

## Immunization of cattle against *Hyalomma anatolicum anatolicum* using larval antigens

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Development of immunity in cross-bred (*Bos taurus* x *Bos indicus*) calves against *Hyalomma anatolicum anatolicum*, vector of bovine tropical theileriosis, was studied using larval antigen (LS) in Freund's complete adjuvant (FCA). Calves immunized with LS + FCA showed significant rejection of larvae ( $57.25 \pm 6.8$ ) and nymphs ( $45.75 \pm 5.16$ ). Abnormally fed larvae ( $11.4 \pm 0.8$ ) and nymphs ( $8.25 \pm 1.2$ ) were also recovered from immunized calves. This abnormal feeding may possibly be attributed to their inability to gain access to the blood vessels owing to the host immunological reactions. Consequently, feeding of extravascular fluid leads to white colour of fed ticks. Sera from all immunized calves after a week of immunization were positive for anti-LS antibodies in ELISA. The investigation indicates that LS in FCA enhanced anti-tick immunity.

Without effective tick control, it would be virtually impossible to raise livestock economically in many areas of the world<sup>1</sup>. A recent estimate put the worldwide losses in livestock production caused by ticks and tick-borne diseases at \$8 billion<sup>2</sup>. The ixodid tick *Hyalomma anatolicum anatolicum* is the most important vector of pathogens to livestock in India and other tropical countries. Conventional methods for control of ticks by the use of acaricides have certain limitations, such as acaricide resistance, environmental pollution and high cost. As an alternative, immunological control of ticks is an emerging area of research for the development of anti-tick vaccines. Development of acquired immunity against ticks by artificial immunization with tick antigens has been reported in different tick-host combinations<sup>3-5</sup>. Very limited work has been done on the immunization of cattle against *H. a. anatolicum*. Development of immunity against this tick species following repeated infestation in rabbit<sup>6</sup> and cattle<sup>7</sup> have been reported. Artificial immunization against *H. a. anatolicum* in cattle using tick salivary gland extract (SGE) antigens have been reported<sup>8,9</sup>. Recently, Ghosh *et al.*<sup>10</sup> reported the immunoprotective potentiality of the larval antigen of *H. a. anatolicum* against adult ticks in rabbits. However, so far no work has been carried out for the immunization of cattle by the use of larval antigen of *H. a. anatolicum*. The present investigation reports immunization of cattle to immature stages of *H. a. anatolicum* with immunogens prepared from larvae.

**Ticks**—Ticks from colonies, propagated and maintained at the Entomology laboratory of the institute as per the method described by Khan *et al.*<sup>11</sup> were used.

**Experimental animals**—New born cross-bred (*Bos indicus* x *Bos taurus*) male calves with no previous exposure to tick were procured from the Institute's dairy farm. These calves were housed in tick-proof shed and treated regularly with coumaphos (0.125%). The tick naive-status of these calves was further confirmed by immunodiffusion and en-

zyme linked immunosorbent assay with larval, nymphal and adult antigens of *H. a. anatolicum*. At the time of immunization these calves were 6-8 months old.

**Preparation of larval antigen**—Antigen extractions was done in an ice-bath and were stored at  $-20^{\circ}\text{C}$ . Laboratory-reared larval ticks were disrupted in 0.15M phosphate buffered saline (PBS), 1mM disodium EDTA (pH7.2), in a glass homogenizer, filtered free of cuticle and debris and sonicated for 10 min, interspersed with periods of cooling. The homogenate was centrifuged at 15000 g for 20 min to yield a supernatant designated as TLE. Total larval extract (TLE) was further centrifuged at 100,000 g for 1hr and separated into larval supernatant (LS) and pellet (LP). The supernatant was mixed with 200 i.u./ ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin and was used for immunization. The protein concentration of the antigen was determined<sup>12</sup>.

**Experimental immunization**—Calves (12) were randomly divided into two groups. The immunization protocol including controls is outlined in Table 1.

**Sera sample**—Blood was collected from the jugular vein of each calf on days 0,7,14,21,28,35,42 and 49. Sera were separated and centrifuged at 5000g for 15 min and stored at  $-20^{\circ}\text{C}$  until screened for antibodies to the LS antigen in

Table 1—Protocol of artificial immunization of calves against *H. a. anatolicum* larvae and nymphs

Immunization schedule	Group A*	Group B
Primary immunization (Day 0)	2 ml LS (6 mg) emulsified in equal volume of FCA, injected sc	2 ml FCA mixed with equal volume of PBS, injected sc
First booster (Day 14)	3 ml LS (9 mg) mixed as above, injected sc	3 ml FCA mixed as above, injected sc
Second booster (Day 28)	1.5 ml LS (4.5 mg) mixed in equal volume of PBS, injected im	3 ml of PBS injected

\*Each group includes 6 calves. The dose specified refer to one calf. FCA, Freund's complete adjuvant

immunodiffusion and ELISA.

**Serological methods**—Immunodiffusion test was carried out according to the procedure of Ouchterlony<sup>13</sup>. Agarose (1%) was used and gel slides were stained with Coomassie brilliant blue.

Microtiter plates were coated with 50 µg LS (5 µg ml<sup>-1</sup>) in carbonate bicarbonate buffer, pH 9.6. After overnight incubation at 4°C and washing the plates 4 times with PBS containing 0.05 % Tween-20 (PBS-T), the plates were blocked with 150 µl PBS containing 3 % BSA per well for 2 hr at room temperature. After washing the plates with 4 changes of PBS-T, 50 µl of 1:1000 dilution of both experimental and control serum in 1 x PBS was placed in quadruplicate wells and kept at room temperature for 2 hr. The plates were again washed with PBS-T for 4 times, 50 µl of a 1:10000 dilution in 1 x PBS of affinity-purified rabbit anti-bovine IgG of horse reddish peroxidase conjugate (Sigma, USA) were added to each well. After 2 hr incubation, the plates were washed and finally 50 µl of substrate solution containing O-phenylene diamine (Sigma, USA) in phosphate citrate buffer, pH 5 and H<sub>2</sub>O<sub>2</sub> were added to each well. The plates were kept in darkness for 30 min and optical density (OD) was measured at 492 nm using Titertek Multiscan plate reader (Flow Labs, U.K).

**Challenge infestation**—After 10 days of last immunization, all the animals were challenged with unfed 1200 larvae and 300 nymphs per ear and secured with cotton bags. Live fed larvae and nymphs collected from the bags after completion of their feeding (3-5 days for larvae and upto 11 days for nymphs) were counted. Anti-tick immunity was assessed by calculating the mean percentage recovery of naturally dropped fed larvae/nymphs at the end of the feeding period. Percentage recovery of dropped stages was calculated by the formula:

$$\frac{\text{Number of ticks dropped}}{\text{Number of ticks released}} \times 100$$

The per cent rejection was calculated by the formula: 100 - % recovery.

The dropped larvae/nymphs were grouped as abnormally fed and normally fed. After counting, both the batches were kept separately.

**Statistical analysis**—Differences between means were established by analysis of variance and individual differ-

Table 2—Feeding and development of *H. anatolicum* larvae and nymphs fed on immunized and control calves

Parameters	[Values are mean ± SE]			
	Challenged by larvae		Challenged by nymphs	
	Immunized	Control	Immunized	Control
Rejection (%)	57.25 ± 6.8a	9.6 ± 1.24	45.75 ± 5.16a	10.8 ± 1.35
Feeding period (d)	3.8 ± 0.3	5.0 ± 0.6	9.26 ± 0.7	6.8 ± 0.3
Abnormally fed ticks (%)	11.4 ± 0.8b	1.2 ± 0.03	8.25 ± 1.2b	1.9 ± 0.4
Moulting period (d)	12.98 ± 0.3	11.0 ± 1.25	16.64 ± 0.2	16.25 ± 1.8

P values: a < 0.01; b < 0.05

ences between groups were analysed by Student's t test<sup>14</sup>.

**Attachment and feeding behaviour of larvae**—Table 2 shows the effect of immunization on larvae. In immunized calves larvae were seen dying *in situ* within 36 hr of attachment and skin hypersensitivity reactions were visible around the bite sites of many larvae. The mean per cent rejection (57.25 ± 6.8) in group A was significantly ( $P < 0.01$ ) greater than that of group B (control). The feeding period of the attached larvae was insignificantly lower in group A. A significantly ( $P < 0.05$ ) higher number of abnormally fed larvae (whitish in colour) was recovered. No significant differences in the moulting period of larvae fed on immunized and control calves were recorded.

**Attachment and feeding behaviour of nymphs**—Table 2 summarizes the effects of immunization on nymphs. In contrast to higher rejection of larvae, nymphs were less affected as mean per cent rejection was 45.75 ± 5.16. However, the value was significantly ( $P < 0.01$ ) greater than the control group. As in the case of larval challenge a significantly ( $P < 0.05$ ) higher percentage of abnormally fed nymphs were recorded from the calves of group A. The moulting period of nymphs fed on both the groups was almost equal.

About 40% abnormally fed larvae and nymphs moulted successfully into nymphs and adults respectively, as did the normally fed larvae and nymphs.

**Detection of anti-LS antibodies**—Immunodiffusion test showed single precipitin band in the sera of calves collected on 14 day post-immunization (dpi). On 21 to 49 dpi 1-2 bands were seen.

Sera from control group calves collected at different days were negative for antibodies to LS antigens in ELISA. However, a significantly ( $P < 0.01$ ) higher antibody response was detected on 28 dpi and maximum response was noted at 42 dpi in immunized calves in comparison to control. The antibody response starts declining at 49 dpi.

Immunization with tick derived antigens has considerable potential in controlling tick infestation of livestock<sup>15-17</sup>. To protect high milk producing cross-bred (*Bos indicus* × *Bos taurus*) cattle with less resistance to ticks and tick-borne diseases, means to enhance acquired immunity by artificial immunization with tick antigens is drawing attention of several research groups.

The present investigations demonstrated significantly higher antibody levels in immunized calves indicating that LS antigen was immunogenic. ELISA being a very sensitive test, can therefore, be used for detecting and monitoring anti-tick antibodies in experimental and in field conditions. Previously, early and late anti-tick antibodies were detected by ELISA<sup>18-20</sup>.

In most of the experiments with multi-host ticks, the immunized animals were challenged with same stage from which the immunizing extracts were made. However, Verma *et al.*<sup>4</sup>, Sran *et al.*<sup>9</sup> and Ghosh and Khan<sup>21</sup> have shown that guinea pigs and cattle are protected against *Rhipicephalus appendiculatus* adults, larvae and nymphs of

*H. a. anaticum* and adults of *Boophilus microplus* by immunizing the animals with antigens from immature stages of *R. appendiculatus*, salivary glands of *H. a. anaticum* and larval antigen of *B. microplus* respectively. In our earlier experiments<sup>10</sup> we have shown that rabbits are protected against adult of *H. a. anaticum* by immunizing with the antigens from unfed larvae and nymphs of the species. It was also suggested that the presence of common antigenic bands of 97.4, 85.0, 66.0, 47.3, 42.0 and 31.0 kDa in the homogenates of larvae, nymphs and adults of *H. a. anaticum* may be responsible for cross-immunity between the stages. Present experiments clearly show for the first time that unfed larvae of *H. a. anaticum* provide an easily available and efficient source of antigen for immunization of calves against both larvae and nymphs of the species. The white colour of the abnormally fed larvae and nymphs is possibly due to lack of red blood cells intake in their meal. This may be of considerable importance in decreasing the transmission of the disease, theileriosis. The immunological cross-reactions between the stages may be due to the presence of common antigenic proteins as reported<sup>10,22</sup>.

In conclusion, the present investigation indicates that the larval antigen can be used efficiently for the development of anti-tick immunity. Enzyme linked immunosorbent assay may be useful to monitor anti-tick immune response in calves.

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