IMMUNOPROPHYLAXIS AGAINST COMMON DOG TICK USING GUT ANTIGEN

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DECLARATION

I hereby declare that this thesis, entitled **"IMMUNOPROPHYLAXIS AGAINST COMMON DOG TICK USING GUT ANTIGEN"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy,
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CERTIFICATE

Certified that the thesis, entitled **"IMMUNOPROPHYLAXIS AGAINST COMMON DOG TICK USING GUT ANTIGEN"** is a record of research work done independently by **Ajithkumar. K.G** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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AJITHKUMAR. K. G.

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Introduction

1. INTRODUCTION

Ticks have long been regarded as constraints to human welfare and to domestic animals. On global basis, ticks represent one of the most important group of arthropods that affect animal health. Estimates of economic loss due to ticks and tick borne diseases are in billion dollars worldwide, although precise figures for most countries are lacking. In India the most commonly faced problem in dogs is external parasitic infestation by ticks. All active stages (larvae, nymph and adult) are obligate blood feeders and further, adults require more blood for sperm and egg production. Ticks can also cause dermal irritation and hypersensitivity reaction. Their bites are not only annoying and painful but also result in localized skin inflammation, secondary infection and possible introduction of disease causing microorganisms. In large number, ticks can cause iron deficiency anemia.

Among dog ticks, the brown dog tick, *Rhipicephalus sanguineus,* the most widely distributed tick species in the world (Pegram *et al.*, 1987) has been linked to many diseases such as ehrlichiosis, babesiosis and haemobartenellosis (Cupp, 1991) which are primarily canine but may sporadically occur in other mammals, including man (Goddard, 1989). It is found on dogs in tropical and sub tropical areas throughout the year. In warm temperate areas where definite seasonal changes occur, ticks are commonly found on host from early spring until autumn; ticks are few on animals during winters.

In Kerala, nine species of hard ticks (Family-Ixodidae) have so far been recorded from domestic animals. They are *Boophilus annulatus, R. sanguineus, R. turanicus, Haemaphysalis bispinosa, H. turturis, H. spinigera, Hyalomma anatolicum anatolicum and Amblyomma integrum* (Sreekrishnan, 1992). A survey on ticks infesting dogs in Thrissur Corporation has not been conducted so far and relatively little is known about the prevalence of tick infestation in dogs.

At present, the major tick control strategy widely available is the use of chemical acaricides. Chemical use was introduced in 1950's for the control of arthropod pests. The disadvantages of acaricide use, such as contamination of animal food products and environment, the ability of ticks to quickly acquire resistance against a wide range of acaricides and the high cost (Wikel, 1988; Willadsen and Kemp, 1988; Nolan *et al.*, 1989) have necessitated the need to develop alternative tick control strategies. Apart from immunisation against tick infestation, several other alternative tick control measures reviewed by Sonenshine (1993) have shown little or no promise in terms of their practicality and sustainability.

In the recent years, there has been renewed interest in immunity to ticks and in host immunisation using a variety of tick derived antigens as an alternative to treatment with insecticides for controlling tick infestation. Current research into immunological control of ticks centres around two different approaches. The first of these, which follows the tradition of immunoparasitology, investigates the complex series of physiological and immunological reactions that follow repeated attachment of ticks to their hosts. The antigens eliciting these responses are typically those of the salivary gland or the mouth parts. The second approach looks at the effects of stimulating immunological interaction that do not occur as a result of natural infestation. The interaction may be with antigens that are not displayed to the host as a result of natural host-parasitic interaction, known as 'concealed' antigen. The most promising results have been with antigenic material derived from the gut of ticks.

The currently available commercial vaccines TickGARD^{PLUS} and Gavac^{TM} produced in Australia and Cuba respectively as an aid in the control of cattle tick, *Boophilus microplus* consists of a recombinant antigen (Bm86) based on a protein from the microvilli of the digest cells in the gut of *B. microplus* (Rand *et al.*, 1989: Willadsen *et al.*, 1989; Gough and Kemp, 1993). Ingestion of blood containing antibody to Bm86 causes lysis of gut cells of tick, leading to reduced number of ticks

engorging, reduced egg production and reduced egg viability (Kemp *et al.,* 1989). Development and commercialization of these vaccines against the cattle tick *B. microplus* has shown that immunising hosts against tick infestation is practical. However, development of a successful vaccine against major species in the genera of *Amblyomma, Hyalomma and Rhipicephalus*, is still far from reality.

Very little work has been done on dog ticks. Repeatedly infested dogs do not develop natural resistance to further infestation with *R. sanguineus* (Garin and Grabarev, 1972; Szabo *et al.,* 1995). But those immunised with tick gut extract in Freund's complete adjuvant, developed resistance (Szabo and Bechara, 1997) suggesting that enhancement of cellular immunity may be one important mechanisms to induce resistance.

Like wise in India a few workers assessed the effect of different antigens from the dog tick *R. sanguineus* (Tripathi *et al.*, 1998; Rath, 1999).

Considering the above problems, the present investigation has been undertaken to embark upon the following salient objectives.

1. To understand the tick infestation in dogs

2. To identify the species involved and

3. To explore the possibility of immunoprotection against the most common dog tick using gut antigen

Review of Literature

2. REVIEW OF LITERATURE

The first account of host immunity to tick appears to be the one written by Nuttal (1911), which refers to the phenomenon of natural immunity in humans. From 1930 onwards, the first investigation into host resistance began to appear, and since then innumerable studies on the immunological aspects of the host tick relationship have been carried out.

2.1 PREVALENCE

Khan and Srivastava (1993) reported that out of the 10 species of *Rhipicephalus* recorded so far in India, *Rhipicephalus haemaphysaloides*, *R. sanguineus* and *R. turanicus* were the most prevalent. *Rhipicephalus haemaphysaloides* showed host catholicity as it infested cattle, buffaloes, sheep, goats, camel, dogs, cats, rabbits and rats while *R. sanguineus* mostly remained confined to dogs. Both the Rhipicephlids showed bimodal activity. The first period of activity was from March to May and the other from mid June to November.

Vazquez *et al.* (1998) reported that the prevalence of *R. sanguineus in* dogs in Cuernavaca was 20 per cent, during a three year study between 1993 and 1995. No significant differences were observed in infestation of dogs with *R. sanguineus* based on their age, sex and by year. Prevalence rate was high throughout the year and peaks were found in April, July and November, whereas prevalence was lower in January (winter season).

Inokuma *et al.* (2001) reported that out of the twenty two free roaming dogs in Ishigaki island examined during July 1998, twelve dogs (54.5 per cent) were infested with *R. sanguineus*.

Jacobs *et al.* (2001) reported that out of the nine ixodid tick species collected from dogs in the central region of the Free State Province, South Africa, *R. sanguineus* was most abundant. The majority of these ticks attached to the back and neck of the dogs, with the proportions being larger in long-haired than in short-haired dogs. Most of them were collected during the warmer months particularly from January to April. Large number of *Haemaphysalis leachi*, the next most numerous species, were also collected from the back and the neck of the dogs. Most of these were present during the period from September to November.

Horak and Matthee (2003) found the occurrence of 13 species of ixodid ticks and one species of argasid ticks on dogs in a survey conducted in Western Cape Province of South Africa and the most numerous of these were *H. leachi, R. gertrudae* and *R. sanguineus.* No clear pattern of seasonality was evident for *H. leachi.* The largest number of adult *R. gertrudae* were generally present from August to October while adult *R. sanguineus* were collected during October 2000, February and March 2001, from January to April 2002 and during October 2002

Neves *et al.* (2004) identified 10 ixodid tick species in dogs in southern Mozambique. *Rhipicephalus sanguineus* was the most numerous of the ten species, and its prevalence and intensity of infestation were significantly higher in city dogs (94.8 per cent) than in rural dogs (18.8 per cent), whereas the converse was true for *H. leachi* (6.9 per cent and 93.8 per cent).

In a survey conducted for a period of two years in Hungary by Foldvari and Farkas (2005), majority of ticks collected from the dogs were adults (91.7 per cent) and others were nymphs. Six species were found namely *Dermacentor reticulatus* (48.9 per cent), *Ixodes ricinus* (43.2 per cent), *I. canisuga* (5.6 per cent), *H. concinna* (2 per cent) and one specimen each of *D. marginatus* and *I. hexagonus*. Single species infestation with *I. ricinus* or *D. reticulatus* was found on 145 (46.8 per cent) and 120 animals (38.7 per cent), respectively. Mixed infestation caused by these two

species was detected on 24 dogs (7.7 per cent). *Ixodes canisuga* and *H. concinna* were found on seven and five dogs respectively. *Dermacentor reticulatus* and *I. ricinus* were collected almost throughout the year. The activity peaked in spring and autumn for both species.

2.1.1 Morphology and Identification

Sen and Fletcher (1962) described the keys for identification of various ticks of animals and birds.

Soulsby (1982) also gave an account on the morphology of different species of ticks infesting animals and birds.

Kettle (1995) also provided the morphological and identifying features of ticks of animals and birds.

Walker *et al.* (2000) developed identification keys for adult species of *Rhiphicephalus* ticks from the afrotropical regions and elsewhere. For nymphs and larvae, unique plates have been compiled in which line drawings of the capitula of similar species were grouped together to facilitate identification. They have also provided full description, host, site of attachment and zoogeography of each species.

2.2 TICK COLONY MAINTENANCE

Sweatman (1967) reported that the longevity of *R. sanguineus* was temperature dependent, and was shortened significantly by a high saturation deficit between 20 and 30^0C.

Tick infestation procedure was reported by Brown *et al.* (1983) where they used plastic Petri dishes, measuring 40 x 12 mm, each containing 200 larval *R. appendiculatus* which were placed inverted on the sheared flank skin of the host and secured with adhesive tape.

Manohar and Banerjee (1992) reared *Hyalomma anatolicum anatolicum* ticks by feeding on rabbit ears using thick cotton bags and then keeping at 28 ± 1^{0}C in BOD incubator in cotton plugged glass tubes inside the dessicator over a 10 per cent solution of potassium hydroxide providing humidity of 85 per cent.

Kumar and Kumar (1996a) maintained *H. dromedarii* and *H. anatolicum* tick colonies in the laboratory at 28^{0}C and 85 per cent relative humidity in BOD incubators following conventional method of tick rearing.

Parmar *et al.* (1996) reared *H. anatolicum anatolicum* and *Boophilus microplus* tick colonies by feeding different life cycle stages on cross-bred calves or rabbits and their subsequent maintenance in the laboratory at 85 per cent relative humidity, 28 ± 2^{0}C until they moulted to the next instar.

Ghosh and Khan (1998) colonized *B. microplus*, *H. anatolicum* and *R. sanguineus* by feeding on calves / rabbits and maintaining at 30 ± 2^{0}C and 80 ± 5 per cent relative humidity.

Laboratory reared *R. sanguineus* ticks were subsequently maintained in the laboratory under controlled temperature of 30 ± 2^{0}C and 80 ± 5 per cent relative humidity (Tripathi *et al.*, 1998).

2.3 TICK ANTIGENS

Trager (1939) carried out pioneering studies that gave evidence of acquired immunity to ticks. Later Feldman-Muhsam (1964) observed that rats after successive infestation with *R. sanguineus,* became immune to ticks, a process that was considered to constitute immunisation of the hosts.

Allen *et al.* (1977) demonstrated that salivary gland antigen of *D. andersoni* was present in the skin of infested guinea pigs.

Immunogen used by McGowan *et al.* (1980) was an extract of homogenized whole tick tissue collected from the tick, *Amblyomma maculatum.*

Heller-Haupt *et al.* (1987) used unfed larvae of *A. variegatum* as an antigen source for immunizing laboratory animals.

Opdebeeck *et al.* (1988) immunised Hereford cattle with membranes and soluble component extracts from the mid guts of *B. microplus*. Membrane vaccine protected cattle (91 per cent) against challenge with 3 x 20000 larval ticks administered at an interval of seven days whereas vaccine made from soluble antigens did not protect cattle.

Wikel (1988) opined that antigens not normally involved in the acquired resistance could be used to induce an antitick immunity. These novel antigens obtained from the tick gut absorptive surface, were not introduced into the host during tick feeding, but exposed to host immune effector elements in the blood meal, resulting in ixodid rejection, prevention of ova production and death.

Membrane protein from mid gut of partially fed females of *B. microplus* were reported to contain antigens capable of inducing immune response in cattle (Willadsen *et al.*, 1988; Wong and Opdebeeck, 1989).

Kimaro and Opdebeeck (1994) prepared extracts from the membranes of eggs (EM) and guts (GM) of *B. microplus* which were used to immunise cattle and challenged twice with 20,000 larval ticks one week apart. EM antigens did not protect cattle against challenge with ticks, despite high levels of anti-egg antibodies in the sera of the vaccinated cattle, detected by an indirect enzyme-linked immunosorbent assay (ELISA). Cattle vaccinated with GM, however, had high levels of antibodies against GM and were protected significantly against challenge with *B. microplus*.

Riding *et al.* (1994) purified and characterized a membrane protein Bm91 with an apparent molecular weight of 86,000 located largely in the salivary gland and gut of *B. microplus* ticks. The protein could not be recognized in the sera from cattle with extensive exposure to ticks under natural conditions. The immunity induced by vaccination, therefore, represented another example of vaccination against a hematophagous parasite with "concealed antigens".

Szabo and Bechara (1997) prepared salivary gland and gut extracts from *R. sanguineus* by dissection, sonication, centrifugation and filtration. Immunisation of guinea pigs with both these extracts along with adjuvant resulted in lower tick recovery and lower engorged female weights. Dogs were inoculated with gut extract alone as this fraction induced a more efficient resistance in guinea pigs.

Da silva Vaz *et al.* (1998) reported that immunisation of cattle with *Boophilus* Yolk pro-Cathepsin (BYC) induced a protective immune response. Measurement of various biological parameters demonstrated a partial protection against the tick.

Garcia-Garcia *et al.* (1998) reported the expression of the recombinant concealed antigen Bm86 in *Pichia pastoris,* candidate for combining vaccines for cattle on account of its dual role as an immunogen and adjuvant.

Following immunisation with homogenised antigens prepared from unfed larvae and nymphs of *H. anatolicum anatolicum* (HLAg and HNAg respectively), rabbits developed significant level of protective immunity to infestation with the adult species (Ghosh *et al.*, 1998).

A 24 kDa protein from *R. sanguineus* (Rs24p) which was common to larvae, nymphs, whole body and salivary gland extracts of males and females was detected specifically in the serum of dogs after repeated infestation with adult *R. sanguineus.*

The duration of antibodies against Rs24p in dogs infested with adults was examined by western blotting analysis. Rs24p antibody was detected in two of four dogs during the period of 40 days in the first infestation. In the second infestation, all the dogs showed positive reaction against Rs24p, but the duration of the antibodies varied greatly among the animals (Inokuma *et al.*, 1999).

Mulenga *et al.* (1999) sequenced and characterized a *H. longicornis* tick salivary gland-associated cDNA coding for a 29-kDa extracellular matrix-like protein, with the structural conservation similar to all known collagen proteins expressed in both unfed and fed immature and mature *H. longicornis* ticks. Immunisation with the recombinant p29 conferred a significant protective immunity in rabbits, resulting in reduced engorgement weight in adult ticks and up to 40 and 56 per cent mortality in larvae and nymphs respectively that fed on the immunised rabbits.

Rath (1999) reported that whole ground and salivary gland antigens of *R. sanguineus* conferred antibody mediated partial immunity to the rabbits. Whole ground antigen was much better than salivary gland antigen in reducing the egg mass weight, increasing engorgement period of adult ticks and nymphs, pre oviposition and oviposition period and delayed attachment period.

Das *et al.* (2000) purified the extracts prepared from unfed larvae of *H. anatolicum anatolicum* by immunoaffinity chromatography using anti-gut IgG as ligand. Immunisation of cross-bred (*Bos taurus* x *B. indicus*) calves of 6-7 months of age with this affinity-purified antigen (Aff-GHLAg) resulted in the rejection of 70.6 per cent larvae, 54.5 per cent nymphs and 61.9 per cent adults. No significant change in the engorged weight of females was observed. However, significant decrease in the engorgement weight of larvae and nymphs was recorded.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of reactive fraction showed a 68 kDa protein in crude larval antigen of *B. microplus* and 62 and 29 kDa proteins in that of *H. anatolicum anatolicum* (Ghosh and Khan, 2000).

Patarroyo *et al.* (2002) reported that on immunisation of cattle with the three synthetic peptides (SBm4912, SBm7462 and SBm19733) derived from the Bm86 glycoprotein from *B. microplus* gut, SBm7462 showed the best efficacy (81.05 per cent), which also reduced the biological parameters significantly.

Bishop *et al.* (2002) cloned a cDNA encoding cement protein, 36 kDa protein, designated as *Rhipicephalus* Immuno-dominant Molecule 36 (*RIM36*)) of the tick *R. appendiculatus*, located in the secretory *H. anatolicum anatolicum e* cell granules of the type III salivary gland acini. This 36 kDa protein induced strong antibody responses in cattle experimentally infested with tick, detected by western blot of antisera by polyclonal antibodies raised against recombinant *RIM36.*

The most abundant protein present in *B. microplus* eggs, vitellin, was isolated and purified as a non-covalent complex of six glyco-polypeptides of Mr 44–107 kDa. An 80 kDa glycoprotein (GP80) independently purified from larvae of *B. microplus* induced immune responses that partially protected sheep from the tick. However, critical protective epitopes were associated with the folding of the protein and/or the oligosaccharides attached to it. (Tellam *et al.*, 2002)

2.3.1 Preparation of Midgut Antigen

Partially engorged *R. sanguineus* females fed on dogs were dissected and guts collected separately, suspended in phosphate buffered saline (PBS, pH 7.4) and stored at -40^0C. Guts were then centrifuged for 15 minutes at 5000 x g, supernatant discarded and suspended in PBS. This procedure was repeated until a clear supernatant was obtained.. Guts were then suspended in Tris-buffer (pH 6.8) 0.1M

and sonicated for 10 minutes with 1/10 s cycles (60W). Extracts were then centrifuged at 15000 x g for one hour at 4^0C. Supernatant was filtered through a 0.22 μm GV filter and stored at -40^0C until use (Szabo and Bechara, 1997).

Laboratory maintained prefed *H. bispinosa, H. marginatum isaaci* and *R. haemaphysaloides* ticks were dissected individually using a stereo microscope and the harvested midguts were placed in 0.1M PBS, pH 7.2. Midguts were homogenized @1500 rotations/minute for 10 minutes using a homogeniser and the homogenate was sonicated at 8 μ amplitude for 10 minutes with 4 cycles/minute, and each cycle lasting 2.5 minutes. The sonicated homogenate was centrifuged at 15,000 x g for 30 minutes at 4^0C. The supernatant, which was used as gut antigen was stored at -20^0C after adding 1mM Phenyl Methyl Sulphonyl Fluoride (Bhaskaran *et al.*, 2003).

2.3.2 Preparation of Whole Tick Extract Antigen

Semi engorged female ticks were surface sterilized and then disrupted in PBS pH 7.3, using sterile mortar and sterilized glass beads .The suspension was filtered through a buchner funnel and the filtrate was homogenized by ultrasonic disintegrator using a standard probe at 200W for 3 minutes on ice. The homogenate was then centrifuged at 30000 x g for one hour at 4^0C to get a supernatant and pellet. The pellet was dissolved with 1 % sodium deoxycholate in PBS, pH 7.3 for 36 hrs at 4^0C, ultrasonically disintegrated and then mixed with the supernatant (Banerjee and Manohar, 1992).

2.3.3 Preparation of Salivary Gland Antigen

Salivary gland antigen (SGA) was prepared from female *D. andersoni*, which had engorged on a rabbit for four days. Ticks were placed in 0.01M PBS pH 7.2 and dissected along their dorsal surface. Salivary glands were dissected free of other tissues and placed into 0.01 M PBS, pH 7.2, at 4^0C. The separated salivary glands

were homogenized and the resultant preparation was centrifuged at 10000 x g for 30 minutes. The supernatant was passed through a 0.22 μm filter and held at 4^0C until used as SGA (Wikel, 1981).

According to Nyindo *et al.* (1989) salivary glands were harvested in 2ml Dulbecco's phosphate buffered saline (PBS) without protease inhibitors. The glands were sonicated at an amplitude of 22 μm for 2 minutes at 4^0C. The sonication process was repeated four times at 3 minutes intervals. Soluble fraction of the extract was obtained after centrifugation at 1,000 x g for 15 minutes.

The salivary glands of *Hyalomma anatolicum anatolicum* were removed intact and were placed in 0.1M at PBS 4^0C and stored at -20^0C for further use. Stored tick salivary glands were thawed and homogenized in sterile mortar and pestle over ice. The homogenate was suspended in 15 mM sodium deoxycholate. The material was further homogenized by an ultrasonicator at 55,000 cycles per second with simultaneous cooling on ice. The homogenate was then centrifuged at 10,000 x g at 5^0C for 30 minutes and the supernatant collected and this constituted the whole salivary gland antigen (SG Ag-I). Stored salivary glands were thawed and homogenized in sterile mortar and pestle over ice. The homogenate was then centrifuged at 10,000 x g at 5^0C for 30 minutes, the supernatant collected as antigen (SG Ag-II). The sediment of antigen-II was suspended in 15mM sodium deoxycholate and the resultant preparation was kept as antigen (SG Ag-III). (Banerjee *et al.*, 1990)

2.4 IMMUNISATION TRIAL USING MIDGUT ANTIGEN

Cattle were immunised with either 3 X 500 μg soluble gut antigen (GS) of *B. microplus* along with Freund's complete adjuvant (FCA) or 350, 100 and 50 μg membrane associated gut antigen along with saponin or both together at the same rate administered intramuscularly at 0, 14, 42 days. The membrane vaccine protected

cattle (91 per cent) against larval challenge but vaccine made from soluble antigen did not protect (Opedebeeck *et al.*, 1988).

Thakur *et al.* (1992) immunised rabbits with 2 ml of saponin–antigen mixture containing 15 mg of protein derived from the midgut of *H. anatolicum anatolicum* and another group with *B. microplus* midgut antigen. Immunisations were carried out four times. Immunised rabbits were protected against homologous as well as heterologous challenges.

Kimaro and Opedebeeck (1994) showed that cattle were protected (88 per cent) significantly against *B. microplus* when vaccinated intramuscularly with 1 mg of soluble fraction of gut membrane (LI-GM) divided into 5 doses (2.5 ml per dose) along with Quil A.

Cattle vaccinated with recombinant vaccine (GAVAC, Heber Biotech S.A) employing 2 ml containing 100 µg of the rBm86 antigen with oil adjuvant in the neck intramuscularly at 0, 4 and 7 weeks effectively controlled *B. microplus* population (Rodriguez *et al.*, 1995a).

Rabbits inoculated subcutaneously with supernatant antigen and gut pellet antigen from *H. dromedarii* partially protected them against *H. dromedarii* female ticks. First two injections were given after emulsification with FCA while the third without FCA (Kumar and Kumar, 1996a).

Szabo and Bechara (1997) reported that guinea pigs acquired resistance to *R. sanguineus* ticks when immunised with gut extract and saponin as adjuvant. Guinea pigs were inoculated three times at the rate of 10, 5 and 5 µg of extract per animal 15 days apart. Dogs were more efficiently immunised against *R. sanguineus* ticks when inoculated with tick gut extract and FCA.

Akhtar *et al.* (1999) inoculated sheep with *B. microplus* midgut cell culture vaccine (2.5 ml / animal) intramuscularly followed by a booster dose three weeks later. The antibody titres against gut membrane antigen (GM) were significantly higher than those of gut soluble antigen (GS).

Holstein-Friesian calves were immunised with supernatant fraction extracted from midgut of *H. anatolicum anatolicum* through subcutaneous route in three divided doses on day 0, 14 and 28. Calves received antigen in FCA as first inoculation. The second and third inoculations of antigen were made with equal amount of Freund's incomplete adjuvant (FIA). Supernatant provided high protection and affected feeding and fertility of adult ticks (Razmi *et al.*, 2003).

2.4.1 Adjuvants

Jackson and Opdebeeck (1995) reported that Quil A was clearly superior to FIA and aluminium hydroxide ($Al(OH)_3$) upon immunisation of sheep and cattle with soluble and membrane midgut antigens of *B. microplus.*

Szabo and Bechara (1997) used 15 µg of saponin as adjuvant for immunisation of guinea pigs with salivary gland and gut extracts of *R. sanguineus.* They also used FCA for immunising dogs with gut extracts initially and the remaining two inoculations with FIA. Dogs were most efficiently immunised against *R. sanguineus* with gut extract and Freund's adjuvant instead of saponin.

2.4.2 Assessment of Immune Response

2.4.2.1 *Humoral Immune Response*

Galun (1978) proposed that antibodies directed against some target antigen in the tick changed the function of the target resulting in either death of tick or no development. This formed the rationale for immunizing cattle against ticks.

Jackson and Opdebeeck (1990) vaccinated cattle with mid gut membrane (GM) antigen derived from *B. microplus*, with Quil A. It resulted in a significant increase in total immunoglobulins mainly in the IgG1 and IgG2 fractions of the serum. Levels of IgG, IgG1 and complement fixing antibodies were significantly correlated to the protection against cattle ticks.

2.4.2.1.1 Agar Gel Immunodiffusion (AGID)

Immunodiffusion studies revealed single precipitation bands between the immunizing antigen and sera collected on day 16, 25 and 28 from calves immunised with midgut antigen derived from *D. andersoni.* Strong multiple bands as well as single bands were obtained from sera collected from calves on day 38 (Allen and Humphreys, 1979).

Following vaccination of cattle against *B. microplus* using extracts derived from adult female ticks, serum antibodies to soluble extracts of adult ticks were detected by gel diffusion and radioimmunoassay but antibody levels in individual cattle were not correlated with their immunity to ticks (Johnston *et al.*, 1986)

Njau *et al.* (1986) used agar gel double diffusion technique to detect circulating precipitating antibodies against *R. evertsi evertsi* salivary gland antigen. Gel diffusion demonstrated precipitin antibodies in most rabbits infested with 20 and 100 ticks by day 21 and 14 respectively, after the first infestation.

Heller-Haupt *et al.* (1987) detected antibodies from serum of guinea pigs immunised with unfed larval homogenates of *A. variegatum* in double immuno diffusion in agarose by the method of Ouchterlony (1958). Strong precipitation lines developed between the serum of a guinea pig immunised with *A. variegatum* unfed larval homogenates and unfed larval and nymphal homogenates of both *A. variegatum* and *A. hebraeum.*

Sera of both immunised and control calves collected on days 0, 7 and 14 showed no precipitation bands either with adult or larval antigens of *B. microplus*. Single precipitation band was observed between the immunising antigens and the sera of immunised calves collected on day 21 and 28 and double bands were seen on day 35 post immunisation (Panda *et al.*, 1992).

Ghosh and Khan (1995) assessed humoral immune response in calves immunised by tick tissue extracts of *B. microplus* by double diffusion. Sera collected from immunised calves on 0, 7 and 14 days post immunisation (DPI) showed no precipitating bands. Single band was, however detected in sera collected from 42 to 80 DPI, which was faint on 95 DPI and no bands could be observed on 110 DPI.

Kumar and Kumar (1996b) reported that immune sera from experimental New Zealand white rabbits gave positive results with partially fed *H. dromedarii* tick derived midgut antigen with double diffusion test. Double diffusion test revealed precipitin bands in response to both supernatant antigens i.e with FCA as well as without FCA on day 14 and 21 post immunisation respectively. The band continued to be prominent up to 84 days post immunisation in response to supernatant antigen with FCA and only up to 49 days post immunisation in response to supernatant antigen without FCA and then bands started fading and were visible only up to day 63 post immunisation.

2.4.2.1.2 Passive Haemagglutination (PHA)

McGowan *et al.* (1980) reported that Passive haemagglutination (PHA) antibody titres of rabbits immunised with extracts derived from homogenised ticks, developed within 7 days and increased to a mean titre of 12(log2) within 28 days.

Njau *et al.* (1986) reported that antibody against salivary antigen could be detected during the first week of initial tick challenge. He also opined that PHA test

was more sensitive than immunodiffusion for serological diagnosis of ectoparasitic infestation.

Akhthar *et al.* (1999) used Indirect Haemagglutinating Antibody (IHA) for determining the anti tick antibody titres in sheep immunised with midgut *B. microplus* cell culture vaccine. The antibody response against GM antigen (1:16 to 1:128) was significantly higher ($P < 0.01$) than that against GS antigen (1:2 to 1:32)

2.4.2.2 *Cell Mediated Immune Response*

2.4.2.2.1 Intradermal Test

Binta and Cunningham (1984) reported that a component of an extract from *R. appendiculatus* larval ticks induced an immediate hypersensitivity reaction in sensitized cattle when inoculated intra-dermally. The skin reaction was absent in steers not previously fed by ticks

Panda *et al.* (1992) reported that intradermal injection of tick-tissue antigens resulted in the development of antigen specific immediate and delayed hypersensitivity skin reactions in calves immunised with *B. microplus* adult and larval extracts. Immediate type of skin reaction attained maximum intensity at 30 minutes with infiltration of large numbers of polymorphonuclear cells and eosinophils. Delayed type of skin reaction was more pronounced at 24 hours with infiltration of large number of lymphocytes and macrophages.

Kumar and Kumar (1996b) reported that skin test of rabbits immunised with *H. dromedarii* tick derived mid gut antigen revealed immediate and delayed type of hypersensitivity reaction in terms of increment in skin fold thickness in response to midgut antigen.

Tripathi *et al.* (1998) reported that rabbits immunised with larval extract (RLAg) and nymphal extract (RNAg) of *R. sanguineus* were positive for immediate hyper sensitivity (ITH) reaction on intradermal inoculation of RLAg and RNAg. All immunised rabbits showed oedematous swelling, characteristic of ITH reaction within 12 minutes of inoculation of RLAg and RNAg. Histological examination of skin biopsies taken from inoculation sites revealed that inflammatory cells were mainly basophils and eosinophils.

2.5 HISTOPATHOLOGY

Theis and Budwiser (1974) reported that a dog not exposed to arthropod of any kind before, reacted histologically to *R. sanguineus* tick exposure with infiltration of polymorphonuclear leukocytes as seen in dogs repeatedly infested.

Histological analysis of larval *R. appendiculatus* feeding sites in naive and actively sensitised guinea pigs at 6, 24, 48, 72 and 96 hour post-tick attachment revealed that the cavity at the entrance of the ticks mouth parts into the uppermost dermis, and the surrounding cellular infiltrate (lesion) increased. Early (6 hours) lesions were dominated by eosinophils, again predominated at 72 hours (44 per cent), and finally basophils were dominant at 96 hours (78 per cent). At the site of secondary feeding in animals expressing acquired resistance, dermal cavities at the site of entrance of the tick mouth parts were occasional in occurrence and were reduced in size indicating altered tick feeding. Basophils were dominant at all observation times ranging from 61 to 91 per cent of the infiltrate followed by eosinophils, ranging in abundance from 7 to 21 per cent. Recipients of immune serum had a smaller cellular filtrate around feeding ticks, but basophils were also dominant. Basophils appeared to be the principal host cell involved in acquired resistance to tick feeding as indicated by the profound cutaneous basophil reaction that characterized the immune response to larval ticks both in actively and passively sensitized hosts. The finding of significant eosinophil

accumulations at tick feeding sites of both hosts indicated that these cells may also contribute to acquired resistance (Brown *et al.*, 1983).

2.5.1 Histopathology of Tick Fed on Experimentally Immunised Animals

Histological examination of the tick *B. microplus* fed on cattle vaccinated with tick extracts showed that the gut was the primary site of damage. Within 24 to 48 hours of attachment, digestive cells were either sloughed off into the lumen or were completely destroyed leaving only the basal lamina and muscle layer (Agbede and Kemp, 1986).

Thakur *et al.* (1992) reported rupture of gut wall and escape of gut contents into haemocoel of ticks fed on rabbits immunised with *H. anatolicum anatolicum.*

2.6 EFFECT OF IMMUNISATION ON BIOLOGICAL PARAMETERS.

Allen and Humphreys (1979) reported that ticks feeding on guinea pigs immunised with antigen I derived from midgut and reproductive organs produced relatively few eggs from which no larvae hatched and those placed on guinea pigs immunised with antigen II derived from all internal organs of tick neither engorged nor laid.

Immunisation of guinea pigs with *D. andersoni* salivary gland antigen resulted in lower resistance expressed by significantly fewer larvae engorging and reduction in the weight of engorged larvae (Wikel, 1981).

Heller-Haupt *et al.*(1987) observed that there was significant reduction in mean engorged weight, recovery rate and moulting of larvae released on guinea pigs immunised with unfed larval homogenate of *A. variegatum.*

There was a reduction in the number of dropped ticks and mean egg weight when ticks fed on Hereford cattle immunised with membrane and soluble antigen component extracted from the midgut of *B. microplus* (Opedebeeck *et al.*, 1988).

Thakur *et al.* (1992) reported that 82 and 62.3 per cent protection of rabbits was observed on the basis of eggs laid by *H. anatolicum* and *B. microplus* respectively along with 0.1 per cent saponin as adjuvant.

Kimaro and Opedebeeck (1994) reported that there was 77 percent decrease in the number of larval ticks that dropped when fed on cattle immunised with extracts prepared from gut of *B. microplus.*

Average weight of ticks dropping after engorgement from cattle vaccinated with particulated Bm 86 antigen (rBm86) purified from *Pichia pastoris* was significantly reduced (50 per cent). The difference in mean weight, percentage damage and total egg production between vaccinated and control groups were statistically significant ($P<0.01$). Seventy percent reduction of reproductive ability of ticks was also observed by Rodriguez *et al.* (1994).

Cattle vaccinated with recombinant Bm 86 (rBm86) preparation GAVAC, Heber Biotech, showed reduction in the weight of ticks and egg laying capacity compared to control. This difference decreased with time. In the vaccinated group ticks were found with a change in grey colour to red colour as a signal of gut damage (Rodriguez *et al.*, 1995b).

Kumar and Kumar (1996a) reported significant reduction in feeding and reproductive index of ticks fed on rabbits immunised with gut supernatant antigen of *H. dromedarii.*

Szabo and Bechara (1997) considered the parameters like engorged female weight (FW), engorging period (EP), pre oviposition period (POP), incubation period (IP), larval hatchability rate (LH) and efficiency rate of female in converting their food reservoir to eggs (ERCE) related to female tick feeding and reproductive performance as indicators of immunisation during each feeding. Among the reproductive and feeding parameters, only weight of engorged female ticks released on guinea pigs immunised with gut extract was significantly lower. Egg mass weight from female ticks of the same experimental group also differed, but it was not significant. Incubation, pre oviposition and engorging period as well as larval hatchability and efficiency rates of female ticks in converting their food reservoir to eggs, did not vary significantly, although ticks from both immunised groups took one day longer to engorge on an average.

De La Fuente *et al.* (1998) found a broad correlation between titre of antibodies to the Bm 86 antigen and the efficacy of vaccination, measured through the direct effect on the fertility of the ticks engorged on vaccinated grazing cattle.

Bm86 containing vaccine affected not only the number of tick completing the life cycle but also the reproductive capacity of the survived ticks. Overall efficacy of vaccine was higher than 99.9 per cent (Fragoso *et al.*, 1998).

Ghosh *et al.* (1998) observed significant reduction in engorged percentage and weight of engorged females and egg masses when fed on rabbits immunised with homogenate from unfed immature *H. anatolicum anatolicum*.

Tripathi *et al.* (1998) reported higher mean percentage rejection of larvae, nymphs and adults and reduction in engorgement period of female ticks, pre oviposition period and egg mass when ticks were fed on rabbits immunised with nymphal extracts (RNAg) and larval extracts (RLAg) of *R. sanguineus*.

Crossbred calves immunised with affinity purified mid gut antigen (Aff-GHLAg) of *H. anatolicum anatolicum* rejected 70.6 per cent larvae, 54.5 per cent nymphs and 61.9 per cent adults. There was no significant change in engorged weight of females but significant decrease in the engorgement weight of larvae and nymph was noticed (Das *et al.*, 2000).

As a result of vaccination with Bm86 and Bm95, ticks engorging on immunised cattle were visibly damaged and had significantly lower weight and reproductive capacity (Garcia-Garcia *et al.*, 2000).

Vaccination of Holstein cattle with Bm86 vaccine TickGARD^{PLUS} resulted in a 56 per cent reduction in tick numbers in field over one generation and 72 per cent reduction in the reproductive efficiency of ticks reared in laboratories (Jonsson *et al.*, 2000).

Nelore calves previously sensitized with *B. microplus* trypsin inhibitors (BmTIs) present in larvae when challenged with tick larvae (2000 larvae/animal) showed 72.8 per cent efficacy and interfered in tick development with 69.7 per cent and 71.3 per cent reduction of both tick numbers and egg weight respectively (Andreotti *et al.*, 2002).

Materials and Methods

3. MATERIALS AND METHODS

3.1 PREVALENCE OF TICK INFESTATION

One thousand two hundred dogs in Thrissur Corporation area were examined for the presence of tick infestation during a period of one year from July 2004 to June 2005 with a monthly average of 100 dogs. Ticks were collected by physical examination of dogs brought to University Veterinary Hospitals, Mannuthy and Kokkalai, from households in the corporation area and from neighbourhood dogs in the Veterinary College campus, Mannuthy. A proforma was designed to record the date of collection, place of collection, breed, age and sex of dogs, stage of tick and their site of attachment and presence of disease if any. Ticks were removed mechanically by a pair of curved mosquito forceps as described by Theis (1968).

3.1.2 Identification of Tick Species

Ticks collected from each dog were placed in separate tubes and brought live or in 70 per cent alcohol to the Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Mannuthy. Identifications were carried out under stereomicroscope using standard keys of Sen and Fletcher (1962) and Walker *et al.* (2000).

3.2 EXPERIMENTAL ANIMALS

3.2.1 Rabbits

Tick colony was maintained on six month old female New Zealand White rabbits purchased from the Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy and Rabbit breeding farm, Centre for Advanced Studies in Animal Genetics and Breeding, Mannuthy. Rabbits were placed in

separate cages in the small animal experimental shed of the Department of Veterinary Parasitology. All animals used were tick bite naive.

3.2.2 Guinea Pigs

Twelve, four month-old female guinea pigs were purchased locally. All guinea pigs were tick bite naive. Immunisations with gut antigen were conducted on six guinea pigs and six animals were used as control.

3.3 COLONISATION OF TICKS IN THE LABORATORY

The most common dog tick found out by prevalence study was colonized in the Department of Veterinary Parasitology following the method of Puttalakshmamma *et al.* (1994) with minor modifications. External ears of rabbits were used as feeding site. Pathogen free engorged female of *R. sanguineus* were collected from the infested dogs and kept individually in specimen tubes of 25 mm x 50 mm (Tarsons Products Pvt. Ltd.) covered with sterile cotton cloth (Gada cloth) and fastened with rubber band. The specimen tubes were kept half immersed in moist sand to facilitate egg laying and hatching. The mouth of the specimen tubes were covered with cloth to provide ventilation. As soon as the eggs started hatching, sterile cotton cloth was replaced by polyester cloth fastened with rubber band and cello tape, which prevented the hungry larvae from escaping. For all the stages except larvae, cotton cloth was used for covering the tube. Emerged larvae were starved for 10 days to harden the mouthparts and legs. These larvae were released on the ears of rabbits restrained in a box by introducing the hair clipped ear into the specimen tube containing the larvae. Then each ear was separately covered with ear bag and tied at the base of the ear using the string attached to it. Ear bags were further secured by adhesive tapes at the base of the ear. Then the rabbits were put back into the cage. Ear bags were daily untied for routine check up and the dropped engorged larvae were separated. They were cleaned and transferred to specimen tubes, then covered

with cotton and kept in moist sand bed for moulting. Likewise various life cycle stages were fed on the external ear of tick bite naive New Zealand White rabbits using thick cotton ear bags. The dropped out engorged stages in the ear bags were collected and allowed to moult in the specimen tubes kept in the moist sand bed. After moulting the next instar was stored in the specimen tube kept in glass desiccators containing 15 per cent potassium hydroxide (KOH) immersed in moist sand in a thermocole box so as to maintain a temperature of 25 ± 2°C and 85 per cent relative humidity. This thermocole box was kept in an enamel tray filled with water so as to prevent the escape of ticks.

3.4 MIDGUT ANTIGEN

3.4.1 Preparation of Midgut Antigen

Gut antigen was prepared as previously described by Szabo and Bechara (1997) with minor modifications.

3.4.1.1 *Reagents*:

Phosphate buffered saline (PBS) 0.15 M, pH 7.4

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate	1.132 g
Potassium dihydrogen phosphate	0.20 g

Dissolved in one litre of distilled water and pH was adjusted to 7.4. Sterilized by autoclaving at 121°C for 15 minutes at 15 lb pressure.

3.4.1.2 *Procedure*

Partially engorged *R. sanguineus* female ticks fed on rabbits for four to five days were collected and surface sterilized by cotton soaked with methanol. These ticks were dissected under binocular dissection microscope. The mid guts were separated and collected in chilled PBS, pH 7.4 after washing in the same. Each serum collection vial was loaded with guts of 15 ticks suspended in 0.5 ml chilled PBS, pH 7.4 and stored at -80^{0}C. Tick guts were then centrifuged for 15 minutes at 5000 x g in cooling centrifuge. Supernatant was discarded and gut was resuspended in PBS. This procedure was repeated till a clear supernatant was obtained. Guts were then suspended in PBS and homogenized in a sterile chilled mortar and pestle. Homogenized gut suspension was sonicated (Branson Sonifier 450) for 10 minutes with 1/10 s cycles over ice. Extracts were then centrifuged at 15000 x g for one hour in a cooling centrifuge at 4^{0}C. Supernatant was filtered through a 0.22 μm syringe filter (Acrodisc® Syringe Filters) and stored at -80^{0}C as 0.5 ml aliquots with phenyl methane sulphonyl fluoride (PMSF; 1 mM) as protease inhibitor.

3.4.2 Estimation of Protein Concentration

Protein concentration of the antigen was estimated as per the method of Lowry *et al.* (1951) using protein estimation kit (Genei Pvt.Ltd, Bangalore).

3.5 IMMUNISATION

Immunisations were carried out using 12 female guinea pigs aged four months. They were allocated randomly to two groups. Group I (n = 6) was inoculated with gut antigen and adjuvant and group II was inoculated with equal volume of PBS and adjuvant. Guinea pigs were inoculated three times with 50 μg, 25 μg and 25 μg of gut antigen per animal on day zero, 14th and 28th day respectively (Table 1)

Zero day

Equal quantities of antigen and Freund's complete adjuvant (FCA) were mixed to make final volume of 0.5 ml suspension, which was inoculated by subcutaneous route on the flank.

14^{th} and 28^{th} day

Equal quantities of antigen and Freund's incomplete adjuvant (FIA) were mixed to make a final volume of 0.5 ml suspension which was inoculated subcutaneously on the left side of the neck on 14^{th} day and on right side of the neck on 28^{th} day.

Table 1. Immunisation protocol for guinea pigs

Group	0^{th} day	14^{th} day	28^{th} day	Challenge infestation
I (n=6)	0.25 ml(50 µg) antigen+0.25 ml FCA (S/C)	0.25 ml(25 µg) antigen+0.25 ml FIA (S/C)	0.25 ml(25 µg) antigen+0.25 ml FIA (S/C)	35 days post immunisation
II(n=6)	0.25 ml PBS +0.25 ml FCA (S/C)	0.25 ml PBS +0.25 ml FIA (S/C)	0.25 ml PBS +0.25 ml FIA (S/C)	

3.6 BLEEDING SCHEDULE

Blood was collected from the experimental animals by cardiac puncture on day 0, 14, 21 and 35 after first immunisation. Serum was separated each time for detection of antigen antibody reaction.

3.7 RAISING OF HYPERIMMUNE SERUM IN RABBITS

One male New Zealand White rabbit aged six months was used for raising hyper immune serum. Rabbit was inoculated intramuscularly with 500 µg of gut antigen along with FCA. Two booster dose of 250 µg each along with FIA were injected intramuscularly on 14^{th} and 21^{st} day post initial inoculation. Blood was collected day 0, 7, 14, 21 and 35 post initial inoculations. Serum was separated each time for detection of antigen antibody reaction.

3.8 ASSESSMENT OF IMMUNE STATUS

Evaluation of immunisation was done by observing humoral and cell mediated immune responses.

3. 8. 1 Humoral Immune Response.

3.8.1.1 *Agar Gel Immuno Diffusion (AGID)*

Agar gel Immunodiffusion was done as per the method of Ouchterlony (1958).

Materials

Gels for AGID

Agarose	1.0 g
Sodium azide	0.01 g
PBS	100 ml

To dissolve the agarose in PBS, solution was boiled for one minute in microwave oven.

Agar coated slides

Clean glass slides were coated by smearing 0.5 per cent melted agarose in distilled water and dried by keeping the slides horizontally over glass rods.

Method

Three milliliters of melted agarose was poured onto precoated glass slides and allowed to set. One central well and five peripheral wells, each with three millimeter diameter were punched out in such a way that the distance between the central well and any peripheral well was three millimeters. Distance between the adjacent peripheral wells was kept equal. The central well was filled with 20 µl of gut antigen. The peripheral wells were filled with 20 µl of serum samples. The slides were incubated at room temperature in a humid chamber for 24 hours and were examined for the presence of precipitin lines.

3.7.1.2 *Passive haemagglutination (PHA)*

The test was carried out according to the method described by Kagan and Norman (1976) and Sawada *et al.* (1982) with minor modifications.

3.8.1.2.1 Gluteraldehyde fixed sheep RBC (GA-SRBC)

Reagents

Alsever's solution.

Glucose	2.05 g
Sodium citrate	0.3 g
Citric acid	0.055 g
Sodium chloride	0.42 g
Distilled water	100 ml

Phosphate buffered Saline (PBS) 0.15 M, pH 7.2

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate	1.132 g
Potassium dihydrogen phosphate	0.20 g

Sheep blood collected in Alsever's solution was washed six times by centrifugation (650 x g) for 20 minutes in 0.85 per cent sodium chloride solution. After the last wash, the packed cells were suspended in PBS (pH 7.2) to yield a 10 per cent suspension (V/V) and chilled to 4^0C in an ice bath. A 25 per cent solution of gluteraldehyde was diluted to one per cent (V/V) with PBS and chilled to 4^0C. The washed sheep RBC (10 percent) was mixed with an equal volume of one per cent solution of gluteraldehyde and the mixture was incubated at 4^0C for 30 minutes with gentle stirring. The mixture was then centrifuged at 650 x g for 10 minutes at 25^0C. The pelleted fixed cells were washed three times with PBS containing 0.1 per cent sodium azide.

3.8.1.2.2 GA-SRBC Sensitized with Sonicated Gut Antigen

Suspension of GA-SRBC (10 per cent) was mixed with an equal volume of diluted gut antigen with a protein concentration of 1 mg/ml. The mixture was incubated at 37^0C for one hour with occasional shaking. The sensitized cells were washed three times in PBS containing 0.25 per cent bovine serum albumin (BSA-PBS) and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (V/V).

3.8.1.2.3 Determination of Optimal Concentration of Antigen

Prepared four dilutions of antigen in PBS, pH 7.4 so as to contain a protein concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml. Sensitized the cells with each dilution of antigen as described above in preparation of gluteraldehyde fixed

sensitized red cells. Checked one negative and positive serum with each concentration of antigen giving the highest titre with the immune serum. No reaction with the negative serum is considered optimal.

3.8.1.2.4 Test Procedure

Passive haemagglutination was carried out in U bottom microtitre plates. Serial two fold dilutions of antiserum in BSA-PBS were taken in 25 µl quantity in 20 wells except for serum on 0^{th} day of immunisation, in which two fold dilution of serum were taken up to 10^{th} well. Twenty five microlitre of the sensitized SRBC was added to each well and the plates were shaken and allowed to stand for 24 hours at 25^0C before SRBC setting patterns were read. The PHA titre was expressed as the reciprocal of the highest dilution of serum showing a definite pattern (flat sediment) compared to the pattern in negative well (Smooth dot in the centre of the well).

Diluent control

Transferred 25 µl of BSA-PBS to four wells in column 11 and added 25 µl of 0.5 per cent suspension of sensitized cells.

Antigen control

Prepared 25 µl of 0.5 percent suspension of unsensitized cells in PBS containing 0.25 per cent bovine serum albumin (BSA-PBS) and 0.1 per cent sodium azide. Prepared serial dilution of sera to be tested in four wells in column 12. To each well, added 25 µl of unsensitized gluteraldehyde fixed cells to obtain a negative reaction.

3.8.2 Cell Mediated Immune Response.

3.8.2.1 *Intradermal Test.*

Cellular immune responses of guinea pigs were determined by performing the skin test as per the method of Losson *et al.* (1988) and Kumar and Kumar (1996b) with minor modification. Test was performed 2 weeks after the last injection of immunisation schedule on all animals both in experimental and control groups. The hairs on the right flank of guinea pig were shaved and skin marked out into two circles of 2 cm diameter separated by 2 cm. Inoculated 0.1 ml of antigen (10 μg) intradermally into the centre of one marked out circle. The other circle on the same animal received 0.1 ml of PBS as control. Skin fold thickness was measured in mm by Vernier caliper at 0, 0.5, 1, 6, 12, 24, 48, 72 and 96 hours post inoculation. The cellular immune response was observed in terms of increased skin fold thickness at the inoculation site of antigen while comparing with that of control animals. Data on skin fold thickness were analysed statistically.

3.8.3 Challenge Infestation.

Guinea pigs in immunised and control group were challenged with five pairs of adult unfed *R. sanguineus* ticks on 35 days post immunisation (DPI). Each rodent was infested with five female and five male ticks. Ticks were placed inside a feeding chamber with porose area on top consisting of a plastic tube (3 cm diameter and 1.2 cm height) glued with a non hazardous preparation (Fevi Kwik, Pidilite industries, Mumbai) on to the shaved back of guinea pigs and further secured with adhesive tape (Paragon adhesive tape, Smith & Nephew Healthcare Pvt. Ltd). Indigenously designed Elizabethan collars were used to prevent natural grooming. Daily observation was made on the biological parameters of the female tick.

3.8.3.1 *Biological Parameters*

The following biological parameters, related to female tick feeding and reproductive performance were observed during each infestation.

3.8.3.1.1 Feeding Parameters

3.8.3.1.1.1 *Feeding or Engorging Period (EP) in days.*

The engorging period was assumed to be the time that elapsed from time of release of ticks on host until their detachment, partially or fully engorged

3.8.3.1.1.2 *Weight of Engorged Female Ticks (FW) in mg.*

Engorged female tick weights were measured immediately after tick detachment.

3.8.3.1.1.3 *Feeding Efficiency Index*

$$\text{Feeding efficiency index} = \frac{\text{Weight of engorged female in mg}}{\text{Feeding period in days}}$$

3.8.3.1.2 Fertility Parameters

3.8.3.1.2.1 *Pre Oviposition Periods (POP) in days*

POP = Date of start of oviposition – Date of detachment engorged tick

3.8.3.1.2.2 *Ovi Position Periods (OP) in days.*

OP = Date of end of oviposition - Date of start of oviposition

3.8.3.1.2.3 *Egg Mass Weight (EW) in mg*

3.8.3.1.2.4 *Incubation Periods (IP) in days.*

IP = Date of emergence of larva – Date of start of oviposition

3.8.3.1.2.5 *Efficiency Rate of Female Ticks in Converting Their Food Reservoir to Eggs*

Efficiency rate of conversion to eggs (ERCE) was calculated as follows :

$$ERCE\ (\%) = \frac{EW \times 100}{FW}$$

3.8.3.1.2.6 *Larval Mass in mg*

3. 9 EFFICACY OF IMMUNISATION

Detached adult female ticks were collected from immunised and control animals to determine the efficacy of immunisation by employing the formulae put forth by De La Fuente *et al.* (1995)

DT (%) =100 (1 - NTV/NTC)

DT (%) = Percentage reduction of adult females

NTV = Number of adult females in the immunised group.

NTC = Number of adult females in the control group.

DO (%) = 100(1-PATV/PATC)

DO (%) = Percentage reduction of mean weight of eggs

PATV = The average weight of eggs per survived tick in the immunised group.

PATC = The average weight of eggs per survived tick in the control group.

DR (%) = 100(1-PMTV/PMTC)

DR (%) = The percentage reduction of mean weight of adult females

PMTV = The mean weight of adult females in the immunised group.

PMTC = The mean weight of adult females in the control group.

DF (%) = 100 (1-PPLOV/PPLOC)

DF (%) = Percentage reduction of fertility

PPLOV = The mean weight of larvae per gram of eggs in the immunised group

PPLOC = The mean weight of larvae per gram of eggs in the control group

E (%) = 100{1-(CRT x CRO x CRF)}

E (%) = Efficacy of immunogens

CRT = NTV/NTC: reduction in the number of adult female ticks as compared to the control group

CRO = PATV/PATC: reduction in egg laying capacity of the survived ticks as compared to the control group.

CRF = PPLOV/PPLOC: reduction in fertility as compared to the control group.

3.10 STATISTICAL ANALYSIS

Data obtained in the present study were analysed using statistical tests like Chi square test, Multiple regression analysis, t-test and ANACOVA (Snedecor and Cochran, 1989).

Results

4. RESULT

4.1 PREVALENCE OF TICK INFESTATION

One thousand two hundred dogs in Thrissur Corporation area were examined for the presence of tick infestation during a period of one year from July 2004 to June 2005. Out of these, 124 tick infested cases were recorded giving a prevalence rate of 10.33 per cent. Six dogs were infested with larvae, 20 with nymph and 101 with adult ticks. (Table 2 and 3)

Species of ticks identified from positive cases were *Rhipicephalus sanguineus, R. haemaphysaloides, Haemaphysalis bispinosa and H. bispinosa* var *intermedia.*

Out of 124 positive cases of tick infestation *R. sanguineus* presented maximum incidence rate (8.58 per cent) while *H. bispinosa* var *intermedia* showed minimum incidence rate (0.08 per cent). The incidence of *R. haemaphysaloides* and *H. bispinosa* were 0.33 per cent and 1.33 per cent respectively (Table 2, Fig. 1). Chi-square test revealed significant differences ($P<0.01$) between species wise prevalence.

The monthwise prevalence rate of tick infestation on dogs varied between a maximum of 15 per cent in October and November 2004 and a minimum of 5 per cent in June 2005. The prevalence rate of tick infestation during other months were 10 per cent (July), 10 per cent (August), 10 per cent (September), 9 per cent (December), 7 per cent (January), 11 per cent (February), 10 per cent (March), 10 per cent (April) and 12 per cent (May) (Table 4). Influence of climatic variables like maximum and minimum temperature, mean relative humidity and rainfall on tick infestation during different months analysed by multiple regression revealed no significant difference ($P>0.05$) (Fig. 2).

Effect of seasonal and climatic variation on the prevalence of tick infestation was also studied during the same period. Prevalence of tick infestation was maximum (11.5 per cent) during post monsoon season (October to January) and minimum (8.75 per cent) during monsoon (June to September) but there was no significant difference between the prevalence rates during these three seasons. Pre monsoon season represented a prevalence of 10.75 per cent (Table 5). No clear pattern of seasonality was observed for *R. sanguineus,* which was present throughout the year with a maximum infestation of 13 per cent in October and a minimum of 3 per cent in June.

Prevalence of tick infestation in male dogs was 11.6 per cent and that in female dogs was 9.05 per cent. Chi square test revealed no significant ($P>0.05$) gender wise variation in tick infestation (Table 6).

Maximum incidence rate of tick infestation was seen in dogs aged between two and four years of age (12.12 per cent) while minimum was in dogs above four years of age (7.87 per cent). Prevalence of tick infestation in dogs below two years of age was 9.63 per cent and variation in tick infestation between different age groups was not statistically significant (Table 7).

There was a highly significant ($P<0.01$) association between breeds and intensity of infestation with maximum intensity of infestation in German Shepherd (GSD) and minimum in Cocker spaniel and Great Dane. Twenty seven GSD dogs showed an infestation below 10 numbers of ticks while 19 GSD showed an infestation above 10 numbers of ticks. Intensity of infestations in positive dogs is shown in table 8.

Predilection sites of ticks on the body were ear, neck, interdigital space, dorsum of the body, whole body, eyelids, perianal region, withers, thorax and hind limbs. Percentage of attachment of ticks in positive cases was maximum on the ear

(84.68 per cent), followed by neck (26.61 per cent), interdigital space (17.74 per cent), dorsum of the body (12.1 per cent), whole body (7.26 per cent), eye lids (3.23 per cent), perianal region (0.81 per cent), withers (0.81 per cent), thorax (0.81 per cent) and finally hind limbs (0.81 per cent) (Table 9).

Table 2. Prevalence of dog infestation with ticks in Thrissur Corporation area from July 2004 to June 2005

Number of dogs examined	Species of ticks	Number of dogs infested with ticks	Prevalence of infestation (%)	Overall prevalence (%)
1200	*Rhipicephalus sanguineus*	103	8.58[a]	10.33
	R. haemaphysaloides	4	0.33[b]	
	Haemaphysalis bispinosa	16	1.33[c]	
	H. bispinosa var *intermedia*	1	0.08[d]	

Figures having different superscript differ significantly (P<0.01)

Table 3. Number of dogs infested with different instars of ticks

Species of ticks	Number of dogs infested with		
	Larvae	Nymphs	Adults
Rhipicephalus sanguineus	6	18	82
R. haemaphysaloides			4
Haemaphysalis bispinosa		2	14
H. bispinosa var *intermedia*			1
Total	6	20	101

Table 4. Monthwise prevalence of tick infestation in dogs in Thrissur Corporation from July 2004 to June 2005

Month	No.of dogs examined	No. of positive cases				Overall prevalence (%)	Mean Max. Temperature (°C)	Mean Min. Temperature (°C)	Mean RH (%)	Total rainfall (mm)
		R. sanguineus	*R. haemaphysaloides*	*H. bispinosa*	*H. bispinosa* var *intermedia*					
2004 July	100	8		2		10	31.8	22.2	85	369.6
2004 August	100	10				10	31.3	23.4	83	386.9
2004 September	100	10				10	32.8	22.2	80	208.8
2004 October	100	13		1	1	15	33.8	23.1	73	493.2
2004 November	100	12		3		15	32.8	23.9	65	71.7
2004 December	100	7	2			9	33.6	21.9	55	0
2005 January	100	4	2	1		7	35	22.6	56	7.6
2005 February	100	10		1		11	37.6	22.3	53	0
2005 March	100	9		1		10	38.2	24.6	42	0
2005 April	100	8		2		10	33.7	24.8	74	171.4
2005 May	100	9		3		12	33.6	25	72	89.2
2005 June	100	3		2		5	30	23.5	86	711.4

Table 5. Seasonal variation on the prevalence of tick infestation

Season	No.of dogs examined	No. of positive cases				Overall prevalence (%)	Mean Max. Temperature (°C)	Mean Min. Temperature (°C)	Mean RH (%)	Total rainfall (mm)
		R. sanguineus	*R. haemaphysaloides*	*H. bispinosa*	*H.bispinosa* var *intermedia*					
Pre monsoon (Feb. to May)	400	36	nil	7	nil	10.75	35.8	24.2	60	260.6
Monsoon (June to September)	400	31	nil	4	nil	8.75	31.5	22.8	84	1676.7
Post monsoon (October to January)	400	36	4	5	1	11.5	33.8	22.9	62	572.5

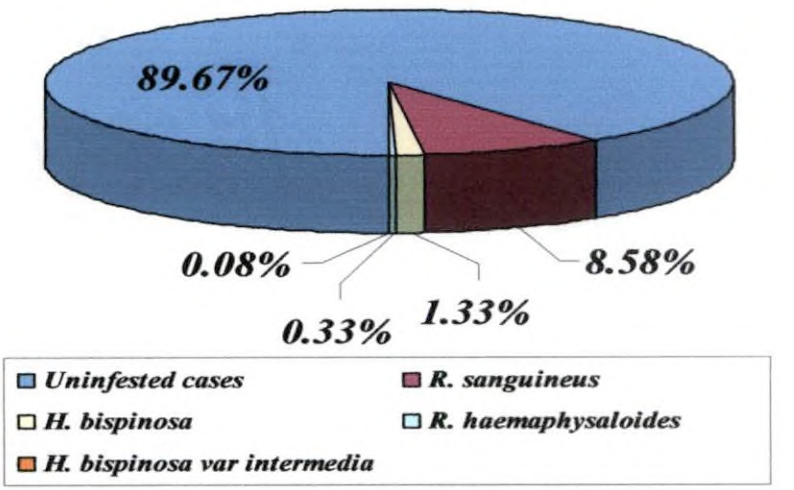


Fig. 1 Species wise prevalence of tick infestation in Thrissur Corporation area during 2004 July to 2005 June.

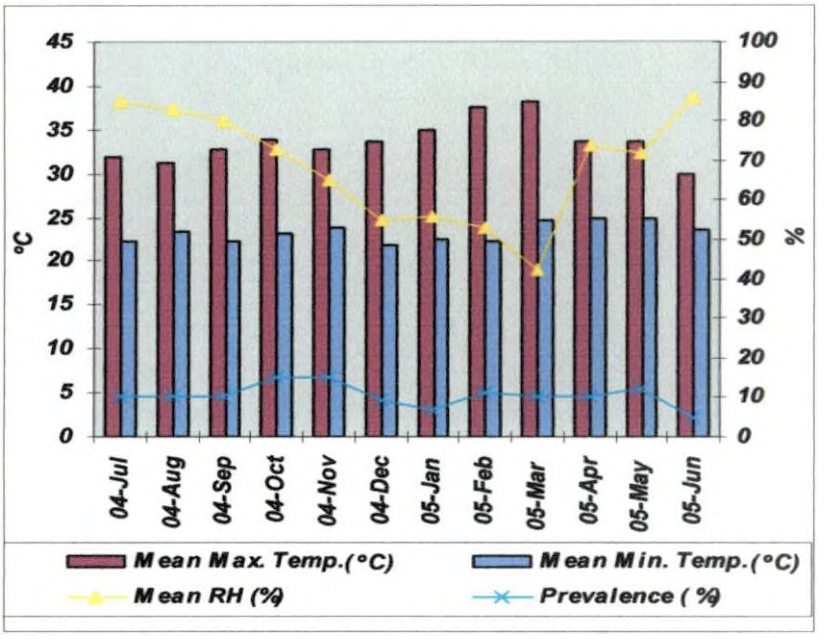


Fig. 2 Effect of temperature and humidity on tick infestation in dogs

Table 6. Gender specific prevalence of dog infestation with ticks in Thrissur Corporation area from July 2004 to June 2005

Period	Tick species	Male dogs		Positive cases (%)	Female dogs		Positive cases (%)
		No. of dogs examined	No. of positive cases		No of dogs examined	No. of positive cases	
2004 July to 2005 June	*R. sanguineus*	592	60	10.14	608	43	7.07
	R. haemaphysaloides		1	0.17		3	0.49
	H. bispinosa		8	1.35		8	1.31
	H. bispinosa var *intermedia*					1	0.16
Total			69	11.66		55	9.05

Table 7. Age wise prevalence of tick infestation in dogs in Thrissur Corporation area during July 2004 to June 2005

Breed	2 years of age			2- 4 years of age			> 4 years of age		
	No of dogs examined	Positive cases	Prevalence (%)	No of dogs examined	Positive cases	Prevalence (%)	No of dogs examined	Positive cases	Prevalence (%)
American cocker spaniel									
Basset hound	6			1					
Beagle	--			1					
Boxer	3			3			1		
Bull Mastiff	2			1					
Crossbred	40	5	12.5	33	1	3.03	9	1	11.11
Cocker spaniel	22			12	1	8.33	3		
Dachshund	68	2	2.94	42	6	14.29	16		
Dalmatian	16			2			1		
Doberman	27	3	11.11	18	1	5.56	2		
Golden retriever	2						1		
Great Dane	3			4	1	25	1		
GSD	175	19	10.86	135	21	15.56	37	6	16.22
Labrador	84	9	10.71	32	4	12.5	10	1	10
Lhasa Apso	5	1	20	5			1		
Non Descript	86	15	17.44	65	11		14		
Poodle	--			1					
Pug	2								
Rottweiler	34	4	11.76	6					
Spitz	68	4	5.88	68	6		31	2	6.45
TOTAL	644	62	9.63	429	52	12.12	127	10	7.87

Table 8. Intensity of infestation in 124 positive dogs

Breed	No of dogs with an intensity of infestation (+)	No of dogs with an intensity of infestation (++)	No of dogs with an intensity of infestation (+++)	No of dogs with an intensity of infestation (++++)
Cross bred	3	1	1	2
Dachshund	7	1	--	--
Great Dane	1	--	--	--
GSD	17	10	8	11
Labrador	10	1	1	2
Lhasa Apso	1	--	--	--
Non Descript	21	3	--	2
Rottweiler	2	2	--	--
Spitz	3	4	2	3
Doberman	--	1	--	3
Cocker spaniel	--	--	1	--
TOTAL	65	23	13	23

+ 0-5 Ticks ++ 5-10 Ticks +++ 11-15 Ticks ++++ >15 Ticks

Table 9. Predilection site of different instars of ticks in 124 positive cases

Predilection site	No of attachments			Total	Percentage
	Larva	Nymph	Adult		
Dorsum		1	14	15	12.1
Whole Body	1		8	9	7.26
Ear	5	20	80	105	84.68
IDS		4	18	22	17.74
Neck		5	28	33	26.61
Eye lid		1	3	4	3.23
Perianal region			1	1	0.81
Withers			1	1	0.81
Thorax			1	1	0.81
Hind limb	1			1	0.81

4.1.1 Identification of Tick Species

4.1.1.1 *Rhipicephalus sanguineus*

A medium sized, pale yellowish-brown or reddish brown tick with a simus pattern of punctuation in the male and scalpel shaped cervical fields in the female. (Plate 1 and 2)

Male

Capitulum broader than long, Basis capituli with acutely curved lateral angles, not overlapping the scapular areas of conscutum, palps short and rounded apically. Anterior process of coxa I was inconspicuous. First coxa was bifid. Ventrally spiracle elongate throughout, each with a narrow dorsal prolongation dorsally. Ventrally adanal plate elongately subtriangular with distinctly broad posterior aspect. Festoons present.

Female

Capitulum broader than long. Basis capituli with broad lateral angles; porose area small, about twice their own diameter apart. Palps longer than those of male, narrowly rounded apically.

4.1.1.2 *Rhipicephalus haemaphysaloides*

Large brown species with the following salient characters(Plate 3)

Male

Basis capitulum twice as broad as long. Adanal shields sickle shaped with external and posterior margins forming a regular curve. Body wall expanded laterally in engorged specimens; in partially engorged specimens the middle festoons protrudes as caudal process.

Female

Punctuation few, sparsely scattered, strongly unequal, larger ones not found in the posterior portion of the median field.

4.1.1.3 *Haemaphysalis bispinosa*

(Plate 4 and 5)

Male

Dorsal retroverted spur on palpal article 3 long and prominent. Scutum inornate without eyes. Sexual dimorphism slight, the male possessing no ventral plate or shields. Capitulum with base sub-rectangular, palps short and conical. Broadest near the posterior end of article 2 which projected laterally beyond the base. Normal coxal armature consisted of a moderate internal spur on Coxa I (subtriangular in shape), a slight spur at the middle of the posterior borders of Coxae II and III, and at the internal angle of Coxa IV. Festoons present.

Female

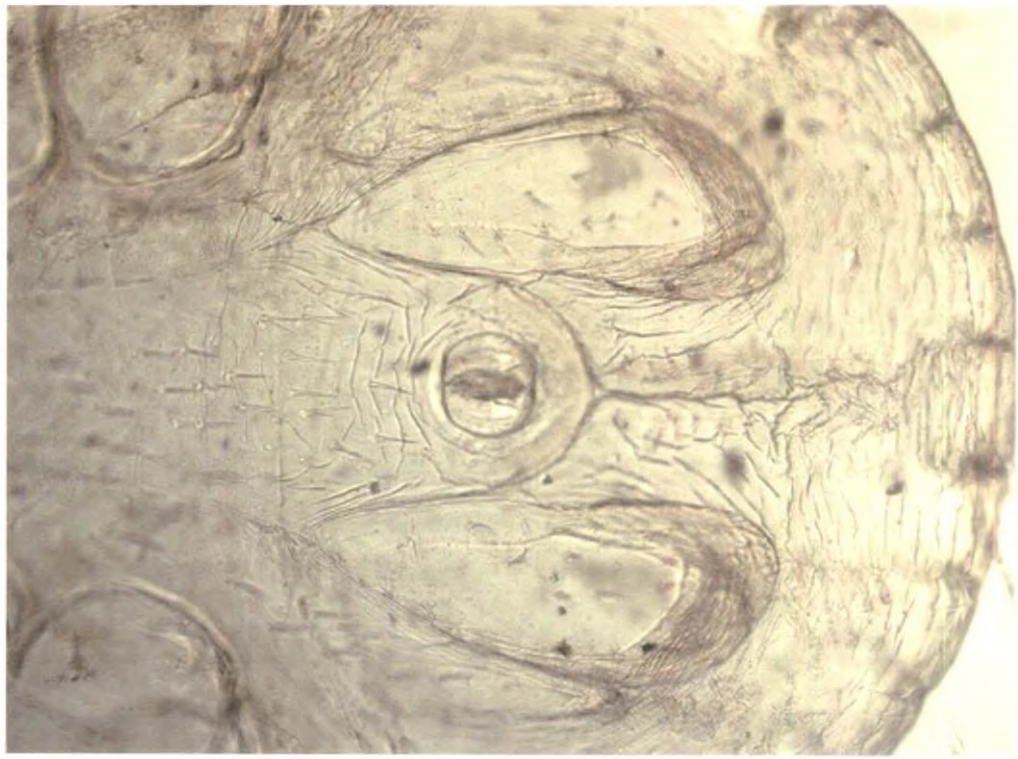
Basis capituli as long as palps, lateral salience pointed. Scutum without lateral grooves. Normal coxal armature. Dorsal spur on palpal article 3 fairly strong.

4. 1. 1. 4 *Haemaphysalis bispinosa* var. *intermedia*

Male

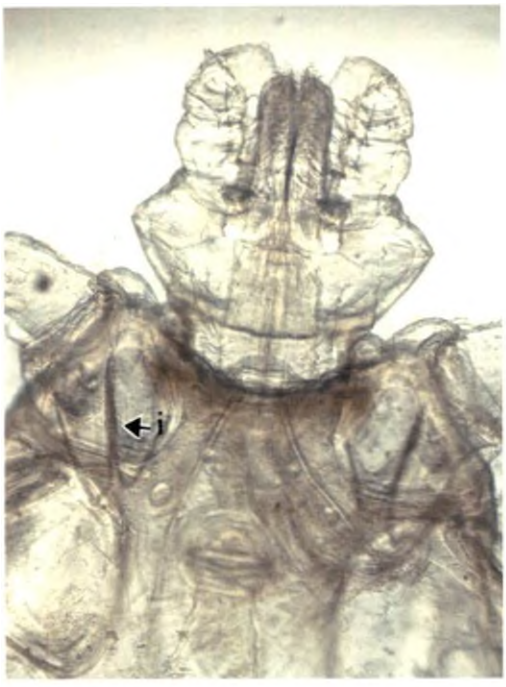
Dorsal spur on palpal article 3 broad, ridge-like and obsolete. Normal coxal armature (Plate 6).

A

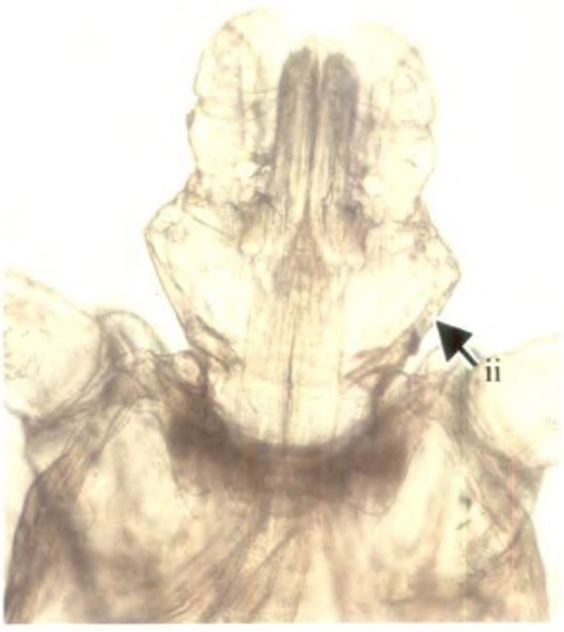


B

Plate 1. *Rhipicephalus sanguineus*- (A) ventral view of male (B) adanal plates



A

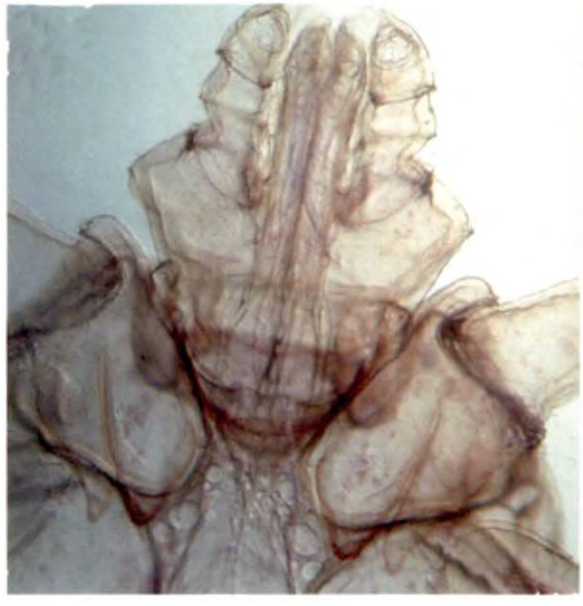


B

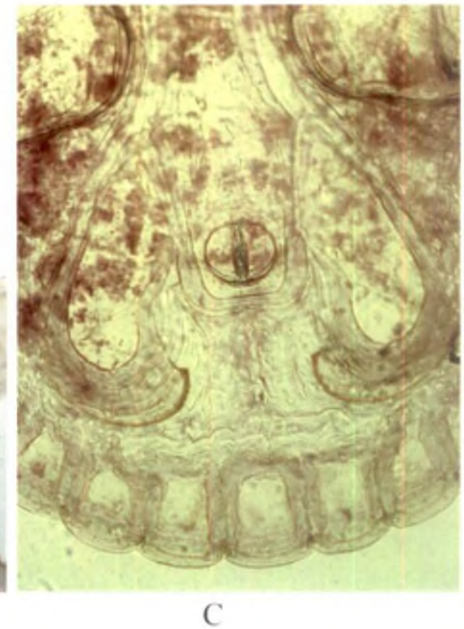
C

Plate 2. *Rhipicephalus sanguineus* Capitulum- (A) dorsal and (B) ventral view - (i) bifid coxae (ii) hexagonal basis capitulum, (C) larvae

A



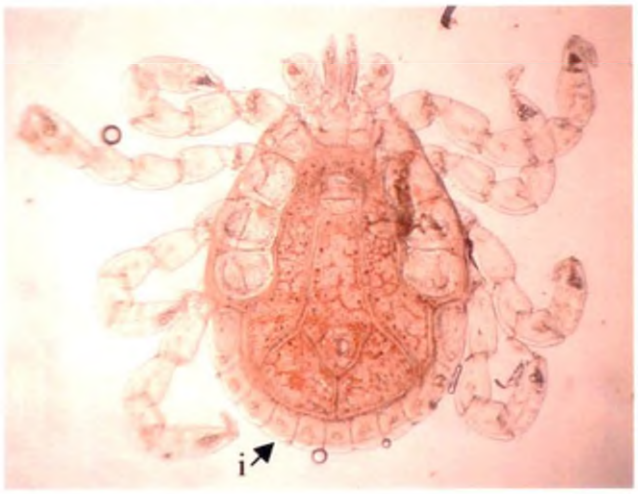
B



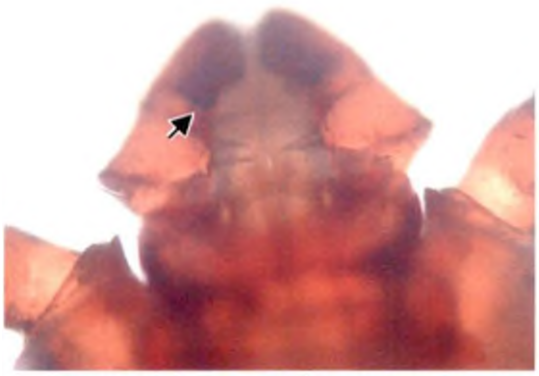
C

Plate 3. *Rhipicephalus haemaphysaloides* - (A) ventral view of male
(B) capitulum and bifid coxae of male
(C) sickle shaped adanal plates

Plate 3 Contd. Engorged adult female *Rhipicephalus haemaphysaloides*



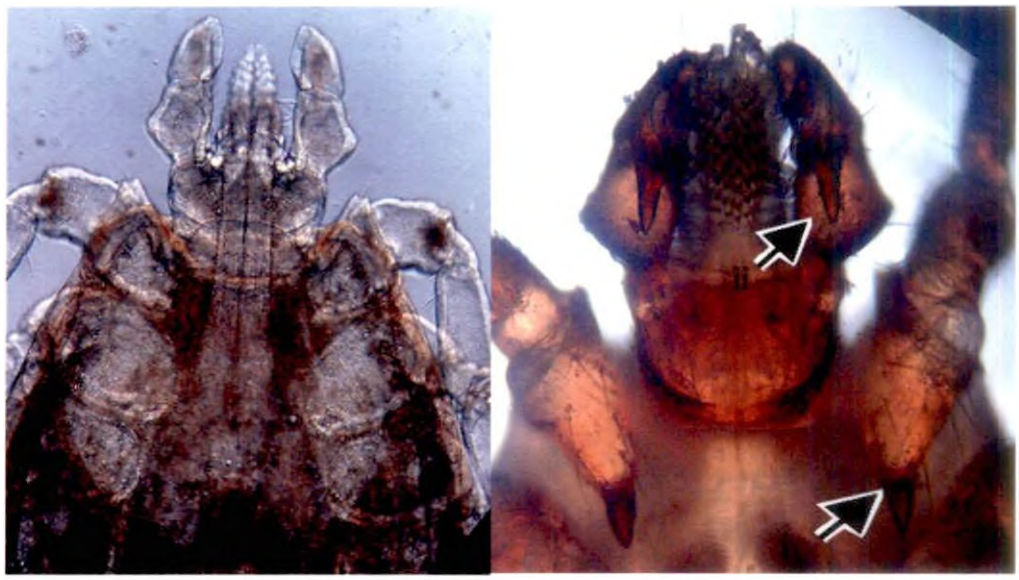
A



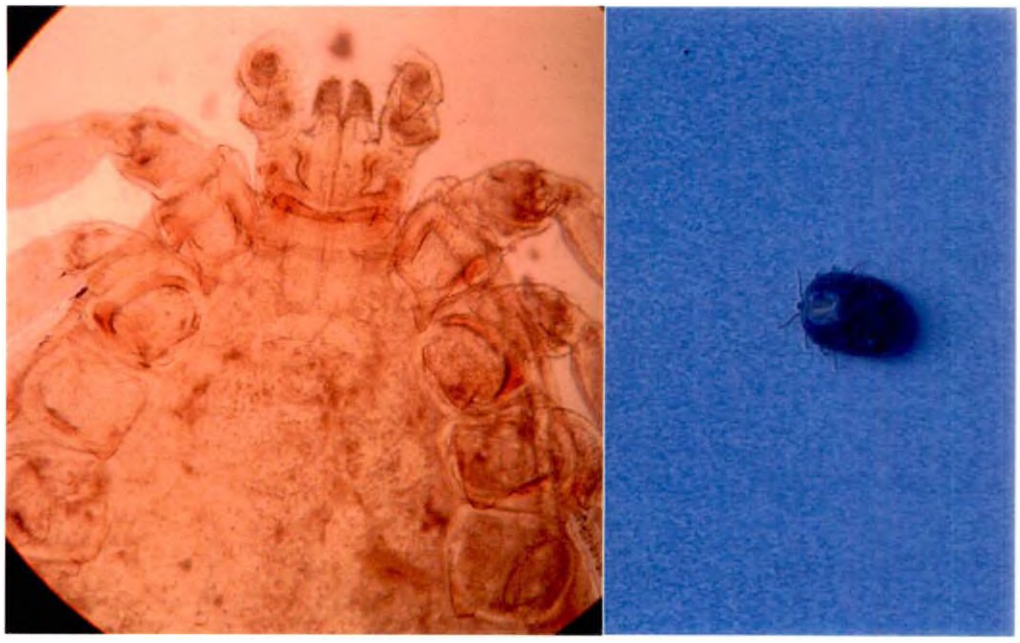
B

C

Plate 4. *Haemaphysalis bispinosa* - (A) ventral view of male-(i) festoons
(B) dorsal aspect of capitulum-dorsal retroverted spur on third pedipalp.
(C) dorsal retroverted spur in an elevated position



A B



C D

Plate 5. *Haemaphysalis bispinosa* - (A) dorsal view of capitulum and scutum of female (B) ventral view of capitulum and coxae - (i) ventral retroverted spur, (ii) coxal spur (C) ventral view of male showing coxal armature (D) engorged female

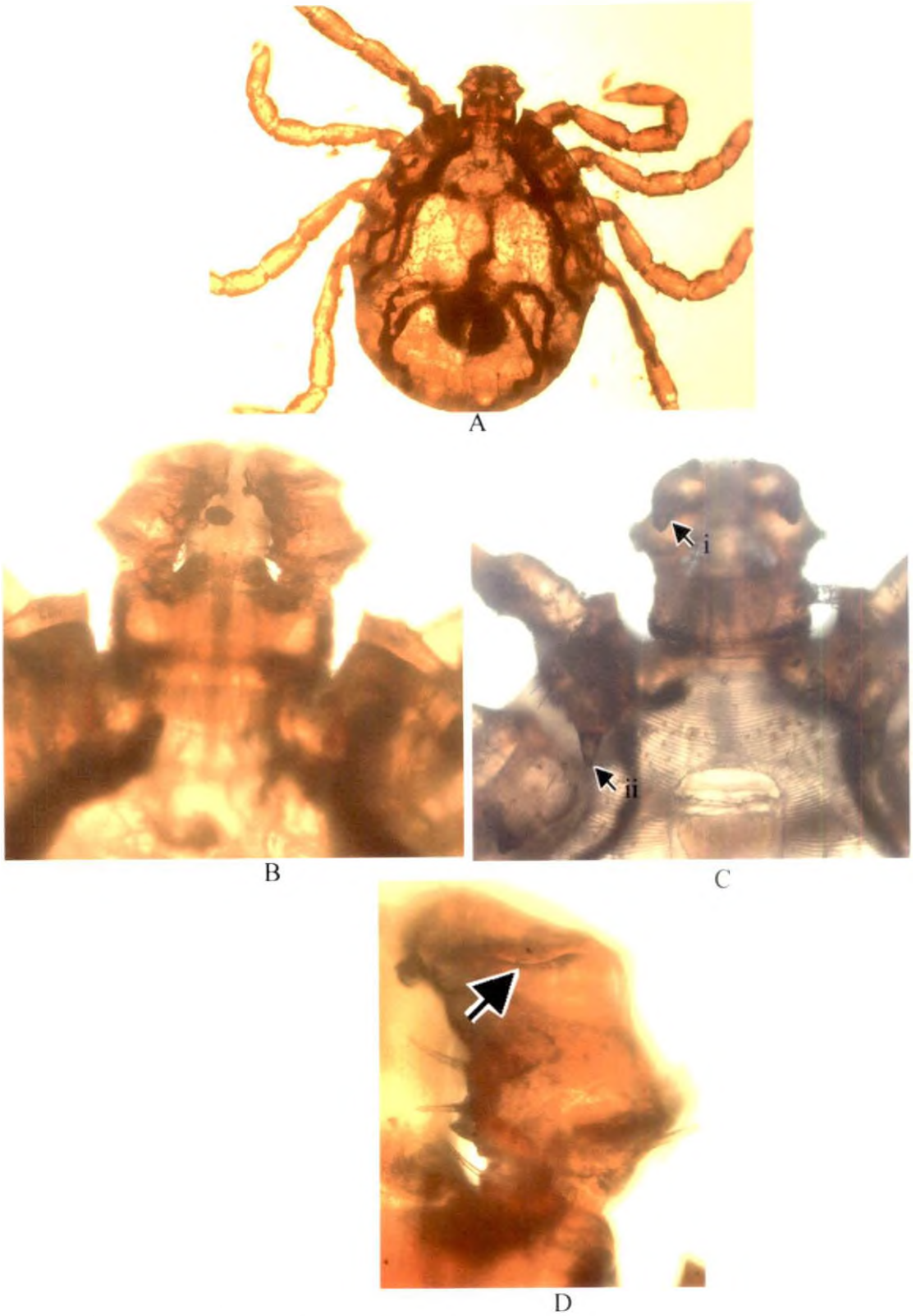


Plate 6. *Haemaphysalis bispinosa* var *intermedia* - (A) dorsal aspect of male (B) dorsal view of capitulum (C) ventral view of capitulum and coxae - (i) ventral retroverted spur (ii) coxal spur (D) broad ridge like dorsal spur

4.2 COLONISATION OF TICK

Rhipicephalus sanguineus was successfully colonised in the laboratory by ear bag method using tick bite naive rabbits. (Plate 7, 8 and 9)

4.3 MIDGUT ANTIGEN

4.3.1 Protein Concentration of Gut Antigen

Protein concentration of midgut antigen prepared from 350 partially fed *R. sanguineus* was estimated to be five milligram per ml.

4.4 IMMUNISATION

4.4.1 Assessment of Immune Status

4.4.1.1 *Humoral Immune Response.*

4.4.1.1.1 Agar Gel Immuno Diffusion (AGID)

Sera collected on 14^{th}, 21^{st} and 35^{th} day post immunisation from immunised guinea pigs were subjected to AGID test but no precipitation reaction was observed. Hence hyperimmune serum was raised in a rabbit by inoculating subcutaneously 500 µg of gut antigen along with FCA followed by 250 µg of gut antigen on 14^{th} and 21^{st} day post immunisation. Serum samples collected on 7, 14 and 21 days post immunisation when diffused against antigen, a faint precipitin line was observed between serum collected on 21 day post immunisation and antigen. Serum collected from the 35 day post immunisation showed thick precipitin bands when observed under illumination.

A

B

C

Plate 7. Colonisation of ticks - (A) Ear bag method of tick rearing
(B) Method of infesting rabbit with ticks
(C) Rabbit inside the restrainer box

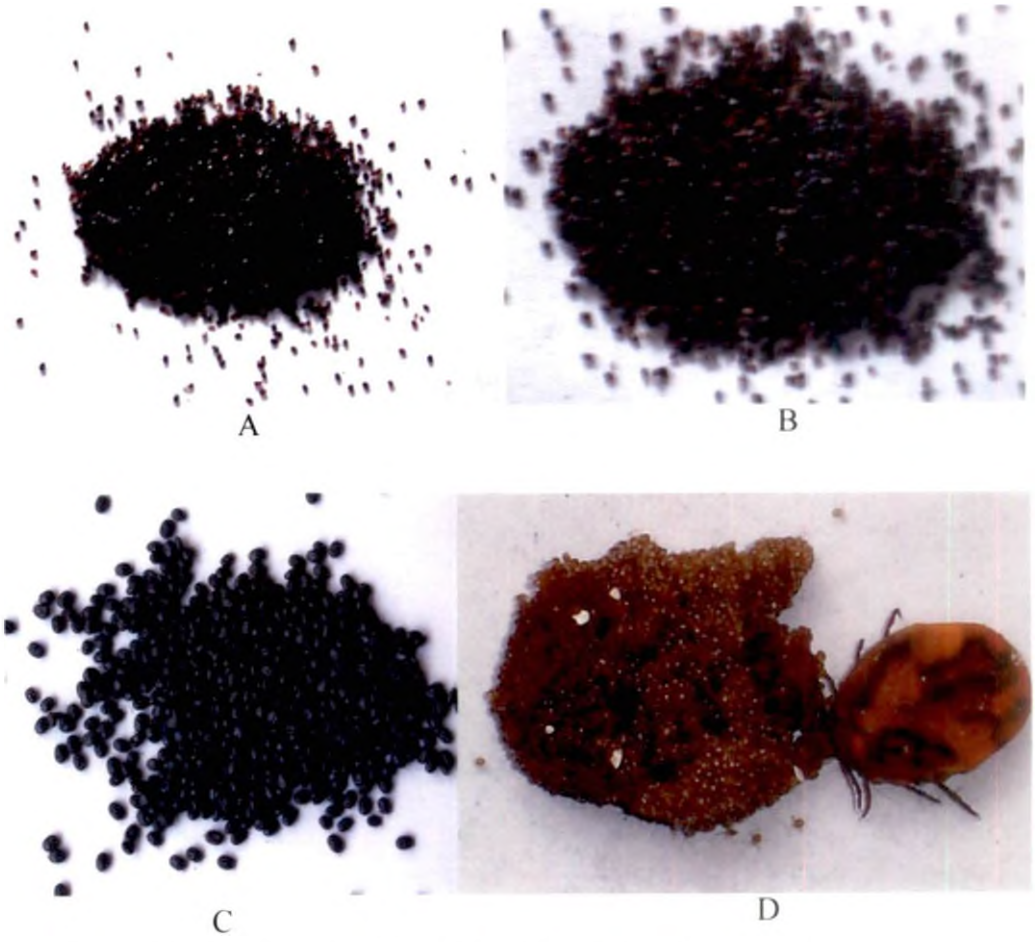


Plate 8. Different stages of tick colonies (A) engorged larvae of *Rhipicephalus sanguineus* (B) engorged nymphs of *R. sanguineus* (C) engorged nymphs of *R. haemaphysaloides* (D) beginning of egg mass hatching

Plate 9. (A) sand bed (B) specimen tube with unfed larvae inside

4.4.1.1.2 Passive haemaglutination (PHA)

Passive haemaglutination assay using gluteraldehyde fixed sheep RBC was done to detect the serum antibody titre of guinea pigs immunised with gut extracts.

The optimal concentration of antigen used for the assay was found to be one milligram per ml.

Immunised guinea pigs developed PHA titre within 14 days which increased to a mean titre of 1:106.67 ± 33.05 by 35 days. Passive haemaglutination (PHA) antibody titre against gut antigen ranged from 1:8 to 1:32, 1:32 to 1:64 and 1:64 to 1:128 in the sera collected on 14th, 21st and 35th day post immunisation respectively. PHA titres are shown in table 10, Plate 10.

Table 10. Antibody titre against gut antigen of *Rhipicephalus sanguineus* in immunised guinea pigs as detected by PHA.

Animal Number	PHA titre			
	0th day	14th DPI	21st DPI	35th DPI
1	0	1:32	1:64	1:128
2	0	1:8	1:32	1:64
3	0	1:8	1:32	1:128
4	0	1:16	1:32	1:64
5	0	1:16	1:64	1:128
6	0	1:16	1:64	1:128
Mean ± SD	0	1:16 ± 8.76	1:48 ± 17.53	1:106.67 ± 33.05

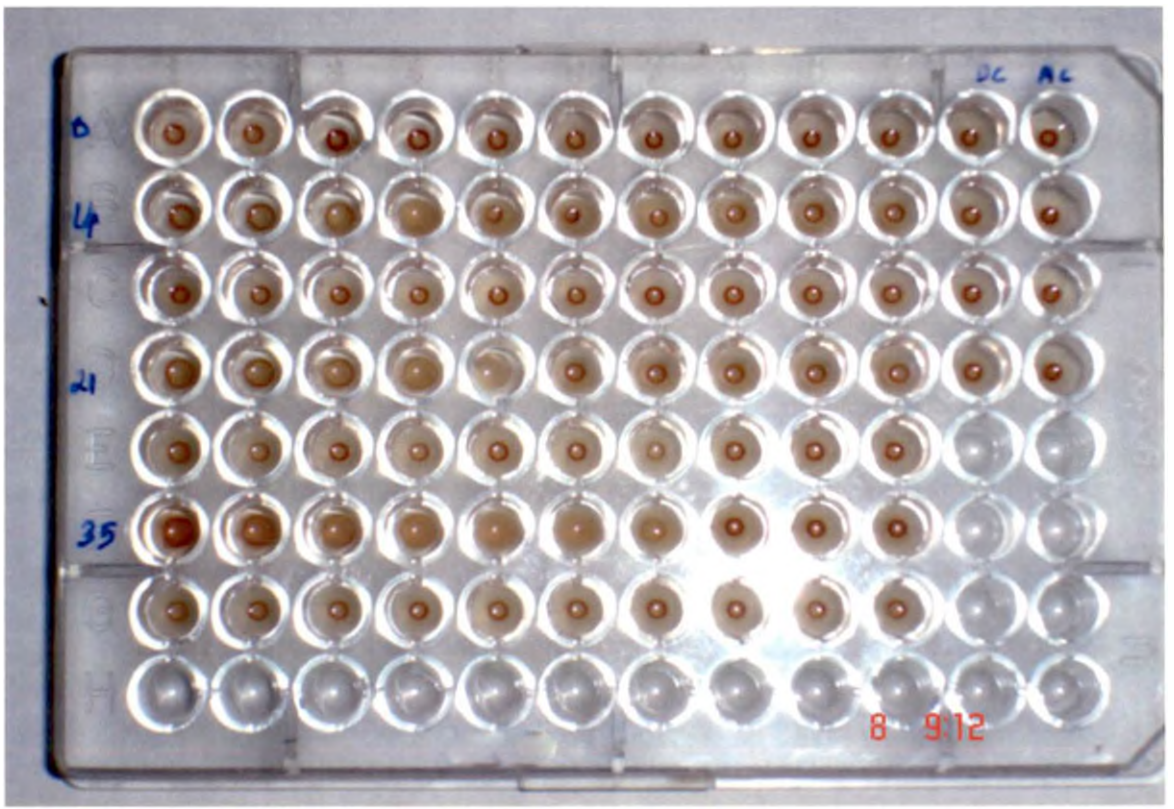


Plate 10. Passive haemagglutination test

Row 1- serial dilution of preimmunisation serum (upto 10th column)

Row 3and 4 -serial dilution of serum collected on 14th day of immunisation

Row 5 and 6 -serial dilution of serum collected on 21st day of immunisation

Row 7 and 8 - serial dilution of serum collected on 35th day of immunisation

DC (column 11)- diluent control

AC (column 12)- antigen control

4.4.1.2 *Cell Mediated Immune Response.*

4.4.1.2.1 Intradermal Test.

One hundred microlitres of *R. sanguineus* gut extracts was injected intradermally into the skin of guinea pigs and 100 µl of PBS was injected as control on a different site to assess the dermal reaction of antigen. There was erythema and circumscribed oedema after inoculation of antigen. Oedematous skin reaction was maximum at 30 minutes in both immunised and control animals. The circular swelling was more pronounced in immunised group indicating immediate type hypersensitivity reaction. By this time animal evinced pain on palpation of the swelling. Swelling became wider and indurated with time and by 12 hours, swelling had externally disappeared. Antigen produced thickening in skin of immunised and control animals. The mean value of skin thickness before and after inoculation of antigen and PBS are given in table 11. Skin thickness on antigen inoculation in immunised group increased to two fold (4.19 ± 0.42 mm) by 0.5 hour from 2.08 ± 0.14 mm (0^{th} hour) and hyperaemia subsided after 12 hour and skin thickness significantly ($P<0.01$) increased gradually up to 48^{th} hour (6.14 ± 0.51 mm) and started regressing thereafter. In control animals skin thickness increased up to 6^{th} hour and then gradually decreased. Skin thickness at the PBS inoculated site in immunised and control animals significantly ($P<0.01$) increased only up to 6^{th} hour and regressed thereafter. (Plate 11) By taking the initial variable (initial skin thickness) as concomitant variable ANACOVA was done after adjusting the mean and found significant ($P<0.01$) difference in skin thickness at the antigen inoculated site between immunised and control animals from 0.5 hour to 72 hours. (Table 12). Maximum skin thickness and induration at 48 hours indicated a delayed hyper sensitivity reaction.

Table 11. Mean skin thickness of guinea pigs after intradermal inoculation of gut antigen of *Rhipicephalus sanguineus*

Time (Hours)	Mean skin thickness in immunised animals (mm)		Mean skin thickness in control animals (mm)	
	Antigen	PBS	Antigen	PBS
0	2.08 ± 0.14	2.10 ± 0.14	2.25 ± 0.35	2.20 ±0.20
0.5	4.19 ± 0.42**	2.75 ± 0.19**	3.00 ± 0.29**	2.78 ±0.16**
1	4.23 ± 0.40**	2.85 ± 0.24**	3.16 ± 0.49**	2.71 ± 0.17**
6	4.44 ± 0.41**	2.86 ± 0.24**	3.34 ± 0.52**	2.71 ±0.17**
12	5.28 ± 1.03**	2.77 ± 0.24**	2.57 ± 0.36*	2.56 ±0.14**
24	6.01 ± 0.66**	2.35 ± 0.16**	2.53 ± 0.32NS	2.42 ±0.11NS
48	6.14 ± 0.51**	2.12 ± 0.19NS	2.37 ± 0.20 NS	2.26 ±0.15NS
72	3.01 ± 0.43**	2.10 ± 0.17NS	2.27 ± 0.15 NS	2.20 ±0.15NS
96	2.36 ± 0.18*	2.10 ± 0.17 NS	2.21 ± 0.15 NS	2.20 ±0.15NS

Means are compared row wise ** Significant at 1% level * Significant at 5% level

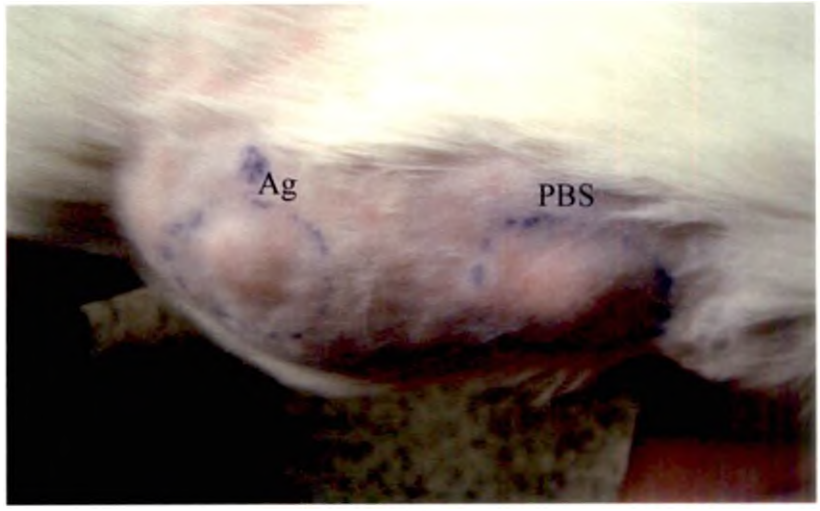
NS- non significant

Table 12. Adjusted mean skin thickness for column wise comparison by ANACOVA

Time (Hours)	Mean skin thickness in immunised animals (mm)		Mean skin thickness in control animals (mm)		Probability
	Antigen	PBS	Antigen	PBS	
0	2.080 [a]	2.100 [a]	2.250 [a]	2.200 [a]	P>0.05
0.5	4.260 [a]	2.910 [b]	2.800 [b]	2.900 [b]	P<0.01
1	4.290 [a]	2.900 [bc]	3.080 [b]	2.680 [c]	P<0.01
6	4.290 [a]	2.900 [bc]	3.280 [b]	2.690 [c]	P<0.01
12	5.360 [a]	2.660 [b]	2.470 [b]	2.510 [b]	P<0.01
24	6.070 [a]	2.400 [b]	2.460 [b]	2.390 [b]	P<0.01
48	6.200 [a]	2.170 [b]	2.300 [b]	2.300 [b]	P<0.01
72	3.040 [a]	2.120 [b]	2.230 [b]	2.180 [b]	P<0.01
96	2.380 [a]	2.120 [b]	2.180 [b]	2.190 [b]	P<0.05

P<0.01 significant at 1% level P<0.05 significant at 5% level

Means in a row having common superscript are not significantly different



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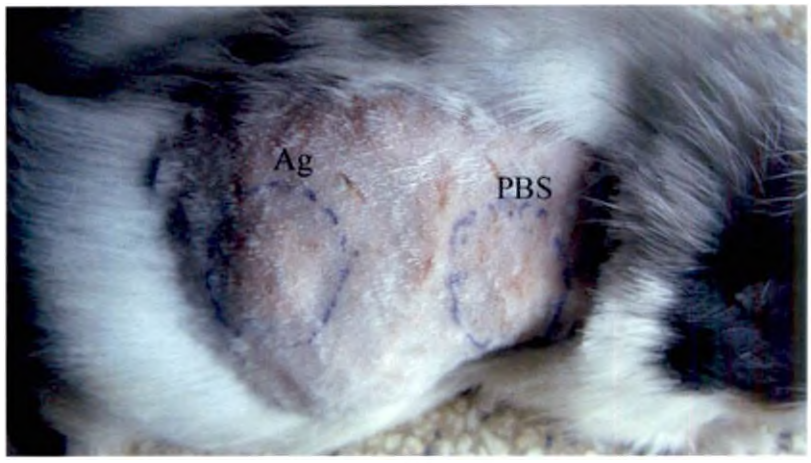

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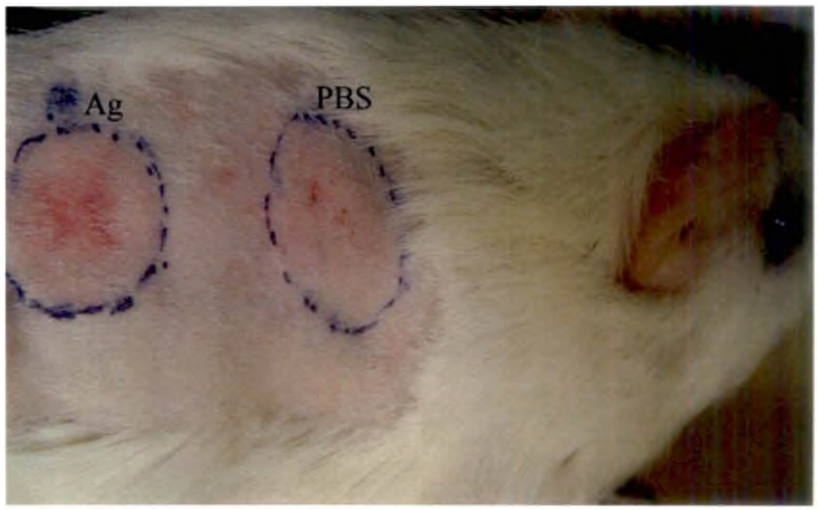

C

Plate 11. Intradermal test - dermal reaction at 30 min. in (A) immunised guinea pig (B) control and (C) at 48 h. in immunised guinea pig

4.5 CHALLENGE INFESTATION.

Both immunised and control guinea pigs were inoculated with five pairs of adult unfed *R. sanguineus* ticks on 35 day post immunisation by plastic feeding chamber method.

Challenge infestation results of guinea pigs are presented in table 13. Ticks recovered from the guinea pigs immunised with gut extracts was 63.33 per cent and that from control animals was 86.67 per cent. Non recovered ticks refer to those which did not attach to host in 15 days, or were found dead or not found at all. Most non recovered ticks did not attach at all and many were found dead, dehydrated or trapped in inflammatory exudates. Many engorged ticks from immunised guinea pigs were darker and so distended that they could not move properly. Among these, three ticks did not oviposit and after death became black and acquired a hard consistency (Plate 12).

4.5.1 Biological Parameters

Data obtained were analysed statistically using t-test by comparing the means in the immunised and control group.

4.5.1.1 *Feeding Parameters*

4.5.1.1.1 Feeding or Engorging Period (EP) in days

Recovered ticks from immunised guinea pigs showed significantly increased ($P<0.01$) feeding period compared to control group. Prolonged engorgement period (12.26 ± 1.24) was recorded in female ticks fed on guinea pigs immunised with gut extracts compared to that in control group (9.69 ± 1.23) (Fig. 3).

4.5.1.1.2 Weight of Engorged Female Ticks (FW) in mg.

The mean weight of engorged female *R. sanguineus* ticks that detached from immunised guinea pigs was significantly ($P<0.01$) lower (211.08 ± 33.14 mg) compared to control animals (314.23 ± 32.68) (Fig. 5). Percentage of ticks that died without ovipositing in immunised group was 6.67 per cent but none of the ticks engorged in the control group failed to lay eggs.

4.5.1.1.3 Feeding Efficiency Index

Mean feeding efficiency index in experimental group (17.41 ± 3.34) was significantly ($P<0.01$) lower compared to the control group (32.91 ± 5.38).

4.5.1.2 *Fertility Parameters*

4.5.1.2.1 Pre Oviposition Periods (POP) in days

The mean pre ovipositon period was significantly ($P<0.01$) extended (6.44 ± 1.21 days) in ticks fed on immunised group compared to the control group (4.5 ± 0.86 days) (Fig. 3).

4. 5. 1. 2. 2 Ovi Position Periods (OP) in days.

The mean oviposition period of engorged female *R. sanguineus* was reduced significantly ($P<0.01$) in immunised group (10.5 ± 1.26 days) compared to control group (14.3 ± 1.83 days) (Fig. 4).

4. 5. 1. 2. 3 Egg Mass Weight (EW) in mg

Mean egg mass produced by ticks fed on immunised guinea pigs was (100.96 ± 14.41 mg) significantly lower ($P<0.01$) compared to the egg mass produced by

ticks fed on the control animals (214.09 ± 22.93 mg) (Fig. 5). Out of the females engorged on immunised animals 15.79 per cent failed to lay eggs.

4.5.1.2.4 Incubation Periods (IP) in days.

Mean incubation period of ticks fed on immunised group was 21 ± 0.68 days and in ticks fed on control group, it was 21 ± 0.98 days. Even though they differed it was not statistically significant ($P>0.05$) (Fig. 4).

4.5.1.2.5 Efficiency Rate of Conversion to Eggs (ERCE)

Mean efficiency rate of females in converting their food reservoir to eggs in immunised group (48.52 ± 4.27 per cent) was significantly lower ($P<0.01$) compared to control group (68.22 ± 3.77) (Fig. 6).

4.5.1.2.6 Larval Mass in mg

Significantly lower ($P<0.01$) larval mass (45.21 ± 6.74) was produced by ticks fed on immunised group compared to that in control group (102.53 ± 10.43) (Fig. 5). Out of the females which laid egg masses, 12.5 per cent failed to hatch.

4. 6 EFFICACY OF IMMUNISATION

Percentage reduction of adult females (DT), mean weight of eggs (DO), mean weight of adult females (DR) and fertility (DF) were 26.92 per cent, 52.84 per cent, 67.17 per cent and 6.5 per cent respectively. Efficacy of immunisation (E) was 67.78 per cent.

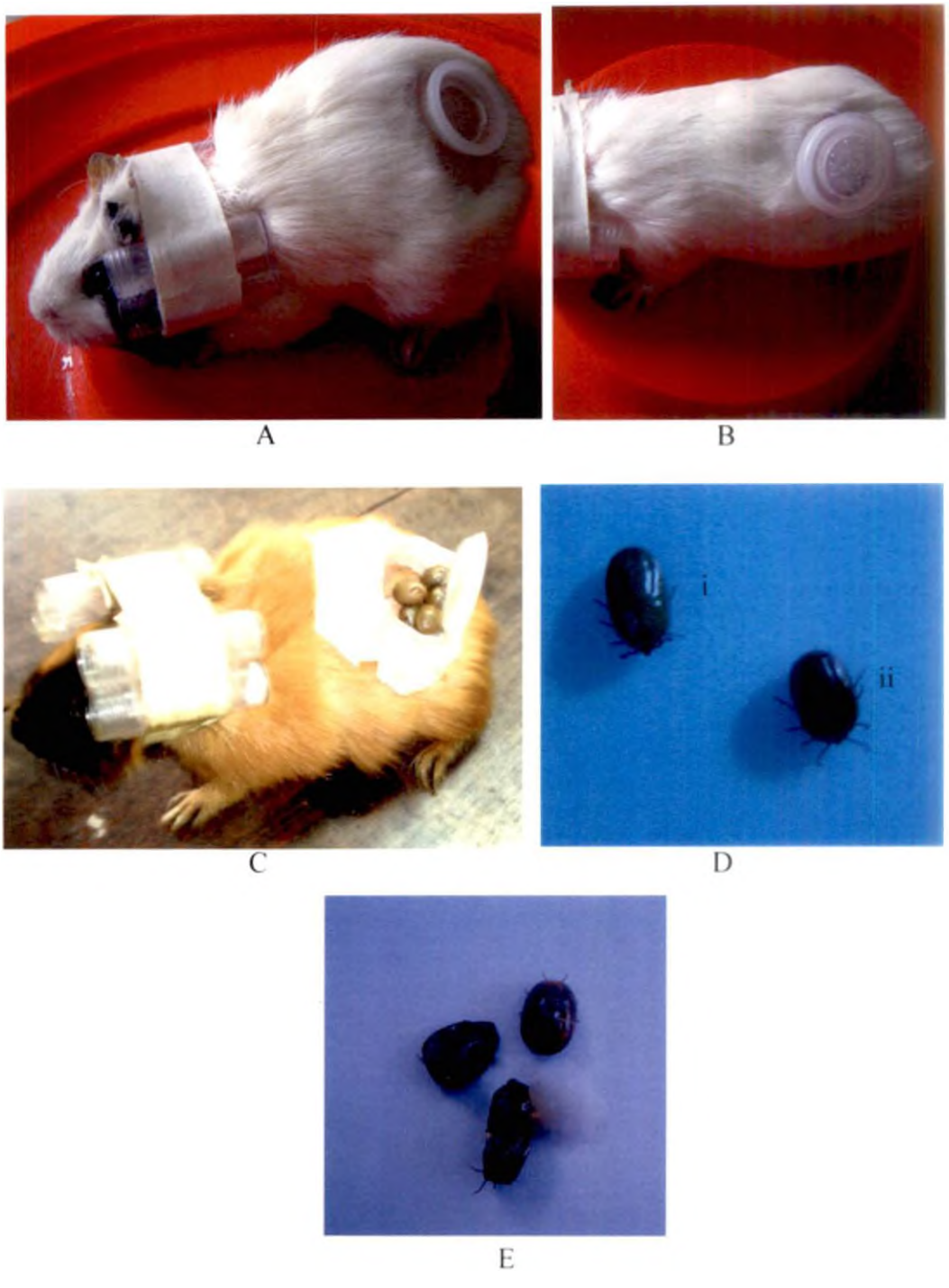

Plate 12. Challenge studies - guinea pig with feeding chamber (A) without cap (B) covered with porose cap (C) engorged tick inside the feeding chamber (D) effect of immunisation-(i) normal and (ii) dark coloured female (E) dead ticks due to immunisation effect

Table 13. Effect of immunisation with gut antigen in guinea pigs during challenge infestation with *Rhipicephalus sanguineus*

Sl. No	Parameters	Control Group	Immunised Group
1	Ticks recovered (%)	86.67	63.33
2	Engorging period (days)	9.69 ± 1.23	12.26 ± 1.24 *
3	Engorged female tick weight (mg)	314.23±32.68	211.08 ± 33.14 *
4	Pre oviposition period (days)	4.5 ± 0.86	6.44 ± 1.21 *
5	Oviposition period (days)	14.3 ± 1.83	10.5 ± 1.26 *
6	Egg mass weight (mg)	214.09 ± 22.93	100.96 ± 14.41 *
7	Incubation period (days)	21 ± 0.98	21 ± 0.68 NS
8	Egg rate conversion efficiency (%)	68.22 ± 3.71	48.52 ± 4.27 *
9	Larval mass (mg)	102.53 ± 10.43	45.21 ± 6.74 *
10	Ticks that died without ovipositing (%)	0	6.67
11	Ticks that failed to lay eggs (%)	0	10

* $P<0.01$ significant at 1% level

NS -non significant

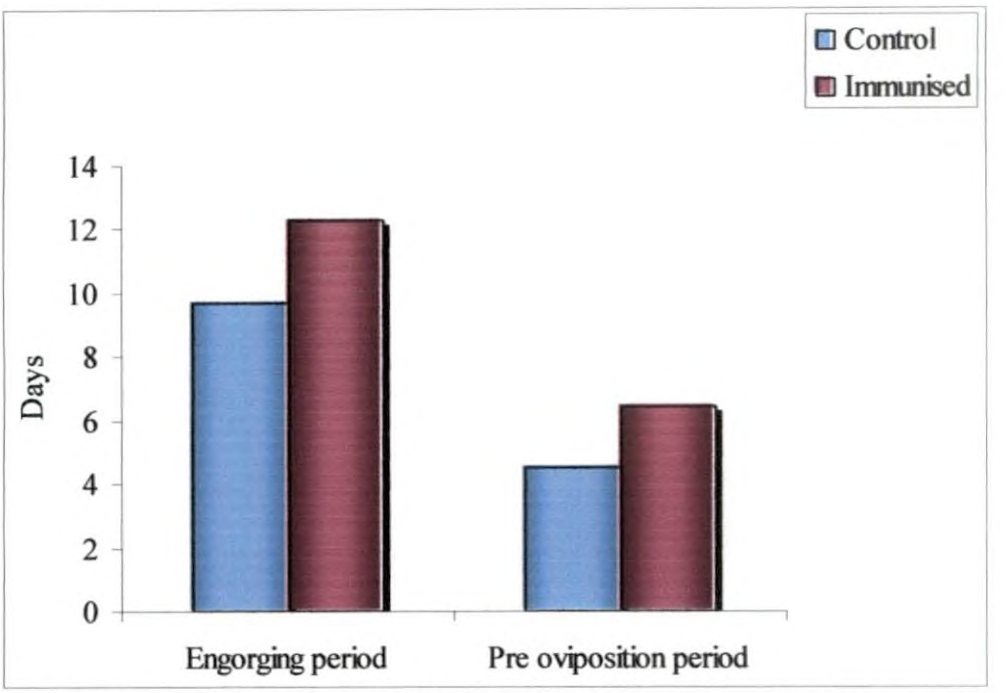

Fig. 3 Effect of immunisation on engorging period and pre oviposition period following infestation of immunised and control guinea pigs

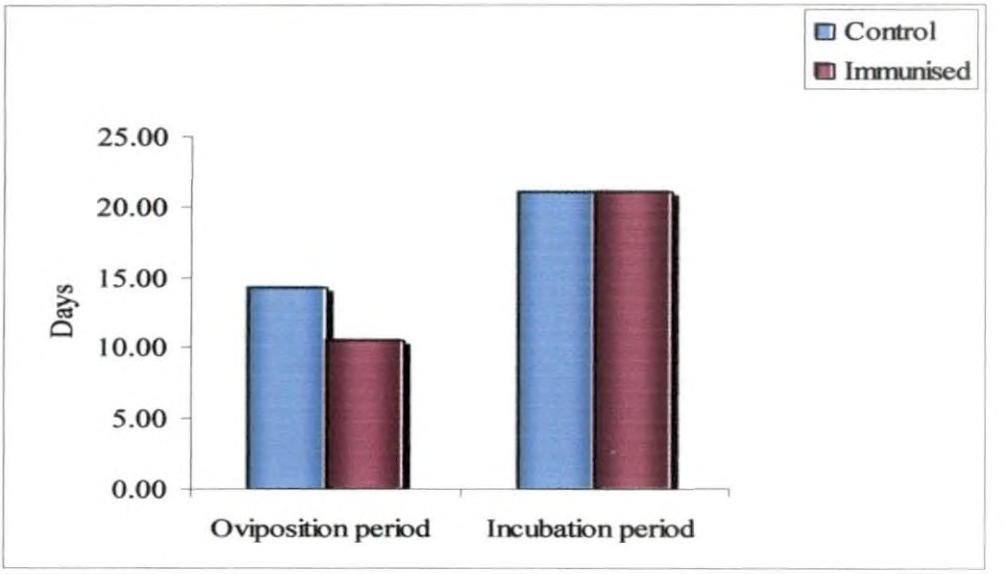

Fig. 4 Effect of immunisation on oviposition period and incubation period following infestation of immunised and control guinea pigs

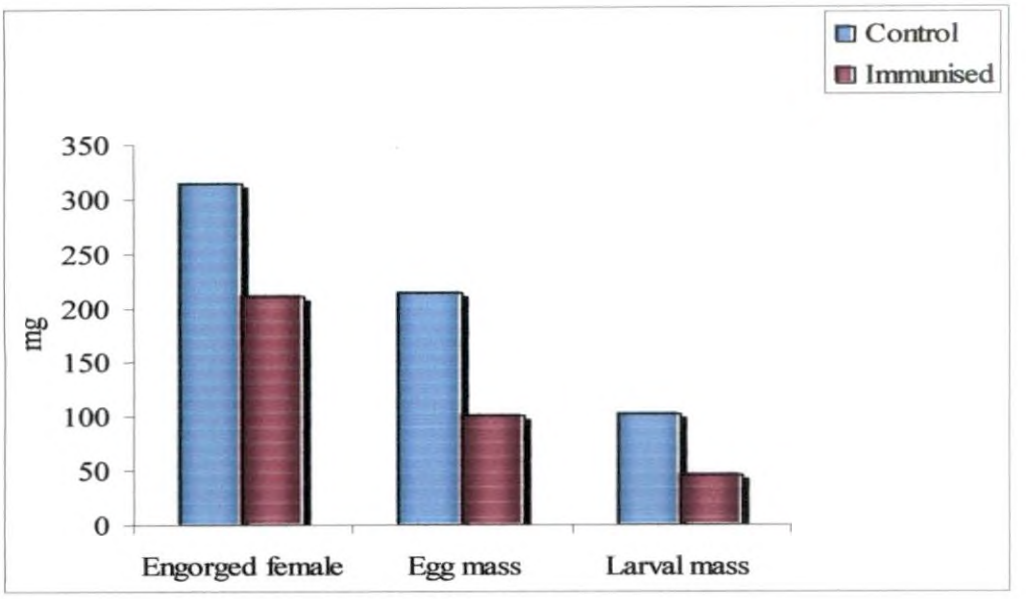

Fig. 5 Effect of immunisation on weight of engorged female , egg mass and larval mass following infestation of immunised and control guinea pigs

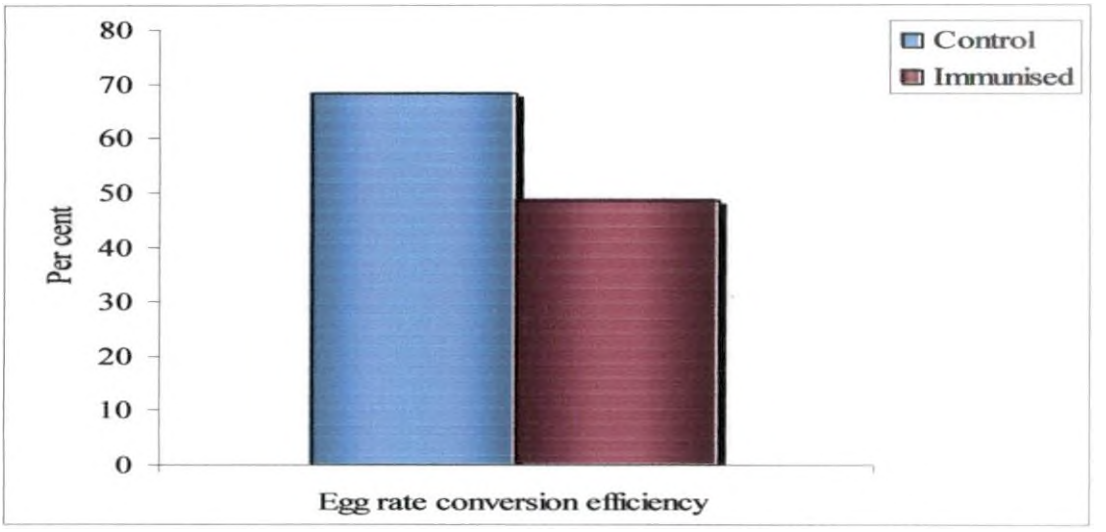

Fig. 6 Effect of immunisation on efficiency rate of female ticks in converting their food reserve to eggs (ERCE) following infestation of immunised and control guinea pigs

Discussion

5. DISCUSSION

5.1 PREVALENCE

This is the first systematic survey of tick species of dogs in Thrissur Corporation. Out of a total number of 1200 dogs screened from July 2004 to June 2005, 10.33 per cent were found positive for tick infestation. In the present study specimens of four hard tick species namely *Rhipicephalus sanguineus*, *R. haemaphysaloides*, *Haemaphysalis bispinosa, H. bispinosa* var. *intermedia* were identified based on the morphology which conforms to the description of many authors (Sen and Fletcher, 1962; Soulsby, 1982; Kettle, 1995; Walker *et al.*, 2000). Significant difference in species wise prevalence of tick infestation was found on 124 positive dogs. About 80 per cent of positive dogs were infested with adults, which can be partly explained as macroscopic examination of animals might have overlooked nymph and larval stages.

In the present study the most prevalent species of tick infested on dogs were *R. sanguineus* (8.58 per cent), which is in agreement with reports of Jacobs *et al.* (2001); Horak and Matthee (2003) and Neves *et al.* (2004) and was present throughout the year, which is in agreement with Vazquez *et al.* (1998). All the developmental stages of *R. sanguineus* preferred domestic dogs as host to the virtual exclusion of all other animal (Walker *et al.*, 2000) which agreed with the present collection of larvae, nymphs and adults from dogs alone.

The second most prevalent species of ticks infesting dogs was *H. bispinosa* (1.33 per cent). The present finding of this species on dogs concurred with the findings of Sen and Fletcher (1962) who reported its infestation on dogs in addition to its main occurrence on cattle and buffaloes in India. The present finding of this species of tick from dogs forms the first record from Kerala.

Prevalence of *R. haemaphysaloides* infestation in dogs was 0.33 per cent. This formed the first record of this species from dogs in Thrissur and so also from Kerala. It commonly occurrs in cattle and buffaloes in India (Shahardar and Narsapur, 2003) and has also been seen rarely in goat, sheep, camel and species of wild animals (Sen and Fletcher, 1962). The presence of this species of ticks on dogs was supported by Khan and Srivastava (1993) and Walker *et al.* (2000).

The least prevalent species was *H. bispinosa* var. *intermedia* (0.08 per cent). Only one specimen was collected from a stray dog, which also formed the first record.

Tick infestation was found throughout the year and the peaks were observed in October and November (15 per cent) with the lowest prevalence in June (5 per cent). No significant difference ($P>0.05$) in tick infestation during different months was observed due to temperature, relative humidity, and rainfall.

Season in Kerala can be divided into pre monsoon, monsoon and post monsoon. Even though ticks were present throughout the season, they were most abundant during the post monsoon (11.5 per cent). Though the mean relative humidity was high and mean minimum and maximum temperatures were low, the prevalence of tick infestation was least during monsoon (8.75 per cent) which is in disagreement with reports of higher tick infestation in goats (Latha *et al.*, 2004) and sheep (Mushi *et al.*, 1996; Latha *et al.*, 2004) during rainy season. This lower prevalence during rainy season may be due to good management practices adopted by dog owners and the constant care the dogs received during monsoon season. No clear pattern of seasonality was observed for *R. sanguineus,* which was present throughout the year.

The different variables considered in the present study such as age and sex were not significantly associated with the presence of tick infestation in dogs, which

is in agreement with Vazquez *et al.* (1998). Maximum incidence of tick infestation was seen in dogs aged between two and four years of age (12.12 per cent) while minimum was in dogs above four years of age (7.87 per cent). Prevalence of tick infestation in dogs below two years of age was 9.63 per cent. Only 10.58 per cent of the dogs screened were above four years of age, which may be the reason for the low prevalence of tick infestation in this age group. Prevalence of ticks in all ages confirms that previous report that dogs do not develop resistance to tick infestation by *R. sanguineus* even if they are continuously reinfested (Bechara *et al.*, 1994).

There was a highly significant ($P<0.01$) association between breeds and intensity of infestation with maximum intensity of infestation in German Shepherd dogs. Majority of dogs screened in this survey were German Shepherds, which may be the possible reason for this high intensity of infestation observed in this breed.

Site of attachment of ticks were ear, neck, interdigital space, dorsum of the body, eyelids, perianal region, withers, thorax and hind limbs in the descending order of intensity. Highly significant variation ($P<0.01$) between attachment sites was observed with maximum at the ear (84.68 per cent) followed by neck and interdigital space, which agreed with Horak and Matthee (2003) who reported that more than 50 per cent of adult *H. leachi* and *R. gertrudae* attached to the head, ear, neck and shoulders of dogs.

5.2 MIDGUT ANTIGEN

5.2.1 Protein Concentration of Midgut Antigen

Protein concentration of *R. sanguineus* mid gut antigen obtained from 350 partially fed ticks in the present study was 5 mg per ml while Szabo and Bechara

(1997) estimated it as 3.2 mg per ml. Razmi *et al.* (2003) obtained 4.2 mg per ml from the midgut of *H. anatolicum anatolicum.* The low concentration of protein in gut antigen in their study may be due to lesser number of ticks dissected.

5.3 ASSESMENT OF IMMUNE STATUS

5.3.1 Humoral Immune Response

5.3.1.1 *Agar Gel Immuno Diffusion*

Gel diffusion failed to demonstrate precipitin antibodies in immunised guinea pig serum but demonstrated precipitin bands by day 21 and 35 in the serum of rabbits immunised with a higher concentration of antigen. The failure of demonstration of precipitin lines in guinea pigs may due to the low concentration of protein used . The present result is supported by Njau *et al.* (1986) who opined that immunodiffusion was a less sensitive serological test for the detection of ectoparasitic infestation compared to PHA and ELISA.

5.3.1.2 *Passive Haemagglutination*

Antibody reaction with tick extracts have been detected by many investigators (Opedebeeck *et al.,* 1988; Jackson and Opedebeeck., 1990; Akhthar *et al.*, 1999; Manohar and Banerjee, 1992). Use of PHA in the present study for the detection of anti tick antibody titres have been supported by the findings of Akhtar *et al.* (1999) who used PHA for the first time to detect the immunogenic activity of the midgut antigen derived from the cell culture. Antibodies were detected in serum of immunised guinea pigs by day 14 of initial immunisation. A mean titre of 1:16 ± 8.76 on day 14 post immunisation increased progressively to 1:48 ± 17.53 on 21st day and then to 1:106.67 ± 33.05 on 35th day post immunisation. A peak antibody titre of 1:128 was observed on 35th day post immunisation in four animals and the remaining two animals developed a titre of 1:64. In the present study PHA test

showed that antibody can be detected during the second week of initial immunisation which is in partial agreement with Njau *et al.* (1986) and McGowan *et al.* (1980) who showed that antibodies can be detected on the 7th day of initial challenge. The present finding that PHA test is more sensitive than immunodiffusion for assessing the humoral immune response was supported by Njau *et al.* (1986)

5.3.2 Cell Mediated Immune Response

5.3.2.1 *Intradermal Test*

The present finding of cellular immune response exhibited by experimental animals through skin test in the form of immediate as well as delayed hypersensitivity reaction as evidenced by significant increase in the skin thickness at inoculated site of antigen are in accordance with the findings of Kumar and Kumar (1996b) who reported increased skin thickness in rabbits immunised with *H. dromedarii* derived midgut antigen. These findings are also in agreement with Panda *et al.* (1992) who reported delayed and immediate hypersensitivity reaction in calves sensitized with adult and larval antigen of *B. microplus*.

Animals in the control group also elicited a slight immediate hypersensitivity reaction during the skin test, which may be attributed to the presence of some vasoactive moiety in the antigen as reported by Wikel *et al.* (1978).

5.4 CHALLENGE INFESTATION

Lower recovery of ticks from immunised group in the present study is in agreement with Szabo and Bechara (1997). Bechara *et al.* (1994) reported that vaccinated guinea pigs had an intense reaction with prominent hyperaemia, oedema and exudation at the site of attachment. Such a local skin reaction might have caused the present impaired attachment and feeding which resulted in reduced tick recovery in immunised animals. Alteration of colour, improper distension and hard

consistency of ticks fed on immunised guinea pigs in the present study may have occurred due to gut damage and escape of host erythrocytes and leucocyctes into haemolymph resulting in further damage of other tissues (Agbede and Kemp, 1986; Thakur *et al.*, 1992).

5.4.1 Biological Parameters

The effect of induced immunity in guinea pig against *R. sanguineus* in response to gut antigen was expressed by the prolonged engorgement period, reduced engorged female weight, prolonged pre oviposition period, reduced oviposition period, reduced egg mass weight, reduced egg rate conversion efficiency and reduced larval mass.

5.4.1.1 *Feeding Parameters*

5.4.1.1.1 Feeding Period

Recovered ticks from immunised guinea pigs showed significantly extended feeding period compared to the controls in the present study which is in partial agreement with Szabo and Bechara (1997) who observed that ticks on immunised group took one day longer to engorge but was not statistically significant. Bechara *et al.* (1994) also reported significant increase in the feeding period when guinea pigs were immunised with crude unfed adult tick extracts of *R. sanguineus*. Tripathi *et al.* (1998) also recorded an increase of 1.3 days in engorgement period of female ticks fed on *Rhipicephalus* nymphal antigen immunised rabbits.

5.4.1.1.2 Weight of Engorged Female Ticks

Highly significant reduction in engorgement weight of female ticks, released on immunised guinea pigs, observed in the present study agrees with Szabo and Bechara (1997). Similar findings were also reported by Kumar and Kumar (1996a)

and Razmi *et al.* (2003) who reported significant reduction in engorgement weight of *Hyalomma* ticks in immunised rabbits and cattle respectively.

5.4.1.1.3 Feeding Efficiency Index

Feeding efficiency index value in the immunised guinea pigs in the present study was very low compared to the value 41.9 ± 2 as reported by Sahibi *et al.* (1997) when calves were immunised with intestinal extracts of *H. marginatum marginatum.*

5.4.1.2 ***Fertility Parameters***

5.4.1.2.1 Pre Oviposition Periods (POP)

Pre ovipositon period in the present study was significantly ($P<0.01$) extended in ticks fed on immunised group as compared to the control group. This concurred with reports of Banerjee *et al.* (2003) who observed significant increase in pre oviposition periods of *H. anatolicum anatolicum* fed on calves immunised with a fractionated midgut supernate antigen.

5.4.1.2.2 Ovi position periods (OP)

Significant reduction ($P<0.01$) in the oviposition period of engorged female *R. sanguineus* in immunised group (10.5 ± 1.26 days) as compared to control group (14.3 ± 1.83 days) is in agreement with Kumar and Kumar, (1996a) who observed significant ($P<0.01$) reduction in oviposition period of *H. anatolicum anatolicum* fed on rabbits immunised with midgut supernate with FCA. However change in oviposition period was non significant in *B. microplus* when cattle were immunised with tick extract supernatant antigen (Ghosh and Khan, 1996) and in *H. anatolicum anatolicum* in cattle immunised with gut extract (Razmi *et al.,* 2003).

5.4.1.2.3 Egg mass Weight (EW)

The significant reduction in egg production reflected by reduced egg mass weight in immunised group is quite similar to the findings of Szabo and Bechara (1997) who observed significant reduction in egg mass weight in dogs immunised with gut extracts of *R. sanguineus* with FCA and Razmi *et al.* (2003) who observed significant ($P<0.05$) reduction in egg mass weight produced by *H. anatolicum anatolicum* in cattle immunised with gut extracts. Similarly Bechara *et al.* (1994) also reported a significant drop in the weight of egg mass laid by ticks fed on hamsters and guinea pigs vaccinated with crude unfed adult tick extracts.

5.4.1.2.4 Incubation Periods (IP)

Mean incubation period of ticks fed on inmunised and control group of guinea pig did not vary significantly which is in agreement with many investigators (Kumar and Kumar, 1996a; Szabo and Bechara, 1997; Jittapalapong *et al.*, 2000)

5.4.1.2.5 Efficiency Rate of Conversion to Eggs (ERCE)

Mean efficiency rate of females in converting their food reservoir to eggs in immunised group was significantly lower in the present study which is in agreement with Szabo and Bechara (1997) who observed a significantly lower ERCE of *R. sanguineus* ticks released on dogs immunised with tick gut extracts and Freund's adjuvant but no significant difference was observed in ERCE of *R. sanguineus* ticks released on guinea pigs immunised with tick gut extracts and saponin.

5.4.1.2.6 Larval Mass

In the present study significantly lower ($P<0.01$) larval mass was produced by ticks fed on immunised group compared to that in control group, which is in

agreement with Fragoso *et al.* (1998) and Patarroyo *et al.* (2002). Out of the females, which laid egg mass, 12.5 per cent failed to hatch.

5.5 EFFICACY OF IMMUNISATION

By employing the formula derived by De La Fuente *et al.* (1995), effects of immunisation on biological parameters were evaluated by calculating percentage reduction of adult females, mean weight of eggs, mean weight of adult females and fertility. In the present study immunisation with gut extracts affected the number of ticks completing the life cycle (DT), percentage reduction of weight of females (DR) and reproductive capacity of survived ticks (DO and DF), which is in agreement with Fragoso *et al.* (1998) and Patarroyo *et al.* (2002). Efficacy of immunisation with gut extracts in the present study was 67.78 per cent. Similarly Fragoso *et al.* (1998) observed an overall vaccine efficacy of 99.9 per cent when cattle were vaccinated with Gavac and 35.87 per cent (Patarroyo *et al.*, 2002) when cattle were immunised with three synthetic peptides derived from the *B. microplus* gut protein (Bm 86).

Summary

6. SUMMARY

A detailed study on the prevalence of tick infestation in dogs in Thrissur Corporation area was conducted during the period from July 2004 to June 2005. Immunisation of guinea pigs using gut antigen derived from the most prevalent dog tick was also carried out.

One thousand two hundred dogs in Thrissur Corporation area were examined for the presence of tick infestation with a monthly average of 100 dogs. Ticks were collected by physical examination of dogs brought to University Veterinary Hospitals, Mannuthy and Kokkalai, at households in the Corporation area and from neighbourhood dogs in the Veterinary college campus, Mannuthy. Out of the total number of 1200 dogs screened from July 2004 to June 2005, 10.33 per cent were found positive for tick infestation. In the present study, specimens of four hard tick species viz *Rhipicephalus sanguineus*, *R. haemaphysaloides*, *Haemaphysalis bispinosa, H. bispinosa* var. *intermedia* were identified. Significant difference in species wise prevalence was found in 124 tick positive dogs. The most prevalent species of tick infesting dogs was *R. sanguineus* (8.58 per cent) followed by *H. bispinosa* (1.33 per cent), *R. haemaphysaloides* (0.33 per cent) and *H. bispinosa* var *intermedia* (0.08 per cent). All the developmental stages of *R. sanguineus* preferred domestic dogs as hosts to the virtual exclusion of all other animal. All the instars of other species of ticks could not be collected from dogs. *Haemaphysalis bispinosa*, *R. haemaphysaloides* and *H. bispinosa* var *intermedia* were recorded from dogs from Kerala for the first time.

Prevalence of tick infestation was found throughout the year and the peaks were detected in October and November (15 per cent) with the lowest prevalence during June (5 per cent). Studies on seasonal and climatic variation on the prevalence of tick infestation in dogs indicated no significant differences ($P>0.05$) between tick

infestations during different months. Even though ticks were present throughout the season, they were most abundant (11.5 per cent) in the post monsoon period. Though the mean relative humidity was high and mean minimum and maximum temperature were low, the prevalence of tick infestation was least during monsoon season (8.75 per cent). No clear pattern of seasonality was observed for *R. sanguineus,* which was present throughout the year.

The different variables considered such as age and sex were not significantly associated with the presence of tick infestation in dogs. Maximum incidence rate of tick infestation was seen in dogs aged between two to four years (12.12 per cent) while minimum was in dogs above four years of age (7.87 per cent). In dogs aged below two years the prevalence rate was 9.63 per cent.

There was highly significant ($P<0.01$) association between breeds and intensity of infestation. The maximum intensity of infestation was observed in German shepherd dogs.

Sites of attachment of ticks were ear, neck, interdigital space, dorsum of the body, eyelids, perianal region, withers, thorax and hind limb in the descending order of intensity. Highly significant variation ($P<0.01$) was observed between attachment sites with maximum on the ear (84.68 per cent) followed by neck and interdigital space.

Rhipicephalus sanguineus, the common dog tick found in the present study, was colonised in the laboratory by feeding different stages on tick bite naïve rabbits by ear bag method. Different stages of unfed ticks were maintained in desiccator containing 15 per cent potassium hydroxide.

Gut antigen was prepared from *R. sanguineus*. Partially engorged female *R. sanguineus* were dissected after surface sterilisation. Guts were separated in

phosphate buffered saline (PBS), sonicated, ultracentrifuged and supernatant was collected. This supernatant was used as gut antigen. Protein concentration of this gut antigen was estimated to be 5 mg/ml.

Six guinea pigs were immunised with 0.5 ml of gut antigen Freund's complete adjuvant (FCA) emulsion with a protein concentration of 50 μg, followed by two booster dose of gut antigen and Freund's incomplete adjuvant (FIA) emulsion containing 25 μg proteins on the 14^{th} and 21^{st} day post initial immunisation. Six guinea pigs were kept as control by inoculating PBS instead of gut antigen along with adjuvants.

Agar gel immunodiffusion (AGID) done to assess humoral immune response in immunised guinea pigs failed to develop precipitin lines but hyper immune serum raised in rabbits immunised with a higher concentration of antigen developed precipitin bands on 21^{st} and 35^{th} day post immunisation. Anti tick antibody was detected as early as 14^{th} day post immunisation in immunised guinea pigs by passive haemagglutination test (PHA), which indicated that PHA was more sensitive than AGID. PHA antibody titres against gut antigen ranged from 1:8 to 1:32, 1:32 to 1:64 and 1:64 to 1:128 in the sera collected on 14^{th}, 21^{st} and 35^{th} day post immunisation respectively.

Intradermal test in immunised animals revealed both immediate and delayed hypersensitivity as indicated by significant increase in skin thickness at 30 minutes and 48 hours respectively compared to the control animals.

Guinea pigs in immunised and control group were challenged with five pairs of adult unfed *R. sanguineus* ticks on 35 days post immunisation (DPI). Ticks were placed inside a feeding chamber with porose area on top consisting of a plastic tube glued with a non hazardous preparation on to the shaved back of guinea pigs and further secured with adhesive tape. Induced immunity resulted in impaired

attachment and feeding, reduced tick recovery, alteration of colour, improper distension and hard consistency of ticks. The effect of induced immunity in guinea pigs against *R. sanguineus* in response to gut antigen was expressed by altered feeding and fertility parameters which included the prolonged engorgement period, reduced engorged female weight and feeding efficiency index, prolonged pre oviposition period, reduced oviposition period, egg mass weight, egg rate conversion efficiency and larval mass. Except the incubation period all the reproductive and feeding parameters differed significantly.

Thus the present systematic study showed the general characteristics of tick infestation in dogs. Data reported herein will facilitate in devising strategic control measures and in the epidemiological studies of the diseases transmitted by dog ticks. Guinea pigs acquired resistance to *R. sanguineus* ticks when immunised with crude midgut antigen along with Freund's adjuvant as indicated by the deleterious effects on feeding and fertility parameters.

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IMMUNOPROPHYLAXIS AGAINST COMMON DOG TICK USING GUT ANTIGEN

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ABSTRACT

The occurrence of tick infestation in dogs in the Corporation of Thrissur, Kerala, India, was studied in 1200 dogs during a period of one year from July 2004 to June 2005. Four hard tick species viz *Rhipicephalus sanguineus*, *R. haemaphysaloides*, *Haemaphysalis bispinosa, H. bispinosa* var. *intermedia* were identified. Significant difference in species wise prevalence was found on 124 tick positive dogs. The most prevalent species of tick infesting dogs was found to be *R. sanguineus* (8.58 per cent) followed by *H. bispinosa* (1.33 per cent), *R. haemaphysaloides* (0.33 per cent) and *H. bispinosa* var. *intermedia* (0.08 per cent). Three species of ticks namely *R. haemaphysaloides*, *H. bispinosa,* and *H. bispinosa* var. *intermedia* have been recorded in dogs for the first time from Kerala. The influence of month, season, age, gender and breed on the prevalence rate were observed. Prevalence rates were calculated by month, season, age, gender and breed. No significant difference ($P>0.05$) of tick infestation during different months was observed due to temperature, relative humidity, and rainfall. No clear pattern of seasonality was observed for *R. sanguineus,* which was present throughout the year. The different variables considered in the present study such as age and gender were not significantly associated with the presence of tick infestation in dogs. There was a highly significant ($P<0.01$) association between breed and the intensity of infestation with maximum intensity of infestation in German shepherd dogs. Sites of attachment of ticks were ear, neck, interdigital space, dorsum of the body, eyelids, perianal region, withers, thorax and hind limbs. Highly significant variation ($P<0.01$) was observed between attachment sites with maximum on ear (84.68 per cent) followed by neck and interdigital space.

Tick-bite naive guinea pigs inoculated with gut extracts and Freund's adjuvant revealed induced immunity against *R. sanguineus* expressed by altered feeding and fertility parameters consisting of the prolonged engorgement period,

reduced engorged female weight and feeding efficiency index, prolonged pre oviposition period, reduced oviposition period, egg mass weight, egg rate conversion efficiency and larval mass. Except the incubation period all the reproductive and feeding parameters differed significantly compared to the control.

Inradermal test done on immunised guinea pigs to assess cell mediated immunity revealed both immediate and delayed hypersensitivity reaction. Humoral immune response assessed by agar gel immunodiffusion (AGID) and passive haemagglutination (PHA) revealed that the former was less sensitive compared to the latter. Passive haemagglutination test detected anti tick antibodies as early as 14^{th} day post immunisation. Peak titre 1:128 reached on the 35^{th} day post immunisation.