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Purification, Characterization and Evaluation of Larval Immunogens of Cattle Tick Boophilus annulatus by Infected Cattle Sera

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Abstract: The present study was conducted to isolate new target immunogenic molecules from the larval stage of the cattle tick, *Boophilus annulatus* (Acari: Ixodidae). CNBr-Sepharose 4B coupled with rabbit infected serum was adopted in purification process. The isolated fraction showed higher diagnostic potency than crude extract of *B. annulatus* infection in *Bos indicus* and *Bos taurus* cattle by ELISA. The electrophoretic profile of the fraction consists of six bands of molecular weight121, 95, 67, 57, 45 and 21 KDa compared with 15 bands of high and low molecular weights associated with crude larval antigen as proved by SDS-PAGE. The immunogenic bands of the fraction that were recognized by *Bos indicus* and *Bos taurus* cattle naturally infected sera were 95 KDa, 67 KDa, 45KDa and 23KDa and 67 KDa and 45KDa respectively using immunoblot. The isolated fraction was used successfully in the diagnosis of *B. annulatus* tick infection in *Bos indicus* cattle (70.6%) and in *Bos taurus* (90%) serum samples. The current study suggested that the isolated fraction contains promising diagnostic immunogens need further purification to optimize its diagnostic value of *B. annulatus* infection.

Key words: Boophilus Annulatus · Larvae · Affinity Chromatography · Cnbr-Sepharose 4B · ELISA · Cattle

INTRODUCTION

Ticks are of great veterinary importance compared to other ectoparasites. They consume large quantities of host blood during their lengthy attachment period (7-14 days), which may be extended depending on the tick species and unique host association. The bovine tick, *Boophilus annulatus* (*B. annulatus*), is a bloodsucking ectoparasite that causes severe production losses in the cattle industry. The average tick burden causes an annual weight loss of 0.7 kg/tick [1]. With the huge number of ticks infesting cattle and other livestock animals, the subsequent effect on beef production is a reduction of hundreds of millions of kilograms annually. Camels, cattle and chickens are severely affected by ticks and may transmit serious pathogenic microorganisms [1]. Serological diagnosis of tick infections was previously reported using different assays as Immunoelectrophoresis, immunofluorescence and enzyme-linked immunosorbent assay (ELISA) [2-4]. Except for the ELISA, these tests have not been standardized and reagents are not available for routine use. So, it was proved that ELISA was more sensitive for serodiagnosis of ticks [5-7]. ELISA showed that calves infested with *B. annulatus* develop antibodies to components of tick as salivary gland, ovary and larva during the early infestation. Antibodies reactive with tick extracts have been described by several researchers [8-12, 28].

Sensitivity and specificity of serological tests were mainly affected by the used antigen. To increase the diagnostic potency of antigens, isolation of their immunodiagnostic fractions would be useful. Larval antigens in a partially pure S-300 Sephacryl protein fraction, BmLF2 have been used to examine serological responses against ticks [13,14]. Many investigators used the Western blot technique to identify relevant antigens of several tick species using sera collected from different animal hosts [1, 7,15, 28]. The adoption of both immunological methods, ELISA and immunoblot is essential to obtain a product of immunogenic properties, Therefore, the objective of the current study was the development of affinity partially purified fraction of larval proteins of cattle tick *B. annulatus* extract to be used for serological evaluation of tick infection by ELISA. Also, structural characterization of this antigen and the detection of its immunogenic bands were undertaken.

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MATERIALS AND METHODS

Tick: Engorged females of *B. annulatus* were collected from the ground of cattle farms in Berkash village, Giza, governorate, Egypt. Identification of females was confirmed in the laboratory according to Hoogstraal [16] and Estrada-Peña *et al.* [17]. The females were incubated at a constant temperature of 24± 2°C with a relative humidity of 75 ± 5% in permanent darkness to obtain eggs and larvae as previously described [18].

Preparation of Larval Antigen: Whole larval antigen of *B. annulatus* was prepared according to the method of Ghosh and Khan [19]. Laboratory-reared, 3 day-old unfed larvae were homogenized in 0.15 M phosphate-buffered saline, pH 7.2. The homogenate was sonicated in ice for 3-4 cycles for 20-25 sec each at 16-micron amplitude and centrifuged at 15000 × g for 60 min at 4°C. The supernatant was collected as the whole larval antigen. The total protein concentration of the antigen was estimated according to the method of Lowery *et al*. [20].

Infected Rabbit Sera: One thousand (Five hundred larvae for each rabbit) laboratory –reared, 3 day-old unfed larvae were fed on two local breed rabbits. Each rabbit weighted 1500gm. After six days, from attached of larvae blood samples were collected after rabbits were scarified and centrifuged at 2000 rpm for 15 min. Sera were collected in Eppendorph tubes and stored at -20°C till use.

Serum Samples of Cattle: Blood samples of *Bos indicus* and *Bos taurus* cattle were collected (without anticoagulant) and centrifuged at 2000 rpm for 20 min. Sera were separated, labeled and stored at -20°C until use.

Antibody-Sepharose 4B Affinity Column Chromatography: Rabbit anti larval antigen antibodies were dialyzed for three days against 100mM NaHCO3 buffer pH8.3 containing 500m M Nacl and 0.02% NaN3 at 4°C and then coupled to cyanogen bromide (CNBr) - activated Sepharose 4B (Pharmacia Fine Chemicals) by strictly following the manufacture instructions. Crude tick larval antigen of *B. annulatus* was applied to the column and bound fraction was eluted using 50mM glycine containing 500mM Nacl pH 2.3. Protein concentrations of unbound and purified antigens were assayed by the method of Lowry *et al*. [20].

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE was performed according to Laemmli [21]. Crude larval antigen and its fraction were separately mixed with sample buffer containing 2-mercaptoethanol before loading to the slab gel using 10% SDS-PAGE under reducing condition. After separation, gel was fixed in 50% methanol and stained with silver stain according to Wray *et al*. [22]. Relative molecular weights of bands were calculated using molecular weight marker.

Enzyme Linked Immunosorbent Assay (ELISA): The immunogenic activities of the crude larval antigen and isolated fraction were monitored by ELISA in experimentally infected rabbit sera. The most potent antigen was adopted to detect tick infection in collected *Bos indicus* and *Bos taurus* cattle serum samples using ELISA. ELISA was performed according to Santiago and Hillyer [23] with little modifications. Antigens concentrations, sera and conjugate dilutions were determined by checkerboard titration. The cutoff value was calculated by the method of Hillyer *et al*. [24].

Western Blot: The profiles of reactive bands were recognized by immunoblot using infected *Bos indicus* and *Bos taurus* cattle serum samples proved by ELISA. Crude larval antigen and isolated fraction were electrophoresed using 10% SDS- PAGE under reducing condition [21]. Gel was electrically transferred into nitrocellulose membrane (NC) according to the method of Towbin *et al*. [25]. NC sheet was cut into two parts followed by blocking in 5% bovine serum albumin in PBS for 2h. The two diluted serum samples at 1: 200 in 5% BSA/TBS were reacted with both transferred antigens on NC of two parts for 2 h. Following washing, peroxidase labeled anti-bovine IgG diluted 1:1000 (Bio- Rad co.) in TBS was added to NC for 1h. The chromogen AEC (Sigma) substrate was added to NC and allowed to develop for 30 min. The reaction was visualized by the naked eye.

RESULTS

Affinity Isolated Fraction: The Purification process resulted in isolation of two fractions; a single specific bound fraction and unbound fraction. The total yield of larval bound immunogens was 4µg/ml which represents around 20 % of the total larval proteins.

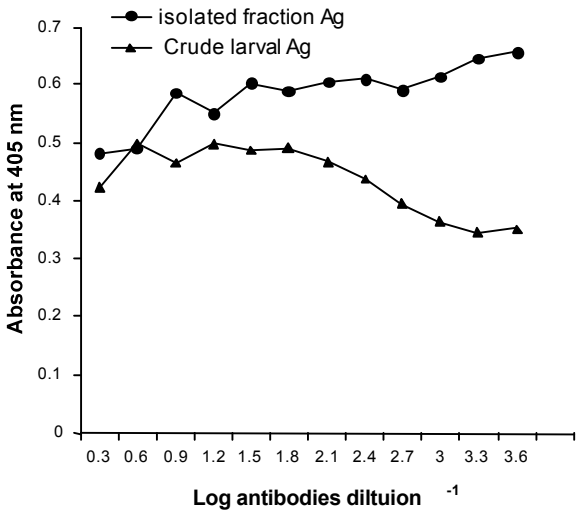

Fig. 1: Potency of crude larval protein and isolated fraction of cattle *B.annulatus* larvae in IgG detection in experimentally infected rabbit sera with tick

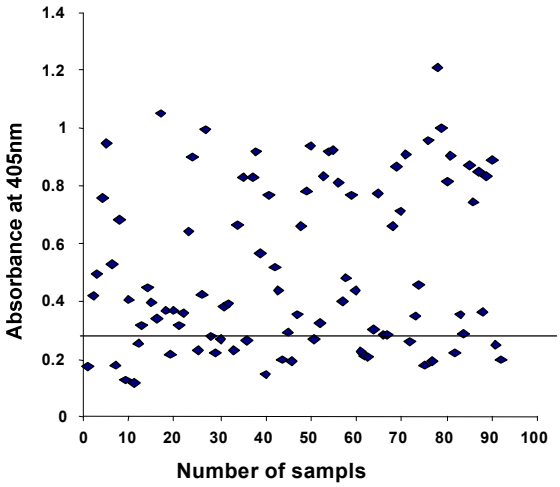

Fig. 2: Potentials of immunoaffinity isolated fraction from Cattle tick *B. annulatus* in IgG detection in Bos indicus sera

Immunogenic Activities: Fig.1 showed higher potency of isolated fraction than crude larval antigen in the diagnosis of tick infection in two-fold serially diluted rabbit sera. The potency of the fraction is directly proportional to antibody dilution; the potency increases gradually by increase the dilution of antibodies. In contrary, the crude antigen was inversely proportional with diluted antisera. Moreover, the isolated fraction showed potency in the evaluation of *B. annulatus* tick infection in *Bos indicus* cattle sera (70.6%) (Fig. 2) and *Bos taurus* cattle sera (90%) (Fig. 3).

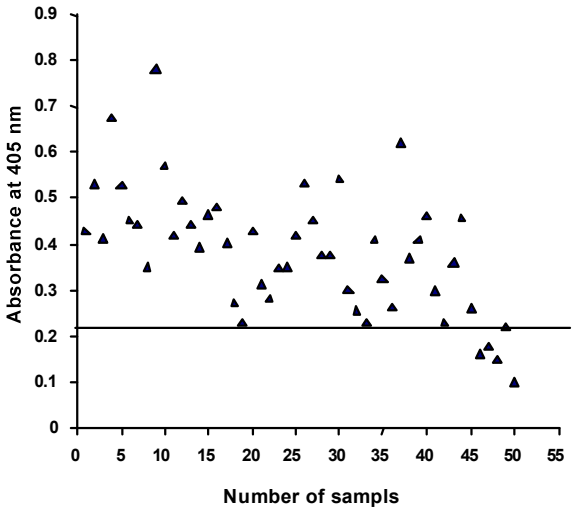

Fig. 3: Potentials of immunoaffinity isolated fraction from Cattle tick *B. annulatus* in detection of IgG in Bos *taurus* sera.

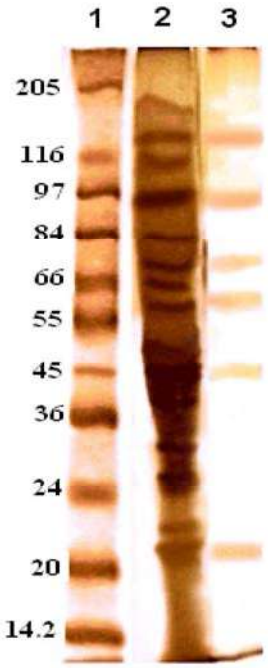

Fig. 4: Comparative electrophoretic profile of crude larval antigen (lane2) and isolated fraction (Lane3). Molecular weight standards in KDa (Lane 1).

Electrophoretic Profiles of Crude Larval Antigen and Isolated Fraction: The crude larval antigen revealed multiple components of molecular weight 178,121,115,95,83,70,67,57,45,40,37,30,25, 23and 21 KDa (Fig.4,Lane2). While isolated fraction was resolved into 121, 95, 67, 57, 45 and 21 KDa (Fig.4, Lane3).

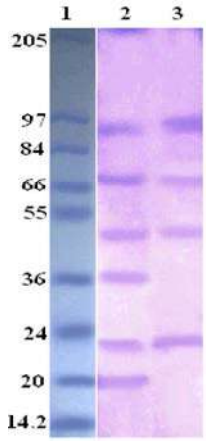

Fig. 5: Immunoreactive bands identified by infected *Bos indicus* cattle sera in crude larval antigen (lane 2) and isolated fraction (3). Molecular weight standards in KDa (lane 1).

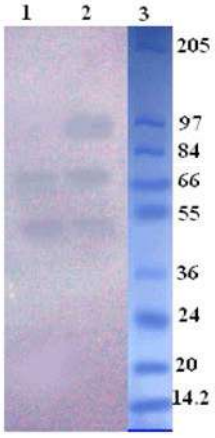

Fig. 6: Immunoreactive bands identified by infected *Bos taurus* Cattle sera.in crude larval antigen (Lane 2) and isolated fraction (lane 1). Molecular weight standards in KDa (lane 3).

Immunogenic Bands: The recognized immunogenic bands in crude larval antigen by naturally infected native Cattle sera with *B. annulatus* were 95KDa, 67KDa, 45KDa, 37KDa, 23KDa and 21 KDa (Fig.5, Lane 2). While 4 of these bands were recognized in isolated fraction; 95 KDa, 67 KDa, 45KDa and 23KDa (Fig. 5, Lane 3), immunoreactive bands of the two antigens that were identified by naturally infected *Bos taurus* sera with *B. annulatus* were different, in crude larval antigen the recognized three immunogenic bands were 95KDa, 67KDa and 45 KDa (Fig.6, Lane2). While the responsible bands for reactivates with *Bos taurus* sera in isolated fraction were only two immunogenic bands 67 KDa and 45KDa as observed in Fig.6, Lane 1.

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 showed 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 and accurate diagnosis [26-29]. Identification of expressed larval proteins of *B. annulatus* that elicit a humoral immune response in the cattle host, as a result of natural exposure, is the principal purpose of the current investigation. *B. annulatus*, as other tick species, introduces multiple components to their hosts. In the present study, larval antigen of *B. annulatus* showed 15 bands of high and low molecular weights as proved by SDS-PAGE. This would most likely include those larval proteins that are exposed to the host via the salivary glands as a result of successful larval feeding. Complicated electrphoretic profile of *B. annulatus* larvae was previously reported [13]. Due to the complex composition of the crude larval protein extract, the approach used in the present study, was based upon a separation of the larval extract into partially purified fractions, in order to identify those larval proteins that elicit host humoral responses. The bound fraction to CNBr-Sepharose 4B showed higher diagnostic potentials than crude extract of experimental *B. annulatus* infection in rabbits using ELISA. Interestingly, highly diluted rabbit antisera (1: 4096) reacted more strongly with isolated fraction than low diluted or concentrated serum which confirm diagnostic potency of the fraction. In contrary, the diagnostic potentials of crude extract decreased with increased dilution of antiserum which reflects its limited capabilities. Moreover, the advantage of the fraction compared to the crude antigen in the diagnosis of experimental tick infection is an indicator of the success of the purification process. The fraction was resolved into only 6 bands compared to 15 bands associated with crude extract by SDS-PAGE. This result is considered as an additionall evidence which supports the success of the purification process. A glycoprotein fraction of 32 and 15KDa was isolated previously from *B. annulatus* larvae using two-step affinity column chromatography by El Hakim *et al*. [1], compared with 6 bands of molecular weights 121, 95, 67, 57, 45 and 21 KDa isolated in the current study. The difference in molecular identity between both fractions is mostly attributed to purification methods adopted for isolation of fractions and to

the identity of fractions themselves (protein or glycoprotein). Similarly, 3 antigens with molecular weights of 86 kDa [30], 63 kDa [31] and 75-80 kDa [32] were isolated form a closely related tick species, *B. microplus*. The immunoaffinity chromatographic purification method was previously attempted for the purification of salivary gland antigens of *Amblyomma americanum* [19] larval antigens [9, 10], salivary gland, gut origin larval,larval antigen [33] and nymphal antigen of *Hyalomma anatolicum* and *Hyalomma dromedarii* [34-36]. The isolated larval glycoproteins of comparatively lower molecular weights of 32 kDa and 15 kDa may enrich the list of purified tick antigens [1]. All these previous studies indicate that affinity column chromatography is commonly appropriate method used in ticks purification.

In the current study, four bands were identified in the isolated fraction using infected native cattle sera; 95 KDa, 67 KDa, 45KDa and 23KDa while only two of them; 67KDa and 45KDa were identified in the fraction by infected *Bos taurus* cattle sera using immunoblot. This observation indicates that the immune system of each host species responds differently to the exposed larval molecules although, both are relevant. Several antigens extracted from *B. annulatus* tick were recognized by sera collected from cattle infested with this species [7] demonstrated that there are common antigens in different tissues of *B. annulatus*; 170, 117, 100, 70, 37, 33 and 30 kDa identified by sera of infested cattle. This variation in bands pattern profile is probably attributed to differences in antigen preparation, differences in separation conditions as reducing or non reducing and gel percentage. Western blot data indicated that *B. annulatus* infested cattle develop antibodies to components of tick such as larvae during the infestation. Using ELISA, the present study confirmed the potency of isolated fraction in the diagnosis of *B.annulatus* tick infection in *Bos indicus* cattle sera (70.6%) and *Bos taurus* cattle sera (90%). The high sensitivity observed in the present study was attributed to both the purity of isolated fraction and the sensitive assay (ELISA).

CONCLUSION

The present study provides some basic information about isolated fraction of *B. annulatus* larvae which includes immunogens elicit hosts immune response in both *Bos indicus* and *Bos taurus* cattle. The significance of these immunogens resides in its promising prospective in the induction of host immunity against *B.annulatus* larval infection.

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