

Figure 4. Diagrammatic cross section showing extent of the Wadda palaeolake at Pithoragarh (inset shows the lithocolumn).

## Characterization of immunogenic components of *Hyalomma anatolicum anatolicum* – the vector tick of bovine tropical theileriosis

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Native polyacrylamide gel electrophoresis (PAGE) and immunoblotting assays were used to characterize the immunogenic proteins of salivary gland extract (SGE) and saliva of *Hyalomma anatolicum anatolicum* tick. Native PAGE of the SGE revealed 10 proteins of molecular weights 56, 60, 64, 66, 120, 148, 220, 264, 300 and >300 kDa. In the immunoblot assay of the tick SGE, mice hyperimmune serum reacted with only one protein of 66 kDa. In contrast to the SGE, the tick saliva revealed only 2 proteins of 264 and 66 kDa. The 66 kDa protein was also observed in the tick larvae extract. These results suggest that the 66 kDa tick antigen may be one of the likely proteins of relevance to anti-tick immunity and subunit vaccine development for *H. anatolicum anatolicum*.

*H. ANATOLICUM ANATOLICUM* is the most common tick vector of *Theileria annulata*, a lymphocytotropic and erythrocytotropic protozoan, causing high mortality in European dairy cattle (*Bos taurus*) and their cross-bred progeny (*B. taurus* × *B. indicus*) in India and other tropical countries. Thus, bovine tropical theileriosis has

Institute of Palaeobotany, Lucknow indicate that the Wadda lake was formed about 44,000 years BP and lasted until about 2,000 years BP.

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become a major constraint in cross-breeding programmes aimed at upgrading the local dairy cattle for high milk production. The use of chemical acaricides for the control of ticks has the obvious disadvantages of development of resistant tick strains, environmental pollution, public health hazards and high costs. A novel approach for control of ticks (ectoparasite arthropods) by immunization with tick antigens is becoming an interesting area of research<sup>1–3</sup>. In *H. anatolicum anatolicum* tick immunity studies in this laboratory, acquired immunity has been demonstrated in cross-bred calves following a single infestation with adult *H. anatolicum anatolicum* resulting in 60–65% rejection of the tick larvae. In addition, a significant proportion of the attached larvae and nymphs failed to feed normally on blood<sup>4</sup>. Subsequently, it was demonstrated that artificial immunization with adult tick salivary gland extract homogenates emulsified with Freund's Incomplete Adjuvant (FIA) produced protection manifested by rejection of tick larvae challenge infestations. The SGE induced anti-tick immunity was enhanced using *Ascaris* helminth extract in the adjuvant emulsion<sup>5</sup>, a known immunomodulator of IgE immune responses<sup>6</sup>.

The present study was undertaken to analyse the salivary gland immunogenic sub-components in the tick salivary gland by polyacrylamide gel electrophoresis (PAGE) and immunoblotting, using hyperimmune serum from inbred Balb/c mice in our pursuit for the development of murine monoclonal antibody probes to tick salivary antigens. An attempt in this direction would help in the development of an effective subunit anti-tick vaccine. Gill *et al.*<sup>7</sup> characterized the salivary gland antigens of *H. anatolicum anatolicum* by SDS-PAGE and

immunoblotting, using hypersensitized rabbit sera. In contrast to this report of the UK group, our study using hyperimmune Balb/c mice sera revealed one protein of 66 kDa on native PAGE gel (non-denatured), while none of the protein bands from SDS-PAGE gels reacted immunologically.

*H. anatolicum anatolicum* tick colonies (eggs, larvae, nymphs and adult stages) were maintained at the Tick Borne Disease Research Centre, Punjab Agricultural University, India, as previously described by Bhattacharyulu *et al.*<sup>8</sup>. Briefly, this involved feeding of different life cycle stages on cross-bred calves or rabbits and their subsequent maintenance in laboratory at 85% relative humidity and a temperature of  $28 \pm 2^\circ\text{C}$ , until they moult into the next instar.

Salivary glands were removed from partially fed adult females of *H. anatolicum anatolicum*, as per the method described by Walker *et al.*<sup>9</sup>. These were triturated in a little volume of saline in a glass homogenizer and subsequently sonicated (Soniprep-150 MSE, England), held under ice bath at 2  $\mu$  amplitude in 5–6 bursts of 30–60 seconds each. The sonicated material was centrifuged (Sorvall 6000B) at 2000 rpm for 5 min and the supernatant salivary gland extract was collected and stored at  $-70^\circ\text{C}$  till further use.

Adult female ticks, which had fed for 96 h, were held onto a glass surface using a double-sided adhesive tape with their dorsal surface facing upwards. Each tick was injected with 10–30  $\mu\text{l}$  of 1:10 dilution of dopamine (3-hydroxytyramine-HCl – 1.5 mg/ml) in saline, and saliva was collected into haematocrit capillary tubes as described by Kaufman<sup>10</sup>. On an average, 10–20  $\mu\text{l}$  of saliva was collected from one tick and pooled saliva stored at  $-70^\circ\text{C}$ .

Two-week-old larvae, free of egg shells and moult castings, were chilled to immobilize them and triturated in a little volume of normal saline, sonicated and centrifuged, as described above. The supernatant was stored at  $-70^\circ\text{C}$  and used as antigen till use. Protein content estimation of all the antigen preparations was done by the method of Lowry *et al.*<sup>11</sup>.

Eight male inbred Balb/c mice (Indian Institute of Nutrition, Hyderabad) were used for immunization. Each was injected with 150  $\mu\text{l}$  of the SGE (3.0  $\mu\text{g}/\mu\text{l}$ ) emulsified with FIA and vitamin E (6:4) by i.p. route. Tengerdy and Lacetera<sup>12</sup> have reported that vitamin E adjuvant formulations are potent immunoenhancers in mice. Booster injections were administered at 15, 21 and 30 days post-immunization with the SGE antigen in saline (at reducing antigen concentrations from half to one fourth and one sixth, respectively of the primary antigen inoculum). Mice were bled from tail for serum collection prior to each booster injection. Blood collected from all mice were pooled for each batch of serum collection and sera were stored at  $-70^\circ\text{C}$ .

Native PAGE discontinuous gels based on the method of Laemmli<sup>13</sup> consisting of resolving (12%) and staching gel (4%) were used. Mini-protean-II dual slab cell (Bio-Rad Labs, Richmond, CA) was used for electrophoresis. Buffer used was 25 mM tris, 192 mM glycine, pH 8.3. The SGE, saliva and LE antigen samples were diluted in sample buffer (12.5% tris-HCl, pH 6.8 and 10% glycerol). Samples were loaded and 0.05% bromophenol blue was added as a tracking dye. Molecular weights were estimated using bovine albumin, monomer-tetramer, standard marker kit (66, 132, 198 and 264 kDa, Sigma Chemical Co, USA). The samples were run at 100 V for 2 h. Gels were stained for 30 min in 0.1% Coomassie blue R-250 in fixative (40% methanol, 10% acetic acid) and destained for 1–3 h in 40% methanol and 10% acetic acid.

Discontinuous gels consisting of resolving (12%) and staching gels (4%) were used. 10% SDS was added to the gels, electrode and sample buffer (sample buffer had in addition 5%  $\beta$ -mercaptoethanol). Molecular weights were estimated using a mixture of standard markers – carbonic anhydrase (29 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa) (Sigma Chemical Co, USA).

The native PAGE gels were run by loading the gels without comb with the SGE antigen sample diluted in the sample buffer. Transfer buffer was 0.7% acetic acid. Transfer of proteins from the gels onto the nitrocellulose membrane sheets (NCM) was carried out using Mini-Transblot Cell (Bio-Rad) according to the procedure of Towbin *et al.*<sup>14</sup>. Transfer was carried out at 100 V for 5 h or at 30 V overnight. One strip of the transblotted NCM was stained with 0.1% amido-black to monitor the transfer of bands. The remaining NCM was blocked in 3% lactogen in 0.15 M phosphate buffer with 0.02% merthiolate, pH 7.2 (PBM), overnight at  $4^\circ\text{C}$ . For SDS-gels, transfer buffer used was tris glycine methanol. Transfer was carried out at 100 V for 90 min.

Subsequently the NCM was cut into thin strips which were processed for the immunoblot assay. One strip was treated with mice anti *H. anatolicum anatolicum* SGE serum for 1 h at  $37^\circ\text{C}$ , followed by three washings with PBM. The strip was further incubated with biotin conjugated goat-anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM, Sigma Chemical Co., USA) at a predetermined optimal dilution of 1:800 in 3% lactogen-PBM for 1 h at  $37^\circ\text{C}$ . Following washings with PBM, the strip was reacted with avidin-HRP (Sigma Chemical Co.) at a predetermined dilution of 1:200 in 6% lactogen-PBM for 1 h at  $37^\circ\text{C}$ . After three washings with PBM, the strip was treated with freshly prepared substrate solution (5 mg of 3–3' diaminobenzidine dissolved in 10 ml of PBM + 20  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$ ) for 2–3 min and the reaction stopped by rinsing the strip in PBM. In the immunoblot assay, control NCM strips from the same transblot were simultaneously treated with negative mice serum and

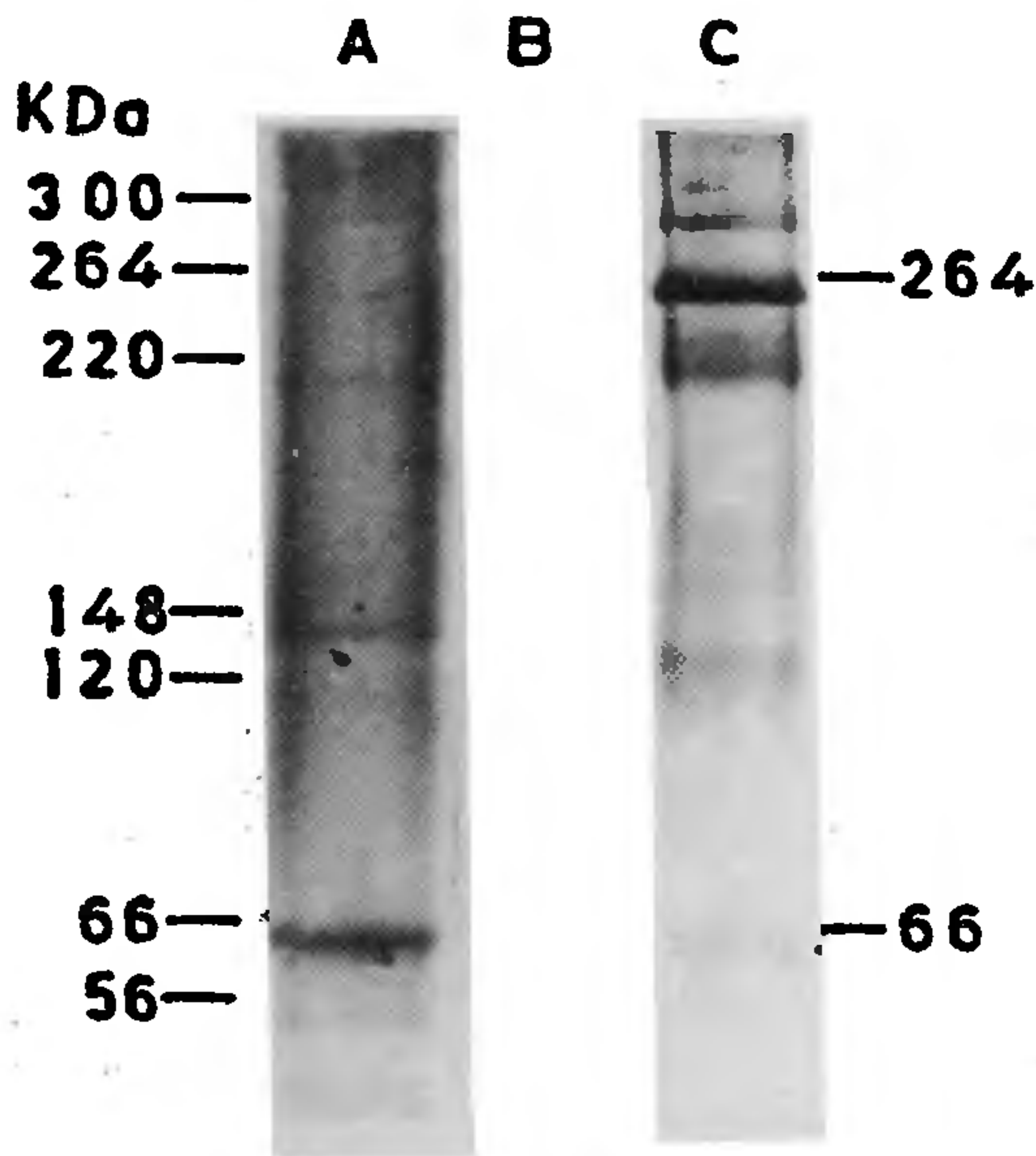


Figure 1. Native PAGE analysis of *H. anatolicum anatolicum* salivary gland extract (lane A), saliva (lane B) and larvae extract (lane C).

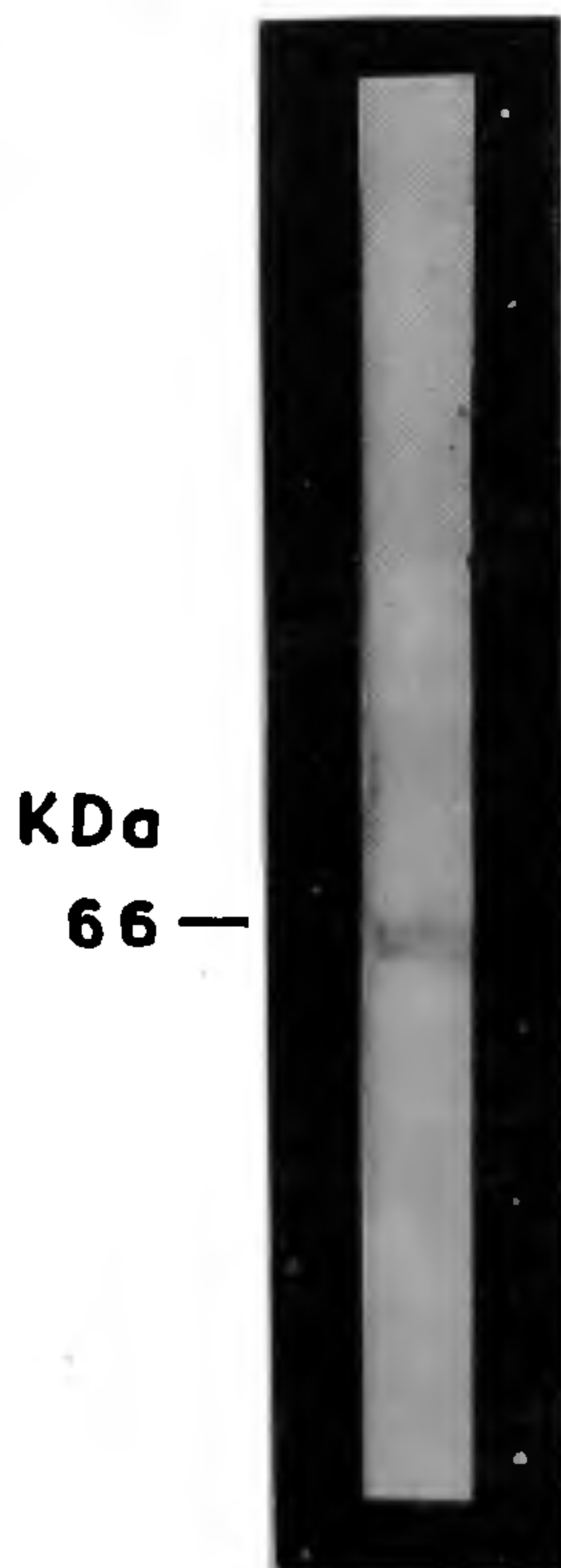


Figure 2. Immunoblot of the *H. anatolicum anatolicum* salivary gland extract native PAGE transblotted nitrocellulose membrane strip with mice anti-*H. anatolicum anatolicum* salivary gland extract hyperimmune serum.

PBM in place of anti-SGE hyperimmune mice serum and processed similarly.

Native PAGE of the SGE revealed 10 proteins of molecular weights 56, 60, 64, 66, 120, 148, 220, 264, 300 and > 300 kDa (Figure 1, lane A). In contrast, the saliva secretion showed only 2 proteins of 264 and 66 kDa (Figure 1, lane B). The LE revealed several proteins including the 264 and 66 kDa bands present in the SGE and saliva (Figure 1, lane C).

In the immunoblot assay, native PAGE of the SGE revealed one immunodominant protein of 66 kDa (Figure 2).

SDS-PAGE of the salivary gland extract (SGE) of *H. anatolicum anatolicum* revealed 12 major and 6 minor bands. Molecular weights of the major bands were 35, 38, 42, 44, 49, 51, 57, 63, 68, 72, 85 kDa. However, in the immunoblot assay, none of these SDS-PAGE polypeptides reacted with the anti-SGE hyperimmune serum (figure, not shown).

In this study on native PAGE gels, it was ascertained that the 264 and 66 kDa proteins were common to the SGE, saliva and larval extracts of *H. anatolicum anatolicum*. Native PAGE of the SGE revealed one protein of 66 kDa in the immunoblot assay.

Previous studies conducted in this laboratory have shown that cross-bred calves mount effective immunity to *H. anatolicum anatolicum* following cutaneous feeding of ticks<sup>4</sup> as well as artificial immunization with adult tick SGE homogenates emulsified with FIA<sup>5</sup>. During the natural process of tick feeding, saliva secretions are injected into the host animal at the site of tick attachment and effective anti-tick immunity induced. The paramount effect of anti-tick immunity is manifested against the tick larvae<sup>4,5</sup>. The 66 kDa antigen was also shown in the saliva, SGE and larvae extract (LE) of *H. anatolicum anatolicum* and its immunodominance in the immunoblot assay of the tick SGE suggests that it may be one of the likely proteins of relevance to anti-tick immunity in *H. anatolicum anatolicum*. For analytical studies requiring minute amounts of the antigen, purification of the relevant antigen from saliva would be simpler because it contains only 2 proteins. However, for bulk production of the antigen, the larval extract would be a better choice due to easier production of larvae colony in millions, while collection of large volumes of saliva and salivary gland dissection is cumbersome.

In the immunoblot assay of SDS-PAGE of the tick SGE, no specific reaction was revealed. In contrast to the present report, the previous report of UK group demonstrated that sera from hypersensitized rabbits reacted with 9 polypeptides in the saliva and 17 in the SGE from 96 h fed females of *H. anatolicum anatolicum* ticks using SDS-PAGE and immunoblotting. In our study the lack of reaction of hyperimmune mice serum in the SDS-PAGE immunoblot suggests that the system

picked up only the conformational but not the linear epitopes. No definite explanation for different results of our laboratory from that of Gill *et al.*<sup>7</sup> can be rendered with ease, they but may be attributed to different immunization protocols and animal species used.

The present study conducted in the inbred Balb/c mice will serve as a prerequisite for the development of murine monoclonal antibody probes to the tick SGE and saliva antigens for future work on characterization and purification of tick immunogens for the development of subunit anti-tick vaccine and specific diagnostic kits to monitor anti-tick immune responses.

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## ***Brugia malayi* in Indian leaf monkey (*Presbytis entellus*) – Response to repeated exposures of infective larvae**

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**Longitudinal studies on the development of disease manifestations and alterations in eosinophil counts in Indian leaf monkey (*Presbytis entellus*) infected with *Brugia malayi* infection were carried out. Monkeys received multiple exposures of infective larvae during prepatency as well as during late patency. All the monkeys showed positivity for microfilaraemia (mf) after 75 days of infection. The period of patency was observed to be around 400 days. Following re-exposure with infective larvae there was development of stage-specific resistance against mf and the monkeys acquired resistance against adult parasites. Increased eosinophil counts coincided with decrease in mf counts and re-exposures with fresh harvest of L<sub>3</sub>. The appearance of limboedema may or may not be associated with raised body temperature, however, low level of microfilaraemia coincided with the development of oedematous swelling.**

OUR knowledge of genesis of lymphatic filarial diseases is scanty. In India, 45 million people are infected with this disease<sup>1</sup>, of which 25 million harbour microfilarae in their blood and 19 million suffer from filarial disease manifestations such as swelling of limbs, hydrocoele, elephantiasis, etc<sup>2</sup>. In spite of certain startling revelations available today on differences in physiological and immunological parameters between carriers and symptomatic groups of subjects, the development of elephan-

tiasis and the risk factors involved for the development of filarial disease is yet to be understood. The principal bottleneck in this area is the non-availability of a simulating model where longitudinal study could be carried out. Several rodents<sup>3,4</sup>, canine<sup>5,6</sup> and feline<sup>4,7</sup> hosts infected with *Brugia* species have been used for studying the lymphatic filarial disease in recent years. However, the major drawback of these models is that the infection does not mimic the human disease in the anatomic localization of adult worms, in the symptomatology or in the immune effector mechanisms that may be involved. Moreover, the information generated so far with different experimental models is perhaps not completely translatable to human lymphatic filarial diseases. Nevertheless, the experimental studies conducted by Mak *et al.*<sup>8</sup> using non-human primate with *B. malayi* would probably explain the cause of filarial diseases. However, the setback in Mak's model is that the *Presbytis* species (with *B. malayi* infection) used does not produce any gross filarial manifestations (acute or chronic). The recent observations made in our laboratory show that Indian leaf monkey (*P. entellus*) when exposed to single or multiple doses of infective larvae (L<sub>3</sub>) of *B. malayi* causes some of them to develop acute filarial manifestations<sup>9</sup>. Therefore, it is worthwhile to carry out longitudinal studies of alternations in various physiological parameters of host (*P. entellus*) harbouring *B. malayi* infection. We report here the results of experimental studies on development of disease manifestations, including alterations in absolute eosinophil counts in Indian leaf monkey, following single or multiple exposure with L<sub>3</sub> of *B. malayi*.

Six male monkeys (3–4 kg) were used for the study. Five of them were exposed to L<sub>3</sub> of *B. malayi* obtained from freshly dissected infected vectors (*Aedes aegypti*)<sup>9</sup>. One unexposed monkey served as an uninfected control.