The Pharma Innovation Journal 2022; SP-11(5): 94-100

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(5): 94-100

www.thepharmajournal.com
Received: 16-03-2022
Accepted: 18-04-2022

Gawande PJ
Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Jumde PD
Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Baviskar BS
Director, Society for Wildlife Conservation, Education and Research, Nagpur, Maharashtra, India

Corresponding Author
Gawande PJ
Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Salivary gland antigen of *Rhipicephalus haemaphysaloides*: Immunological studies in rabbits

Gawande PJ, Jumde PD and Baviskar BS

Abstract
The immunological studies of salivary gland antigen of *Rhipicephalus haemaphysaloides* in rabbits was carried out in the department of Parasitology, Nagpur Veterinary College, Nagpur. Maharashtra Animal and Fishery Sciences University, Nagpur. Partially fed *R. haemaphysaloides* ticks were used for collection of salivary gland. The concentration of protein was estimated to be 2.1 mg/ml of antigen. Crude salivary gland antigen of *R. haemaphysaloides* was characterized by SDS-PAGE, which revealed 17 protein bands. It is followed by alkaline, acidic and sodium phosphate buffer treatment. Raising of hyperimmune sera was done in New Zealand white rabbits in divided doses on day 0, 14 and 28 followed by blood collection. Second booster dose was given at 2 weeks interval and final blood collection was performed 2 weeks post second booster.
The test was conducted for group I and II (control group) by using sera collected from animals on 0, 14, 28 and 42 days following immunization. Prominent precipitation line appeared only from 10 days – II booster which persisted 42 days post II booster in animals belonging to group I. No such reaction was observed in group II control rabbits.

Keywords: Salivary gland, antigen, rabbit, *Rhipicephalus haemaphysaloides*

Introduction
Agricultural resources and animal wealth play a vital role in the economic upliftment of the country and to the small farmers and laborers in particular. Ticks and tick borne diseases affect 80% of the cattle population and are widely distributed throughout the world, which cause significant production losses (de Castro, 1997) [8] in cattle annually with the production of never ending display of acaricides resistant ticks besides other effects on environmental pollution (Narsapur, 2003). The economic losses due to tick population are increased from 600 million to several billion dollars per annum globally (Ervin *et al*. 1989) [9]. The feasibility of controlling tick infestations through immunization of hosts with selected tick antigen was demonstrated with the development of vaccines that reduced *Boophilus* ticks on cattle (Willadsen, 1997). Several proteins identified from ticks are considered to be potential vaccine candidates for control of tick infestations (Willadsen, 2002).
In India, number of reports are available on use of different tick crude extracts for immunization of rabbits, *viz*., the antigen extracted from whole tick and salivary glands of *Hyalomma anatolicum anatolicum* and immunized the rabbits (Manohar and Banerjee, 1992a), with use of extract of midgut of *H. a. anatolicum* (Kumar *et al*., 2003). Similarly immunological reactivity study conducted by using larval, nymphal and adult antigens of *Boophilus microplus, H. a. anatolicum* and *Rhipicephalus sanguineus* (Ghosh and Khan, 1998) [13], besides midgut antigen of different ticks infesting small ruminants (Latha *et al*., 2003). In Tamil Nadu, immunogenicity of crude midgut antigen of *Rhipicephalus haemaphysaloides* had been conducted in rabbits with encouraging results (Aruljothi, 2003). Although, the immunological response against midgut antigen of *Rhipicephalus haemaphysaloides* is available.
Centurier *et al*. (1981) [6] reported that rabbits immunized or not immunized with corpuscular and soluble salivary gland components of *Ornithodoros moubata* were infested 1 to 5 times with 2nd nymphal instars of the tick. He found no differences in weight gain and weights of replete ticks. Johnston *et al*. (1986) [18] reported the preparation of whole adult extract antigen from *Boophilus microplus*. Agbede and Kemp (1986) [1] reported that ticks which fed on cattle vaccinated with whole tick extracts of *B. microplus* showed ruptured digest cells. Gill *et al*. (1986) [11] separated nine proteins from saliva and seventeen from salivary gland extract.

Brown and Askenase (1986) derived crude salivary gland proteins from *Amblyomma americanum* ticks and analyzed by gel filtration and ion exchange chromatography. Rechav and Dauth (1987) [32] stated that *Rhipicephalus appendiculatus* showed a positive correlation between the levels of gamma globulin in the serum and the resistance developed by the host. Brown (1988) [5] observed that the serum from rabbits immunized with *A. americanum* derived SGA emulsified with complete Freund's adjuvant recognized most of the proteins identified by active serum, whereas serum from animals immunized with SGA in incomplete Freund's adjuvant did not. Willadsen *et al.* (1989) isolated a membrane protein Bm 86 with molecular weight of 89,000 from gut membrane of *B. microplus.* The supernatant was separated and mixed with wheat germ lectin-sepharose column 2.5 × 26 cm. The eluted supernatant fractions were chromatographed on a 1.5 × 12 cm column of Con A-Sepharose in 0.005 M Tris chloride buffer Ph 7.5, subjected to isoelectric focusing and purified by HPLC gel filtration.

Fivaz (1989) [10] stated that rabbits developed resistance to *R. appendiculatus* instars following repetitive infestations. Rejection was accompanied by elevated IgM and IgG titres. Extracts of salivary gland, mouth-part cement and whole ticks induced a dose-related lymphocyte transformation. Skin-provocation tests with tick extracts elicited an immediate type-I hypersensitivity reaction with a delayed time-course which was influenced by antihistamines. Passive-transfer studies indicated that resistance was partially transferred with serum.

Christine *et al*. (1991) [7] detected enormous antibodies in rabbits and rats against salivary gland homogenates exposed to *I. dammini* that cross react with the antigens of closely related species, *I. scapularis* and significantly with *Dermacentor variabilis* antigens. Lee and Opdebeeck (1991) [23] reported that the precipited antigen from gut membrane of *B. microplus* solublised with Triton X-100 and obtained significant protection against *B. microplus*. They further separated the protective gut antigen of *B. microplus* by SDS-PAGE and detected the proteins of 200, 80, 77, 62, 57 and 30 kDa. Essumen *et al.* (1992) reported that the solublised midgut membrane fraction represented a promising source of immunogen for the development of an anti-tick vaccine for *R. appendiculatus.*

Tembo and Rechav (1992) [36] stated that beta and gamma globulin levels and numbers of eosinophils and neutrophils increased significantly in inoculated rabbits. The recipient animals developed resistance to homospecific nymphal infestations of *Amblyomma hebraeum*. Manohar and Banerjee (1992a) immunized rabbits with various ticks following challenge with adult *H. a. anatolicum* significant decrease in engorgement weight and egg mass weight and an increase in engorgement period and preoviposition period were observed in whole tick extract antigen immunized rabbits. The sera of immunized rabbits showed single precipitation band on 14 and 21 day. On 28, 35, 49th day one or two bands were seen.

Thakur *et al*. (1992) [37] separated midgut antigen from *H. a. anatolicum* and *B. microplus* female ticks and immunized the rabbits by using saponin. On challenging the rabbits with hungry larvae and adults of *H. a. anatolicum* revealed reduced feeding rate and poor reproductive performance of ticks, along with extensive damage of gut lining with high mortality. Manohar *et al*. (1992b) [25] prepared whole tick extract antigen from semi engorged female *H. a. anatolicum* ticks and antibodies were detected on day 14th and gradual enhancement in the titre recorded upto 28th day in immunized rabbits. They detected antibodies from 14 to 63 days post immunization by DID. Das *et al*. (1993) reported progressive decrease in serum glucose and albumin levels after four inoculations of *B. microplus* in rabbits.

Mbogo (1994) [26] stated that rabbits were rendered resistant to all the instars of the tick. He observed that the resistance was more pronounced in adult ticks than in the immature stages. The resistance was manifested as a reduction in the number of ticks that fed successfully to engorgement, reduced engorgement weights and reduced fecundities. Rechallenging the resistant experimental rabbits with all the developmental instar stages of *R. appendiculatus* showed that resistance was maintained in subsequent infestations.

Riding *et al.* (1994) [33] isolated a membrane protein Bm91 from crude membrane and particulate fractions of *B. microplus*. Smith (1994) [34] described sodium dodecyl sulphate gel electrophoresis technique in which proteins were reacted with anionic detergent SDS to form negatively charged complexes. The amount of SDS bound by protein and so the charge on the complex, is roughly proportional to its size. The proteins of either acidic or basic form negatively complexes that can be separated on the basic of differences in charges and sizes by electrophoresis through a sieve like matrix of polyacrilamide gel. Kumar and Kumar (1995) reported the presence of serum antibody in rabbits after their immunization with midgut antigen from partially fed *Hyalomma dromederii* ticks with double immunodiffusion (DID) and capillary tube agglutination tests. Wozniak *et al*. (1996) [40] infested the rabbits with *Ornithodoros* sp. ticks and assayed for anti-tick antibody by ELISA against salivary gland extract (SGE) of ticks. Western blot analysis demonstrated tick antisera to contain antibodies. Ghosh and Khan (1996) observed only one precipitation band upto 21 day post immunization and two days from 28-84 DPI by DID test in four animals immunised with tick extract supernatant antigen of *B. microplus.*

Nyindo *et al*. (1996) reported the effect of naturally acquired resistance on vaccination with SGA. They conducted SDS-PAGE and immunoblotting analyses and revealed that antibodies with high avidity to SGA were directed to a 39-kDa polypeptide. Sera from rabbits that were first vaccinated with SGA consistently reacted with the 39-kDa polypeptide. Sera from rabbits that were infested recognized strongly a 42-kDa polypeptide among 5 polypeptides in the SGA, and found that resistance resulting from adult tick infestation is not augmented by immunity caused by vaccination with SGA. Ghosh and Khan (1997) [35] reported that the tick extract supernatant antigen of *B. microplus was* purified by gel filtration chromatography. On SDS-PAGE analysis of first peak they detected four bands of 105.4, 92.2, 67.0, and 62.0 kDa. Szabo and Bechara (1997) [35] reported the acquired resistance to *R. sanguineus* ticks in guinea pigs when immunized with tick gut and salivary gland extracts subcutaneously.

Ghosh *et al*. (1998) [13] prepared homogenized antigens from unfed larvae and nymphs of *H. a. anatolicum*. Five rabbits each were inoculated with 8.56 mg HLAg and 9.34 mg HNAg in 3 divided doses subcutaneously. Following immunisation rabbits developed significant level of protective immunity to infestation with adults of this species. Significant reduction in engorged percentage and weights of engorged females, egg masses and increased engorgement period were observed in females fed on immunized rabbits, compared to that of female

ticks fed on control rabbits. Larval antigen immunized rabbits showed significant antibody level from 28-126 days while with HNAg elevated antibody levels were recorded up to 112 days. Anti-HLAg and anti-HNAg sera recognized common antigenic bands of 97.4, 85, 66, 47.3, 42 and 31 kDa in homogenates of larvae, nymphs and adults.

Tripathi *et al*. (1998) [39] immunized rabbits with homogenates of unfed larvae and nymphs of the tick *R. sanguineus* and observed significant level of protective immunity to infestation with all the stages of the species. Sera from larval extract (RLAg) immunised rabbits were strongly positive for anti-RLAg antibodies after 3 weeks of immunisation while nymphal extract (RNAg) immunised rabbits were positive for anti-RNAg antibodies after 2 weeks of immunisation by ELISA. The immunised rabbits were also positive for immediate type hypersensitivity reaction on intradermal inoculation of RLAg and RNAg. Latha (1998) [21] showed prominent precipitation bands by agar gel immuno diffusion test, only from day twenty one post immunization with midgut antigen of *H. marginatum isaaci* which persisted till 63 DPI. No such reaction was observed on 0, 7 and 14 days.

Zhu-QingXian *et al*. (1999) [41] reported that the resistance to tick infestation was greatest in rabbits vaccinated with purified whole tick antigen of *I. sinensis* as compared to midgut, salivary gland and whole tick crude antigen. Ticks fed on rabbits vaccinated with tick egg antigen showed similar resistance as those fed on adjuvant control vaccinated rabbits, which revealed infestation of Ixodes sinensis could induce effective resistance in the host against the tick by destroying its midgut digestive cells

Omar (1999) [27] vaccinated five Boscat white rabbits with *H. dromedarii* tick egg antigen. Immunized rabbits developed variable serum antibody titres. Ticks removed from immunized rabbits, two weeks after challenge showed significant reduction in ticks weight, egg mass weight, oviposition period and percentage of egg hatchability. Raman (2000) performed SDS-PAGE analysis of partially purified midgut antigen of *B. microplus* and revealed twelve protein bands on 20.6, 24.4, 29.4, 34.2, 41.6, 46.8, 62.2, 67.7, 86.1, 92.4, 100.0 and 140.0 kDa while the purified antigenic fraction had four bands at 62.2, 67.4, 87.2 and 92.2 kDa.

Govindarajan *et al*. (2001) reported the protein profile of the crude midgut antigen of *Haemaphysalis bispinosa* by SDS-PAGE. The antigen revealed a protein profile consisting of 9 bands. Prominent bands were visible at 73, 65, 48 and 40 kDa, while faint bands were observed at 90, 80, 75, 35 and 33 kDa. Lawrie and Nuttall (2001) investigated antigenic responses of different host species that were repeatedly infested with *Ixodes ricinus* ticks by using Western blot analysis. Antigenic profiles of larval and nymphal whole tick homogenates were compared with the respective salivary gland extract (SGE) samples using sera from rabbits repeatedly infested with adults, nymphs or larvae. The pattern of antigenic tick-molecules recognized by infested host species varied with the period of feeding, developmental stage and the particular host species parasitized. Ramprakash *et al*. (2002) [31] used two New Zealand white rabbits to raise hyperimmune sera against midgut antigen of *Haemaphysalis bipsinosa*. Further they observed that Western blot of the midgut antigen showed antigenic bands at 90, 65, 40 and 35 KDa. In their studies the highly antigenic specific proteins were observed at 90 and 65 KDa.

Anil-Rana *et al*. (2003) reported the immunological cross reactivity between the salivary gland antigen and midgut antigen of *H. bipsinosa*. SDS-PAGE revealed common proteins of molecular weight 32 kDa in the midgut and salivary gland extracts. Aruljothi *et al*. (2005) [3] conducted experiment on Western blot analysis of midgut proteins of *R. haemaphysaloides*, which revealed that the crude midgut antigen had five immunogenic polypeptides ranging from 25.3-66.0 kDa, while partially purified midgut antigen revealed four immunogenic proteins ranging from 25.4-58.4 kDa.

Das and Ray (2005) reported the purification of larval antigen of *H. a. anatolicum* by two step affinity chromatography using anti tick gut specific rabbit IgG and IgG from immunized cattle. In their studies the purified antigen showed the presence of single polypeptide of 37 KDa on SDS PAGE.

Imamura *et al*. (2005) described the application of anti-tick vaccine which emerged as a most promising alternative tick control strategy compared to the current use of acaricides that suffer from a number of limitations. Vaccination of rabbits with rHLS2 conferred protective immunity against ticks, resulting in 44.6 and 43.0% mortality in nymphal and adult ticks, respectively.

Prevot *et al.* (2007) [29] reported the rise of neutralizing antibodies following immunization of rabbits and mice, which revealed significant protective immunity against ticks in rabbits only, resulting in a 30% mortality rate and diminution of weight gain in both nymphs and adults and a prolongation of blood feeding time in adults. Kavitha *et al*. (2007) [19] reported the isolation of a 35 kDa midgut polypeptide of *R. haemaphysaloides* from rabbits. After tick challenge, reduction in the number, mass and oviposition capacity of engorged females indicated a high efficacy of 83.3%, demonstrating the efficacy of the immune response elicited by 35 kDa midgut antigen to control the *R. haemaphysaloides*.

Materials and Methods

We have done experiment on New Zealand white rabbits with no any tick exposure, were used in the present experimental trails. Ticks were dissected according to the standard procedure described by Till (1961) and Blewett and Branagan (1973). Partially fed female *R. haemaphysaloides* ticks were surface sterilized. The salivary gland was taken out with the help of forcep on a glass slide and washed it with a drop of Phosphate Buffer Saline (pH 7.2) and then placed in PBS at 4 ^{0}C.

Preparation of salivary gland antigen (SGA)

The isolated salivary glands were homogenized in presence of 2mM (PMSF), 2mM (EDTA), 3mM (EGTA) and 0.01% merthiolate. Homogenate was subjected to sonication. Sonicated homogenate were centrifuged at 14 000 g for 45 minutes at 4 ^{0}C in a refrigerated centrifuge (Remi C-24). The protein concentration was determined by slandered method [Lowry *et al.* 1951]. The prepared antigen was used in the immunization trial as crude salivary gland antigen.

Characterization of salivary gland antigen (SGA)

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The crude salivary gland antigen of *R. haemaphysaloides* was characterized by 10.0% SDS-PAGE using a discontinuous system (Laemmli 1970) [20].

Protocol

A vertical slab gel electrophoresis system (Regular model, Banglore Genei) was used.

Sample loading
Molecular weight protein marker in the range of 97.4-14.3 kDa was used.

Electrophoresis
A power supply of 50 V was applied for the migration of protein through the stacking gel and 100 V through the separating gel. Run was stopped when the tracking dye reached the borderline of agarose ceiling.

Staining and Destaining
After electrophoretic run, the gel was removed carefully and immersed in 0.25% Coomassie Brilliant blue R-250 staining solution for one hour and then followed by destaining until the bands of polypeptide became unclear. The molecular weights of the unknown polypeptides were determined using the Syngene programme.

Dialysis
Washed the sac in 50% Ethanol with pinch of sodium bicarbonate and EDTA and heated the solution. Dialysis of SGA was performed to remove PBS and added 20mM sodium phosphate buffer solution in the SGA.

Treatment of Glass Wool
Warmed the glass wool in concentrated HCl for 2 min. transferred it to beaker containing distilled water and kept at 4 ^{0}C.

Regeneration of DEAE Sepharose
Mixed the DEAE Sepharose with the equal volume of 0.2 M NaOH for 30 min with intermittent stirring. Then washed by decantation using large volumes of distilled water until pH of the supernatant is approximately neutral. A 5 ml glass syringe was fitted in the burette stand. Glass wool was packed at the bottom of the column just above the knob and DEAE Sepharose beads were carefully loaded without air bubbles and allowed to settle down.

Alkaline treatment
Overloaded the column with 0.2 M NaOH FOR 30 min, then washed it by distilled water until pH comes to neutral (pH 7.0).

Acidic treatment
Overloaded the column with 0.2 M HCl for 30 min, then with distilled water to make pH neutral (pH 7.0).

Sodium Chloride Treatment
A clear solution of 1 ml SGA was carefully loaded to couple with beads of DEAE-Sepharose for 10 min and washed with the various concentrations of 20 ml of Sodium chloride solution *viz.* 0.05 M, 0.1M, 0.15 M, 0.2 M, 0.25 M, 0.3 M, 0.5 M. 2 ml of 10 fractions were collected from each concentration in a storage vials and the optical density (OD) values were recorded for each sample in spectrophotometer at 280 nm absorbance. The elution profile of the SGA was arrived by plotting a graph based on the OD values of each sample fraction.

Raising of hyperimmune sera
Immunization trails were carried out using eight New Zealand white rabbits. They were divided into two groups of four rabbits each. Animals in immunization groups received 500 mg of antigen per rabbit. Salivary gland antigen was mixed with equal volume of Freund's Complete Adjuvant (FCA) and was injected at multiple sites in rabbits in divided doses on day 0, 14 and 28 followed by blood collection. Second booster dose was given at 2 weeks interval and final blood collection was performed 2 weeks post second booster. The control rabbits received Freund's complete and incomplete adjuvant (FIA), alone on day 0, 14 and 28. All experimental rabbits were aseptically bled from heart before immunization on day 0, 14, 28, 42 post infection and subsequently at weekly interval upto 10 weeks post immunization**.** 5 ml of blood was collected from each animal and allowed to clot at room temperature for 2 hrs. followed by 4 hrs incubation at 4 ^{0}C and finally serum was separated and centrifuged at 4 ^{0}C for 30 minutes at 2500 rpm. Serum was aliquated and labeled properly and stored at -20 ^{0}C after adding 0.01% Merthiolate (thiomersol).

Sera
Blood from all experimental animals were collected from 0 day at weekly intervals to day 63 post immunization. The sera were separated and preserved with 1:1, 00,000 thiomersol and kept at -20 ^{0}C.

Assessment of humoral immune response
Agar gel immunodiffusion test (AGID) was conducted to assess humoral immune response in the experimental rabbits.

Agar Gel Immuno Diffusion Test
It was based on the precipitation reaction between antigen and antibody. The antigen and antibody migrate in the gel and form a precipitation line.

Protocol
The test was done according to the method of Ouchterlony (1958) with minor modifications. Slide was coated with 1% Agarose allowed it to solidify and then 4.5 ml of 1% Agarose solution was poured. Following solidification, seven wells were cut using a gel cutter. The inner well was at a distance of 4 mm from the outer ones. To the centre well salivary gland antigen (Ag) added. The outer wells were charged with the sera samples collected on 14 days post-II booster from the rabbits belonging to group II (control animals). Similarly, the wells B, C, E and F were charged with the sera samples collected on 14 days post-II booster from the rabbits belonging to group I. The slides were then incubated for about 24 hours in a petridish containing cotton soaked in water for maintaining moist condition. Following the formation of precipitation line, wells were sealed by agarose, slide was kept for washing in normal saline solution (NSS) for 24 hrs in order to remove non-precipited or unreacted protein. Finally it was washed with distilled water for 1 hr. The slide was covered with wet filter paper and kept at 57 ^{0}C for 18 hrs. Filter paper was removed and slide was stained with amido black stain.

Staining
The slides were then stained with Amido black staining for 30 minutes and destained using destaining solution.

Results and Discussion
Salivary Gland Antigen (SGA)
Preparation
The salivary gland antigen was prepared as per the method of

Johnston *et al*. (1986) [18] and the concentration of protein was estimated to be 2.1 mg/ml of antigen. Ghosh *et al.* (1998) [13] prepared homogenized antigens from unfed larvae and nymphs of *H. a. anatolicum* he inoculated rabbits with 8.56 mg Hylomma Larval antigen and 9.34 mg Hylomma Nymphal antigen subcutaneously.

Characterization

Characterization of Salivary Gland Antigen of *R. haemaphysaloides* was carried out by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The SDS-PAGE profile of the crude salivary gland antigen revealed 17 protein bands with Coomassie blue staining at 25.8, 30.1, 37.5, 40.1, 44.0, 59.2, 64.3, 68.0, 76.2, 81.2, 87.4, 92.8, 104.0, 117.2, 130.4, 140.3, 146.9 kDa. Smith (1994) [34] responses sodium dodecyl sulphate gel electrophoresis technique in which proteins were reacted with anionic detergent SDS to form negatively charged complexes. Jongejan *et al*. (1989) SDS-PAGE of SGE and ME from *A. variegatum* revealed the presence of 48 protein bands in SGE and 29 bands in midgut extract.

Although reports on such studies on salivary gland antigen of *R. haemaphysaloides* are absent. However the reports on other ticks include of Lee and Opdebeeck (1991) [23] who fractionated the purified fraction of the soluble midgut antigen of *B. microplus* Qu 13 by SDS-PAGE and observed protein bands from 30 to 200 kDa. Similarly, Ghosh and Khan (1997) [14] characterized midgut antigen of *B. microplus* and identified 16 protein bands ranging from 34.2 to 105.4 kDa in the crude antigen and 4 protein bands in fraction of TESA. While Raman (2000) revealed twelve protein bands ranging from 20.6 to 140.0 kDa of partially purified and 62.2 to 92.2 kDa of purified antigenic fraction of *B. microplus* midgut.

Purification

The crude salivary gland antigen was purified by ion exchange chromatography using beads of DEAE-Sepharose. The graph plotted based on the OD value of each of the 10 antigenic fractions revealed a peak and the protein concentration of 8 antigenic fractions forming the peak was estimated.

Similarly size exclusion ion exchange chromatography has been used for purification of *Ixodes ricinus* by Ganapamo *et al.* (1997) [12] and for *Dermacentor andersoni* by Gordon and Allen (1987). Similarly, Brown and Askenase (1986) also purified the salivary gland antigen of *Amblyomma americanum* by ion exchange chromatography, while the purification of midgut antigen of *B. microplus* by gel filtration chromatography has been reported by Ghosh and Khan (1997) [14] and Raman (2000).

Assessment of humoral immune response

The humoral immune response was assessed by Agar Gel Immuno Diffusion Test (AGID). The agar gel immuno diffusion test was conducted for group I and II (control group) by using sera collected from animals on 0, 14, 28 and 42 days following immunization. Prominent precipitation line appeared only from 10 days – II booster which persisted 42 days post II booster in animals belonging to group I. No such reaction was observed in group II control rabbits. Ghosh and Khan (1996) immunized calves with tick extract supernatant antigen and assessed humoral immune response by double immuno diffusion test wherein a single band was seen upto 21 DPI and 2 bands were noticed from28-84 DPI. This is similar to the present study with the exception that only one band was noticed from 21 to 63 DPI. Earlier reports on double immuno diffusion (DID) are that of Allen and Humphreys (1979), Johnston *et al*. (1986) [18]. Similarly, Kumar and Kumar (1996a) showed that DID was positive upto 84 DPI in response to supernatant midgut antigen of *H. dromedarii.* With *B. microplus* Panda *et al*. (1992) noticed single precipitation bands on 21 and 28 DPI and double bands on 38 DPI.

Manohar and Banerjee (1992) immunized rabbits with *Hylomma anatolicum anatolicum.* The sera of immunized rabbits showed single precipitation band on 14 and 21 day. On 28, 35, 49th day one or two bands were seen. Latha (1998) [21] showed prominent precipitation bands by agar gel immuno diffusion test, only from day twenty one post immunization with midgut antigen of *H. marginatum isaaci* which persisted till 63 DPI. No such reaction was observed on 0, 7 and 14 days

Kumar and Kumar (1995) reported the presence of serum antibody in rabbits after their immunization with midgut antigen from partially fed *Hyalomma dromederii* ticks with double immunodiffusion (DID) and capillary tube agglutination tests by giving three inoculations subcutaneously on days 0, 14 and 21 at a dose rate of 1 mg antigen per animal.

Ghosh *et al*. (1998) [13] Larval antigen immunized rabbits showed significant antibody level from 28-126 days while with Hylomma nymphal antigen (HNAg) elevated antibody levels were recorded up to 112 days. Anti-Hylomma larval antigen (HLAg) and anti-HNAg sera recognized common antigenic bands of 97.4, 85, 66, 47.3, 42 and 31 kDa in homogenates of larvae, nymphs and adults.

Conclusions

Salivary Gland Antigen of *Rhipicephalus haemaphysaloides* tick provoked immune response in rabbits. On characterization by SDS-PAGE, the SGA 17 protein bands ranging from 25.8 to 146.9 kDa were detected. On Partial purification SGA revealed protein concentration of 8 antigenic fraction ranging from 0.003 to 0.646 kDa. AGID test detected prominent precipitation line from 38 days DPI of salivary gland antigen. Thus, the salivary gland antigen of *Rhipicephalus haemaphysaloides* can constitute major immunological molecule as a vaccine candidate against tick parasitism of bovines.

Acknowledgment

Authors are thankful to The Associate Dean, Nagpur Veterinary College, Nagpur for providing all necessary facilities.

References

1. Agbede RIS, Kemp DH. Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Histopathology of ticks feeding on vaccinated cattle. Int. J Parasitol. 1986;16:35-41.
2. Allen JR, Humphreys SJ. Immunization of guinea pigs and cattle against ticks. Nature. 1979;280:491-493.
3. Aruljothi A, Latha BR, John L. Western blot analysis of midgut proteins of *Rhipicephalus haemaphysaloides*. Journal of Veterinary Parasitology. 2005;19(2):127-129.
4. Blewett DA, Branagan D. The demonstration of *Theileria parva* infection in intact *Rhipicephalus appendiculatus* salivary glands. Trop. Anim. Health. Prod. 1973;2:27-34.

5. Brown SJ. Highlights of contemporary research on host immune responses to ticks. Veterinary Parasitology. 1988;28(4):321-34.
6. Centurier C, Weiland G, Seubert S. Immunobiological studies on the host parasite relationship between rabbits and the soft tick *Ornithodoros moubata*. Berliner-und-Munchener-Tierarztliche-Wochenschrift. 1981;94(11/12):238-241.
7. Christine MW, James L, Coleman, Jorge LB. Salivary Gland Antigens of *Ixodes dammini* Are Glycoproteins That Have Interspecies Cross-Reactivity. The Journal of Parasitology. 1991;77(6):965-973.
8. De Castro JJ. Sustainable tick and tick born disease control in livestock improvement in developing countries. Veterinary Parasitology. 1997;7:77-97.
9. Ervin RT, Eppin FM, Ford RF, Hair JA. Estimation and economic implication of lonestar ticks infestation of night gain of cattle *Bos taurus* X *Bos indicus*. J Econ. Ento. 1989;12:343-345.
10. Fivaz BH, Norval A. Observations on successive infestations of the rabbit host by the ticks *Rhipicephalus appendiculatus* and *R. zambeziensis* (Acari: Ixodidae). Experimental Appl Acarology. 1989;7(4):267-79.
11. Gill HS, Boid R, Ross CA. Links Isolation and characterization of salivary antigens from *Hyalomma anatolicum anatolicum*. Parasite Immunol. 8(1):11-25.
12. Ganapamo F, Rutii B, Brossard M. Identification of an *Ixodes ricinus* salivary gland fraction through its ability to stimulate CD4 T cells present in BALB/c mice lymph nodes draining the tick fixation site. Parasitology. 1997;115(1):91-6.
13. Ghosh S, Khan MH, Gupta SC. Immunization of rabbits against *Hyalomma anatolicum anatolicum* using homogenates from unfed immature ticks. Indian Journal of Experimental Biology. 1998;36(2):167-70.
14. Ghosh S, Khan MH. Identification of tick antigen immunogenic in calves. Ind. J Anim. Sci. 1997;12:249-252.
15. Gordon JR, Allen JR. Isolation and characterization of salivary antigens from the female tick, *Dermacentor andersoni*. Parasite Immunol. 1987;9(3):337-52.
16. Govindarajan T, Ramprakash P, Velmurugan R, Latha BR, John L. Isolation and characterization of midgut antigen of *Haemaphusalis bispinosa*. Ind. J Small Rum. 2001;7(1):55-56.
17. Imamura S, da Silva Vaz Junior I, Sugino M, Ohashi K, Onuma M. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an anti-tick vaccine. Vaccine. 2005;23(10):1301-11.
18. Johnston LAY, Kemp DH, Pearson RD. Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Effects of induced immunity on tick populations. Int. J Parasitol. 1986;16:27-34.
19. Kavitha KT, Latha BR, Raj GD, John L. Immunoprophylaxis of rabbits against *Rhipicephalus haemaphysaloides* ticks using immunoaffinity purified 35 kDa midgut antigen. Veterinarski arhive. 2007;77(2):147-157.
20. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T_4 Nature. 1970;227:630-684.
21. Latha BR. Immunological strategies in the control of ixodid ticks. Ph. D., thesis submitted to TANUVAS, 1998.
22. Lawrie CH, Nuttall PA. Antigenic profile of *Ixodes ricinus*: effect of developmental stage, feeding time and the response of different host species. Parasite Immunology. 2001;23(10):549-56.
23. Lee RP, Opdebeeck JP. Isolation of protective antigen from the gut of *Boophilus microplus* using monoclonal antibodies. Immunol. 1991;72:121-126.
24. Lowry DH, Rosenbrough NJ, Ferry AC, Randall RJ. Protein measurement with the folin phenol reagent. Journal of Veterinary Parasitology. 1951;193:265-275.
25. Manohar GS, Banerjee DP. Cross resistance of heterologus tick species in rabbits immunized with whole antigen extract of *Hyalomma anatolicum anatolicum.* Ind. J Ani. Sci. 1992b;62:513-516.
26. Mbogo SK. Immunisation of rabbits against the brown ear tick, Rhipicephalus appendiculatus using tick haemolymph. Exp Appl Acarol. 1994;18(5):281-91.
27. Omar SFA. Experimental studies on vaccination of rabbits against hard ticks (*Hyalomma dromedarii*). Veterinary-Medical-Journal-Giza. 1999;47(4):457-466.
28. Panda DN, Ansari MZ, Sahai BN. Immunization of cattle with *Boophilus microplus* adult female and larval extracts; I. Antibody and cutaneous responses. Journal of Veterinary Parasitology. 1992;6(1):33-36.
29. Prevot PP, Couvreur B, Denis V, Brossard M, Vanhamme L, Godfroid E. Protective immunity against *Ixodes ricinus* induced by a salivary serpin. Vaccine. 2007;25(17):3284-92.
30. Raman M. Immunoprophylaxis against Boophilid ticks. Ph. D. thesis submitted to TANUVAS, 2000.
31. Ramprakash P, Govindarajan Velmurugan T, Bhaskaran Ravi Latha, Lalitha John. Western blot analysis of midgut antigen of *Haemaphysalis bispinosa*. Indian Journal of Animal Science. 2002;72:863-864.
32. Rechav Y, Dauth J. Development of resistance in rabbits to immature stages of the Ixodid tick *Rhipicephalus appendiculatus*. Medical Veterinary Entomology. 1987;1(2):177-83.
33. Riding GA, Jarmey J, Mckenna RV, Pearson R, Cobon GS, Willadsen P. A protective concealed antigen from *Boophilus microplus*: purification ionization and possible function. J Immunol. 1994;153:5758-5166.
34. Smith H. SDS Polyacrylamide Gel Electrophoresis of Proteins. In: Methods in molecular biology, Basic protein and peptide protocols Edited by: J. M. Walker, Humana press Inc., Totowa, New Jersey. 1994;32:23-34.
35. Szabo MPJ, Bechara GH. Immunization of dogs and guinea pigs against *Rhipicephalus sanguineus* ticks using gut extract. Vet. Parasitol. 1997;68:283-294.
36. Tembo SD, Rechav Y. Immunization of rabbits against nymphs of *Amblyomma hebraeum* and *A. marmoreum* (Acari: Ixodidae). Journal of Medical Entomology. 1992;29(5):757-60.
37. Thakur M, Singh DK, Varshney BC, Raghav PRS. Experimental Immunization of Rabbits against *Hyalomma anatolicum anatolicum.* J Vet. Parasitol. 1992;6(1):15-20.
38. Till WM. The contribution to the anatomy and histology of Brwon Ear Tick *Rhipicephalus appendiculatus* Neumann. Memories of Entomological Society of Southern Africa. 1961;6:124.
39. Tripathi P, Ghosh S, Khan MH. Immune response in rabbits against *Rhipicephalus sanguineus.* Indian Journal of Experimental Biology. 1998;36(5):476-82.

40. Wozniak EJ, Butler JF, Zam SG. Immune recognition of Ornithodoros tick (Acari: Argasidae) salivary antigens by anti-*Psoroptes cuniculi* antibodies. J Parasitol. 1996;82(2):375-8.
41. Zhu-Qing Xian, ZhiGang Liu, BingHui Ye, GuoQiang Xiong, Jing Li. Morphometry and DNA analysis of midgut digestive cells of *Ixodes sinensis* after infesting rabbits. Systematic-and-Applied-Acarology. 1999;4:21-30.