ATTEMPTED IMMUNIZATION OF CROSS-BRED CALVES AGAINST IXODID TICK, *HYALOMMA ANATOLICUM ANATOLICUM* KOCH (1844)

By

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Dissertation submitted to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

in

VETERINARY PARASITOLOGY

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CERTIFICATE I

This is to certify that this dissertation entitled, "Attempted immunization of cross-bred calves against Ixodid tick, Hyalomma anatolicum anatolicum Koch (1844)" submitted for the degree of Ph.D. in the subject of Veterinary Parasitology of the Haryana Agricultural University, is a bonafide research work carried out by Dr. R.R. Momin under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

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CERTIFICATE II

This is to certify that this dissertation entitled: "Attempted immunization of cross-bred calves against Ixodid tick, Hyalomma anatolicum anatolicum Koch (1844)" submitted by Dr. R.R. Momin to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of Ph.D., in the subject of Veterinary Parasitology, has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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Dedicated To

My

Beloved Wife

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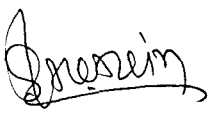

(R.R. MOMIN)

C O N T E N T S

CHAPTER-I

INTRODUCTION

Ticks are the largest and most important group of ectoparasites of domestic animals in tropical and sub-tropical countries including India. The ixodid ticks are most important as vectors, and next to mosquitoes in importance as vectors to man (Balashov, 1972; Bram, 1975). It is apparent that as the epidemiological pattern of some of the diseases of animals and man are changing, an increasing number of tick-borne diseases in them are being reported recently (Hoogstraal, 1981).

Ticks and tick-borne diseases are major animal health problems, particularly in the developing countries like India, where cross-breeding programmes with exotic cattle are practiced. It has been estimated that the ticks affect roughly 80 per cent of cattle population (Snelson, 1975) and cause annual world economic loss of 7 billion US dollars (McCosker, 1979), through reduced meat and milk production, mortalities, damaged hides and the cost of tickicides. Ticks harm the animals by tick worry, unthriftiness, delayed growth, anaemia, abortion, calf losses, reduced fertility, toxicoses, lowered resistance against infections, incidence of tick-borne diseases (babesiosis, theileriosis, anaplasmosis), and even death. Tick bite may serve as portals of entry for bacteria, parasites and other pathogens. Injection of toxins with tick bite can result in paralysis, sweating sickness or toxicosis.

Hyalomma anatolicum anatolicum Koch, (1844), is the most common tick infecting Indian livestock. Out of seven species of Hyalomma occurring in India, H.a.anatolicum is most widely distributed (Kaiser and Hoogstraal, 1964). Besides causing direct damages, this tick acts as vector of various diseases like equine babesiosis caused by Babesia caballi and B. equi in Greece (Enigk, 1943), East coast fever of cattle caused by Theileria parva in East Africa (Lewis and Fotheringham, 1941), and theileriosis of cattle caused by Theileria annulata in Asia (Delpy, 1949), Egypt (Daubney and Said, 1951), and Soviet Central Asia (Serdyukova, 1946).

For controlling tick population, the most reliable method until today was the use of potent insecticides. However, large scale and indiscriminate use of various insecticides has led to the emergence of insecticide resistance in ticks (Wharton and Roulston, 1970; Wharton, 1976). Moreover, the presence of chemical residues in animal products, and their effects on the environment leading to acute or chronic toxicity to man, animals and plants are matters of great concern and must be considered when new acaricides are developed or put to use. These factors and the cost-benefit ratio of developing a new acaricide discouraged the researchers to develop new compounds for control of ticks (Durand, 1976). Tick resistance has been reported against almost all chemicals used for control (Solomon, 1983).

The cost of developing new insecticides, their application, the availability of potent insecticides, emergence of resistance etc. have warranted investigation on alternative methods and an integrated approach by which the tick-animal association could be effectively reduced.

Alternative measures of tick control do exist, and it is important to further develop such measures leading to new approaches of control. Towards this end, attempts have been made in recent years for inducing some resistance in animals against the attachment and establishment of ticks. No work on these lines seems to have been carried out in India so far. Studies conducted elsewhere showed the possibility of producing some significant resistance in laboratory animals as well as in large animals against tick attachment, employing different species of ticks. This seems to be very promising and new line of approach in controlling tick-borne infections in animals that are rampant in India.

The present investigation was, therefore, undertaken with the following objectives:

1. To study the development of immunity, if any, in the young cross-bred calves by repeated tick attachments.
2. To immunize the cross-bred calves by inoculation of the salivary gland antigen.

CHAPTER – II

REVIEW OF LITERATURE

I. Historical background

The investigation of acquired immunity to ticks has a long history. It was observed early this century that different breeds of cattle tend to carry different numbers of the tick, Boophilus microplus.

Development of acquired resistance to ixodid tick infestation was first reported by Johnston and Bancroft (1918), when they described development of resistance to Boophilus sp. by cattle given repeated infestations. Resistant cattle developed a lymph-like exudate at tick attachment sites, and it was speculated that the resistance developed in response to substances introduced by the tick during feeding.

A genetic basis for tick resistance was hypothesized, when it was observed that asiatic breeds of cattle were more resistant to infestation. Subsequent studies led to the development of the concept that resistance observed in bovines consists of innate and acquired aspects, and animals of Bos indicus genetic composition were innately more resistant than Bos taurus (Kelley, 1943; Riek, 1962; O'Kelly and Spiers, 1976; Wharton et al., 1970). However, Wagland (1975, 1978) and Hewetson (1971) reported that Bos taurus and Bos indicus cattle not previously infested with B.microplus were equally susceptible to infestation, a finding that cast doubt on the validity of the concept of innate resistance.

Trager (1939a) demonstrated that laboratory animals (guinea pigs, deer mice and rabbits) acquired resistance to ixodid tick infestation. Subsequent reports described acquisition and expression of tick resistance for these laboratory animals, but only few investigators (Allen, 1973; Boese, 1974) examined the immunological basis of host resistance.

II. Expression of resistance against ticks

Cattle and laboratory animal species (guinea pigs, rabbits and mice) developed resistance to infestation by ixodid tick species (Willadsen, 1980; Wikel, 1982, 1983, 1984). Resistance was expressed by: (a) prolonged period of engorgement; (b) death of ticks on the host; (c) reduced number of engorged ticks; (d) reduction in engorgement weight; (e) inhibition of egg laying and reduced number and viability of the eggs; (f) reduced hatching per cent, etc.

It is convenient to distinguish between the two immunological approaches to the control of ticks (Willadsen, 1981). It has been reported frequently that the hosts acquire an immunologically mediated resistance after repeated infestation with ticks. The first approach involves the exploitation of this acquired resistance through selection of hosts capable of exhibiting a high level of resistance. The second approach involves immunization with isolated tick antigens to induce resistance.

III. Expression of resistance to repeated tick infestations

A. BOVINE:

The immunity investigated in detail was in cattle against B. microplus ticks. Roberts (1968a) observed that the Bos taurus cattle infested daily with a constant number of B. microplus larvae yielded a relatively constant number of engorged adult females. All the animals rejected the larval attachment within 24 hours of infestation and the effect was more in tick resistant animals. The other subsequent instars were similarly affected. The ability to reject ticks was an acquired one and appears about eight days after initial infection by the larvae of a previously unexposed animal.

Roberts (1968b) mentioned that initially about 50 per cent B. microplus larvae mature on most cattle that had no previous experience of tick infestation. According to the degree of resistance, 0 to 30 per cent of infesting larvae eventually matured to adults on the animals. It was, therefore presumed that this response was immunologically mediated.

Hewetson (1971) stated that there was prolongation of engrogement period, reduction of egg laying period and egg viability in B. microplus ticks fed on immune Bos indicus cattle but these effects were small on this host species as reported by Wagland (1975), and were absent from those of Bos taurus or Bos taurus x Bos indicus cattle (Hewetson and Nolan, 1968; Roberts, 1968b).

Roberts (1971) observed that B.microplus larvae behave in similar fashion on cattle possessing different degrees of resistance and suggested that the larvae remained unaffected and rarely drown in serous exudate but, Riek (1962) mentioned that some ticks may drown in serous exudate.

Strother et al. (1974) reported that Bos taurus, Bos indicus and cross-bred cattle, repeatedly infested with Amblyomma americanum, developed acquired immunity, as shown by the reduction in the number of female ticks engorging and a fall in their engorged weights. However, many of the ticks which did not engorge fully, died on the host at various ages and levels of engorgement.

Doube and Kemp (1975) mentioned that cattle on exposure to Ixodes holocyclus showed removal of the ticks by grooming, death of the ticks in situ, or reduction in their engorgement weight.

Wagland (1975) studied the response of previously unexposed purebred Brahman and beef Shorthorn cattle to four infestations with 20,000 larvae of B.microplus. On first infestation, the yield of engorged female ticks on all animals was about 25 per cent of the larvae applied. After three further infestations, the mean yield of engorged females on the Brahman decreased to 7.5 per cent, whereas, there was no decrease in the yield of ticks on the Shorthorns. On the Brahman cattle, development of larvae to engorged females took one to two days more and the engorged females weighed less.

Wagland (1978) mentioned that the weight of fully engorged female B. microplus ticks reduced by 30 per cent on immune Bos indicus cattle.

Wagland (1979) observed similar effects of B. microplus on Bos indicus, as mentioned by Roberts (1968a,b) though gradual loss of ticks occurred throughout the instars.

Sutherst et al. (1979) studied the development of resistance in Bos taurus cattle to Haemaphysalis longicornis. The small but variable proportions of larvae, nymphs and adult female ticks matured on previously exposed hosts and larger proportions on the control hosts. This suggested that the animals acquired and expressed different levels of resistance against each instar of the ticks.

Garris and Hair (1980) reported that heifers of Bos taurus (Hereford breed) and other heifers of Bos taurus x Bos indicus (Hereford x Brahman (Braford)) were compared in further tests of resistance in different breeds of cattle to lone star ticks (A. americanum). Attachment, feeding time, amount of blood taken and hatching and moulting rates were similar in ticks on heifers of both types, but Braford animals were less favourable than Hereford heifers to the development of A. americanum as indicated by egg mass weight, number of tick feeding and detaching, average weight of ticks on detachment and fecundity rates.

Hair and Garris (1980) observed the development of resistance by Brahman and Hereford cattle to A. americanum in terms of tick attachment and engorgement rates, average

tick weight and moulting, oviposition and facundity rates. Brahman cattle were generally less favourable than Hereford cattle for tick development.

Wikel and Osburn (1982) examined the effect of low level infestation with adult Dermacentor andersoni on the immune responsiveness of Bos taurus cows and calves. Each animal was exposed for one to four infestations, with 10 female and five male ticks. Tick feeding was separated by ten-day tick free period. Thus, the immune system of bovine could be stimulated significantly by repeated low level ixodid tick infestations.

Brown et al. (1984a) reported that purebred Holstein calves acquired resistance to A. americanum adult ticks after one infestation and expressed resistance during a challenge infestation 26 days later. Tick yield from resistant animals were normal, but mean tick weight and egg mass weight were reduced significantly (31 and 32 per cent, respectively).

Fivaz et al. (1984) reported that repeated sensitization of Friezland x Hereford cattle to Rhipicephalus appendiculatus resulted in reduced rates of recovery of the tick at each instar.

George et al. (1985) exposed purebred and crossbred Bos indicus calves to one, two or three infestations with 10 female and five male A. americanum ticks. Resistance was acquired by all the calves after first infestation showing decrease in the number of engorged females, mean weights of engorged females, and mean weight of egg masses. Purebred Bos indicus showed a stronger resistance as compared to

crossbreds. However, all calves showed similar levels of resistance during a third exposure.

Amin-Babjee and Riek (1986) observed the development of resistance to experimental tick (B. microplus) infestation in Bos taurus. Five calves, previously unexposed to ticks, were infested repeatedly for six times with 20,000 larvae. Except for the fourth infestation, all calves developed resistance after a primary exposure. On the first infestation the mean yield of engorged female ticks was 39.2 per cent of the larvae applied. The mean yields of engorged female from second to the sixth infestation were 8.3, 10.3, 11.8, 6.3 and 4.6 per cent, respectively. The weight of the engorged ticks fell in infestations after the primary one, with no change in egg production or their viability.

Gill (1986) observed that cattle (Bos taurus) acquired resistance to H. a. anatolicum feeding after a single infestation. This resistance was manifested by prolongation of engorgement period, mean weight reduction of engorged females and of egg mass, failure to lay eggs and sometimes death of ticks (unengorged) on the host. The degree of resistance increased with successive infestation, and was stronger on tertiary than on secondary infestation.

B. LABORATORY ANIMALS:

Trager (1939a) was the first to observe that when a first batch of larvae of Dermacentor variabilis was placed on a guinea pig, nearly all reached full engorgement,

whereas, the number of larvae reaching full engorgement during subsequent infestations on the same animal was greatly reduced. This suggested that a single infestation induced an effective resistance to subsequent infestations. Resistance was manifested by fewer larvae engorging on the resistant animal and those which engorged had much less blood meal. Moreover, the resistance was not limited to the site of previous tick feeding, thus suggesting systemic resistance rather than a localized alteration of the skin.

Bowessidjaou et al. (1977) demonstrated that repeated infestation in rabbits with Ixodes ricinus caused decrease in the percentage of ticks engorging, the engorged weights, the percentage of female laying eggs and the viability of the eggs. However, the length of feeding increased. Similarly, effects on the percentage of ticks engorging and engorgement weight have been observed by Kohler et al. (1967) for Hyalomma anatolicum excavatum and Rhipicephalus sanguineus in rabbits; Garin and Grebarew (1972) for R. sanguineus in rabbits; Allen (1973) for D. andersoni in guinea pigs; Branagan (1974) for R. appendiculatus in rabbits; Wikel et al. (1978) for D. andersoni in guinea pigs; McTier et al. (1981) for D. andersoni and A. americanum in guinea pigs; Rahman (1984) for Hyalomma rufipes in rabbits; Gill and Walker (1985) for H. a. anatolicum in rabbits and Whelen et al. (1986) for D. andersoni in guinea pigs. Fujisaki (1978), however, reported that immunity in rabbits to H. longicornis was somewhat different in that although the weights of engorged female ticks were reduced,

the number of ticks engorging, length of engorgement and hatching rate of eggs were all unaltered.

Allen (1973) and Wikel and Allen (1976a) stated that a single infestation of D. andersoni and guinea pigs produced almost complete immunity, as shown by less than 20 per cent larval engorgement and frequently reaching almost zero. Wikel and Allen (1976b) also mentioned a six to seven fold weight reduction of the larvae after five days of engorgement and the immunity was immunologically mediated and its acquisition could be blocked by the prior or concommitant treatment of the host with immunosuppressants like methotrexate and cyclophosphamide.

Norval (1978) mentioned that rabbits did not acquire resistance to larvae and nymphs of Amblyomma hebraeum. After repeated infestations of hosts showed no progressive decline in either the tick yield or the engorged weight of fed ticks. Changes in the host physiology - as a result of seasonal changes appeared responsible for the fluctuations.

C. OTHER ANIMALS:

Berdyev and Khudainazarova (1976) infested the lambs with adults of Hyalomma asiaticum asiaticum, either in a single operation, or at intervals of two or three days. The ticks desensitised the local sensory response mechanism of the host and engorged without causing any noticeable irritation, continuous and prolonged parasitism of cattle by ticks, as occur

under field conditions and increase in the amount of saliva injected apparently reduce the sensitivity of the host to the bite. However, laboratory studies demonstrated that the resistance to the bite of ixodids can develop if a simultaneous initial feed by all the ticks of a batch is followed by a second feed after an interval of not less than two to three weeks, and if the number of ticks at the second feed equals or scarcely exceeds that at the first.

Norval (1978) indicated that sheep were unable to acquire a resistance to larvae and nymphs of A. hebraeum. After repeated infestations of sheep, there was no progressive decline in either the tick yield or the engorged weight of fed ticks. Seasonal fluctuations of engorged weights did occur however, with the weight declining in early to midwinter and increasing in early to midsummer. Changes in host physiology, as a result of low temperature acclimatization, appeared responsible for such fluctuation. Tick yield is determined by the amount of grooming activity by the hosts. The feeding period of larvae and nymphs are dependent on the skin temperature of the hosts.

IV. Induction of resistance by using various tick tissues

A number of attempts have been made to artificially immunize hosts with tick extracts to prevent infestation with ixodid ticks and interest in this area of investigation has increased significantly in recent years. There is increasing awareness of the needs to develop alternative methods

for tick control, since acaricide resistance is assuming a serious problem.

A. BOVINE:

Brossard (1976) subcutaneously injected two calves at birth with 100 salivary glands extract prepared from partially engorged adult female of B. microplus to induce resistance. When challenged after two and five months, they gave a lower yield of engorged ticks than did two controls. However, the number of animals was too small for arriving at meaningful conclusion.

Allen and Humphreys (1979) immunized Hereford-cross calves against D. andersoni with extracts of midgut and reproductive organs of ticks. Ticks fed on immunized animals showed reduction in live weight, egg production and larval hatching, though the total recovery of ticks was not affected.

McGowan et al. (1981) immunized Jersey and Hereford calves against A. americanum with larval extracts (3 and 18 mg/kg, respectively). Weight of engorged females fed on immunized animals was singificantly reduced. The number and weight of egg masses and hatching rates were also reduced in ticks fed on Hereford calves.

Johnston et al. (1986) immunized Bos taurus and Bos taurus x Bos indicus cattle against B. microplus using whole extracts derived from adult (18-20 days after infestation of larvae) female ticks. The immunity persisted even after 14 weeks of daily challenge with 1000 larvae and tick population

were reduced by 70 per cent as compared to control. When the animals were challenged twice with 20,000 larvae each time, tick populations reduced by over 90 per cent.

Kemp et al. (1986) immunized Bos taurus cattle against B. microplus using extracts derived from adult (18-20 days after infestation with larvae) female ticks. The moulting of larvae on immunized cattle was delayed by up to 12 hours but otherwise they were unaffected. On two immunized cattle, there was progressive death of female ticks throughout their association with the host and up to 60 per cent of them had damaged gut. These females either failed to engorge, or if they did, many died before egg laying. Male ticks also suffered gut damage. No hypersensitivity reaction or serous exudation was seen at the site of tick attachment on any animal.

B. LABORATORY ANIMALS:

Trager (1939a) obtained immunity in guinea pigs against a challenge with D. variabilis larvae by intracutaneous injection of an extract of whole larvae.

Gregson (1941) immunized two guinea pigs against D. andersoni by using an extract of half engorged nymphs. However, the significance of the results was questionable as only two animals were taken.

Kohler et al. (1967) immunized one rabbit against H. a. excavatum by using salivary gland extract, and the number of ticks maturing was reduced.

Bagnall (1975) immunized 60 guinea pigs by subcutaneous injection of 1.4 mg of larval extract protein of I. holocyclus with or without adjuvant (FCA). Following challenge with larvae three weeks later, the larval rejection varied from 29 to 68 per cent. Three injections of larval extract induced more significant resistance.

Allen and Humphreys (1979) immunized guinea pigs against D. andersoni with extracts of either midgut and reproductive organs (antigen-I) or all internal organs (antigen-II). Ticks from the hosts immunized with antigen-I (1.2 mg/kg body weight with FCA on days 0 and 14), produced significantly fewer eggs than those from controls, and no larvae hatched from the eggs laid. The effects were more in the guinea pigs immunized with antigen-II (similar doses as antigen-I with FCA), since the ticks failed to engorge and produced no eggs.

Wikel (1981) induced significant resistance in guinea pigs against D. andersoni larvae by immunization with salivary gland antigen (SGA) prepared from partially engorged, unfertilized females. The SGA was administered through different routes with or without adjuvant. The induced resistance was expressed by significantly fewer larvae engorging, and the weight of larvae which had engorged was reduced. With 1 µg SGA with FCA and a second dose after 12 days showed statistically significant results as compared to other procedures adopted.

Ackerman et al. (1980) administered whole tick extract or midgut extracts of D. variabilis to rats. Female ticks infesting rats treated with midgut extracts showed delayed attachment, reduced engorgement weights, prolonged oviposition periods and reduced egg production, and the eggs showed reduced hatching rate. Similar effects were, however, not obtained in ticks fed on host immunized with extracts of whole ticks.

Brown et al. (1984b) reported that A. americanum salivary gland-derived antigens induced resistance to a subsequent A. americanum challenge in guinea pigs. Guinea pigs immunized by subcutaneous injection of an emulsion of incomplete Freund's adjuvant (IFA) containing tick salivary gland antigen (SGA) from partially fed female ticks, expressed a significant level of tick rejection when challenged 17 days later. Ticks that fed on animals immunized with SGA+IFA or SGA+FCA expressed significant reductions in engorgement weight. The minimum effective immunizing dose of SGA was between 100 and 280 µg per animal.

Binta et al. (1985) observed immunogenicity of larval extract of R. appendiculatus ticks in rabbits. This extract was immunogenic in rabbits in inoculation with or without FCA. Female ticks feeding on these rabbits had longer engorgement period. The engorgement weight of treated and untreated ticks showed no difference, but hatching of eggs from females fed on immunized rabbits was lower as compared to control ticks.

Wikel (1985) immunized guinea pigs with primary tissue culture cells of developing larvae of A. americanum, by subcutaneous injection of 1 x 10^6 cells on day 0,7 and 21. Challenge was done with male and female A. americanum on day 35. Induced resistance was expressed by reduced engorgement weight and oviposition period of female ticks, and subsequently by death of such ticks at their attachment sites. Moreover, such immunized animals showed significant resistance to infestation with D. andersoni adult ticks.

V. Type of immunological response

As per the current concept, antibody mediated, cell-mediated and complement-dependent immune effector mechanisms are active in the expression of acquired resistance to tick infestation. It is also reported by Willadsen (1980) and Wikel (1982, 1983, 1984) that vasoactive amines might also play a role in rejection of the feeding ticks by the host.

A. HUMORAL IMMUNITY TO TICK INFESTATION:

There is evidence that antibody is involved in immunity to ticks. Trager (1939a) passively transferred resistance by using serum from tick-resistant guinea pigs to unexposed guinea pigs, though presence of antibody could not be demonstrated. Participation of complement fixing antibodies were subsequently reported by Trager (1939b).

Wikel and Allen (1976a) reported that transfer of serum from guinea pigs resistant to D. andersoni, to unexposed recipients , @ 1.5 ml/100 gm. body wt. did not confer any immunity, though there was a slight reduction of engorgement weight of the larvae. Later, Wikel and Allen (1976b) administered cyclophosphamide to resistant guinea pigs and blocked the expression of resistance. Inhibition of resistance was attributed largely to suppression of the host antibody response.

Brossard (1976) detected that serum gamma globulin concentration was significantly increased following B. microplus infestation in Bos taurus cattle. The presence of specific and non-specific antibodies to the salivary gland of adult female ticks was demonstrated by indirect immunofluorescent technique, although the antibody concentration had no correlation with the degree of resistance developed. Similarly, Willadsen et al. (1978) indicated that these antibodies were present at the time when resistance was expressed. He measured the antibody response to a purified tick antigen by indirect haemagglutination test, but the antibody concentration had no correlation with the degree of resistance developed.

Roberts and Kerr (1976) transferred blood plasma @ 40 ml/kg body wt. from highly immune, poorly immune or unexposed cattle to groups of unexposed calves, which were then exposed to B. microplus ticks. Only about half the number of ticks matured out of those engorged on the highly immunized group.

Brossard (1977) passively transferred resistance to I. ricinus with two intravenous injections of serum, @ 0.25 ml kg body wt., from resistant to susceptible rabbits. The engorgement weights were reduced by 25 per cent, though the engorgement period was not affected. Further, Brossard and Girardin (1979) found that 50 per cent female ticks which fed on the recipients of serum from resistant host, produced eggs in contrast to 94 per cent of females from control hosts.

Bowessidjaou et al. (1977) observed that antibody to I. ricinus SGA, as measured by the indirect immunofluorescence, appeared at the end of first infestation and reached high titres on second infestation. No further increase in titre occurred thereafter.

Allen and Humphreys (1979) through immunodiffusion studies revealed single precipitation bands between the immunising antigen and sera collected on day 16, 25 and 28 after inoculation with extracts of midgut and reproductive organs of D. andersoni ticks, from immunized Hereford-cross calves. Strong multiple bands were obtained on day 38.

Rubaire-Akiki and Mutinga (1980b) reported mild resistance in rabbits to repeated R. appendiculatus ticks infestations and this was passively transferred through serum from resistant rabbits. Resistance to A. americanum and R. sanguineus could passively be transferred through respective serum, between the guinea pigs as reported by Brown and Askenase (1981). Such passively transferred

resistance was weaker than that expressed by actively sensitized animals.

McGowan et al. (1980) observed haemagglutinating antibody titres within seven days in rabbits immunized with A. maculatum homogenates and the mean titer reached high within 28 days. However, the above authors (1981) found no passive haemagglutination test positivity in calves immunized by A. americanum larval extract.

Wikel and Osburn (1982) observed precipitating antibodies in immunodiffusion test against SGA of D. andersoni females. Most of the cows had antibodies before and after first infestation, but by the end of the second infestation most cows had lost detectable precipitating antibodies.

Whelen et al. (1984) characterized by Dot-enzyme linked immunosorbent assay (Dot-ELISA), the antibody responses of Bos taurus infested with D. andersoni, and purebred and crossbred Bos indicus infested with A. americanum. Significant antibody titres against SGA were detected.

Whelen and Wikel (1985) passively transferred resistance to guinea pigs to a subsequent infestation with D.andersoni larvae.

Johnston et al. (1986) detected serum antibody to soluble tick extract using gel diffusion test four week following the first injection of tick extract and precipitin lines persisted after the second and third injection in the vaccinated cattle.

Njau and Nyindo (1987) detected humoral immune response in rabbits infested repeatedly with R. appendiculatus and R. evertsi evertsi by ELISA.

B. CELL - MEDIATED IMMUNITY TO TICK INFESTATION:

It is apparent that besides antibody other mechanisms are active in eliciting protective response. Evidence has been provided (Wikel and Osburn, 1982; George et al., 1985) that cell-mediated immune effector mechanism is stimulated by ixodid tick infestation.

1. DELAYED HYPERSENSITIVITY REACTION:

Tritschler (1965) reported one case of allergy in a horse to A. americanum. A macerated tick extract gave a swelling 6 to 36 hours after injection. Similarly, Gregson (1970) demonstrated on himself an irritating wheal reaction to D. andersoni 24 hours after tick attachment, or after injection of saliva. His peripheral blood lymphocytes were stimulated, whereas, those from a non-sensitive person were not.

Bagnall (1975) adoptively transferred resistance to I. holocyclus larvae with lymph node cells (2.5×10^8 cells/animal) from resistant guinea pigs to recipients unexposed to ticks and the larval rejection rate was 38 per cent.

Wikel and Allen (1976a) transferred highly significant levels of resistance to D. andersoni larvae with 10^8 viable

lymph node cells from resistant guinea pigs to previously unexposed guinea pigs. Nineteen per cent larvae engorged on recipient guinea pigs, whereas, 75 per cent on the controls.

Wikel et al. (1978) found that resistant guinea pigs gave intense skin reactions after 24 hours of inoculation with 50 µg of D. andersoni SGA. Unexposed control animals showed little reaction. Although there was a slight immediate reaction 30 minutes after injection, this was non-specific. Lymph node cells from guinea pigs, resistant to D. andersoni larvae, proliferated in an antigen-specific manner, when cultured in vitro with D. andersoni SGA. However, participation of cell types i.e., whether T and/or B - lymphocytes could not be ascertained.

Brown and Askenase (1981) adoptively transferred a significant degree of host resistance to A. americanum and R. sanguineus between guinea pigs with viable peritoneal exudate cells. However, resistance was more in an actively sensitized host. Askenase et al. (1982) reported the same findings for guinea pigs exposed to I. holocyclus and R. appendiculatus.

McTier et al. (1981) confirmed the development of cross skin reactivity in guinea pigs resistant to different ixodid species (D. andersoni, D. variabilis, A. americanum and I. scapularis). But, the skin tests on guinea pigs receiving tick SGA did not conclusively reflect the cross resistance.

Investigation on the role of cell-mediated immunity in affording resistance in bovines to ixodid tick infestation has been neglected. Wikel and Osburn (1982) observed that Bos taurus calves given one to four infestations with limited numbers of adult D. andersoni, reacted with an antigen-specific, delayed skin reaction by an intradermal injection of D. andersoni SGA. Peripheral blood lymphocytes, obtained prior to skin testing, from calves and cows given three to four infestations-responded in vitro to salivary gland components, while similar cells derived from unexposed cattle were not responsive in vitro to these antigens.

George et al. (1985) when gave purebred and crossbred Bos indicus calves one, two or three infestations with A. americanum, had 30 minutes, five hours and 24 hours skin reactions to A. americanum, A. cajennense and D. andersoni SGA. After all three infestations, purebred calves showed proliferation of peripheral blood lymphocytes in vitro in the presence of Amblyomma antigen. It therefore, indicates that Amblyomma species share cross-reactive salivary gland immunogens.

2. IMMEDIATE HYPERSENSITIVITY REACTION TO TICKS:

Riek (1956, 1962) reported that cattle exposed to the ticks were intensely irritated by the larvae. Papular reactions were seen around the attached nymphs and adults on

resistant cattle and a transient increase in blood histamine levels within 24 hours of infestation in exposed cattle. Cattle resistant to B. microplus infestation developed immediate (30 minutes) skin hypersensitivity reaction to extracts of B. microplus larvae or eggs.

Irritation of the host appears to be an important consequence of the cutaneous hypersensitivity reaction which occurs at the site of tick attachment on resistant hosts. Several authors (Riek, 1962; Hewetson and Nolan, 1968; Bennett, 1969; Hewetson, 1971; Koudstaal et al., 1978) reported an increase in grooming activity of tick infested resistant animals in an attempt to reduce the tick burden.

Schleger et al. (1976) observed accumulations and degranulation of eosinophils in epidermal vesicles at the attachment site of B. microplus larvae on tick-resistant Bos taurus; in addition, basophil degranulation was also observed. The more resistant the host, the more intense were these cellular reactions. It seems that an immediate hypersensitivity type of reaction is operational in the rejection of ticks.

Immediate hypersensitivity may be involved in immunity to other ticks. Allen (1973) and Allen et al. (1979) suggested immediate hypersensitivity reaction for D. andersoni, though, except for the observed eosinophil infiltration and basophil degranulation, which are typical of allergic reactions, evidence is lacking.

Willadsen et al. (1978, 1979) correlated immediate skin reactivity to the degree of resistance to B. microplus larval extract. They further observed that the amount of histamine in the skin of cattle resistant to B. microplus correlated directly with the degree of resistance. Wikel (1982) also reported the same findings in guinea pig resistant to D. andersoni.

IV. Histology of skin during tick feeding

Histological studies provide information regarding possible mechanisms involved in acquired resistance at the skin level of the previously sensitized animal.

Trager (1939a) examined the tick attachment sites on guinea pig on the fourth day of a second infestation with D. andersoni larvae and observed large accumulation of leukocytes, containing few eosinophils, under the attached mouth parts. The epithelium was thickened and the cutaneous tissues were oedematous. Similarly, Allen (1973) observed the same pattern in guinea pigs. However, the gross appearance of the ear at the attachment sites of larvae on resistant animals showed a serous exudate which trapped many tick subsequently, and at the attachment sites large vesicles were formed with infiltration of basophils. It was, therefore, suggested that guinea pig resistant to D. andersoni manifests basophil mediated cutaneous hypersensitivity reaction.

Cutaneous basophil accumulations in the host skin were observed at the attachment sites of I. holocyclus ticks in guinea pigs (Bagnall, 1975), A americanum in guinea pigs (Brown and Knapp, 1980), I. ricinus in rabbits (Brossard and Fivaz, 1982) and R. appendiculatus in guinea pigs (McLaren et al., 1983). The number of circulating basophils, sensitized to salivary gland antigens of female I. ricinus, increased during repeated infestations in rabbits (Brossard et al., 1982).

Theis and Budwiser (1974) found leucocytic infiltration, oedema and mast cell degranulation, in both previously exposed and unexposed dogs, to R. sanguineus ticks.

Rubaire-Akiki and Mutinga (1980a) observed epidermal hyperplasia, vesiculation, and eosinophil infiltration at R. appendiculatus attachment sites on resistant rabbits.

Gill and Walker (1985) mentioned that adult H. a. anatolicum feeding sites on rabbits indicated no significant differences in nature and sequence of cellular events at the feeding sites of male and female ticks, although the lesions produced by the feeding males were five to ten times smaller than those of females. Mast cells, basophils and eosinophils were the principal cells involved in this process.

Riek (1962) observed that cattle resistant to B. microplus had accumulations of lymphocytes and polymorphonuclear leukocytes, particularly eosinophils at the attachment sites. Tatchell and Moorhouse (1968); and Moorhouse and

Tatchell (1969) observed mast cell degranulation, eosinophil infiltration and the development of epidermal vesicles in cattle exposed to B. microplus.

Schleger et al. (1976) observed eosinophils and degranulated mast cells at B. microplus attachment sites and reactions were more intense in resistant hosts. Pavlovskii and Alfreva (1941) observed mast cells accumulati- at I. ricinus attachment sites on cattle and Allen et al. (1977) a cutaneous basophil hypersensitivity response at I. holocyclus infestation of previously exposed Bos taurus cattle. Subsequent studies of Brown et al. (1984a) revealed that Bos taurus resistant to A. americanum had cutaneous basophil hypersensitivity reactions at the tick attachment sites.

Hales et al. (1981) observed greatly increased skin capillary blood flow in cattle exposed to B. microplus larvae, and the degree of hyperemia was directly related to the level of tick resistance.

Schleger et al. (1981) suggested that Bos taurus that were highly resistant to B. microplus had more arteriovenous anastomoses in their skin than did cattle exhibiting resistance. It is quite likely that the increased blood flow contributes to antigen distribution to immunologically competent cells or facilitates traffic of cells to tick feeding sites.

Gill (1986) investigated the cutaneous cellular reactions associated with aquisition of resistance in cattle by comparing the cellular events at H. a. anatolicum feeding sites, following primary and tertiary infestations. The acquisition of resistance was characterised by epidermal vesiculation and a significant change in cellular infiltration at tick feeding sites, indicating the development of cutaneous hypersensitivity. On primary infestation the cellular infiltrate was dominated by the neutrophils, followed by mononuclear cells. Basophils showed a consistent increase as the tick feeding advanced.

CHAPTER - III

MATERIALS AND METHODS

The experiments were carried out at the Department of Veterinary Parasitology, Haryana Agricultural University, Hisar.

Experimental animals

Studies were carried out on cross-bred (Bos tarus x Bos indicus) healthy male cattle calves, below one year old, and with no previous exposure to Hyalomma anatolicum anatolicum. These animals were purchased locally and kept under tick-free environment during immunization or until they were deliberately infested. They were sprayed with 0.5 per cent Malathion (Sunray Chemical Company, Agra) before housing. Regular spraying of the animal houses was conducted at fortnightly intervals. The cracks and crevices inside the animal house were burnt from time to time using blow lamp. Every precaution was taken to keep the animals free from any extraneous parasitic or microbial infections. Adequate quantity of normal ration consisting of green fodders, wheat bhusa, gram churi, concentrate like Hafed pellets, mineral mixture and common salt was supplied to all the animals throughout the period of investigation.

The calves were divided into different groups, for use in three experiments as detailed in Table-1.

TABLE-1

SCHEDULE OF EXPERIMENTS IN CROSS-BRED CALVES

Experiments	No.animal	Remarks
I. Repeated tick attachments.	7	Ten times infestation with 50 pairs of ticks on each ear.
II. Immunization with salivary gland antigen.		
Group 1	7	Immunized with SGAg-I and FCA.
Group 2	10	Immunized with SGAg-II and FCA.
Group 3	10	Immunized with SGAg-III and FCA.
Group 4	7	Immunized with SGAg-I only.
Group 5	7	Inoculated with FCA only (control).
III. Passive immunization.		
Group 1	3	Blood plasma (SGAg-I immunized) used.
Group 2	3	Unimmunized (control).

SGAg-I = Whole salivary gland antigen

SGAg-II= Supernatant salivary gland antigen

SGAg-III=Sediment salivary gland antigen

Ticks

Adult H. a. anatolicum used in the studies were originally obtained from a pathogen-free colony maintained in this department. They were maintained at 28±1°C and 85 per cent relative humidity.

Tick rearing procedure

All adult ticks used during these experiments were derived from nymphs that had engorged simultaneously on a New Zealand white rabbit (Fig.1). Ticks were kept at 28±1°C in a B.O.D. incubator in cotton plugged glass tubes in desiccator over a 10 per cent solution of potassium hydroxide to maintain the required humidity.

Preparation of Salivary Gland Antigen (SGAg)

Salivary gland antigen was prepared by slight modification of the method of Wikel (1981). The salivary glands of the adult female ticks were allowed to engorge for four to seven days on calves. Thereafter, the ticks were placed in 0.01 M phosphate buffered saline (PBS), pH 7.2, and the ventral surface of the tick was placed on a small area of wax-filled Petri dish, melted by the heated tip on an electric rod under a dissecting microscope. The dorsal surface of the tick was separated and removed from the body by cutting the lateral edges of the cutical with a No.11 scalpel blade. Gut diverticula were removed and salivary glands were exposed, removed and dissected free of tracheal

elements and other tissues. The salivary glands were removed intact with fine-tipped forceps and were placed in 0.1 M PBS at 4°C and stored at -20°C for further use.

Antigen-I

The stored salivary glands were then thawed and homogenized in a sterile pestle and mortar kept on ice. The sediment was dissolved with 15 mM sodium desoxycholate (Hi-Media Laboratories Pvt.Ltd., Bombay). The material was further homogenized by sonicator (Vibronics Pvt. Ltd., Bombay)(55,000 cycles per second) with cooling on ice. The homogenate was then centrifuged at 5°C at 10,000 g for 30 minutes, supernatant collected and this constituted the whole salivary gland antigen (SGAg-I).

Antigen-II

The stored salivary glands were thawed and homogenized in pestle and mortar kept on ice. The homogenate was centrifuged at 5°C at 10,000 g for 30 minutes. The supernatant collected was kept as supernatant salivary gland antigen (SGAg-II).

Antigen-III

The sediment of antigen-II was dissolved with 15 mM sodium desoxycholate and the resultant suspension was kept as sediment salivary gland antigen (SGAg-III).

Protein concentration of the antigens were determined by the method of Lowry et al. (1951).

Immunization procedures

Experiment-I

In this experiment the calves were infested ten times with 50 pairs of adult ticks on each ear at 15 days intervals (Fig.2). Ticks were released to the ears using thick cotton bags. As the female ticks engorged and later detached, first ten ticks were collected, weighed, placed individually in glass tubes and kept under similar conditions as followed for colony maintenance. These ticks were observed daily. Their pre-oviposition and oviposition periods were recorded. When oviposition was completed, the egg mass was weighed and total number of eggs counted. The per cent hatch of larvae was also estimated. During tenth infestation, tail was also exposed to 50 pairs of ticks infestation (as followed for the ears) for comparative studies (Fig.3).

Ten ml of venous blood was collected from each calf prior to first infestation and thereafter before each subsequent infestation, for determination of E-rosettes. Merthiolate (1: 10,000) was added as preservative to sera samples and stored at -20°C for further use.

Experiment-II

In this experiment the calves were divided into five groups:

Group-I

Each calf of this group was injected subcutaneously with 43.32 mg (7.22 mg/ml) of antigen-I with Freund's Complete

Adjuvant (FCA) (Difco Laboratories, Detroit, Michigan, U.S.A.) in 1:1 ratio, at a final volume of 12 ml, on first day. A second injection was given 14 days later. Thus, each calf received the total amount of antigen which was prepared from 200 salivary glands. Seven days after the second injection, they were challenged with 50 pairs of adult H. a. anatolicum ticks on each ear.

Group-2

Each calf of this group was injected subcutaneously with 42.43 mg (7.07 mg/ml) of antigen-II with FCA in 1:1 ratio, at a final volume of 12 ml, on first day. A second injection was given 14 days later. Thus, each calf received the total amount of antigen which was prepared from 200 salivary glands. They were challenged as above.

Group-3

Each calf of this group was injected subcutaneously with 22.92 mg (3.82 mg/ml) of antigen-III with FCA in 1:1 ratio, at a final volume of 12 ml, on first day. A second injection was given 14 days later. Thus, each calf received the total amount of antigen which was prepared from 200 salivary glands. They were challenged as above.

Group-4

Each calf of this group was injected subcutaneously with 38.82 mg (6.67 mg/ml) of antigen-I only, at a final volume of 6 ml, on first day. A second injection was given 14 days later. Thus, each calf received the total amount of antigen which was prepared from 200 salivary glands. They

were challenged as above.

Group-5

This was the unimmunized control group. The calves received similar injection of FCA (but not antigen) on same days and challenged as above.

Blood and sera samples were collected from these animals on days 0,7, 14, 21, 28 and 35 for determination of E-rosettes, serum gamma globulin estimation, capillary tube agglutination and double diffusion tests.

Further observations on the ticks, engorged and detached from the animals after challenge, were recorded as per the first experiment.

Experiment-III

Collection of plasma:

The immunized (SGAg-I with FCA) donor calves were bled from the jugular vein and blood collected in anticoagulant (Heparin @ 5 I.U./ml). The plasma was separated by centrifugation at 2,000 g for 30 minutes, and stored at -20°C until required.

Administration of plasma:

The plasma was thawed, warmed to 38°C and injected intravenously to three cross-bred calves @ 20 ml/kg body weight, in aliquots of about 200 ml, with one hour rest between the doses to prevent any untoward effect.

Challenge with ticks:

Fifty pairs of unfed adult of H. a. anatolicum were released on each ear within 24 hours after transfusion.

Mechanisms of immune response

A. humoral immune response

1. Capillary tube agglutination test (CAT)

(i) Antigen preparation:

Tick salivary gland antigen was prepared as described earlier.

(ii) Test procedure:

The CAT was performed with undiluted sera. Glass capillary tubes (100 x 0.5 mm) were filled one-third with the antigen by the capillary action. The outside of the tubes was wiped with cotton and remaining two-third of the tube was filled with the serum to be tested. The tube was again wiped and set in a vertical position on a plasticin base with the antigen end at the bottom. The top ends of the tubes were sealed with finger nail polish or molted paraffin to avoid evaporation of the materials. Test was performed at room temperature (25°C) and the results recorded after 24 hours. Positive reactions appeared as agglutination mass at different levels in the capillary tubes.

Test was also put up with known negative (day old calf) serum and with PBS, to know the specificity of the antigen. Serum and antigen controls were also put up.

2. Double diffusion test

(i) Antigen preparation:

Tick salivary gland antigen was prepared as described earlier.

(ii) Test procedure:

One gm of Noble agar (Difco Laboratories, Detroit, Michigan, U.S.A.) was mixed with 50 ml of glass distilled water and dissolved in a boiling water bath and then over a bunsen flame to remove all lumps without charing the agar. Fifty ml of hot (90°C) barbitone buffer, pH 8.2 (Appendix-I) was added to it, mixed and stored at 4°C.

For precoating of the slides (75 x 50 mm), 0.2 gm agar was dissolved in 100 ml of distilled water and poured on the slides, dried and stored.

For preparing gel slides, 1 per cent agar in barbitone buffer (kept at 56°C) was poured on precoated slides, cooled at room temperature (25°C) and wells were cut. The wells were then filled with respective antigen and antisera. Refilling of the wells was done after half an hour, one hour and thereafter when the wells were empty during the first 24 hours. Slides were then put in moist chamber Petri plates (containing water soaked filter papers) at 37°C for 48 hours and the lines of precipitation, if any, observed. After 48 hours, the test was considered terminated.

The slides were then washed for 72 hours with several changes of normal saline solution containing merthiolate (1: 10,000) to remove free proteins from the agar. The slides were then covered with filter paper and dried overnigh The filter paper was then removed after dampening it slightly with water. The slides were stained with Coomassie Brilliant Blue dye (SISCO Research Laboratories Pvt. Ltd., Bombay) (Appendix-II) for five minutes and differentiated

in the destaining solution (Appendix-III). The slides were then dried either in the open or at 37°C.

3. Determination of gamma globulin

Gamma globulin concentration in the serum was measured as per the method of Oser (1976).

B. Cellular immune response

1. Skin test:

Animals after 10th repeated tick attachment and after seven days of challenge following immunization with salivary gland antigen, and the unimmunized controls were used. Salivary gland antigen, (50 µg protein/0.05 ml) from H. a. anatolicum, H. marginatum isaaci and Boophilus microplus was injected intradermally on both sides of the neck, two injections on each side, 15 cm apart. As control, 0.05 ml of 0.01 M PBS, pH 7.2, was injected. Skin thickness was measured by the dorso-ventral and antero-posterior diameters of the area.

2. E-rosette test:

The animals used in the skin test was also used here. Per cent T-cell rosettes were determined following the technique of Grewal and Babiuk (1978) with some modifications as described by Madsen and Johnsen (1979).

Preparation of peripheral blood lymphocytes (PBL) was followed as per the method of Rouse and Babiuk (1974).

For preparation of sheep red blood cells (SRBC), blood was collected from the same sheep throughout the study.

Five ml of blood was collected in heparinized (10 I.U./ ml) tubes and centrifuged at 400 g for 10 minutes. The cells were washed thrice in isotonic saline solution and buffy coat completely removed. The SRBC were then treated with 2-aminoethyl isothiouronium bromide (AET) (Sigma Chemical Co; St.,Louis, U.S.A.). The SRBC were then treated with AET as per the method of Kaplan and Clark (1974).

For detection of the rosette forming cells (RFC), equal volumes (0.2 ml) of PBL preparation (2.5×10^6 cells/ml) and one per cent SRBC treated with AET, were gently mixed in glass tubes and centrifuged at 50 g for five minutes and the tubes were kept overnight at 4°C. Immediately before counting, one drop of toluidine blue (0.1 per cent in sterile isotonic saline) was added to each tube, pellet gently resuspended, and a drop was put to a slide, coverslipped and examined under the microscope. A total of 200 lymphocytes were counted from each preparation. Lymphocytes with three or more closely attached SRBC were considered a RFC (Fig.4). All tests were done in duplicate.

Skin biopsy

The skin biopsies (4x4 mm) with attached ticks were taken from the animals under local anaesthesia (two per cent Anacain, Industrial and Research Institute Pvt. Ltd., Bombay) and fixed in 10 per cent neutral formol saline (pH 7.0) for histopathological studies. Paraffin sections were cut (5 μ), stained with haematoxyline and eosin, and also by toluidine blue.

Criteria for assessing resistance

Aquisition of resistance was recorded and compared by the per cent engorgement of adult female ticks, engorgement period, engorged weight, weight of the egg mass laid, number of eggs laid and per cent hatch of larval ticks.

Statistical analyses

In repeated tick attachment (Expt.I) group, the data were analysed statistically by the analysis of variance and Duncan's multiple range test. In salivary gland immunization (Expt.II) and passive transfer immunization (Expt.III) groups, the performance of ticks on immunized and unimmunized (control calves were analysed by the student's 't' test.

Per cent rejection of ticks (1 - [% engorgement from experimental/% engorgement from control] x 100), per cent weight reduction of engorged tick (1-[mean wt. from experimentals/mean wt. from controls] x 100), per cent weight reduction of egg mass (1-[egg wt. from experimentals/egg wt. from controls] x 100) and per cent hatch reduction of larval ticks (1-[% hatch from experimental/% hatch from control] x 100) associated with acquired resistance were calculated.

CHAPTER-IV

RESULTS

Experiment:I

Repeated tick attachments

The results have been summarised in the accompanying tables- 2A,B,C.

1. Per cent engorgement:

Adult ticks feeding on calves during first infestation (this served as control parameters) showed the mean yield of engorged ticks as 74.71±2.69 per cent. On subsequent infestations, the engorgement per cent gradually reduced and during tenth infestation it was 40.85±1.68. Statistically significant ($P < 0.01$) differance in mean engorgement per cent was observed between first to tenth infestation. First infestation was statistically significant ($P < 0.05$) from fourth to tenth infestation, likewise second from fourth to tenth; third, fourth and fifth from sixth to tenth; and sixth from tenth infestation.

2. Engorgement period (days):

The engorgement period between first (6.1±0.24) to tenth (6.2±0.21) infestation was statistically non-significant ($P > 0.05$).

3. Engorged weight (gm):

Engorgement weight between first (0.428±0.010) to tenth (0.201±0.012) infestation was statistically significant ($P < 0.01$). Progressive reduction of engorged weight to ticks from repeatedly infested calves were seen (Fig.5). Furthermore,

TABLE - 2A

EFFECTS OF HOST'S RESISTANCE TO REPEATED TICK ATTACHMENTS (Mean±S.E. values).

Tick attachment	% engorgement	Engorged wt.(gm.)	Egg mass wt.(gm.)	No.eggs laid	% hatch
1.	74.71 ± 2.69^{a}	0.428 ± 0.010^{a}	0.288 ± 0.009^{a}	3543 ± 168^{a}	99.68 ± 0.04^{a}
2.	76.29 ± 1.93^{a}	0.389 ± 0.012^{ab}	0.239 ± 0.012^{ab}	2969 ± 100^{b}	92.78 ± 2.37^{ac}
3.	69.44 ± 1.75^{ab}	0.344 ± 0.008^{bc}	0.216 ± 0.010^{bc}	2631 ± 72^{bc}	91.66 ± 2.72^{ac}
4.	63.86 ± 2.82^{b}	0.312 ± 0.008^{cd}	0.186 ± 0.006^{cd}	2145 ± 81^{cd}	94.16 ± 2.13^{ac}
5.	64.86 ± 2.26^{b}	0.290 ± 0.014^{cd}	0.182 ± 0.010^{cd}	2055 ± 84^{d}	89.45 ± 2.90^{ac}
6.	55.57 ± 1.65^{c}	0.284 ± 0.011^{de}	0.169 ± 0.012^{cde}	1904 ± 64^{d}	87.22 ± 2.87^{ad}
7.	55.85 ± 1.49^{c}	0.270 ± 0.009^{de}	0.158 ± 0.007^{d}	1888 ± 90^{de}	87.77 ± 1.79^{ae}
8.	47.43 ± 1.50^{c}	0.229 ± 0.011^{ef}	0.124 ± 0.013^{e}	1392 ± 134^{e}	82.90 ± 4.36^{bcde}
9.	47.28 ± 1.78^{c}	0.211 ± 0.016^{f}	0.110 ± 0.015^{f}	1248 ± 149^{f}	84.46 ± 3.95^{bcde}
10.	40.85 ± 1.68^{d}	0.201 ± 0.012^{f}	0.109 ± 0.007^{f}	1238 ± 78^{f}	75.47 ± 2.74^{bde}
	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$

Mean not followed by a common letter are statistically different $P< 0.05$. Data were analyzed by analysis of variance and Duncan's multiple range test for difference among means.

TABLE-2B

EFFECTS OF HOST'S RESISTANCE TO REPEATED TICK ATTACHMENTS (Mean±S.E. values)

Tick attachment	% rejection of attach.	% eng.wt. reduction	% egg mass wt. reduction	% hatch reduction
1.	-	-	-	-
2.	-2.11	9.11	17.01	6.92
3.	6.69	19.63	25.34	8.05
4.	14.52	27.10	42.36	5.54
5.	13.18	32.24	37.15	10.26
6.	25.62	33.64	41.31	12.50
7.	25.24	36.91	45.13	11.95
8.	36.11	48.83	56.94	16.83
9.	37.70	50.70	61.80	15.27
10.	45.32	53.03	62.15	24.29
	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$

Data were analyzed by analysis of variance.

TABLE - 2C

EFFECTS OF HOST'S RESISTANCE TO REPEATED TICK ATTACHMENTS (Mean ± S.E. values).

Tick attachment	Engorgement pd. (days)	Pre-oviposition pd. (days)	Oviposition pd. (days)
1.	6.1±0.24	6.3±0.14^{ac}	17.9±0.21^{a}
2.	6.3±0.21	6.9±0.35^{ac}	16.9±0.50^{a}
3.	6.6±0.21	7.5±0.20^{acd}	18.1±0.39^{a}
4.	6.6±0.07	7.9±0.21^{bcd}	17.6±0.86^{a}
5.	6.4±0.22	7.7±0.28^{acd}	16.9±0.46^{a}
6.	6.4±0.25	7.5±0.30^{acd}	15.9±0.66^{ab}
7.	5.8±0.12	7.5±0.22^{acd}	16.8±0.42^{a}
8.	6.3±0.17	9.0±0.57^{bd}	13.9±0.66^{b}
9.	6.4±0.26	8.0±0.24^{bd}	15.6±0.42^{a}
10.	6.2±0.21	9.8±0.48^{b}	13.5±0.66^{b}
	$P > 0.05$	$P < 0.01$	$P < 0.01$

Mean not followed by a common letter are statistically different $P < 0.05$. Data were analyzed by analysis of variance and Duncan's multiple range test for difference among means.

engorged weight of first infestation significantly varied ($P < 0.05$) from third to tenth infestation; second from fourth to tenth; third from sixth to tenth; fourth and fifth from eighth to tenth; and sixth and seventh from ninth and tenth infestation.

4. **Pre-oviposition period (days):**

Pre-oviposition period significantly ($P < 0.01$) increased from first (6.3±0.14) to tenth (9.8±0.48) infestation. Further, it was observed that first infestation significantly ($P < 0.05$) decreased from fourth and eighth to tenth, likewise second from eighth to tenth; and third to seventh from tenth infestation.

5. **Oviposition period (days):**

The oviposition period between first (17.9±0.21) to tenth (13.5±0.66) infestation was significantly ($P < 0.01$) decreased. Moreover, first to fifth and seventh infestation significantly ($P < 0.05$) increased from eighth and tenth infestation. Some female ticks that failed to oviposit were of normal engorged weight, but turned black (Fig.6) within a few days of drop off from the host, and died shortly thereafter.

6. **Egg mass weight (gm):**

The egg mass weight between first (0.288±0.009) to tenth (0.109±0.007) infestation was significantly ($P < 0.01$) decreased. The first infestation was significantly ($P < 0.05$) increased from third to tenth infestation, likewise second from fourth to tenth; third from seventh to tenth; fourth and fifth from eighth to tenth; and sixth from ninth and tenth infestation.

7. Number of eggs laid:

The number of eggs laid by the ticks between first (3343±168) to tenth (1238±78) infestation was significantly ($P < 0.01$) decreased. The number of eggs laid during first infestation was significantly ($P < 0.05$) increased from second to tenth infestation, likewise second from fourth to tenth; third from fifth to tenth; fourth to sixth from eighth to tenth; and seventh from ninth and tenth infestation.

8. Per cent hatch:

The per cent hatch between the first (99.68±0.04) to tenth (75.47±2.74) infestation was significantly ($P < 0.01$) decreased. The per cent hatch during first infestation was significantly ($P < 0.05$) increased from eighth to tenth; and second to fifth from tenth infestation. Some of the ticks which laid eggs from second to tenth infestation did not hatch at all, became black and lost the characteristic shining (Fig.7).

Comparative studies of tenth attachment on ears and tail:

During tenth infestation, tail was also infested, in addition to ears, with 50 pairs of ticks. The data on engorgement period, engorged weight, pre-oviposition period, oviposition period, egg mass weight, number of eggs laid and per cent eggs hatch from the ticks infested on ears as well as on tail were statistically non-significant ($P > 0.05$) but per cent engorgement between these two sites was statistically significant ($P < 0.05$). The results are summarised in table-3.

TABLE-3

COMPARATIVE STUDIES OF TENTH ATTACHMENT ON EARS AND TAIL IN CALVES (Mean±S.E. values).

Parameters	Ears	Tail	't' test
% engorgement (% rejection)	40.85±1.86 (45.32)	52.00±1.87 (30.40)	$P < 0.01$
Engorgement pd. (days)	6.2±0.21	7.3±0.10	$P > 0.05$
Engorged wt.(gm.) (% wt.reduction)	0.201±0.012 (53.03)	0.195±0.016 (54.43)	$P > 0.05$
Pre-oviposition pd. (days)	9.8±0.48	9.8±0.51	$P > 0.05$
Oviposition pd. (days)	13.5±0.66	14.1±0.39	$P > 0.05$
Egg mass wt.(gm.) (% wt.reduction)	0.109±0.007 (62.15)	0.106±0.008 (63.19)	$P > 0.05$
No.eggs laid	1238±78	1196±98	$P > 0.05$
% hatch (% hatch reduction)	75.47±2.74 (24.28)	74.17±5.06 (25.59)	$P > 0.05$

Humoral immune response:

The humoral immune response was studied in all the calves during first to tenth infestation by capillary tube agglutination test, double diffusion test and by estimation of serum gamma globulin levels.

Capillary tube agglutination and double diffusion tests failed to detect antibodies in any of the calves.

In pre-infested (control) calves the level of serum gamma globulin was 1.422±0.146 gm/100 ml, whereas, during second, fourth and tenth infestation it was 1.420±0.084, 1.570±0.107 and 1.661±0.056 gm/100 ml, respectively. The difference in these values between the pre-infested and subsequently infested calves were statistically non-significant ($P > 0.05$).

Cell-mediated immune response (CMIR):

1. Delayed cutaneous hypersensitivity reaction (Intradermal test)

Inoculation of H. a. anatolicum, H. m. isaaci, B. microplus SGA and buffered saline diluent into the skin of the previously tick unexposed calves (control) showed no swelling except for a mild, transient (lasted for two hours) reaction to H. a. anatolicum SGA. Saline injection in control calves had also nil reaction (Table-4).

In calves infested ten times with H. a. anatolicum, the inoculation of SGA produced initially an immediate hypersensitivity response to Hyalomma antigens, followed by a delayed reaction. However, a mild immediate reaction (lasting for eight

TABLE -4

AREA (Mean cm^2 ±S.E.) OF SKIN REACTION OF TICK-NAIVE CALVES.

Hrs.post-ino.	Salivary gland antigen			
	H.a.anatolicum	H.m.isaaci	B.microplus	PBS
0.5	0.65±0.08	0.60±0.05	0.61±0.10	0.56±0.10
2	0.75±0.07	0.66±0.09	0.57±0.04	0.56±0.06
5	0	0	0	0
8	0	0	0	0
24	0	0	0	0
48	0	0	0	0
72	0	0	0	0

hours) was noticed against B. microplus SGA. On the other hand, the hypersensitivity response lasted up to 48 hours with Hyalomma antigens, and then the reaction was rapidly subsiding. Oedema was extensive and the inoculation site turned into a highly indurated area. Necrotic lesions did not occur at the sites. The saline injected area was slightly distended and persisted only for two hours (Table-5).

Histological examination of the skin biopsies taken during the maximal reaction showed mild to moderate accumulation of oedematous fluid in the deeper dermal layers and excessive infiltration of mononuclear cells specially lymphocytes and macrophages and at times a few polymorphonuclear leucocytes with perivascular cuffing at some places (Fig.8) were also observed.

2. 'E' rosette test:

The per cent 'E' rosettes in pre-infested control calves was 21.50±4.01, and during second, fourth, sixth and tenth infestation it was 30.00±2.94, 30.00±2.16, 28.50±1.93 and 37.25±1.84, respectively. The differences in these values between pre-infestation and subsequent infestation were statistically significant ($P < 0.05$).

Experiment: II

Immunization with tick salivary gland antigens

Group-1: Immunization with whole salivary gland antigen (SGAg-I) and FCA.

Immunization with SGAg-I and FCA to calves induced

TABLE-5

AREA (Mean cm^2±S.E.) OF SKIN REACTION OF TICK RESISTANT CALVES INFESTED TEN TIMES WITH TICKS.

Hrs.post-ino.	Salivary gland antigen			
	H.a.anatolicum	H.m.isaaci	B.microplus	PBS
0.5	1.61±0.31	1.18±0.20	0.65±0.08	0.48±0.05
2	4.05±0.42	2.27±0.17	1.39±0.11	0.46±0.05
5	7.93±1.38	4.21±0.56	1.09±0.09	0
8	7.46±1.60	4.51±0.50	0.87±0.11	0
24	2.56±0.47	2.27±0.47	0.49±0.05	0
48	1.32±0.21	0.65±0.08	0	0
72	0	0	0	0

significant resistance to a challenge infestation with H. a. anatolicum ticks. The data are summarised in table-6. Statistical analysis of difference between mean of the immunized and unimmunized groups showed the engorgement period, engorged weight, pre-oviposition period, egg mass weight, number of eggs laid were statistically highly significant ($P < 0.01$) whereas, the per cent engorgement, per cent hatch and oviposition period were non-significant ($P > 0.05$).

Group-2: Immunization with supernatant of salivary gland antigen (SGAg-II) and FCA.

Immunization with SGAg-II and FCA to calves induced significant resistance to a challenge infestation with H. a. anatolicum ticks. The data are presented in table - 7. Statistical analysis of difference between mean of immunized and unimmunized groups showed that the engorged weight, pre-oviposition period and egg mass weight were highly significant ($P < 0.01$). The number of eggs laid was also found significant ($P < 0.05$). However, the per cent engorgement, engorgement period, oviposition period and per cent hatch were non-significar ($P > 0.05$).

Group-3: Immunization with sediment of salivary gland antigen (SGAg-III) and FCA.

The data are summarized in table-8. Statistical analysis of difference between mean of immunized and unimmunized groups showed that all parameters were statistically non-significant ($P > 0.05$).

TABLE - 6

IMMUNIZATION OF CALVES WITH TICK WHOLE SALIVARY GLAND ANTIGEN (Ag-I) (Mean±S.E.values).

Parameters	Immunized group*	Unimmunized (control)	't' test
% engorgement (% rejection)	67.57±2.90 (8.99)	74.25±2.96	$P > 0.05$
Engorgement pd. (days)	7.4±0.08	6.4±0.05	$P < 0.01$
Engorged wt.(gm.) (% wt.reduction)	0.241±0.015 (42.07)	0.416±0.012	$P < 0.01$
Pre-oviposition pd. (days)	12.1±0.67	8.1±0.35	$P < 0.01$
Oviposition pd. (days)	18.9±0.40	21.4±0.25	$P > 0.05$
Egg mass wt.(gm.) (%Wt. reduction)	0.126±0.006 (48.57)	0.245±0.005	$P < 0.01$
No. eggs laid	1479±81	3083±112	$P < 0.01$
% hatch (% hatch reduction)	94.47±2.78 (4.38)	98.80±0.35	$P > 0.05$

* Antigen-I was administered with FCA.

TABLE - 7

IMMUNIZATION OF CALVES WITH SUPERNATANT OF TICK SALIVARY GLAND ANTIGEN (Ag-II) (Mean±S.E. values).

Parameters	Immunized group*	Unimmunized (control)	't' test
% engorgement (% rejection)	66.80±3.17 (10.03)	74.25±2.96	$P > 0.05$
Engorgement pd. (days)	6.9±0.12	6.4±0.05	$P > 0.05$
Engorged wt.(gm.) (% wt.reduction)	0.290±0.024 (30.29)	0.416±0.012	$P < 0.01$
Pre-oviposition pd. (days)	11.8±0.46	8.1±0.35	$P < 0.01$
Oviposition pd. (days)	20.1±0.33	21.4±0.25	$P > 0.05$
Egg mass wt.(gm.) (% wt. reduction)	0.164±0.015 (33.06)	0.245±0.005	$P < 0.01$
No.eggs laid	2045±211	3083±112	$P < 0.05$
%hatch (% hatch reduction)	94.12±2.29 (4.73)	98.80±0.35	$P > 0.05$

* Antigen-II was administered with FCA.

TABLE -8

IMMUNIZATION OF CALVES WITH SEDIMENT OF TICK SALIVARY GLAND ANTIGEN (Ag-III) (Mean±S.E. values).

Parameters	Immunized group*	Unimmunized (control)	't' test
% engorgement (% rejection)	74.50±2.37 (-0.34)	74.25±2.96	P>0 05
Engorgement pd. (days)	6.7±0.07	6.4±0.05	P>0.05
Engorged wt.(gm.) (% wt.reduction)	0.379±0.009 (8.89)	0 416±0.012	P>0.05
Pre-oviposition pd. (days)	10.3±0 82	8.1±0.35	P>0 05
Oviposition pd. (days)	20.6±0.42	21.4±0.25	P>0.05
Egg mass wt.(gm.) (% wt. reduction)	0.219±0.009 (10.61)	0.245±0.005	P>0.05
No. eggs laid	2753±128	3083±112	P>0 05
% hatch (% hatch reduction)	98.38±0.56 (0.43)	98.80±0.35	P>0 05

*Antigen-III was administered with FCA.

Group-4: Immunization with whole salivary gland antigen (SGAg-I) only.

Immunization with SGAg-I only to calves induced non-significant resistance to challenge infestation with H. a. anatolicum ticks. The data are presented in table-9. Statistical analysis of difference between mean of immunized and unimmunized groups showed that only pre-oviposition period was significant ($P < 0.05$). However, the per cent engorgement, engorgement period, engorged weight, oviposition period, egg mass weight, number of eggs laid and per cent hatch were non-significant ($P > 0.05$).

Humoral immune response:

1. Capillary tube agglutination test (CAT):

CAT was performed in all the calves from 21 days onwards after immunization and positive reaction recorded after 24 hours (Fig.9), in SGAg-I (with FCA) only. Other immunized and the control calves were negative.

2. Double diffusion test:

The precipitin lines were observed from 21 days onward in the calves immunized with SGAg-I, II and III with FCA. In SGAg-I (with FCA) immunized group, three calves showed double precipitin lines (Fig. 10). Calves of all other groups showed single line (Fig.11 and 12). Control and SGAg-I (without FCA) immunized calves showed no lines. In SGAg-I (with FCA) immunized calves the lines started fading out after 70 days of first immunization (Fig.13).

TABLE-9

IMMUNIZATION OF CALVES WITH TICK WHOLE SALIVARY GLAND ANTIGEN (Ag-I) (Mean±S.E. values).

Parameters	Immunized group	Unimmunized (control)	't' test
% engorgement (% rejection)	71.28±2.85 (4.00)	74.25±2.96	P > 0.05
Engorgement pd. (days)	7.1±0.24	6.4±0.05	P > 0.05
Engorged wt.(gm.) (% wt. reduction)	0.363±0.015 (12.74)	0.416±0.012	P > 0.05
Pre-oviposition pd. (days)	10.6±0.60	8.1±0.35	P < 0.05
Oviposition pd. (days)	18.2±0.66	21.4±0.25	P > 0.05
Egg mass wt.(gm.) (% wt. reduction)	0.207±0.011 (15.51)	0.245±0 005	P > 0.05
No. eggs laid	2670±190	3083±112	P > 0.05
% hatch (% hatch reduction)	97.21±0.76 (1.61)	98.80±0 35	P > 0.05

3. Serum gamma globulin:

The values of gamma globulin in immunized and unimmunized (control) calves are presented in table-10.

In groups immunized with SGAg-I, II and III (all with FCA), the difference in gamma globulin values between 0 to 35 days was statistically significant ($P < 0.05$).

In groups immunized with SGAg-I (without FCA) and also unimmunized (control) calves, the difference in values between 0 to 35 days was statistically non-significant ($P > 0.05$).

Cell-mediated immune response (CMIR):

1. Delayed cutaneous hypersensitivity reaction (Intradermal test):

Results are summarized in the tables 11,12,13 and 14. All immunized calves when inoculated with Hyalomma antigens, initially showed an immediate hypersensitivity response lasting for eight hours. However, a mild immediate reaction (lasting for eight hours) was noticed against B. microplus SGA. The saline injected area remained slightly distended for two hours only.

2. 'E' rosette test:

The per cent 'E' rosetting T-lymphocytes in immunized and unimmunized (control) calves are depicted in table-15.

In groups immunized with SGAg-I, II (both with FCA), and SGAg-I (without FCA), the difference in per cent 'E' rosettes values between 0 to 35 days was statistically significant.

TABLE - 10

SERUM GAMMA GLOBULIN LEVELS IN CALVES IMMUNIZED WITH SALIVARY GLAND ANTIGENS (SGAg)*.

Days post-immu.	Gamma globulin (gm./100 ml) (Mean±S.E.)				
	SGAg-I e FCA	SGAg-II e FCA	SGAg-III e FCA	SGAg-I	Unimmunized (Control)
0[a]	1.679±0.047	1.551±0.078	1.495±0.057	1.157±0.056	1.275±0.106
7	2.071±0.067	2.185±0.057	1.435±0.184	1.206±0.034	1.217±0.103
14[b]	2.072±0.070	2.290±0.073	1.550±0.077	1.202±0.028	1.296±0.013
21[c]	2.319±0.069	2.309±0.078	1.846±0.046	1.262±0.041	1.266±0.054
28	2.385±0.039	2.363±0.050	1.881±0.076	1.235±0.072	1.434±0.075
35	2.387±0.047	2.521±0.092	1.960±0.064	1.276±0.035	1.322±0.090
	$P < 0.01$	$P < 0.01$	$P < 0.05$	$P > 0.05$	$P > 0.05$

* SGAg-I = Whole Ag; SGAg-II = Supernatant Ag; SGAg-III = Sediment Ag.
a = 1st immunization; b = 2nd immunization; c= Challenge.

TALBE -11

AREA (Mean cm^2 ±S.E.) OF SKIN REACTION OF TICK RESISTANT CALVES IMMUNIZED WITH WHOLE SALIVARY GLAND ANTIGEN (Ag-I) WITH FCA.

Hrs.post-ino.	Salivary gland antigen			
	H.a.anatolicum	H.m.isaaci	B.microplus	PBS
0.5	3.17±0.67	2.71±0.83	1.64±0.31	0.53±0.28
2	10.55±2.92	5.09±1.22	2.36±0.35	0.63±0.34
5	16.61±1.79	8.79±0.64	1.21±0.19	0
8	21.80±2.04	14.18±1.09	0.81±0.38	0
24	5.99±1.73	6.66±1.29	0	0
48	3.13±1.56	1.75±0.41	0	0
72	0	0	0	0

TABLE-12

AREA (Mean cm^2 ±S.E.) OF SKIN REACTION OF TICK RESISTANT CALVES IMMUNIZED WITH SUPERNATANT SALIVARY GLAND ANTIGEN (Ag-II) WITH FCA.

Hrs.post-ino.	Salivary gland antigen			
	H.a.anatolicum	H.m.isaaci	B.microplus	PBS
0.5	1.99±0.41	1.58±0.18	0.83±0.10	0.47±0.06
2	4.43±0.80	3.68±0.42	2.36±0.28	0.50±0.06
5	12.95±1.28	12.32±1.11	1.02±0.12	0
8	16.49±1.79	15.14±1.27	0.73±0.10	0
24	4.33±0.70	4.80±0.49	0	0
48	1.38±0.34	1.52±0.33	0	0
72	0	0	0	0

TABLE -13

AREA (Mean cm^2 ±S.E.) OF SKIN REACTION OF TICK RESISTANT CALVES IMMUNIZED WITH SEDIMENT SALIVARY GLAND ANTIGEN (Ag-III) WITH FCA.

Hrs.post-ino.	Salivary gland antigen			
	H.a.anatolicum	H.m.isaaci	B.microplus	PBS
0.5	1.07±0.23	0.70±0.07	0.70±0.08	0.79±0.32
2	3.21±0.59	2.27±0.41	1.39±0.13	1.07±0.17
5	8.50±1.42	4.64±1.03	2.07±0.34	0
8	7.26±1.42	5.84±1.74	2.09±0.33	0
24	1.12±0.23	0.94±0.22	0	0
48	0.49±0.04	0.48±0.07	0	0
72	0	0	0	0

TABLE-14

AREA (Mean cm^2±S.E.) OF SKIN REACTION OF TICK RESISTANT CALVES IMMUNIZED WITH WHOLE SALIVARY GLAND ANTIGEN (Ag-I) ONLY.

Hrs.post-ino	Salivary gland antigen			
	H.a.anatolicum	H.m. isaaci	B.microplus	PBS
0.5	0.47±0.07	0.49±0.11	0.37±0.06	0.48±0.11
2	1.37±0.37	1.71±0.59	0.87±0.25	0.46±0.10
5	6.90±1.32	5.35±1.10	1.33±0.36	0
8	3.28±0.71	5.24±0.96	1.20±0.66	0
24	0.88±0.11	1.69±0.33	0	0
48	0.34±0.10	0.46±0.08	0	0
72	0	0	0	0

TABLE - 15

'E' ROSETTE TEST IN CALVES IMMUNIZED WITH TICK SALIVARY GLAND ANTIGENS (SGAg)*.

Days post-immu.	% T-Lymphocytes (Mean±S.E.)				
	SGAg-I e FCA	SGAg-II e FCA	SGAg-III e FCA	SGAg-I	Unimmunized (control)
0[a]	26.50±2.26	25.00±2.47	23.75±3.64	20.25±2.77	24.50±1.82
7	29.50±3.36	28.25±0.56	23.00±2.12	19.25±2.07	24.50±0.90
14[b]	30.00±2.29	25.50±2.19	26.50±2.30	23.25±2.51	26.75±1.13
21[c]	37.75±3.94	31.50±1.68	28.00±2.89	28.75±1.29	25.50±0.55
28	38.25±1.24	38.00±1.62	32.25±3.68	30.50±2.49	28.50±1.95
35	38.75±0.74	36.00±1.87	34.50±2.08	27.50±1.68	25.50±1.03
	$P<0.05$	$P<0.01$	$P>0.05$	$P<0.05$	$P>0.05$

* SGAg-I = Whole Ag; SGAg-II = Supernatant Ag; SGAg-III = Sediment Ag.
a = 1st immunization; b = 2nd immunization; c = Challenge.

In groups immunized with SGAg-III (with FCA) and unimmunized (control) calves, the difference in per cent 'E' rosette values between 0 to 35 days was statistically non-significant ($P > 0.05$).

Experiment:III

Passive transfer of tick resistance with plasma from immunized calves.

The data of passively immunized and unimmunized group are presented in table-16. Statistical analysis of difference between mean of passively immunized and unimmunized group showed that all the parameters were statistically non-significant ($P > 0.05$).

Skin reaction to ticks

1. **Gross reactions:**

After detachment of the engorged females, the attachment site appeared swollen and the cavity where the mouth parts were inserted became conspicuous. In immunized calves a more intense skin reactions occurred around the attachment sites. Twenty-four hours after attachment, epidermal vesicles were formed around all the attached ticks. These vesicles measured 2 to 4 mm in diameter. Vesiculation was accompanied by an excessive serous exudation, hyperaemia and oedema of the ear. Material resembling dried serous exudate encrusted the skin around the site of tick bite.

TABLE - 16

TRANSFER OF TICK RESISTANCE WITH PLASMA FROM IMMUNIZED CALVES (Mean±S E. values)

Parameters	Passively Immu.	Unimmu (control)	't' test
% engorgement (% rejection)	72.33±4.28 (-3.33)	70.00±4.64	P >0.05
Engorgement pd (days)	6.4±0.19	6.5±0.22	P >0 05
Engorged wt.(gm.) (%wt. reduction)	0.372±0.012 (-0.269)	0.371±0 021	P >0.05
Pre-oviposition pd. (days)	7.0±0.14	6.7±0.27	P >0.05
Oviposition pd (days)	19.3±0.29	20 0±0.42	P >0 05
Egg mass wt (gm.) (% wt. reduction)	0.203±0.014 (5.58)	0.215±0.025	P >0.05
% hatch (% hatch reduction)	95.74±2 96 (0.66)	96.38±2.66	P >0.05

2. Histopathology:

Histology of normal calf skin is illustrated in Fig.14. During primary infestation the histological examination of H. a. anatolicum feeding sites showed that the mouth parts of the female H. a. anatolicum penetrated deeply into the dermis and hypostome got ensheathed by the attachment cement (Fig.15) along the entire length. The collagen bundles in contact with the cement cone had lost their fibrous structure and appeared as a homogenous tissue mass. From initial attachment until well after the fully engorged female tick had detached, the cement substance secreted by the tick and serving as an anchoring material, extend over the surface of the epidermis. In some of the feeding sites the mouth parts had penetrated right through the cartilage (Fig.16).

The lesion produced by feeding of male ticks (Fig.17) were 5 to 10 times smaller than those of females. However, the general histological changes, nature and sequence of cellular events at male feeding sites were the same as those seen with female ticks.

In immunized calves during initial attachment, leucocytes appeared at the site of attachment (Fig. 18). The extent and intensity of the infiltration by neutrophils and lymphocytes increased during the succeeding feeding (Fig.19). The maximum reaction was attained when the tick reached the final engorgement stage (Fig.20). Massive

infiltration and necrosis of neutrophils completely destroyed the portions of the dermis immediately below the mouth parts forming a feeding cavity (Fig. 21). As the final rapid engorgement proceeded, the cavity gradually became depleted of the great mass of tightly packed leucocytes, many of which were necrotic. This resulted in a fluid filled cavity which contained some leucocytes and haemorrhage from the damaged blood vessel.

In contrast to the reactions to primary infestation, the reactions in the immunized calves were different. The lesion in immunized calves consisted of epidermal vesiculation. Histologically, the vesicles (Fig.22) were located in between the stratum corneum and stratum granulosum. The vesicle mainly contained neutrophils, basophils (Fig.23), debris of epidermal cells, a few eosinophils (Fig.24) and mononuclear cells, thus giving the appearance of a pustule. In immunized calves, at tick feeding sites infiltration of basophils and eosinophils increased.

CHAPTER-V

DISCUSSION

Repeated tick attachments

The calves developed resistance against primary infestation of H. a. anatolicum ticks, and this resistance was expressed during the second and up to tenth infestation. However, acquired resistance observed (Wagland, 1975, Amin-Babjee and Riek, 1986) with B. microplus were somewhat higher than the present studies. The reason for the difference may be due to continuous association of B. microplus tick (one host tick) to host. Acquisition of resistance against A. americanum in Bos indicus, Bos taurus, and cross-bred (Strother et al., 1974), in Bos taurus and cross-bred (Garris and Hair, 1980), in Bos taurus and Bos indicus (Hair and Garris, 1980) and in Bos indicus and cross-bred (George et al., 1985) also developed after repeated infestations.

In the present studies, significant reduction in average engorgement per cent, and decreased egg masses paralleled decreased engorged weight. But, Brown et al. (1984a) concluded in general terms that the tick yield from resistant animals was normal, but mean tick weight and egg mass weight were reduced significantly. This finding suggests a dual mechanism to the immune response, with one component directed against the tick attaching phase and the other against the engorging phase. Strother et al. (1974) and George et al. (1985) observed that bovine

immune response apparently acted during both the attaching and feeding phases, as observed in the present study. The present finding also agrees with that of Gill (1986) that acquired resistance to H. a. anatolicum feeding was developed in Bos taurus after a single infestation. However, he had not observed engorgement per cent. He also observed higher resistance than that obtained in the present study. This could be due to the difference in the breed of the animal used.

Using 300 pairs of ticks in Bos taurus, Bos indicus and cross-bred (Strother et al., 1974), 50 pairs in Bos taurus (Brown et al., 1984a) or 15 ticks i.e., ten females and five males in Bos indicus and cross-bred calves (George et al., 1985) with A. americanum and 30 pairs of H. a. anatolicum in Bos taurus (Gill, 1986), better resistance was developed. In the present studies, 50 pairs of H. a. anatolicum were used on each ear. It, therefore, indicates that the degree of acquired resistance can be generated by variable number of ticks.

Acquired resistance of the animals in the present studies was manifested at the tick attachment sites, since the animals were restricted from self-grooming. However, the effect of host grooming was not studied as the ticks were restricted on the ears in cotton bags. Riek (1962) and Bennett (1969) reported that host grooming activity was an important factor in natural reduction of tick burdens,

and animals restricted from grooming had more engorged ticks. Cutaneous immune reactivity to the attached tick could cause irritation and thus stimulate host grooming. Stebbing (1974) speculated that immediate hypersensitivity reactions to arthropod bite could have a protective role in stimulating host avoidance behaviour. In this study, cutaneous reactivity at tick attachment site at the end of a second and subsequent infestation, was characterised by the presence of a serous exudate. Such type of reaction were also reported for cattle resistant to B. microplus (Riek, 1962) and I. holocyclus (Allen et al., 1977).

In the present studies some female ticks failed to oviposit. However, such females showed normal engorgement, and their body colour turned black within one week of dropping off the host and died shortly thereafter. In spite of normal engorgement, fertility and fecundity got impaired. Moreover, several adult male ticks feeding on repeatedly infested calves, died prematurely or became immobilized in the exudates from the lesions which formed at the feeding sites; and a number of female ticks failed to engorge and produce eggs. Impaired fertility and fecundity of female ticks due to adverse effect on male ticks and lack of mating with female, was observed (Brown et al., 1984a) in A. americanum during repeated infestations in Bos taurus cattle. Failure of a number of female ticks to become engorged, as a result of premature death of male ticks was stated (McGowan et al., 1980) in

A. maculatum ticks after inoculation with tick homogenates.

Our results showed reduction in hatching per cent from first to tenth infestation. Johnston and Bancroft (1918) stated that failure of engorged Boophilus ticks either to lay normal number of eggs or to lay eggs with abnormal fertility is a sign of resistance by animals. Similarly, Riek (1962) observed that the resistant cattle dropped B. microplus ticks which laid eggs of lower fertility.

Except per cent engorgement, the effect of resistance on all other parameters was same whether the tenth infestation was given on the ear or on the tail, thus suggesting the operation of a systemic resistance rather than a localized alteration of the skin (Trager, 1939a).

Immunization with tick salivary gland antigen (SGAg)

The immunogen used in the present study consisted of whole, supernatant and sediment fractions of tick salivary gland extract (antigen). The use of such extracts to induce an immune response have been described earlier in cattle using B. microplus (Brossard, 1976), in guinea pigs using D. andersoni (Wikel, 1981), in cattle using A. maculatum (McGowan et al., 1981) and in guinea pigs using A. americanum (Brown et al., 1984b) ticks.

Trager (1939a) and Boese (1974) reported that ticks fed on animals immunized with tick SGAg or exposed to previous tick infestations, often failed to engorge completely with consequent reduction in engorgement weight or egg mass weight of the female ticks. On the whole, our observations

on responses of the adult ticks to immunized calves support these findings.

In the present study, incorporation of FCA with SGAg-I afforded better resistance than when the antigen was used alone.

The immune response to ticks is accompanied by a cutaneous basophil response and is mediated partly by IgG_1 antibodies (Brown et al., 1982). In the present studies, calves immunized with antigen and FCA showed precipitating bands in gel diffusion test, increased gamma globulin levels, and cutaneous basophil response at the site of tick attachment. Therefore, the role of adjuvant like FCA is important for production of antibody and specific cellular responses.

In the present study, SGAg-I with FCA proved most potent as compared to SGAg-II or III with FCA, indicating that both the supernatant (SGAg-II) and sediment (SGAg-III) components present in the SGAg-I (whole) gave synergistic response.

An immunologic approach to tick control might be feasible for any tick species which remain in contact with the host (Willadsen, 1980). Allen and Humphreys (1979) immunized calves against D. andersoni, using extracts of midgut and reproductive organs (Ag-I). Similarly, they immunized guinea pigs using extracts of mid gut and reproductive organs

(Ag-I) and all internal organs (Ag-II). Likewise, induction of resistance in guinea pigs to D. andersoni (Wikel, 1981) and A. americanum (Brown et al., 1984b) was observed with salivary gland antigen. However, Johnston et al. (1986) immunized cattle against B. microplus using extract derived from adult female ticks which conferred resistance to tick infestation. Moreover, in other arthropods, Schlein and Lewis (1976) showed that flight muscle proteins were effective in inducing resistance in rabbits to Stomoxys calcitrans. In our study, the whole salivary gland antigen (SGAg-I) with FCA was the best immunizing agent. It, therefore, suggests that immunologic control of tick infestation deserves careful and critical examination, since through such approaches the resistant animals could be protected against tick transmitted pathogens (Bell et al., 1979; Wikel, 1980).

It would, therefore, seem practical to utilize tick antigens for immunizing the animals. The antigen could consist of tick internal organs, like, salivary gland, gut and reproductive organs. However, the whole tick tissue extract as immunogen could not induce significant host immune responses (Wikel, 1981).

In the present study, feeding and reproductive performances of the ticks infesting the immunized calves were significantly reduced particularly in calves of SGAg-I (with FCA) group. The functional antigen(s) of the tick(s) are yet to be identified. The purification of the most

potent antigen is, however, necessary to have better immunogenic response. There could be difficulties in producing large quantities of purified antigen, and once this hurdle is cleared, then immunization of animals against ticks could be a possible proposition in the forseeable future.

Humoral immune response

In the present study of repeated tick infestation, humoral antibody response was not observed from first to tenth attachment. But, using the purified larval tick antigen, antibody titres in repeatedly tick infested animals was observed by Willadsen et al. (1978) by the indirect haemagglutination test in cattle exposed to 20,000 B. microplus larvae; using SGA by indirect immunofluorescence (Bowessidjaou et al., 1977) in rabbits exposed to I. ricinus ticks, using SGA by Dot- ELISA (Whelen et al., 1984) in Bos taurus cattle with D. andersoni and Bos indicus cattle with A. americanum, and using SGA by ELISA (Njau and Nyindo, 1987) in rabbits exposed to 50 pairs of R. appendiculatus. However, the above workers could not directly correlate the higher titres of antibody with higher level of resistance and the humoral immune response they observed could be due to the large number of ticks used per infestation or highly sensitive techniques used. The present study failed to detect the antibody response, presumably because that in repeated tick infestations circulating serum precipitins might got complexed with introduced saliva components and cleared from the

circulation by the reticuloendothelial system, or unresponsiveness might have been induced by the host to recurrent exposures (Wikel and Osburn, 1982). Furthermore, the precipitating antibodies, if developed during initial infestation, tend to disappear as the frequency of tick challenge is increased (Fujisaki, 1978). Moreover, it was observed (Bendyev and Khudainazarova, 1976) that the host becomes desensitized to long, repeated infestations.

In the present study of repeatedly infested calves, gamma globulin showed non-significant changes. However, Brossard (1976) found that serum gamma globulin concentration was significantly increased following tick infestation, though a causal relationship between the gamma globulin level with acquisition of resistance, could not be established

During the present studies by salivary gland immunogens (SGAg-I, II and III) with FCA, all calves exhibited antibody response with double diffusion test, 21 days after first immunization. In SGAg-I (with FCA) immunized calves, two precipitin bands were also formed. It indicates that the humoral antibody response was relatively higher in SGAg-I (with FCA) immunized calves. It appears that incorporation of FCA with antigen gave a better response as the calves immunized with SGAg-I (without FCA) showed no precipitating bands. With CAT the antibody response was observed in SGAg-I (with FCA) immunized group 21 days after first immunization. The acquired resistance to tick infestation by the host after immunization with various ticks tissues,

has been studied in cattle by immunodiffusion (Allen and Humphreys, 1979), passive haemagglutination (McGowan et al., 1981) and gel diffusion (Johnston et al., 1986) tests. The present study showed that the double diffusion test was probably more efficient than the CAT.

In the present studies antibodies were detected up to 70 days after first immunization with SGAg-I (with FCA). The precipitin lines started to fade out after 70 days, indicating decrease in antibody response.

The significantly elevated gamma globulin levels in SGAg immunized (with FCA) animals in the present study indicate the possible protective role of gamma globulin.

Ticks from the immunized calves were lighter in weight indicating the effect of host response or antibody on their feeding. The host antibody directed against some target antigen in the tick will change the function of this target and the tick will either die or not reproduce (Galun, 1978). Accordingly, in the present study, the host antibody response to SGA was monitored by the gel diffusion test and level of serum gamma globulin was estimated.

Host immunoglobulin binding to ixodid tissue may interfere with tick salivation process and gut absorption. Antibody dependent lysis involving complement or antibody dependent cellular cytotoxicity could also alter tick engorgement (Whelen et al., 1986). Ackerman et al. (1981) demonstrated the ability of antibody to cross the gut of D. variabilis tick into the haemolymph. They further explained

that the antibody could react with internal organs of the feeding tick and moulting or oviposition processes inhibited.

Cell-mediated immune response

Cell-mediated immune responses (CMIR) to tick infestation have only recently been examined. During the present studies, the CMIR was assessed *in vivo* and *in vitro* by intradermal test and determination of E-rosettes, respectively. All immunized calves showed immediate and delayed skin hypersensitivity reactions to antigens of both *Hyalomma* species, and immediate reaction to *B. microplus* SGA. Control animals showed mild immediate reaction to both *Hyalomma* species and *B. microplus* SGA. Immediate type skin reaction was observed (Riek, 1962) in cattle repeatedly infested with *B. microplus* larval extract. The immediate and delayed skin reactions were observed in *Bos taurus* cows and calves (Wikel and Osburn, 1982), that initially received a low level infestation with *D. andersoni*, after administratic of *D. andersoni* SGA. In *Bos indicus* and cross-bred calves also, which were immunized with *A. americanum*, immediate and delayed skin reactions were observed (George *et al.*, 1985) to *A. americanum* and *A. cajennense* SGA, whereas, a mild immediate type reaction occurred to *D. andersoni* SGA. The present study shows similar skin reaction as of the above observations. A higher degree of cross-resistance of a host would be expected between the most closely related tick

species (McTier et al., 1981). In the present studies, skin test response was also seen to the heterologous Hyalomma antigen. The salivary gland antigen also elicited delayed skin reaction, an in vivo analogue of CMIR (Nelson and Boyden, 1964). However, the presence of antibody producing cells in these lesions could not be eliminated, since Askenase et al. (1975) have demonstrated their role in delayed skin reactions. It has also been reported (Collins et al., 1970) that delayed skin reactions in some cases are related to the presence of homocytotropic antibodies at the skin test site and occurrence of such a possibility cannot be ruled out in the present study. Delayed skin reaction is a common feature in arthropod parasitic infestation (Allen, 1966; Benjamini et al., 1961).

Salivary gland antigen elicited immediate type skin reaction in both tick-resistant and control calves, suggesting a vasoactive property of some components of this antigen. Pharmacologically active substances have been isolated from the saliva of the ixodid tick, B. microplus (Dickinson et al., 1976).

In the present study, the per cent 'E' rosettes increased significantly after the first tick attachment (in the repeatedly tick infested group) and after first immunization with SGAg-I, II (all with FCA) and SGAg-I (without FCA) indicating the participation of CMIR in acquire

immune response in calves against ticks. Soluble concanavalin A (con-A) and phytohaemagglutinin (PHA) are the known T-lymphocyte mitogens (Greaves and Janossy, 1972). Reduced *in vitro* responsiveness of the lymphocytes to con-A and PHA, as a result of tick infestation, suggests that the tick-host interaction altered the reactivity of T-lymphocytes. This observation is important, since it has been demonstrated that resistance in guinea pigs to *D. andersoni* is governed by a CMI component (Wikel and Allen, 1976a; Wikel *et al.*, 1978). The vast majority of antigen require T-lymphocyte interaction for the generation of an immune response (Miller and Mitchell, 1968). Therefore, impaired T-lymphocyte function could alter the host defences against arthropod-borne pathogens. Substances introduced to the host by the tick might act directly on T- lymphocytes, or initially on the macrophages with the observed changes reflected at the T-lymphocyte level (Wikel, 1982).

Histology of skin response to tick feeding

Hyalomma, *Amblyomma*, *Ixodes* and *Aponomma* ticks have long mouth parts which are fully inserted into the host tissue, whereas, *Dermacentor*, *Haemaphysalis*, *Boophilus*, and *Rhipicephalus* ticks have relatively shorter mouth parts and their penetration is superficial (Tatchell, 1969). As a result, vascular damage and a greater histopathological response by the host are more likely to occur as a result of feeding by the ticks with long mouth parts e.g. *H. a. anatolicum*

In the present studies, the female H. a. anatolicum inserted their mouth parts well into the lower dermal layers and some time passed through the cartilage layer of the ear. This explained the histolytic action of tick saliva.

Insertion of the mouth parts if facilitated by the cheliceral digit which cuts away the host tissue as the hypostome is thrust against the skin. This penetration process causes considerable tissue damage. The extent of tissue damage from tick feeding may be dependent upon mast cell damage and the release of granules, that contain heparin and histamine. These substances play a significant role in the inflammatory or defense responses characterized by neutrophil infiltration (Bloom and Fawcett, 1975). In the present study, the involvement of cellular response was observed through the formation of cavities and occurrence of lesions around the invading mouth parts of both male and female ticks.

During the present investigation, neutrophils were the predominant cells at tick feeding sites. At B. microplus feeding sites on cattle (Tatchell and Moorhouse, 1968), R. sanguineus on dogs (Theis and Budwiser, 1974), and A. americanum on guinea pigs (Brown and Knapp, 1980), also neutrophils were the predominant cells. This appears to be due to the host response to tissue insult caused by the tick salivary gland secretions (Berenberg et al., 1972).

The microscopic appearance of the epidermal vesicles in relation to morphology and cellular constituents in the immunized animals of the present study, were similar to those reported in cattle resistant to B. microplus (Riek, 1962), I. holocyclus (Allen et al., 1977), A.americanum (Brown et al., 1984a) and H. a. anatolicum (Gill, 1986). In contrast to the basophil rich vesicle observed at I. holocyclus (Allen et al., 1977) and A. americanum (Brown et al., 1984a) tick feeding site, the present study reveals few basophils and more neutrophils. Vesicle rich in neutrophils and few basophils was also observed (Gill, 1986) in resistant cattle (Bos taurus) at H. a. anatolicum feeding sites. Such vesicle formation could supposedly explain the inability of the ticks to engorge normally on resistant hosts (Allen, 1973; Tatchell and Moorhouse, 1968; and Trager, 1939a).

In the present study, the intimate contact of epidermal vesicles with cement around tick's mouth parts suggests that the cement material is antigenic and initiates the immune response. The accumulation of basophils is an index of the cutaneous basophil hypersensitivity (CBH) reaction (Dvorak et al., 1970; Allen, 1973).

Resistance apparently depends on the release of inflammatory mediators from the basophils. In sensitized animals, basophil degranulation at most tick feeding sites (Brown and Askenase, 1981; McLaren et al.,1983) could have resulted from the interaction of tick salivary antigens with basophil bound IgE or provoked by the liberation of any anaphylatoxins (Hook et al., 1975). Anaphylactic

degranulation of basophils releases vasoactive amines, especially histamine, 5-hydroxytryptamine and other haemoattractants like eosinophil chemotactic factors for other inflammatory cells. These amines increase vascular permeability thus facilitating the inflow of humoral factors and immune effector cells to tick feeding sites which may contribute to tick resistance. Basophil degranulation may also promote immunity by recruiting eosinophils to tick feeding sites. However, eosinophil recruitment, mediated by antigen-antibody complex and cell mediated mechanisms, can not be excluded (Weller and Goetzl, 1979). The epidermal vesiculation, therefore, appeared as a manifestation of these reactions.

The role of eosinophils, in conjunction with basophils, in the expression of resistance to ticks is not clear. A protective function of eosinophil major proteins has been demonstrated in a number of helminthic infections (Butterworth et al., 1979). It is possible that the cheliceral receptors or the gut epithelium of the tick get damaged by either eosinophil major proteins or other enzymes, leading to poor feeding.

CHAPTER-VI

SUMMARY AND CONCLUSIONS

The present investigation was carried out on cross-bred (*Bos taurus* x *Bos indicus*) healthy male calves, below one year old, to study : (1) the development of immunity, if any, in the calves by repeated tick infestations, and (2) to immunize the calves by inoculation of the salivary gland antigens of adult *H. a. anatolicum* ticks.

In repeated tick attachment experiments, except the engorgement period of the ticks, all other parameters like per cent engorgement, pre-oviposition period, oviposition period, engorged weight, egg mass weight and per cent hatch of eggs showed statistically significant difference from first to tenth infestation.

Three types of salivary gland antigens (SGAg), viz., SGAg-I (whole), SGAg-II (supernatant) and SGAg-III (sediment), were used for immunization. Significant resistance developed in calves of SGAg-I and SGAg-II (both with FCA) groups. Of all, SGAg-I with FCA proved as most potent immunogen as compared to SGAg-II and III (both with FCA) and SGAg-I (without FCA).

Passive transfer of plasma from animals immunized with SGAg-I containing FCA showed no significant resistance in recipients upon challenge. But, capillary tube agglutination test with SGAg-I (with FCA) and double diffusion test with SGAg-I, II, and III (all with FCA) gave positive reactions 21 days after first immunization. The SGAg-I with

FCA group showed positive gel diffusion reaction 70 days after first immunization. All SGAgs (with FCA) immunized animals had significantly increased gamma globulin levels after first immunization.

All repeatedly infested and SGAgs (with or without FCA) immunized animals showed immediate to delayed skin reactions to Hyalomma antigens (H. a. anatolicum and H. m. isaaci). Inoculation of B. microplus antigen showed mild immediate reaction lasting up to eight hours.

There was significant increase in the per cent 'E' rosettes in repeatedly infested, and SGAg-I, II (both with FCA) and SGAg-I (without FCA) immunized groups.

Upon challenged tick attachments on the immunized animals, histopathological observation of skin biopsies revealed formation of cavities, lesion around the invading tick mouth parts, and epidermal vesicles. The infiltration of basophils and eosinophils was also observed.

These observations indicated that probably both humoral and cellular responses are operational in the acquired resistance to ticks.

CHAPTER – VII

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APPENDICES

APPENDIX-I

BARBITONE BUFFER

Barbitone sodium	12.00 gm
Barbitone	4.40 gm
Merthiolate	0.15 gm

1. Dissolved 12.00 gm barbitone sodium in 800 ml glass distilled water.
2. Dissolved 4.40 gm barbitone in 150 ml glass distilled water.

Mixed 1 and 2 and adjusted at pH 8.2 with hydrochloric acid (5 N), added 0.15 gm merthiolate and the final volume was adjusted to 1000 ml.

APPENDIX -II

COOMASSIE BRILLIANT BLUE DYE

Coomassie brilliant blue	0.5 gm
Glacial acetic acid	10 ml
Ethanol	40 ml
Distilled water	40 ml

APPENDIX-III

DESTAINING SOLUTION

Glacial acetic acid	10 ml
Ethanol	40 ml
Distilled water	40 ml

Fig.1. New Zealand White rabbit used for maintenance of tick colony.

Fig.2. Cross-bred calves with ear bags for holding the ticks.

Fig.3. Cross-bred calves with bags on the ears and tail.

Fig.4. Rosette test - showing clustering of erythrocytes around the lymphocyte (X 700).

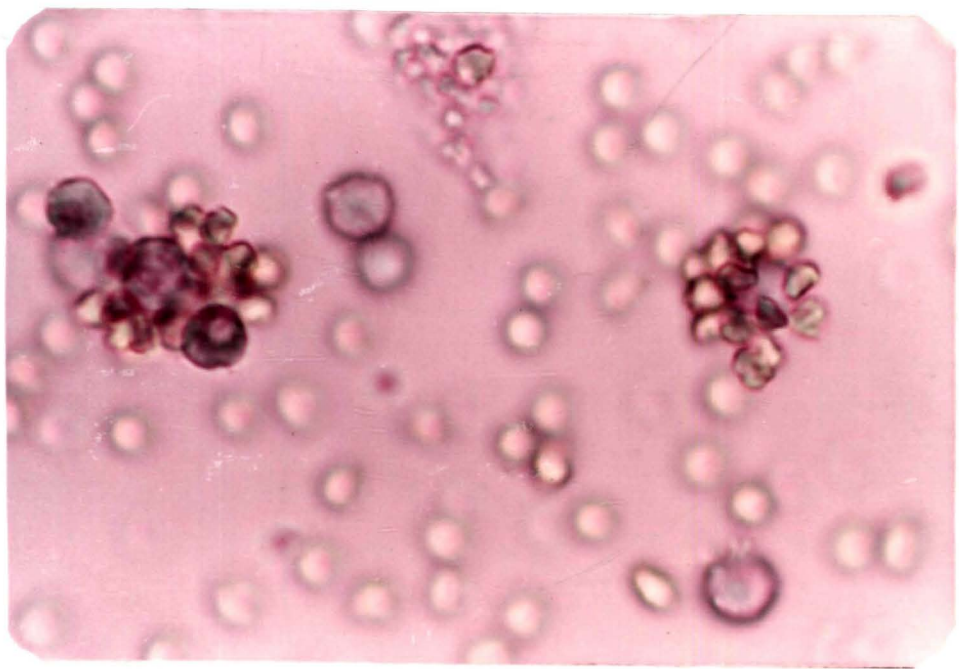


Fig.5. Reduced size of the female ticks engorging on immunized (A) and unimmunized (B) calves.

Fig.6. Some ticks engorged on immunized calves did not lay eggs, latter died and turned black.

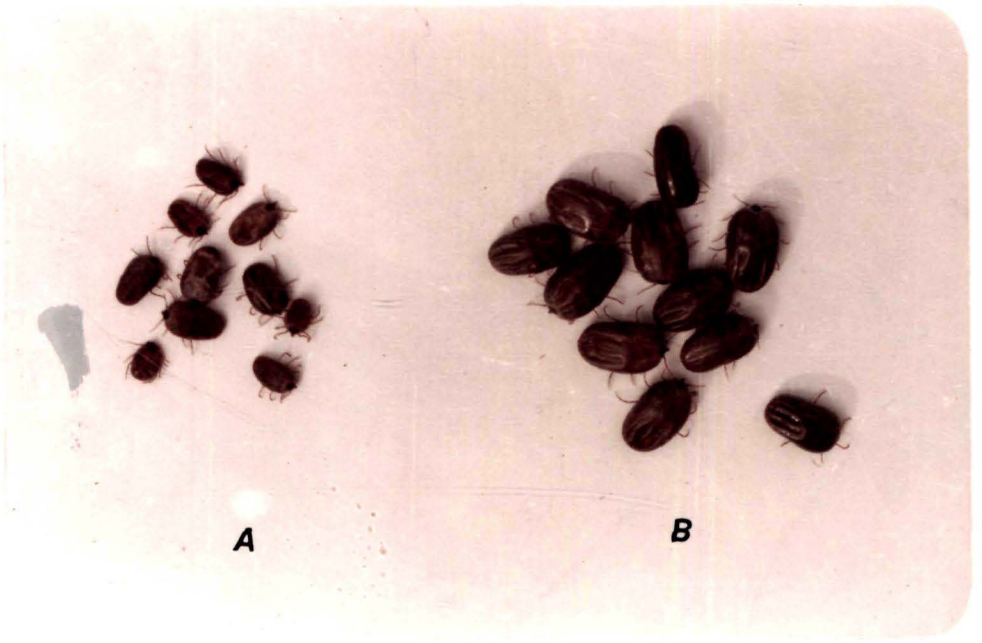
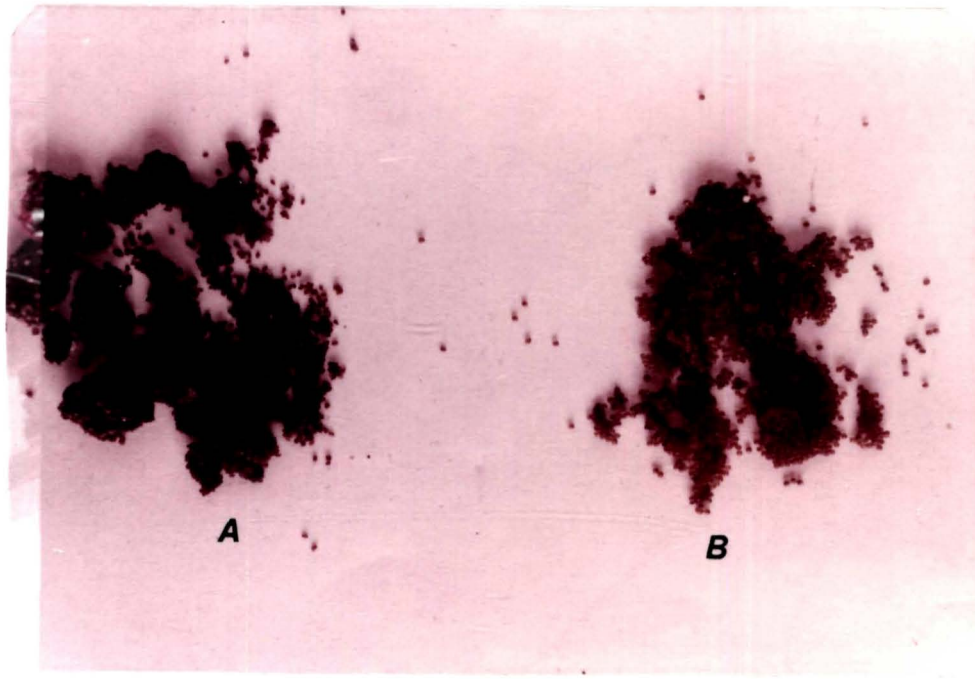

A
B

Fig.7. Egg mass from ticks engorged on immunized (A) calves showing black discolouration; and brownish, shining egg mass of unimmunized (B) calves.

Fig.8. Skin biopsy showing delayed hypersensitivity reaction with marked cellular infiltration (H&E stain X 60).


A
B

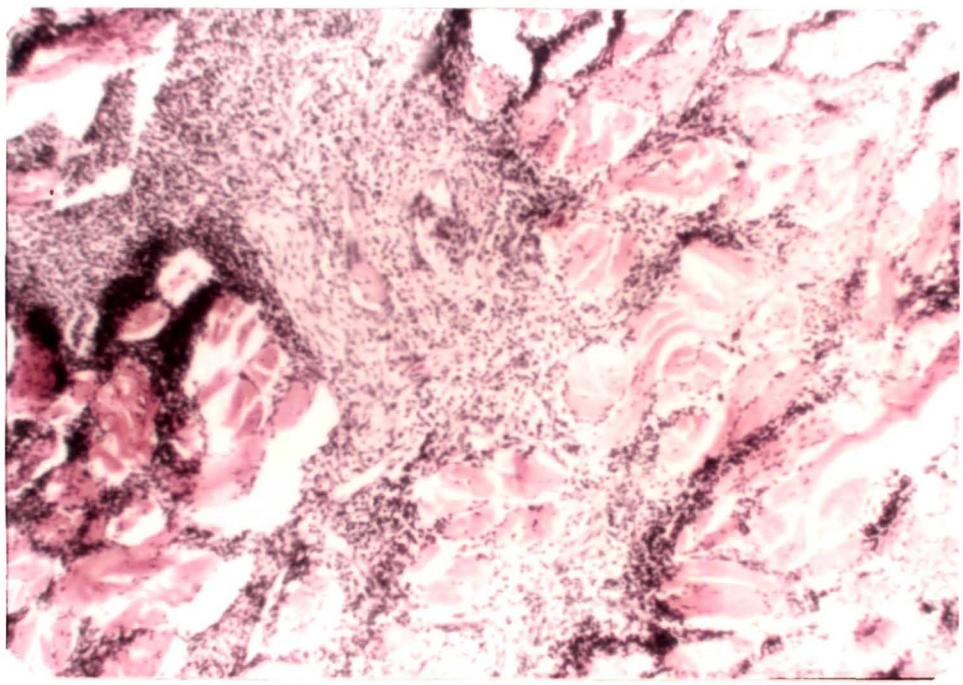


Fig.9. Capillary tube agglutination test.

A = Positive reaction in immunized calves.

B = Negative reaction in unimmunized calves.

C = Antigen control

D = Serum control.

Fig.10. Double diffusion test with group-I (immunized with SGAg-I and FCA) on 21,28 and 35 days after first immunization.

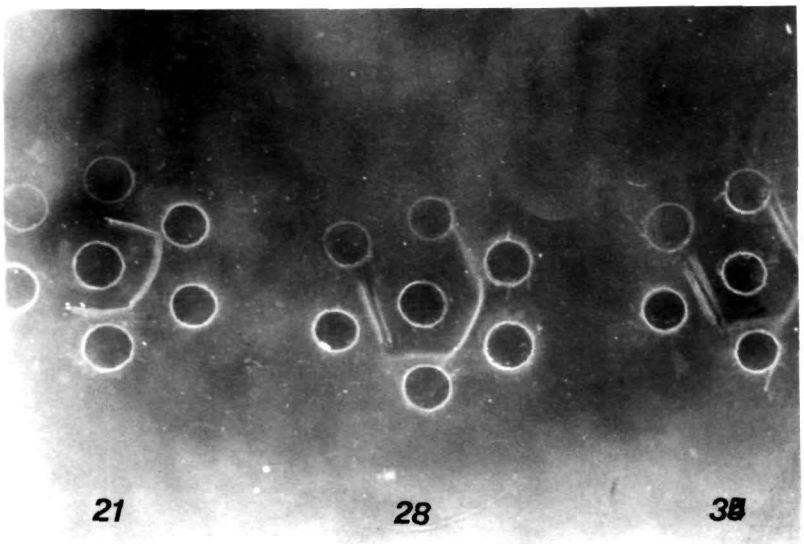
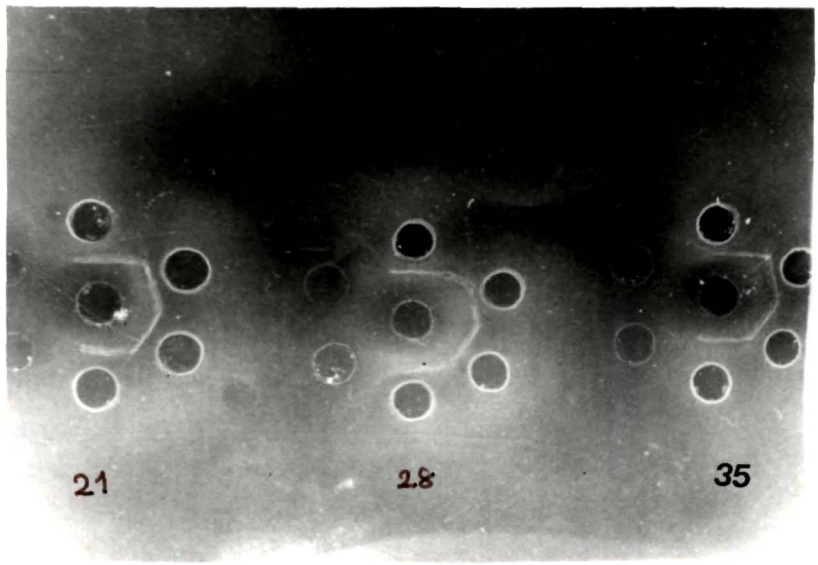

21
28
35

Fig.11 Double diffusion test with group-2 (immunized with SGAg-II and FCA) on 21, 28 and 35 days after first immunization.

Fig.12 Double diffusion test with group-3 (immunized with SGAg-II and FCA) on 21, 28 and 35 days after first immunization.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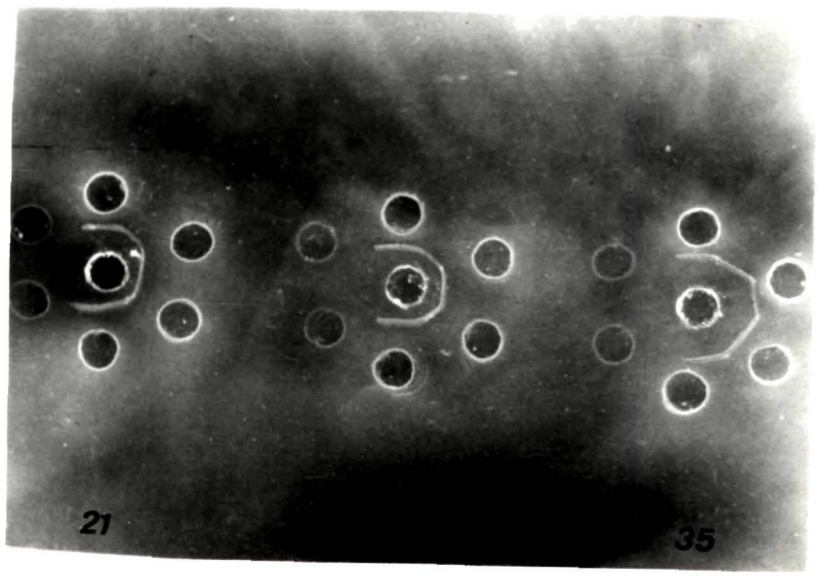
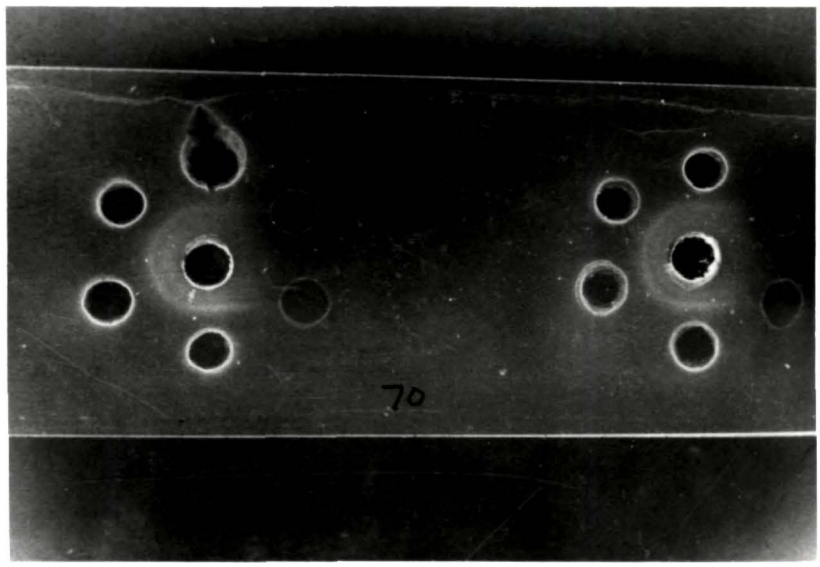

21
35

Fig.13. Double diffusion test with group-I (immunized with SGAg-I and FCA) on 70 days after first immunization.

Fig.14. Normal calf skin showing Epidermis (ED), Dermis (D), Vessels (V), Sebaceous gland (SG) and Hair Shaft (HS) (H & E stain X 60).


70

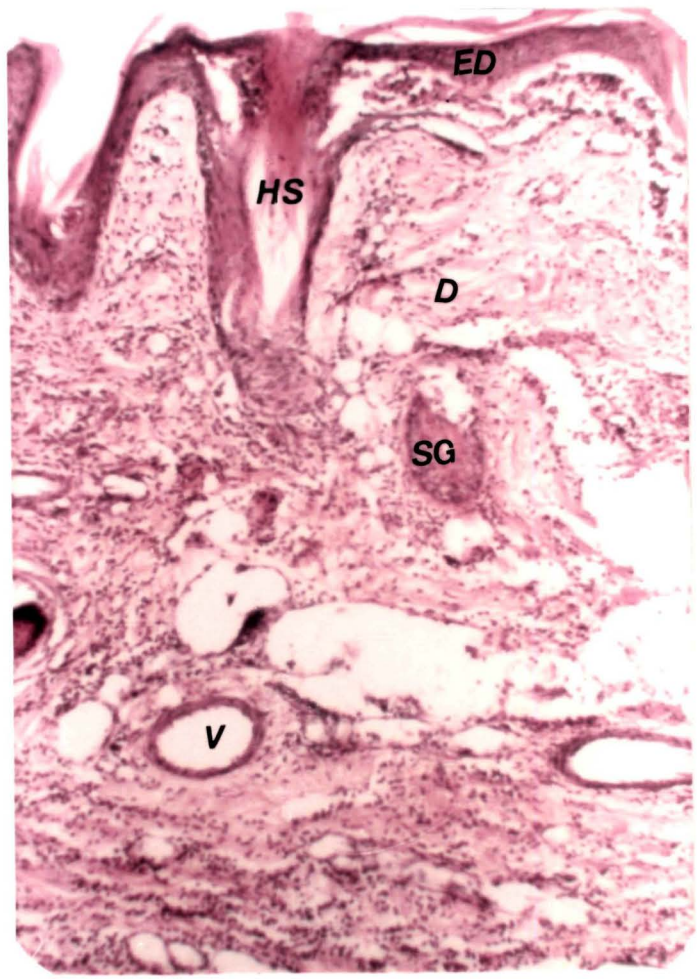
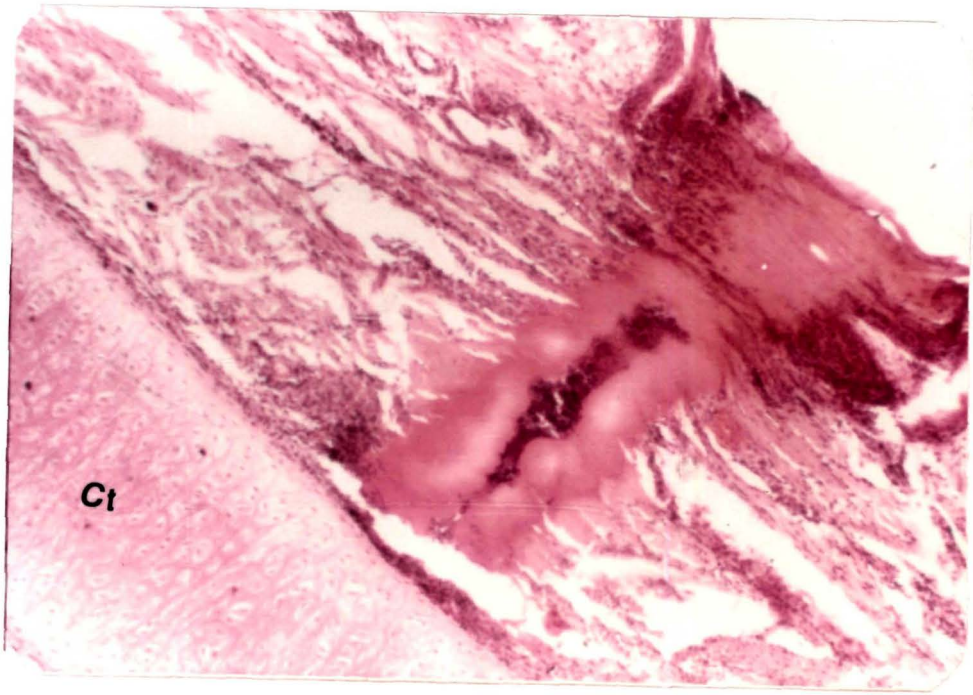

ED
HS
D
SG
V

Fig.15. Female tick mouth parts reaching deeper into the skin layer and approaching the cartilage (Ct) (H & E Stain X 60).

Fig.16. Female tick mouth parts piercing through the cartilage (Ct) showing lysis of tissue and deposition of cement (C) (H & E stain X 60).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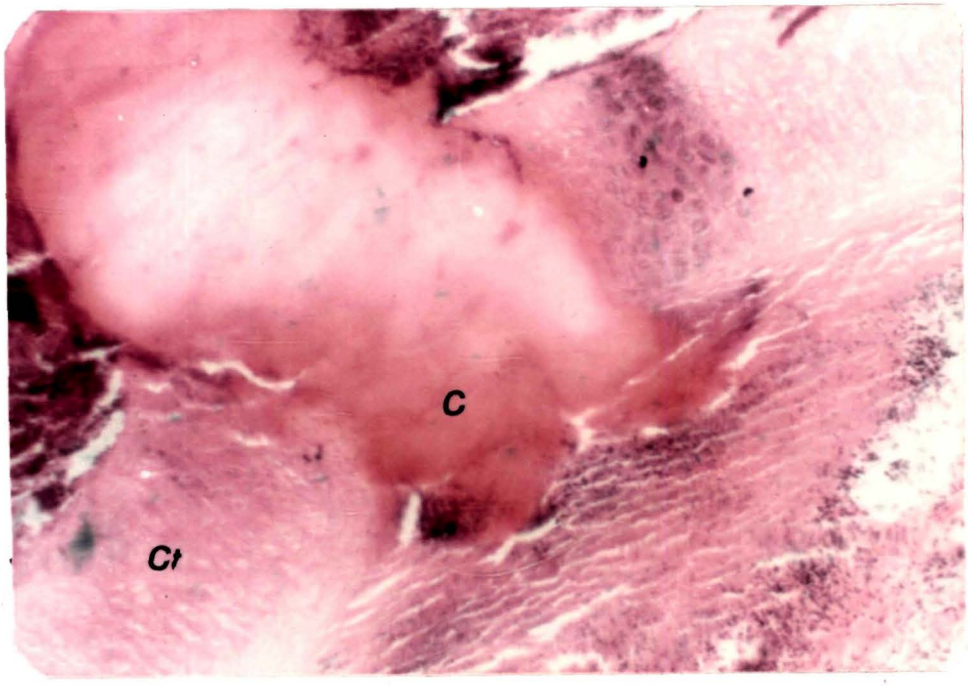
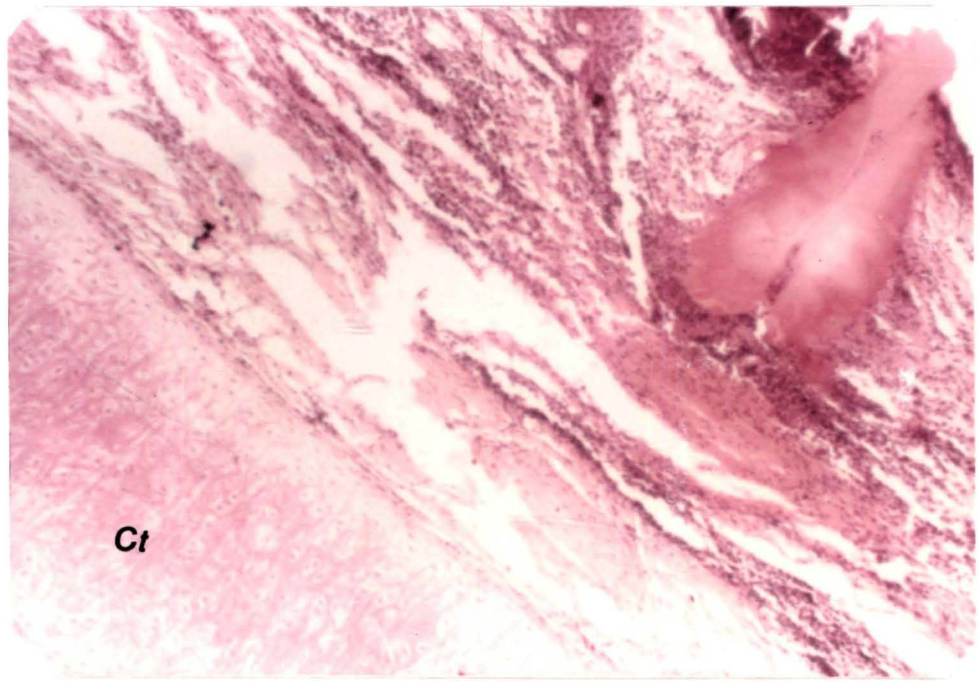

C
Ct

Fig.17. Male tick mouth parts reaching superficial skin layers much above the cartilage (Ct) (H & E stain X 60).

Fig.18. Penetration of female tick mouth parts (MP) and inducing celluar infiltration (H & E stain X 60).


Ct

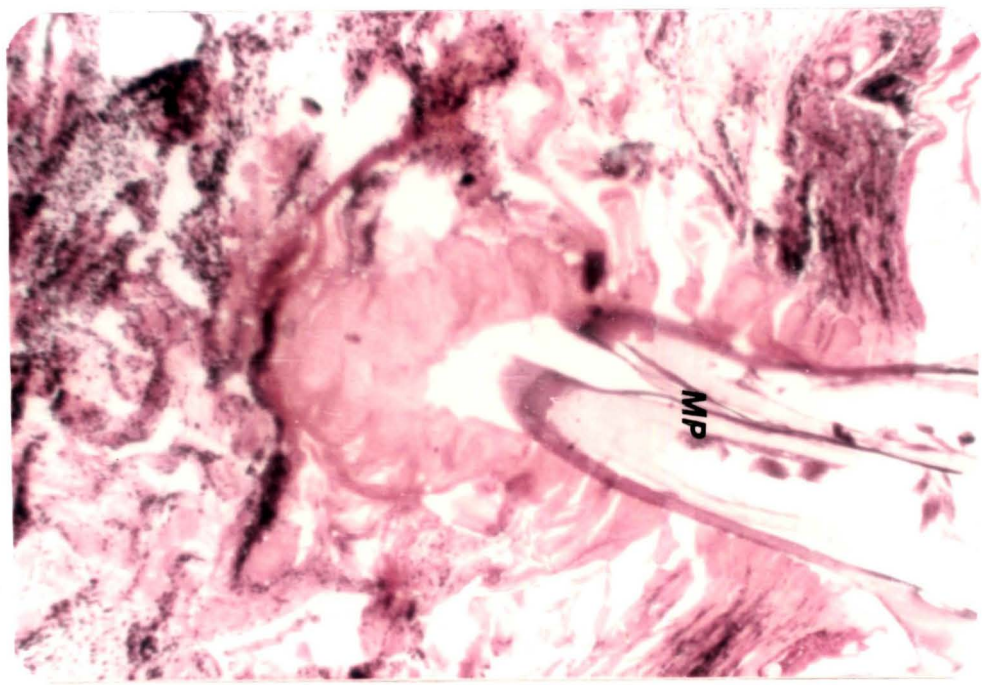
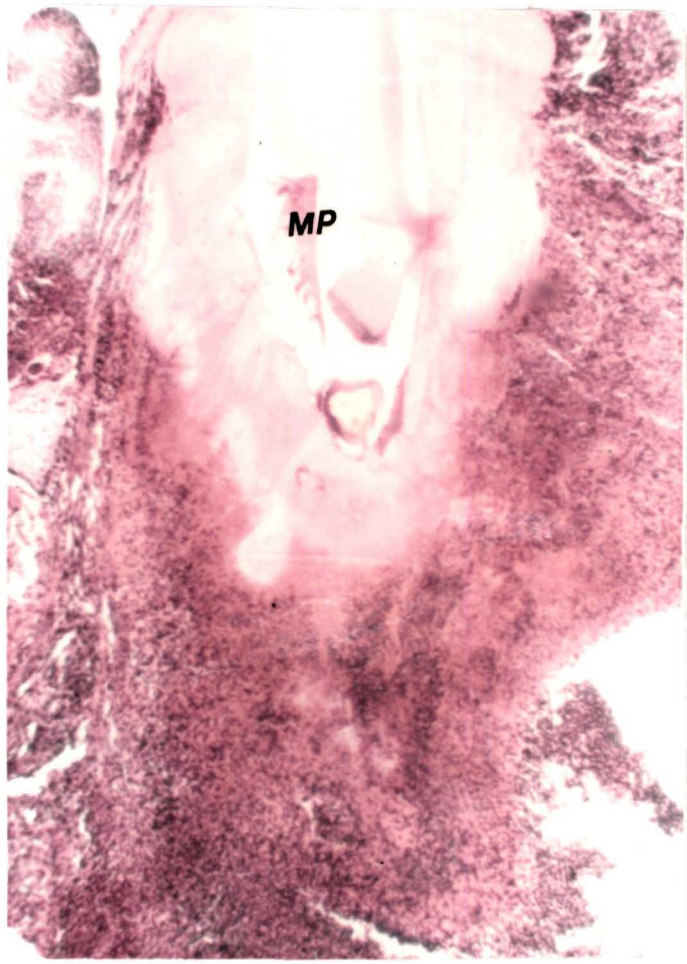

MP

Fig.19. Cellular infiltration intansifies as the feeding progresses and mouth parts (MP) penetrates into dermal layers (H & E stain X 60).

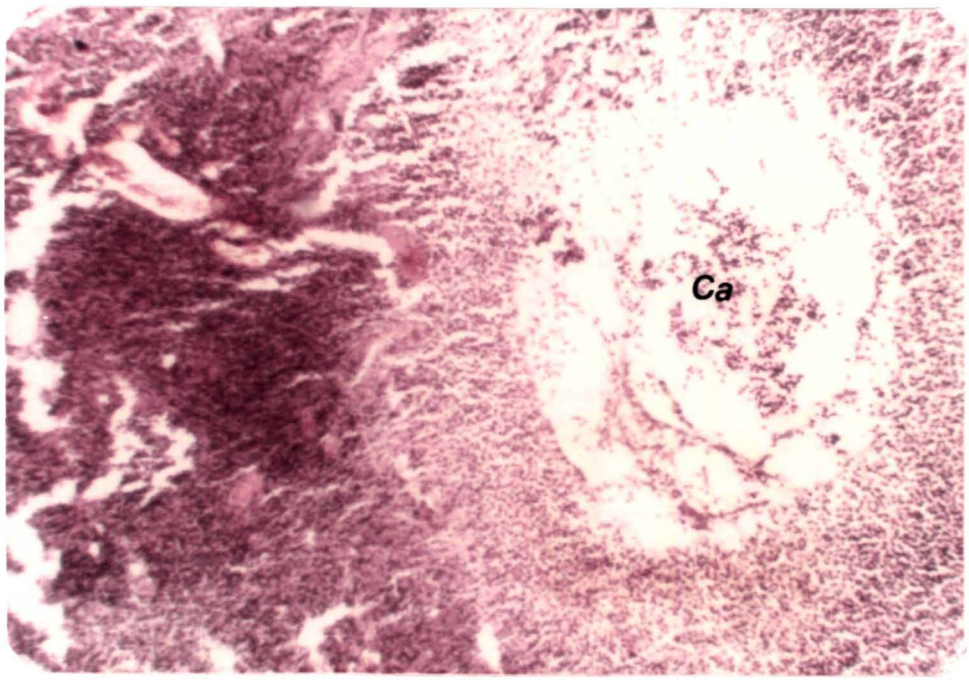
Fig.20. Extensive cellular infiltration during final engorgement with cavity formation (H & E stain X 60).


MP


MP

Fig.21. Complete destruction of skin tissue and formation of feeding cavity (Ca) below the mouth parts (H & E stain X 60).

Fig.22. Epidermal vesicle (Ep.V) at the tick attachment site and leucoyte infiltration especially of neutrophils, basophils and few eosinophils (H & E stain X 60).


Ca

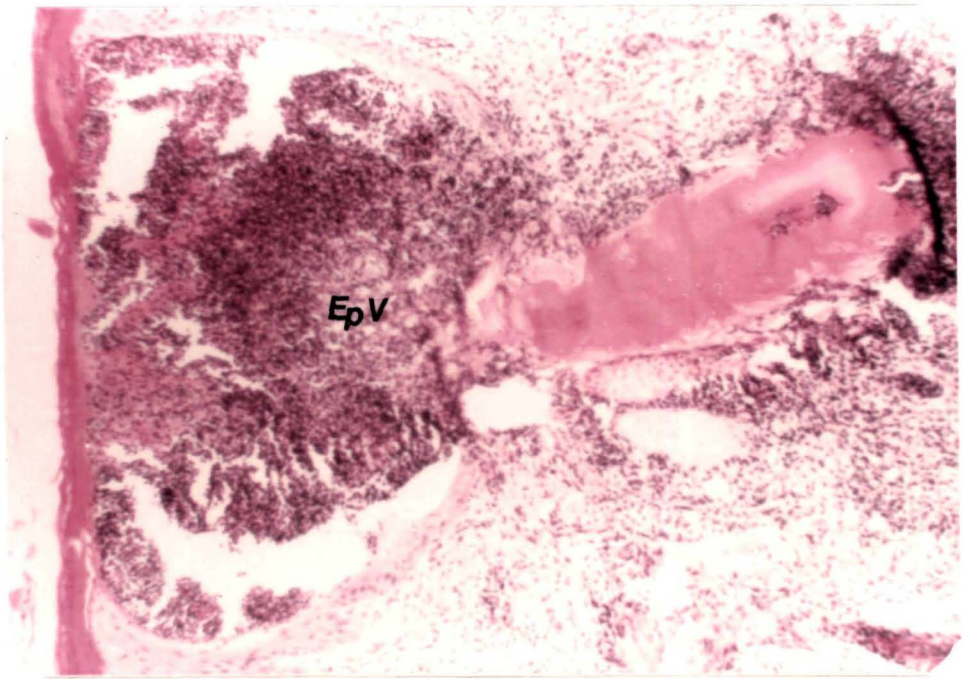
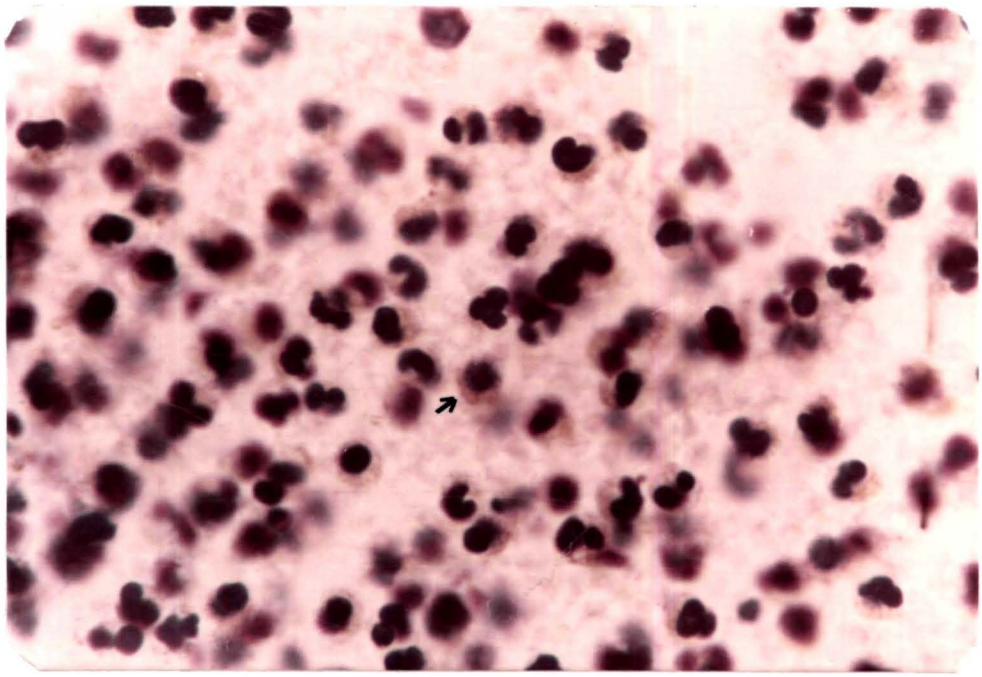
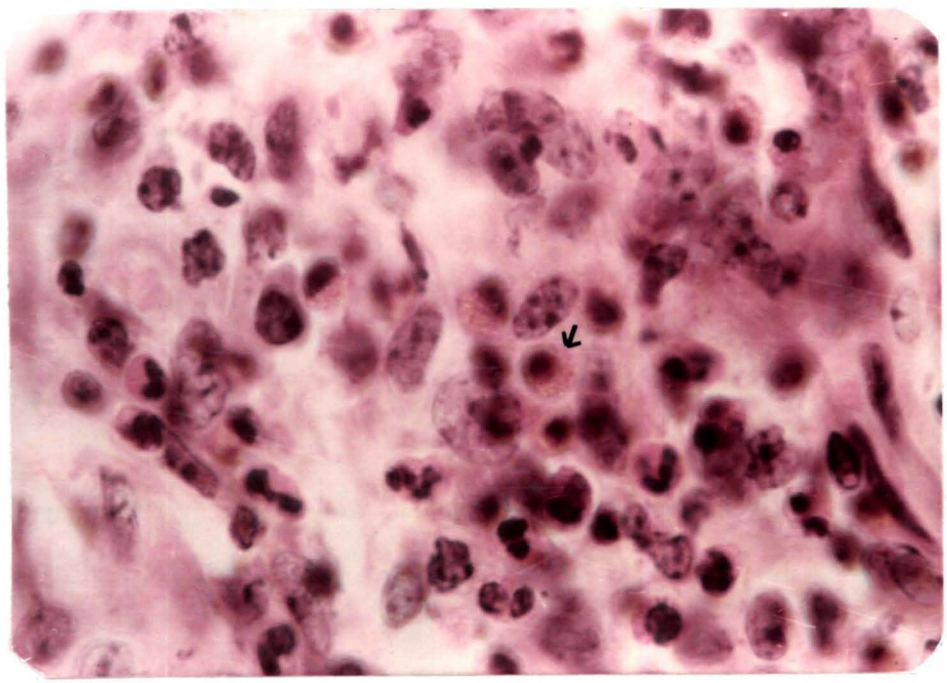

EpV

Fig.23. Basophil infiltration in the dermis beneath the tick mouth parts (Toluidine blue stain X 1000).

Fig.24. Eosinophil infiltration in the dermis beneath the tick mouth parts (H & E stain X 1000).





ABSTRACT

ATTEMPTED IMMUNIZATION OF CROSS-BRED CALVES AGAINST IXODID TICK, Hyalomma anatolicum anatolicum KOCH (1844).

By

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(An abstract of the dissertation presented in partial fulfilment of the requirements for the degree of Ph.D., Haryana Agricultural University).

The present investigation was carried out on 54 cross-bred (Bos taurus x Bos indicus) healthy male calves, below one year old, to study:

(1) The development of immunity, if any, in the calves by repeated tick infestations, and

(2) To immunize the calves by inoculation of the salivary gland antigens of adult Hyalomma anatolicum anatolicum female ticks.

In repeated tick attachment experiments, the calves were infested ten times with 50 pairs of adult ticks on each ear at 15 days intervals. Except the engorgement period of the ticks, all other parameters like per cent engorgement, pre-oviposition period, oviposition period, engorged weight, egg mass weight and per cent hatch of eggs showed statistically significant difference from first to tenth infestation.

Three types of salivary gland antigens (SGAg), viz., SGAg-I (whole), SGAg-II (supernatant) and SGAg-III (sediment), were used for immunization. The calves were divided into five groups. First three groups were immunized with SGAg-I, II and III (all with FCA), respectively. Fourth group was immunized with SGAg-I (without FCA), and fifth group remained as unimmunized control and inoculated with FCA only. In all the groups, immunization was carried out on day 1st and 14th and the animals were challenged on 21st day. Significant resistance developed in the calves of SGAg-I and SGAg-II (both with FCA) groups. Of all, SGAg-I with FCA proved as most potent immunogen as compared to SGAg-II and III (both with FCA) and SGAg-I (without FCA).

Both humoral and cellular immune responses were demonstrated by suitable *in vivo* and *in vitro* tests.

Passive transfer of plasma from animals immunized with SGAg-I containing FCA showed no significant resistance in the recipients upon challenge. But, the capillary tube agglutination test with SGAg-I (with FCA) and double diffusion test with SGAg-I, II and III (all with FCA) gave positive reactions 21 days after first immunization. The SGAg-I with FCA immunized group showed positive gel diffusion reaction 70 days after first immunization. All SGAgs (with FCA) immunized animals showed significantly increased gamma globulin levels after first immunization.

All repeatedly infested and SGAgs (with or without FCA) immunized animals showed immediate to delayed type skin reactions to Hyalomma antigens (H. a. anatolicum and Hyalomma marginatum isaaci). Inoculation of Boophilus microplus antigen showed mild, immediate type reaction lasting up to eight hours. The skin biopsies at peak of reaction showed excessive infiltration of mononuclear cells in the dermal layer.

There was significant increase in the per cent 'E' rosettes in repeatedly infested, and SGAg-I, II (both with FCA) and SGAg-I (without FCA) immunized groups.

Upon challenge tick attachments on the immunized animals, histopathological observations of skin biopsies revealed formation of cavities, cellular infiltration and epidermal vesicles at the site of tick attachment. The infiltration of basophils and eosinophils was also observed.

These observations indicated that probably both humoral and cellular immune responses are operational in the acquired resistance to ticks.