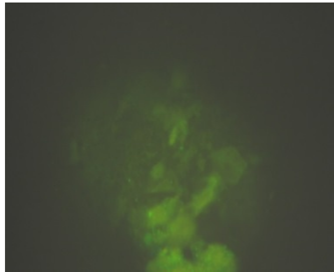

Figure 5b

Figure 5. Midgut Single cell IFA test showed speckled distribution of bm86 like molecules on tick midgut epithelial cells Figure 5a. diffuse and Figure 5b. spot like immunofluorescent pattern

4. Discussion

Ticks still remain as one of medically and veterinary immense important vectors for infectious disease and their control are highly important.

Introduction of vaccine against tick opens a new gateway to control this ecto-parasites and avoidance of side effects created by usage of acaricides chemical. However, among different introduced molecules for tick vaccine, Bm86 was the best known commercialized vaccine which introduced into market as a successful candidate [2,5,7].

In our experiment we found 3 major bands of 208, 97 and 47 kDa , other experiment showed different bands for example in one study [25] they found four bands with molecular weights of 97, 84, 66 and 55 kDa with SDS-PAGE and the band with molecular weight of 66 kDa was dominant. Our SDS-PAGE also revealed similar 97 kDa band. The difference may be due to the use of gut supernatant antigen (GSA) by them and midgut antigen directly in our study.

Ghosh and his colleagues [26] 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*. Their reported 97 and 47 kDa bands were conformed to our study. Das and his colleagues [27] 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 which again their 37 kDa bands report are similar to our study result. The only SDS-PAGE band which is absent in those

studies is our 208 kDa band. Interestingly in a study done by Kumar et al. [28] 26 discrete polypeptide bands were reported 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. Based on their immunoblotting study with rabbit antisera against gut supernatant, eight immunogenic polypeptides with molecular weights between 51.7 and 185.8 kDa were observed.

The basis of our study was using the known molecule of Bm86; and employing the mAb against Bm86 to find similar structure in *Hyalomma anatolicum anatolicum* which is the predominant species of countries like IRAN [14]. Our study revealed similar structure of 37 and 40 kDa in midgut epithelial cells of tick which may link to HA98; a known Bm-like molecule which need further investigation.

Recently Bm86 ortholog of *H.anatolicum anatolicum* like Haa86 [29] were introduced. They also showed promising results for tick control and in particular for control of transmission of *Theileria annulata.*. Moreover, a long list of newly discovered candidate molecules has been reviewed for tick vaccine [30] and combination of different tick antigens with pathogen-derived antigens to form multivalent combination vaccines is very interesting approach which can facilitated by studies like our study to discover new similar molecule based on known vaccine molecule.

5. Conclusion

Immunological control by vaccine is not completely successful on some ticks like *Hyalomma anatolicum anatolicum* and finding a candidate molecule for development of new vaccine is highly desirable. However, known molecules like Bm86 could be used as a successful basis via development of monoclonal antibody, homologues structural analysis, etc., for finding similar structure and facilitate vaccine development. Meanwhile, this study was conducted to find out similar Bm86 molecules in *Hyalomma anatolicum anatolicum* in Iran isolates but we highly recommended for further study on similar and other tick species isolated from other regions for finding universal candidate tick vaccine molecule.

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