IMMUNIZATION STUDIES IN RABBITS USING MIDGUT MEMBRANE BOUND PROTEIN DERIVED FROM RHIPICEPHALUS APPENDICULATUS NEUMANN, RHIPICEPHALUS EVERTSI EVERTSI NEUMANN AND AMBLYOMMA VARIEGATUM FABRICIUS

BY

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A thesis submitted in fulfilment of the requirements
for the Degree of Master of Science (Parasitology) in
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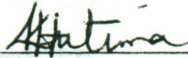
DECLARATION

This thesis is my own original work and has not been submitted for a degree in any other university.
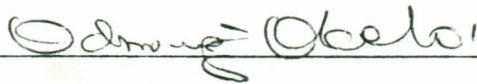
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DEDICATION

Dedicated to:

My parents, Nasiche and Mukonyi

My husband, Kutima

Our children, Lwile, Lyaka and Nasiche.

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

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ABSTRACT

The objective of this study was to immunize
rabbits with midgut membrane-bound proteins derived
from partially engorged Rhipicephalus appendiculatus,
R. evertsi evertsi and Amblyomma variegatum female
ticks and assess whether the immunity elicited was
protective against both homologous and heterologous
tick instars and to isolate and identify the protective
antigens.

Sodium dodecyl sulphate-polyacrylamide gel
electrophoresis of the Gut Membrane-Bound Protein
(GMBP) antigens demonstrated protein bands with
molecular weights ranging from 14 to 140 kDa.
Approximately 37 protein bands were fractionated from
R. appendiculatus GMBP antigens, approximately 45
protein bands were fractionated from R. evertsi evertsi
GMBP antigens and approximately 39 protein bands were
fractionated from A. variegatum GMBP antigens. Twenty-
two of the isolated proteins were shared among the
three tick species. The ability of rabbits to acquire
resistance to R. appendiculatus, R. evertsi evertsi and
A. variegatum was determined by injecting three
separate sets of rabbits with respective GMBP antigens.

Resistance was manifested by prolonged feeding,
reduction in engorgement weights, egg mass weights,
moulting and percentage hatchability and increased

mortality. Cross-resistance was evaluated by dividing R. appendiculatus, R. evertsi evertsi and A. variegatum resistant rabbits into three groups each and challenging them with homologous and heterologous live stages. Considerably high cross-resistance was apparent among the three groups. Cross-protection was more pronounced in the homologous than heterologous systems.

Enzyme Linked Immunosorbent Assay (ELISA) technique detected circulating antibodies in the immune sera to GMBP from homologous and heterologous systems one week after the primary dose. Ouchterlony double immunodiffusion reactions with anti-tick GMBP sera formed 2 to 4 precipitin lines with homologous GMBP antigens and 1 to 2 precipitin line(s) with each heterologous GMBP antigens. A line of complete identity was observed when immune sera to GMBP antigens reacted with GMBP from homologous and heterologous tick species, suggesting common antigenic epitopes.

Western blot analysis on GMBP of R. appendiculatus, R. evertsi evertsi and A. variegatum with sera from immunized rabbits detected protein bands specific to the homologous GMBP antigens, and revealed considerable cross-reactions in the heterologous systems.

In conclusion, there was prolonged feeding periods, reduced engorged weights, egg mass weights

hatchability and moulting and increased death rate of both homologous and heterologous challenge ticks which

fed on resistant rabbits. This was due to the presence of common antigens. The presence of cross-reacting antigens conferred cross-protection. These results have pointed out that it is possible to protect livestock from R. appendiculatus, R. evertsi evertsi and A. variegatum using an antigen from any one of the three tick species hence reducing the expence of having to develop an antigen to control each tick species as there are in existence.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1: Economic importance of ticks

Ticks and tick-borne diseases are of world-wide importance (Balashov, 1972; Bram, 1975; Steelman, 1976). Ticks are responsible for severe losses caused by either the effect of the tick through mortality or debility due to the diseases transmitted, blood loss, damage to the hides and udders, tick worry, the injection of toxins and low weight gain (FAO, 1984; Sutherst et al., 1979; Gothe, 1981). Of all external parasites that infest livestock, ticks cause the greatest economic losses in the world today, with an estimate of 80% of the world's 1,226 million cattle affected (FAO, 1984; Wellcome, 1980). Although different species of ticks and tick-borne diseases occur in different ecological regions, their impact on the animal production is similar in nature and importance (FAO, 1984). In East Africa, the FAO Livestock Survey (1962; 1967) described the losses as the "single largest drawback to livestock sector development".

Ticks have been shown to transmit several pathogens which cause fatal diseases to livestock. The diseases include; East Coast Fever (ECF) due to Theileria parva parva, Babesiosis caused by Babesia

bovis, Anaplasmosis caused by Anaplasma marginale,
(Steelman, 1976). East Coast Fever, transmitted by
Rhipicephalus appendiculatus is the most important
tick-borne disease in East and Central Africa (FAO,
1984). Where the disease is endemic, for example Coast
Province, 10 to 50% of the calves born are lost from
ECF and other tick-borne diseases annually. The
mortality rate is higher in the exotic and the
susceptible indigenous cattle thus, more than 90% of
cattle die when infected with the disease (Dolan, 1981;
FAO, 1984).

In 1977, there were 5000 cases of ECF confirmed by
microscopic diagnosis of blood samples in Kenya, and an
almost equivalent number of Anaplasmosis cases and less
than 1000 cases of Babesiosis (FAO, 1984). The number
of calves that die from ECF and other tick-borne
diseases is estimated to be 100,000 annually (Duffus,
1976). Osogo (1981) reported that ECF has high
morbidity and mortality rates, estimated to kill one
cow every minute in areas where the disease is endemic
such as Nyanza and Coast Provinces.

Several other investigators have carried out
research to establish the actual mortality levels in
some areas in East Africa. In one of the most detailed
studies done in Kenya, Barnett (1957; 1961) selected
two areas, at Lela (Nyanza Province) and at Bungoma
(Western Province). At Lela, Barnett (1957; 1961)

observed 461 Zebu calves over a period of 4 years. On average 28.6% of the calves died annually, with no significant variation over the years. A similar proportion of mortality was observed at Bungoma, where on average, 29% of calves died annually. In the two areas, ECF due to R. appendiculatus contributed 80% to the total calf mortality. Exotic cattle introduced into the two areas all died of ECF and other tick-borne diseases a few days after exposure. McCulloch et al. (1968) carried out a similar study in Sukumaland, Tanzania and reported that 45.7% of the calves and 9.0% of the adult population was lost through mortality in the ECF enzootic regions. A smaller proportion of cattle, 4.3% calves and 5.1% adults, died in the regions where tick-borne diseases were less endemic. Ferguson and Poleman (1973) reported further that calf mortality ranged from 10 to 50% in tick endemic areas where tick control is not practised. A heavy tick infestation hinders and shunts the growth of calves and thus reduces their potential as future milk producers (FAO, 1984).

Ticks penetrate the hides of cattle during the process of feeding and the lesions cause formation of scars. When the hide is tanned, the scar tissue disfigures its surface grain, thus reducing its value by 10% (Sutherst et al., 1986). In Australia for instance, consistently heavy infestations of Hereford

cattle by Boophilus species cause loss in economic
value of hides (Sutherst et al., 1986). Injury
following tick bites may result in severe secondary
infections; such as by Dermatophilus or Chrysomyia or
the injury may result in loss of one or more quarters
of the udder (Sutherst et al., 1986). Livestock lose a
lot of blood due to the feeding ticks leading to
anaemia. Three-host adult female ticks, for instance,
account for 60 to 80% of the total amount of blood
taken from a host (Sutherst, 1981). Animals also
suffer from "Tick worry" a condition in which hosts
constantly carry large populations of ticks on various
parts of the body despite the absence of diseases.
Livestock in this state, seldom enjoy good health
(Wellcome, 1980) as they spend a lot of time grooming
rather than foraging (De Castro et al., 1985).

While feeding, ticks inject toxins into their
hosts causing either paralysis such as by Ixodes
rubicundus, Ixodes holocyclus and Dermacentor
andersoni, or sweating sickness caused by Hyalomma
truncatum (Bezuidenhout and Malherbe, 1981) or general
toxicosis sometimes leading to death due to
R. appendiculatus and R. evertsi evertsi (Gregson,
1970, 1973; Gothe, 1981; FAO, 1984).

Boophilus microplus is responsible for 0.0007 Kg
weight loss per female tick completing engorgement
(FAO, 1984). The population of ticks may vary from

10,000 to more than 100,000 ticks per host per year
hence the associated estimated annual weight losses for
B. microplus therefore, range from about 7.0 to more
than 70.0 Kg (FAO, 1984). With large ticks such as
Amblyomma species (FAO, 1984) about five engorging
female ticks per day cause serious losses in unexposed
cattle, weight losses ranging from 7.0 to more than
70.0 Kg.

The United States Department of Agriculture (1965)
estimated that tick-induced losses were US$60,000,000
annually to cattle production and US$700,000 annually
to sheep production in the United States. Earlier than
1906 it had been estimated that B. microplus and the
transmission of Babesia bigemina cost the United States
cattle industry US$100,000 anually (Steelman, 1976).
More recent studies by Rinkanya and Tatchell (1988)
showed that global losses of livestock due to ticks
and the cost of control of ticks by use of acaricides
is in the order of US$7,000,000,000 annually.

Rhipicephalus appendiculatus is the chief vector
of T. parva parva, the pathogen which causes ECF. It
also transmits T. parva lawrencei the causative agent
of "Corridor" disease in cattle which may be fatal;
Theileria taurotragi, Ehrlichia bovis, Rickettsia
conori and Nairobi Sheep disease virus (FAO, 1984).
Severe toxaemia results from heavy infestations of
hosts by R. appendiculatus. This condition leads to

reduced host immunological competence and resistance to other infections and may result in death (FAO, 1984).

Rhipicephalus evertsi evertsi transmits B. bigemina, T. parva parva and also transmits Borrelia theileri of both cattle and horses and R. conori (FAO, 1984). While feeding, R. evertsi evertsi secretes toxins which cause paralysis in lambs (Gothe, 1981; FAO, 1984).

Amblyomma variegatum is the principal vector of Cowdria ruminatium and also transmits T. mutans, T. velifera, Coxiella burneti and R. conori (FAO, 1984). Amblyomma variegatum and other African Amblyomma species are the etiological agents of cutaneous streptothricosis caused by Dermatophilus congolensis (Norval, 1976). Amblyomma variegatum also transmits several livestock viral infections such as Dugbe, Nairobi sheep-disease, Crimean-Congo haemorrhagic fever (Nairovirus), Thogoto, Bhanjia and Yellow fever (Flavivirus) (FAO, 1984; Wellcome, 1980). The long mouthparts of Amblyomma cause abscess formation which may lead to udder damage and serious secondary infections (FAO, 1984).

1.2: Control of ticks

Tick control can be achieved by attacking one or more instars in the life cycle. The most appropriate control method varies according to the species of tick.

Total reliance on any one method of tick control often fails to provide stable, long term control (FAO,1984; Wharton and Roulston, 1970; Norval, 1979).

Several methods have been employed in attempt to control ticks. These include, acaricide application and biological control. The current and most common method used to control ticks is the acaricide application (Wharton, 1976; Solomon, 1983; Matthewson, 1984). Acaricides are usually applied topically, by dipping the animals, running them through spray races, hand spraying or hand dressing. These practices are carried out as often as two times a week (Wellcome, 1980; FAO, 1984).

There are many drawbacks associated with the use of acaricides in an attempt to control ticks. The rigorous application of acaricides has led to the development of acaricide resistant ticks (Newton, 1967; Wharton and Roulston, 1970; Wharton, 1976; Norval, 1979; Solomon, 1983). Development of new acaricides with different formulation is expensive (Cunningham, 1981). This drawback poses a threat to livestock health and production in many areas of the world. Resistance shows its highest incidence in one-host tick of the genus Boophilus probably because a much larger fraction of the total tick population of such a species is under chemical challenge at any one time than two-

host or three-host ticks (Wellcome, 1980; Wharton and
Roulston, 1970).

Resistance to acaricides has also been confirmed
in Rhipicephalus sanguineus, B. decoloratus, B.
microplus, R. appendiculatus and R. evertsi evertsi
(Wharton and Roulston, 1970). In addition, all
acaricides may lead to environmental degradation and
incorporation of their residues in the animal products.
Apart from being toxic, acaricides are also expensive.
For instance, in the period between 1971 and 1976, the
value of dip rose from KSh.13.3 to KSh.30.1 million, an
average increase of 14.5% (Kenya Statistical Abstract,
1978). Moreover, acaricides are cumbersome to use and
time consuming. Besides, there are expensive demands
on the management of their application. Thus, the
labour force involved and the maintenance of the
livestock dips require a considerable capital
expenditure.

Biological control of ticks is another method that
has been employed to a limited extent even though its
potential utility in tick control is still speculative.
The method includes the use of sterile-male-release
technique; habitat modification and use of natural
enemies of ticks. The sterile-male-release technique
may be effected through mass release of irradiated
males which inseminate wild females with sperms
incapable of effecting normal embryogenesis and as a

result, eggs so "fertilized" fail to hatch. The use of sterile-male-release technique has proved extremely effective in the control of certain insect parasites of veterinary importance (Matthewson, 1984).

Sterilization in ticks can be achieved by irradiation and has been demonstrated in a number of species such as Hyalomma anatolicum excavatum (Beuthner, 1975; Srivastava and Sharma, 1976); R. appendiculatus (Beuthner, 1975; Purnell et al., 1972); Amblyomma hebraeum (Spickett, 1978); Amblyomma americanum (Darrow et al., 1976); Haemaphysalis longicornis (Fujisaki et al., 1974; Han et al., 1971) and B. microplus (Han et al., 1971). This method is difficult to use for tick control because ticks are more difficult to breed in large numbers and being less mobile cannot assist much in their own dispersal after release. In addition, female ticks can be mated a number of times with different males so an infertile mating will not influence the productivity of the female if she is mated later by a fertile male (Matthewson, 1984).

Habitat modification through pasture spelling has been utilized as a way of manipulating the environment to control ticks. This technique involves starving the ticks to death by depriving them of a host. This is accompanied by fencing the grazing areas to keep the hosts off the pastures until such time as the ticks are

considered dead. This method has been successful against one-host tick, Boophilus species where only the larvae have to be dealt with (Ellenberger and Chapin, 1919; Wilkinson, 1955; Hair and Howell, 1970; Sutherst and Comins, 1979). Fencing is rarely adequate and there is a risk of substantial losses in production if pastures are left ungrazed for a long time. Another problem associated with pasture spelling is over-grazing in the holding paddock (Hair and Howell, 1970). This method is not practical for control of ticks that feed on more than one host to complete their cycle.

Burning of grass contributes to tick control by lowering the tick populations (Milne, 1944). However, it is not efficient enough to be used as the sole means of tick control because, its effects though severe, are short-lived and infested animals soon contaminate burnt areas. Burning also encourages desertification and indiscriminate elimination of other non-target, beneficial arthropods.

Natural enemies of ticks have been utilised to control them. The most popular though often less successful, has been the use of predators, parasitoids and pathogens (Laird, 1980). This use of predators, parasitoids and pathogens in the area of tick control has been examined but never seriously practiced presumably because of the associated problems in rearing or otherwise procuring the predators,

parasitoids or pathogens in sufficient quantities to
distribute over the environment. Examples of tick
predators are found within a number of insect groups
such as hymenopteran wasps, Hunterellus hookeri (Cheong
and Rajamanikam, 1978), the fire ant, Solenopsis
geminata (Butler et al., 1979) and Solenopsis invicta
(Oliver et al., 1979) that parasitize ticks. However,
the effort to utilize them as biological control agents
for ticks is yet to be investigated. The red-billed
and yellow-billed oxpeckers, Buphagus erythrorhynchus
(Bezuidenhout and Stutterheim, 1980; Stutterheim and
Stutterheim, 1980) are reported to be predators of hard
ticks in Southern Africa whilst predation by lizards
has also been implicated (Norval, 1976). Predators
tend to be poor control agents because they are rarely
sufficiently prey-specific and if they are then, their
own numbers are too closely bound and dependent upon
those of their prey (Matthewson, 1984).

Several plant species have been used to control
haematophagous arthropods. Molasses grass, Melinis
minutiflora and gamba grass of Anthropogon species
reduce tick survival with low infestation on cattle
(Thompson et al., 1978). Some highly productive and
nutritious varieties of the tropical perennial pasture
legumes, Stylosanthes species are covered with
glandular trichomes which secrete viscous fluids

(Sutherst et al., 1982). These fluids immediately immobilize larvae of B. microplus when they come across the cultivar and are killed in the toxic vapour (Sutherst et al., 1982). The problem with this method is that, many varieties of Stylosanthes species are susceptible to fungal infections, making it difficult to choose the right one to utilize in tick control (Sutherst et al., 1982). Zimmerman et al. (1984) reported similar results with A. variegatum larvae which were poisoned within 24h by vapour from the secretions of Stylosanthes. The legumes not only trap the questing ticks but also improve cattle nutrition, a factor which is important in their immune response to ticks (Sutherst et al., 1982).

1.3: Host resistance

Host resistance may be defined as the animal's ability to allow fewer numbers of ticks to attach, prolong their feeding duration and reduce engorgement weights, egg mass weights, percentage hatchability, moulting of larvae and nymphs as well as the reproductive potential of female ticks (Wharton and Roulston, 1970). Resistance, therefore, seems to offer a potential means of tick control. The idea here is to manage tick population by utilizing host resistance as a major mortality factor (Wharton and Roulston, 1970).

Acquired resistance by cattle to tick infestation was first reported by Johnston and Bancroft (1918). They observed that some cattle in their herd persistently carried fewer ticks than others. Since then, several other investigators have reported on the acquired immunity of hosts to tick infestation (Trager, 1939a,b; Kelley, 1943; Riek, 1962; Wagland, 1975, 1978; Allen et al., 1977; Dipeolu and Harunah, 1984; Clarke et al., 1989; Fivaz and Norval, 1989; Newson and Chiera, 1989). Cattle resistance to tick infestation has been reported to consist of innate and acquired components. The innate component has been shown to vary with different breed of cattle (Kelley, 1943). Bos indicus or those with a significant B. indicus genetic background have been shown to be more innately resistant to tick infestation than cattle of Bos taurus genetic composition (Kelley, 1943; Riek, 1962; Francis and Little, 1964; Wharton and Roulston, 1970). However, Wagland (1975; 1978) reported that B. taurus and B. indicus cattle not previously exposed to B. microplus were found to be equally susceptible to infestation. Although tick resistance might have an innate component which seems to vary with breed differences, a significant component of tick resistance is acquired (Branagan, 1974; Allen et al., 1977; Wagland, 1975; Kemp et al., 1976; Willadsen et al., 1978). Riek (1962) observed that B. microplus applied

to tick susceptible animals did not produce visible
signs of host reaction to tick attachment sites.
However, ticks applied to resistant animals elicited
strong cutaneous reactions which caused the animals to
groom the tick attachment sites vigorously.

Bos taurus, B. indicus and cross bred cattle
repeatedly infested with A. americanum acquired
immunity (Riek, 1962). This was manifested by a
reduction in the number of females engorging and
decrease in the engorged weights (Riek, 1962). These
results were found to be comparable with those found
for B. microplus. However, it was reported that many
of the ticks which did not engorge fully died on the
host at various ages and levels of engorgement
(Strother et al., 1974). Similarly, cattle on exposure
to I. holocyclus were shown to acquire an immunity that
results in removal of the ticks by grooming, death of
the ticks in situ or reduction in engorgement weights
(Doube and Kemp, 1975). Significant immunity was also
expressed by cattle towards each instar of the three-
host tick, H. longicornis (Sutherst et al., 1979).
Highly resistant Bos taurus cattle rejected upto 90%
of H. longicornis larvae. Wagland (1979) obtained similar
results on Brahman cattle with B. microplus. He showed
that resistance though gradual, was directed against
all instars of the tick. The weights of fully engorged
female ticks fed on immune B. taurus cattle were

reduced by 30% (Wagland, 1978). These kinds of responses therefore, are presumed to be immunologically mediated (Roberts, 1968b).

Sheep and goats resistance to tick infestation has received very little attention in Kenya. However, Wishitemi (1983) reported that the East African Red Maasai sheep acquired resistance by repeated tick infestations with R. appendiculatus. The acquired resistance was manifested by a significant reduction in percentages of nymphs and adult R. appendiculatus engorging, their weights, moulting, eggs batch sizes and hatchability with successive infestations. Similar observations were reported by Maranga (1983) in the local East African and Toggenburg breeds of goats, but the levels of resistance appeared to vary with the different breeds.

Many studies have established that cattle acquire resistance to tick infestation. However, the use of cattle in an attempt to characterize host resistance to ticks is not as practical as using laboratory animal species as hosts. The well characterized immunity response of most laboratory animal species permits the use of many immunomanipulations which would not be practical with cattle as hosts in the initial studies (Wikel and Allen, 1982).

Several authors have reported the acquisition of resistance to the feeding of ixodid ticks by laboratory

animal hosts (Trager, 1939a,b; Gregson, 1941; Kohler et
al., 1967; Allen, 1973; Branagan 1974; Bagnall, 1975;
Wikel and Allen, 1976a,b, 1977; Wikel et al., 1978).
The pioneering observations regarding laboratory animal
resistance to ixodid tick infestation were those of
Trager (1939a). He noted that guinea pigs acquired
resistance after a single infestation with Dermacentor
variabilis and the resistance was manifested by
reduction in the number of ticks reaching full
engorgement during subsequent infestations and those
which reached full engorgement obtained a reduced blood
meal. With the systemic nature of host resistance
established, Trager (1939b) attempted to passively
transfer resistance by the use of serum from tick-
resistant guinea pigs to tick naive-guinea pigs.
Recipient animals allowed 1 to 70% of the larvae to
engorge while the control animals allowed from 41 to
76% of the larvae to engorge.

Allen (1973) reported that guinea pigs developed
marked resistance after three consecutive 7-day
infestations as manifested by significantly fewer
larvae engorging during the second and third
infestations. Rabbits developed resistance to the
feeding of nymphs of Haemaphysalis leporispalustris
(Boese, 1974). Bagnall (1975) infested guinea pigs
with larvae of I. holocyclus and demonstrated the
development of resistance response which was lethal to

the ticks. Wikel (1976) showed that one 5-day
infestation with 100 D. andersoni larvae would induce a
solid resistance which was expressed during the second
infestation.

Dipeolu and Harunah (1984) reported that rabbits
exposed to larvae, nymphs and adults of A. variegatum
acquired resistance to the subsequent infestation with
adults of this tick species. The acquired resistance
was lowest in rabbits exposed once to larvae and
highest in those exposed twice previously to adults.
In another experiment, Dipeolu and Harunah (1984)
showed that rabbits also acquired resistance after
primary infestation with B. decoloratus. Resistance
reached a climax during the third repeated feeding.
Den-Hollander and Allen (1985) assessed the ability of
D. variabilis larvae to feed on mice during four
repeated infestations using known numbers of larvae.
In secondary infestations larvae feeding appeared to be
enhanced, but in subsequent infestations the mice
expressed acquired tick resistance, manifested by
reduction in numbers and weights of engorged larvae.

Although the studies cited above were conducted
using laboratory animal hosts, the potential for this
type of control in cattle may be feasible. Despite all
these studies, no single host-tick interaction is
clearly understood (Willadsen, 1980). Rechav and Dauth
(1987) showed that repeated infestation of rabbits with

R. appendiculatus larvae evoked a typical response to
antigenic challenge resulting in reduction in engorged
weights of larvae and nymphs fed on resistant rabbits.
Rabbits acquired resistance accompanied by anti-tick
antibodies when repeatedly infested with different
numbers of R. evertsi evertsi larvae (Njau et al.,
1988). The resistance was associated with a drastic
reduction in the number of ticks that attached and
reduced to below 50% the proportion of nymphs which
emerged from the larvae. Anti-tick antibodies were
detected by enzyme-linked immunosorbent technique as
early as 7 days after primary infestation in all hosts.
It has been demonstrated that rabbits develop strong
immunity to infestations with three life stages of
R. appendiculatus (Nyindo et al., 1989). Immunity
following larval infestation was shown to be less
potent in limiting tick feeding of nymphs and adults.
Successive infestations of rabbit hosts by the instars
of R. appendiculatus resulted in a progressive decline
in engorgement and egg weights of adult females and a
reduction in percentage recovery of nymphs and larvae
(Fivaz and Norval, 1989). Successive infestations with
Rhipicephalus zambeziensis caused a more decline in
engorgement and egg weights of adults than for R.
appendiculatus. Percentage recoveries of larvae and
nymphs were also significantly reduced (Fivaz and
Norval, 1989). Cattle repeatedly infested with R.

appendiculatus nymphs acquired resistance manifested by shorter feeding periods and correspondingly reduced engorged weights of the challenge nymphs (Chiera and Newson, 1989).

1.4: Mechanisms of host resistance to tick infestation

Acquired resistance to ixodid tick infestation has an immunological basis involving antibody, cell mediated, complement effector mechanisms and hypersensitivity reactions (Willadsen, 1980; Wikel, 1982b).

During the course of tick feeding, foreign material (antigens) is injected into the host along with the saliva (Krolak et al., 1982). This results in production of circulating antibodies (Allen, 1973; Wikel and Allen 1976a, b). The presence of serum factors with anti-tick activity has been suggested to be an important factor in host immune responses. Cattle and laboratory animals have been shown to develop circulating antibodies to tick antigens (Willadsen, 1980; Wikel, 1983, 1984; Wikel and Whelen, 1986). Roberts and Kerr (1976) reported that plasma collected from cattle with high resistance to B. microplus if passively administered to naive calves conferred a significant degree of resistance to a challenge infestation. They transferred plasma from highly immune, poorly immune or unexposed cattle to

groups of unexposed calves which were then exposed to ticks. The numbers of adult females engorging on the last two groups were not significantly different, but on the first group, only about half the number of ticks matured and the difference was statistically significant.

Several other investigators have reported the production of antibody in response to tick infestation. Rabbits have been shown to develop an IgG antibody to H. longicornis (Fujisaki, 1978). Precipitating antibodies have been found in rabbits with H. anatolicum excavatum and R. sanguineus (Kohler et al., 1967).

Acquired resistance to ixodid tick infestation can be adoptively transferred using several laboratory animal-tick associations (Willadsen, 1980; Wikel, 1983, 1984; Wikel and Whelen, 1986). Bagnall (1975) adoptively transferred resistance with viable lymphocytes from syngeneic guinea pigs. The passive transfer of serum from guinea pigs primed the recipient animal to respond with a mild cutaneous reaction. However, the rejection of larvae from serum recipients was very slight when compared to resistant controls. Adoptive transfer of both viable lymphocytes and serum from resistant animals gave an enhanced response compared to either component alone. This observation

suggested a synergistic effect for humoral and cellular effector mechanisms in the expression of resistance.

Antibodies, inhibit the feeding enzymes of ticks. Tracey-Patte (1979) showed that the activity of the enzyme esterase from B. microplus secreted into the hosts' skin within one hour of attachment, can be removed by a host previously exposed to the tick. In unexposed hosts, removal does not occur.

The use of cobra venom factor to deplete complement was shown to block acquisition of tick resistance (Wikel and Allen, 1977). This observation strongly suggests that complement is essential for acquisition of resistance. Parasites activate complement using either the classical or alternative pathways (Santoro et al., 1979). The classical complement pathway is suggested to be responsible for acquisition of tick resistance (Willadsen, 1980). However, Wikel (1979) showed that the development and acquisition of tick resistance was similar in guinea pigs totally lacking in complement C4 and immunologically competent controls. This would on the other hand, indicate that the alternative pathway of complement activation was involved in the expression of resistance. The exact pathway of complement activation involved in acquisition of resistance remains uncertain.

Cell-mediated immune mechanisms also play a role in acquisition of resistance. Langerhans cells have been associated with salivary gland antigens at tick attachment sites on tick-resistant animals (Allen et al., 1979). They have been shown to be capable of presenting tick salivary gland antigens to syngeneic lymphocytes. They also showed that destruction of these cells by ultraviolet irradiation impaired acquisition of resistance. Trager (1939b) compared the histology of tick attachment sites on guinea pigs receiving their first infestation of D. variabilis larvae with comparable sites on tick resistant guinea pigs. There were slight changes noted during an initial infestation. There was a haemorrhagic pool at the base of the mouthparts and a "fibrin mass" observed at the site of tick attachment. Histologic examination of tick attachment sites on resistant animals revealed a large inflammatory reaction beneath the tick attachment sites and a marked hypodermal hyperplasia. Neutrophil accumulations at tick attachment sites have been reported by Tatchell and Moorehouse (1970) for R. sanguineus on dogs and by Berenberg et al., (1972) for D. variabilis on albino rats. Resistance to D. andersoni in guinea pigs has been characterized by predominance of basophils in the vesicle and the reaction is typical of cutaneous basophil hypersensitivity response (Allen, 1973).

Bagnall (1975) showed that ticks feeding on resistant guinea pigs contained many leucocytes rather than erythrocytes in their guts. This finding suggested that the alteration in meal composition was due to the fact that ticks attached to a resistant guinea pig fed on a skin site containing basophil infiltrate and a few erythrocytes. Basophils normally comprise less than 1% of circulating leucocytes in rabbits, guinea pigs, cattle and human beings and are not residents of the tissue. Their presence in the dermis and in the intra-epidermal vesicles is the most dramatic feature of the pathology of the tick feeding sites in resistant animals (Askenase, 1977). Histamine appears to be involved in the alterations in tick feeding associated with resistance. It is possible that other basophil, eosinophil and mast cell-associated moieties have an influence on tick attachment sites on cattle (Willadsen and Riding, 1979) and guinea pigs (Wikel, 1982b). Mast cells induce similar responses (Askenase, 1977). Basophils therefore, have an anti-tick role which may reside in their ability to maintain vasopermeability and allow other mediators to enter the site of tick feeding (Wikel, 1982a). Early death may occur because basophil-derived mediators may make the skin unfavourable for attachment possibly due to the development of oedema. Hosts depleted of basophil

express no immunity, so a basophil mediator is required
(Brown and Askenase, 1984). However, Maranga (PhD
Thesis, 1988) showed that there was no skin reaction at
the R. appendculatus attachment sites on goats
characteristic of immediate or delayed type
hypersensitivity response.

Immediate hypersensitivity in response to tick
infestation has been investigated in much detail in the
association between cattle and B. microplus (Riek,
1956; 1962). He showed that cattle exposed to the tick
were intensely irritated by larvae while papular
reactions were seen on feeding nymphs and adults on
resistant cattle. Transient increase in blood
histamine levels was found in exposed cattle during
tick infestation (Riek, 1956; 1962). Intradermal
injection of extracts of B. microplus eggs or larvae
gave immediate oedematous dermal reactions and these
reactions could be passively transferred locally by
serum (Riek, 1962).

Schleger et al., (1976) compared cellular
responses to B. microplus larval attachments on cattle
with various degrees of resistance 3h after the larvae
had attached. There were eosinophil accumulation and
mast cell degranulation in highly resistant cattle.
These were less pronounced in cattle of low resistance
and little of either occurred in unexposed animals.
Neutrophils were found occasionally in 3h lesions, more

commonly in 5h ones and they seemed to be more numerous in cattle of high resistance.

Delayed type hypersensitivty reactions have been elicited by intradermal inoculation of salivary gland antigens from partially fed adult female ticks into tick resistant guinea pigs (Wikel et al., 1978). Using the same antigenic material they were able to stimulate lymphocyte blastogenesis with lymphocytes from immune donors but not from non-immune controls (Wikel et al., 1978).

1.5: Artificial immunization

Artificial immunization of hosts against tick feeding has received considerable attention by several investigators (Willadsen, 1980; Wikel, 1988; Opdebeeck et al., 1988a,b; Jongejan et al., 1989; Nyindo et al., 1989; Willadsen et al., 1989). Artificial induction of host resistance has largely been attempted using extracts of whole ticks or organs of ticks, and it has only been recently shown that more purified antigens have been used to induce host resistance (Willadsen and Kemp, 1988; Opdebeeck et al., 1988b, 1989). Identification of more specific antigens, the use of improved adjuvant systems and manipulation of dose may provide better results and should be explored (Willadsen, 1980; Matthewson, 1984; Wikel, 1988; Willadsen and Kemp, 1988).

Several workers have attempted to immunize hosts
artificially with tick extracts derived from unfed,
partially fed or fully fed ticks (Willadsen, 1987;
Wikel, 1988). Trager (1939a) protected guinea pigs
against challenge of D. variabilis larvae by
administering an extract of whole larvae by
subcutaneous injection. Extracts prepared from
different organs of partially fed female D. variabilis
gave partial protection (Trager, 1939b). Gregson
(1941) reported similar results to those of Trager
(1939b) in two guinea pigs injected with extracts of D.
andersoni.

Bagnall (1975) reported that guinea pigs immunized
with an extract of I. holocyclus were protected 29-69%
against subsequent larvae challenge compared with the
control group. The number of H. anatolicum excavatum
maturing on one rabbit were greatly reduced by prior
injection with salivary gland extract of the tick
(Kohler et al., 1967). Garin and Grabarev (1972)
reported that resistance to challenge infestation with
R. sanguineus was induced in rabbits by immunization
with salivary gland extracts. Brossard (1976)
subcutaneously injected two calves at birth with 100
salivary glands from partially engorged adult female
B. microplus. These calves were infested at 2 and 5
later months and subsequently allowed fewer ticks to
engorge than did two controls. Wikel et al. (1978)

reported that small doses of salivary gland antigens
protected guinea pigs against D. andersoni when
inoculated together with Freunds complete adjuvant
(FCA). Allen and Humphreys (1979) immunized guinea
pigs against D. andersoni with extracts of either
midgut and reproductive organs (antigen I) or all
internal organs (antigen II). Ticks from the host
immunized with antigen I produced significantly fewer
eggs than those from controls. No larvae hatched from
the eggs laid by the immunized hosts. The effects were
more dramatic in guinea pigs immunized with antigen II
since the ticks failed to engorge hence no eggs were
produced. The antigens were prepared from partially
engorged female ticks. Extracts from tissues of unfed
ticks were ineffective, suggesting that important
antigens were produced only during the late development
of the tick. The number of ticks obtained from calves
immunized with antigen I did not differ from controls.
However, engorgement weights, egg mass and number of
larvae emerging were reduced for ticks which fed on
guinea pigs immunized with antigen I.

Rubaire-Akiki and Mutinga (1980a,b) reported that
rabbits inoculated with extracts from R. appendiculatus
larvae were resistant to the feeding of the larvae.
The resistance was manifested by a reduction in the
number of larvae feeding on the immunized rabbits but
there was no observable effect on the feeding

performance of nymphs or adults. McGowan et al. (1980)

observed early feeding lesions on rabbits inoculated

with Amblyomma maculatum male extracts as compared to

the non-immunized animals. Wikel (1981) showed that

guinea pigs injected peritoneally with D. andersoni

female salivary gland antigen in Freund's incomplete

adjuvant (FIA) became resistant. Resistance was

manifested by reduced number of larvae engorging and

the weight of the larvae which did engorge was reduced.

Salivary gland extracts and cement from A. americanum

female ticks emulsified in FIA conferred protection in

guinea pigs which was manifested by tick rejection and

reduced tick weights (Brown and Askenase, 1984).

Johnston et al. (1986) achieved dramatic

protection of between 65-80% in both B. taurus and B.

taurus X B.indicus breeds with fractionated antigens of

B. microplus adult female ticks. The immunity induced

was still evident after 14 weeks of daily challenge

with 1000 larvae. In another experiment, Johnston et

al. (1986) challenged the cattle with 20,000 larvae and

the tick populations on the vaccinated group were

reduced by over 90% compared to a matched control

group. Kemp et al. (1986) immunized three breeds of

European cattle against B. microplus using extracts

from adult female ticks and showed that the moulting of

larvae fed on vaccinated cattle was delayed. On two of

the vaccinated cattle, there was progressive death of

female ticks throughout feeding and upto 60% of the females had damaged guts. Host erythrocytes were also observed to leak through the damaged tick guts. These females either failed to engorge, or if they did, many of them died before egg laying. Males also suffered gut damage. In contrast, the females which survived on the first day on control cattle usually completed engorgement and neither females nor males showed damaged guts.

Vaccines made from gut and gut syngalion tissue dissected from B. microplus produced 87 and 80% protection respectively compared with adjuvant injected controls in cattle against three infestations with 20,000 larvae administered over 14 days (Opdebeeck et al., 1988a). A vaccine from syngalion alone did not protect cattle. Ticks collected from vaccinated cattle produced 95 and 91% fewer eggs respectively, than ticks from control animals. Vaccinated cattle were protected (37%) 7 months after they had been immunized with tick antigens. In another experiment, Opdebeeck et al. (1988b) showed that Hereford cattle immunized with membrane extracted from midgut of B. microplus protected cattle upto 91% against challenge with 3 times 20,000 larval ticks administered at 7 days intervals.

Despite the well documented literature in this field, the immunological nature, location and number of

the tick antigens involved in the acquisition of resitance, as well as the tissue organs involved in their reproduction, have not been clearly described (Willadsen, 1980; 1987). Almost all the studies reported so far have used extracts of either whole, macerated ticks or tick salivary glands as antigens and the latter has been the most common source. Although it is reasonable to expect antigens to be in the salivary glands, this might not be the case, nor need they be confined to this organ (Willadsen, 1980). Riek (1958) suggested further that hyperimmunization with salivary gland antigen (SGA) may result in exaggerated hypersensitivity which may have harmful effects on the host. Berdyev and Khudainazarova (1976) suggested further that repeated exposure of hosts to tick saliva or tick SGA may result in immunotolerance. Immunization with extracts of internal organs that the host has never encountered naturally thus "concealed'' antigens such as the gut and reproductive organs do not only eliminate the risk of hypersensitivity and immunotolerance but also allow natural resistance to continue playing its role without being affected by immunization.

Concealed antigens are suggested to play an important role in immunity to challenge infestation. Galun (1978) suggested that ticks could be controlled using antibodies raised against the moulting hormone.

Mbogo (personal communication), has reported the, success of Betaecdysone moulting hormone in the control of larvae, nymph and adults R. appendiculatus challenge tick instars.

Immunofluorescence staining showed that serum from rabbits infested with both H. anatolicum anatolicum and R. sanguineus had antibodies to antigens in the digestive system as well as the salivary gland (Kohler et al., 1967). Opdebeeck et al. (1988a,b; 1989) have shown that cattle immunized with vaccines extracted from tick syngalion-gut and tick gut were almost totally protected against repeated challenge with B. microplus. Calves were protected against D. andersoni using midgut and reproductive organs as a vaccine, and recovered a significant number of dead and partially fed ticks from vaccinated cattle (Allen and Humphreys, 1979). Wishitemi (1988) showed that sheep vaccinated with solubilized midgut membrane protein and reproductive organs from partially engorged R. appendiculatus female ticks were protected by over 80% following challenge infestation. Live ticks were smaller and laid fewer eggs compared to those from the control group. Maranga (1988) reported that goats immunized with midgut and salivary gland antigens from partially engorged R. appendiculatus female ticks were protected by over 80%. Ackerman et al. (1981) reported that midgut extracts of D. variabilis induced

significant resistance to infestations while whole tick
homogenate did not induce protective reponse. Ackerman
et al. (1981) demonstrated that host antibody was able
to cross the digestive tract of D. variabilis and this
was responsible for delayed attachment, reduction in
engorgement weight, egg mass and lengthened
preoviposition period.

Histological analysis of ticks obtained from
cattle immunized with crude extracts of adult ticks
adjuvanted in FIA showed that the primary immunological
damage to the tick was located in the digestive cells
of tick gut (Johnston et al., 1986; Agbede and Kemp
1986; Willadsen and Kemp, 1988; Maranga, 1988;
Wishitemi, 1988; Morrison, 1989). Ticks dropped from
vaccinated cattle frequently showed abnormal morphology
and were a bright red in colour, caused by damage to
the tick gut and leakage of gut contents into the
haemocoel (Agbede and Kemp 1986; Kemp et al., 1986;
Opdebeeck et al., 1988a,b). Willadsen and Kemp (1988)
showed microscopically that the most striking feature
about the ticks that fed on vaccinated cattle was the
severe damage to the gut cells. This resulted in
sufficient damage to the gut for intact bovine
erythrocytes to appear in the tick haemolymph, giving
the parasite a distinctive red colour (Johnston et al.,
1986; Kemp et al., 1986; 1988; Opdebeeck et al.,
1988a,b). Wikel (1988) showed that guinea pigs

immunized with gut absorptive surface integral membrane proteins from partially engorged A. americanum could stimulate a host response that resulted in damage to the integrity of the gut of the feeding ticks. Willadsen and Kemp (1988) reported that cattle vaccinated with material derived from semi-engorged female B. microplus produced an immunity to the parasite different from the immunity acquired naturally. Tick Gut antigens from partially engorged B. microplus female ticks of molecular weights 89, 000 Daltons was able to induce effective protection in cattle (Willadsen et al., 1989). This was manifested by the decreased survival of ticks on vaccinated cattle and a reduction in engorgement weight and egg laying capacity of the female ticks. Immune sera to the Gut antigen reacted with the surface of the digestive cells in the tick gut. Histologic examination of ticks engorging on vaccinated cattle with crude and purified gut antigens showed that there was destruction of the digestive cells (Willadsen et al., 1989).

Glycoproteins located on the luminal surface of the plasma membrane of the tick B. microplus gut epithelial cells when used to vaccinate cattle were shown to stimulate an immune response that protected cattle against subsequent tick infestation (Rand et al., 1989). Therefore, it is reasonable to suggest that the

"gut based-antigens" may produce better protection against subsequent tick infestations.

1.6: Cross-protection

Control of ticks by cross-protection using concealed antigens supplements the acquired immunity. Animals that express immunity to one species of tick may be partially or completely immune to another species or even genus of tick (McTier et al., 1981). Though livestock are subject to challenge by more than one tick species under natural conditions, there are only a few cases in which cross- protection between tick species have been studied.

Trager (1939a) reported that guinea pigs first infested with either D. variabilis or D. andersoni showed cross-immunity to larvae of the other tick species. Similarly, rabbits first infested with either D. variabilis or H. leporispalustris produced cross immunity to larvae of the other tick species. Cross-resistance has been reported between H. leporispalustris and D. andersoni; A. americanum and D. variabilis in guinea pigs (McTier et al., 1981). Cattle resistant to H. anatolicum anatolicum infestation were also found to be resistant to R. evertsi evertsi (Latif, 1984a). Larbathe et al. (1985) reported that antibodies induced against B. microplus cross reacted with extracts of Stomoxys calcitrans.

Cross-reactive antibodies in serum from ixodid-infestated animals indicating shared antigen determinants has been reported (Whelen et al., 1984; George et al., 1985) and may be involved in cross-protection (McTier et al., 1981). Cross-reaction detected in vitro with lymphocyte proliferation reinforced evidence from skin tests that A. americanum and A. cajennense salivary gland antigens share components (George et al., 1985). Cross-resistance studies between R. appendiculatus and R. pulchellus in cattle and rabbits infested with nymphs revealed that there exists low cross-resistance between these two closely related tick species (De Castro et al., 1989). A cross-infestation study indicated that R. appendiculatus and R. zambeziensis instars share antigenic moieties (Fivaz and Norval, 1989). Shapiro et al. (1989) reported that antisera from R. appendiculatus resistant guinea pigs also recognized some SGA in ticks of Rhipicephalus. pulchellus, R. evertsi evertsi, A. variegatum and A. gemma. A 94-kDa purified from R. appendiculatus appeared to have a broader cross-reactivity (Shapiro et al., 1989). Rabbits infested with R. evertsi evertsi generated significant cross-protection against a challenge with all the three instars of R. appendiculatus and the larvae and adults of A. variegatum (Njau, 1985).

Cross-resistance was manifested by reduction in the number successfully engorging, and reduced weight of those ticks that engorged compared to the controls. The most significant interspecies cross-resistance was found in R. evertsi evertsi infested rabbits challenged with R. appendiculatus (Njau, 1985; Njau and Nyindo, 1987). More recent studies by Jongejan et al. (1989) showed that western blot analysis on salivary gland extract of A. variegatum and R. appendiculatus revealed considerable cross-reactions. There is no study to date on cross-protection in rabbits using midgut membrane-bound proteins from partially engorged R. appendiculatus, R. evertsi evertsi and A. variegatum female ticks.

The objectives of this study were to investigate the possibility of immunization of rabbits with midgut membrane-bound proteins derived from partially engorged R. appendiculatus, R. evertsi evertsi and A. variegatum females, and to assess whether the immunity so elicited was protective against both the homologous and heterologous challenge infestation. It was also the objective of this study to isolate and identify the antigens involved in the protection.

CHAPTER 2

MATERIALS AND METHODS

2.1:   Experimental animals

2.1.1: Ticks

Rhipicephalus appendiculatus, R. evertsi evertsi and A. variegatum ticks were obtained from the laboratory tick colony of the International Centre of Insect Physiology and Ecology (ICIPE).   The ticks were maintained by feeding them in batches, enclosed in ear bags according to the method described by Bailey (1960) on the ears of the white strain New Zealand rabbits (Figure 1).   The ticks were maintained off the host in cotton-plugged vials measuring 75mm by 25mm in a desiccator, over a solution of potassium chloride (Serva Fine Biochemicals Inc., N.Y., USA.) of relative humidity 85% (Chiera et al., 1985) and kept in a Hotpack (Philadelphia, PA., USA) set at 28°C.   The ticks used in these studies were 3 to 4 weeks old.

2.1.2: Rabbits

New Zealand white strain rabbits, weighing 2 to 3 Kg were purchased from Sasumua Estates Ltd., Njoro. The rabbits were housed in twos, in cages measuring 60cm by 60cm each. The rabbits were injected subcutaneously with 1.5 ml of Sulphadimidine (Bimadin(R), Bimada, UK.) to prevent them against

Figure 1. A photograph showing ticks feeding

on the ears of the white strain New

Zealand rabbits enclosed in ear bags

coccidiosis on three consecutive days following their

arrival at the ICIPE.

2.2: Dissection of the midgut from partially engorged
     female ticks

Rhipicephalus appendiculatus, R. evertsi evertsi,

to posterior end on both ends. The dorsal cuticulum

was then removed to expose the midgut. The midgut was

then washed four times with PBS. Using a fine pair of

forceps the clean midgut was collected in Bijou bottles

filled with cold PBS containing 1mM

phenylmethylsulphonyl fluoride (PMSF), 1mM

iodoacetamide, 5mM aprotinine, 10mM glutathione and 1mM

diisopropylfluorophosphate (DFP) as protease inhibitors.

The harvested midguts were then stored at -20°C until

required.

coccidiosis on three consecutive days following their
arrival at the ICIPE.

2.2: Dissection of the midgut from partially engorged female ticks

Rhipicephalus appendiculatus, R. evertsi evertsi
adults were allowed to feed in batches on rabbit ears,
in cloth ear bags (Bailey, 1960) for five days while
A. variegatum were allowed to feed for seven to ten
days. The ticks were harvested by traction using a
fine pair of forceps and washed under tap water on a
sieve. The ticks were dried on blotting paper and
separated into males and females. The female ticks
were partially embedded live on melted paraffin wax,
the ventral side facing down on the wax in a petri-
dish. The embedded ticks were covered with 0.15M
phosphate-buffered saline (PBS, pH 7.2) at 4°C. An
incision was made along the lateral line from anterior
to posterior end on both sides. The dorsal integument
was then removed to expose the midgut. The midgut was
then washed four times with PBS. Using a fine pair of
forceps the clean midgut was collected in Bijou bottles
filled with cold PBS containing 1mM
phenylmethylsulphonyl fluoride (PMSF), 1mM
iodoacetamide, 5mM aprotinine, 10mM glutathione and 1mM
Diisopropylflurophosphate (DFP) as protease inhibitors.
The harvested midguts were then stored at -20°C until
required.

2.3: Antigen preparation

Tick midguts were disrupted by freeze-thawing three times in plastic vials placed in liquid nitrogen at -70^{0}C and ice respectively (Mongi et al., 1986). The disrupted midguts were washed three times in 15 ml homogenization buffer; HB (10mM PBS, 10mM disodium EDTA) composed of protease inhibitors. To the washed midguts 15 ml of HB was added and homogenized three times at 4^{0}C for 30 seconds on a Polytron Setting at an intensity of 6. The homogenate was centrifuged at 8,000 x g for 20 min at 4^{0}C and then separated into supernatant and pellet. The supernatant was designated gut soluble fraction and saved. The pellet was washed five times in 15 ml of HB and centrifuged after every wash at 8,000 x g for 20 min at 4^{0}C. After the last wash the pellet was mixed with 15 ml of HB containing protease inhibitors and 1% Nonident P-40 detergent (Wishitemi, 1988) was added to the mixture and incubated for 15 min on ice. The mixture was homogenized three times for 30 seconds at 4^{0}C. The homogenate was centrifuged free from cells and debris at 45,000 x g for 20 min on a Sorval$^{(R)}$ R-C Automatic Superspeed refrigerated centrifuge (Sorval Instruments, DuPont Company, CT., USA). The pellet was saved and the supernatant was designated "gut membrane-bound protein" (GMBP) later used as the antigen. The GMBP was aliquoted in 1 ml volumes and stored at -20^{0}C until

Figure 2. Diagrammatic representation of the
techniques used to homogenize and
solubilize midgut antigens from
partially engorged R. appendiculatus,
R. evertsi evertsi and A. variegatum
female ticks.

Midguts

Freeze-thaw 3X
centrifuge at 8,000 xg for 20 min
at 4°C.

Pellet-1   Supernate-1 (Saved)

Washed 4 x with
15 ml HB
Centrifuge at 8,000 x g for
20 min at 4°C

Supernate-2   Pellet-2
(saved)

Homogenize 3X for 30 seconds
at 4°C.

Homogenate 1
Centrifuge at 8,000 x g for
20 min at 4°C

Supernate-3   Pellet-3
(saved)

Add 15 ml HB
Plus 1% NP-40
Incubate for 15 min at 4°C
Homogenize

Homogenate 2

Centrifuge at 45000 x g
for 20 min at 4°C

Pellet-4   Supernate-4
(saved)   (Gut membrane bound
                 protein, GMBP)
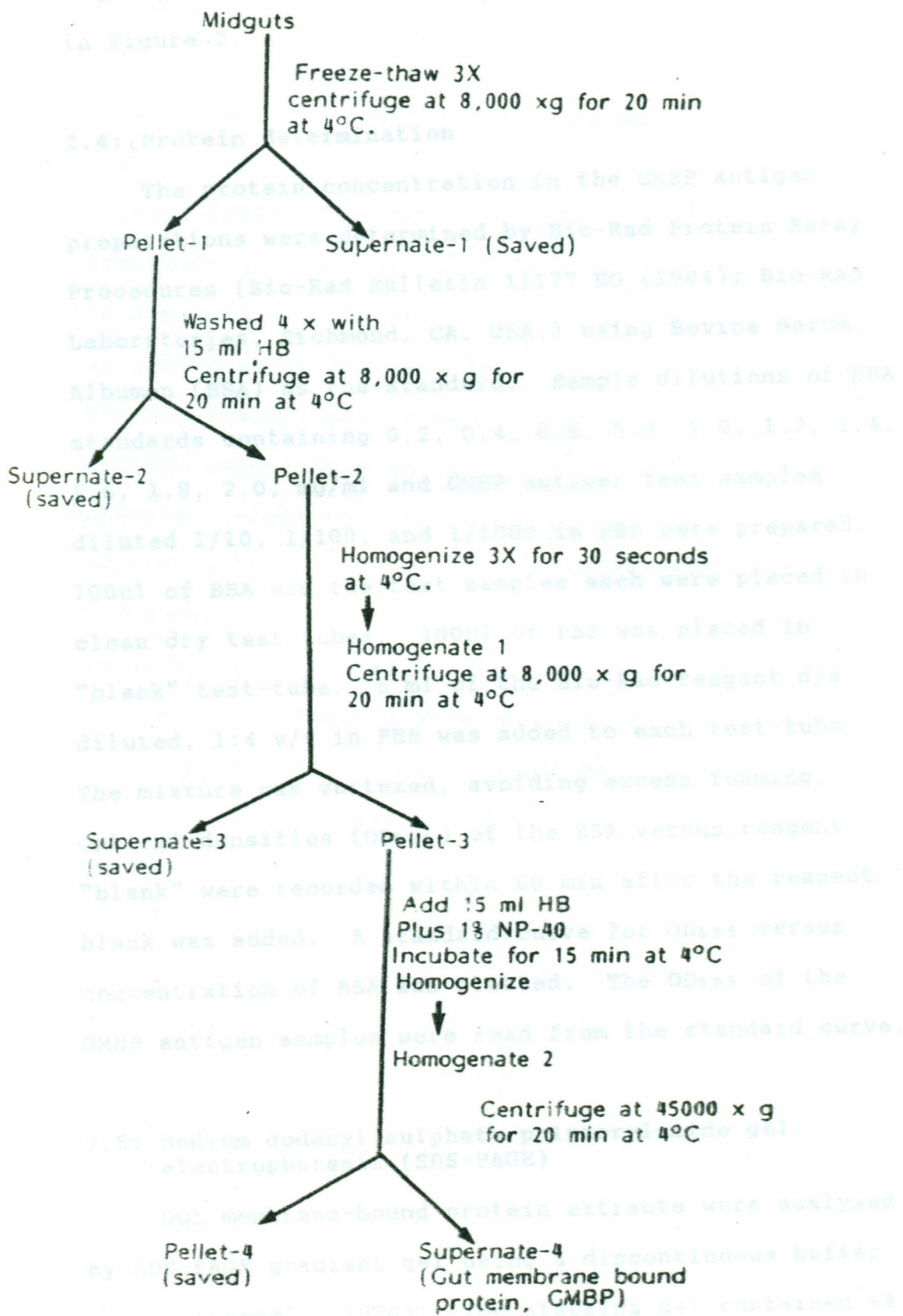
required. The procedure is represented by a flow chart in Figure 2.

2.4: Protein determination

The protein concentration in the GMBP antigen preparations were determined by Bio-Rad Protein Assay Procedures (Bio-Rad Bulletin 11177 EG (1984); Bio-Rad Laboratories, Richmond, CA. USA.) using Bovine Serum Albumen (BSA) as the standard. Sample dilutions of BSA standards containing 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, mg/ml and GMBP antigen test samples diluted 1/10, 1/100, and 1/1000 in PBS were prepared. 100ul of BSA and the test samples each were placed in clean dry test tubes. 100ul of PBS was placed in "blank" test-tube. 5 ml of the Bio-Rad reagent dye diluted, 1:4 v/v in PBS was added to each test-tube. The mixture was vortexed, avoiding excess foaming. Optical densities (OD_{595}) of the BSA versus reagent "blank" were recorded within 60 min after the reagent blank was added. A standard curve for OD_{595} versus concentration of BSA was plotted. The OD_{595} of the GMBP antigen samples were read from the standard curve.

2.5: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gut membrane-bound protein extracts were analysed by SDS-PAGE gradient gel using a discontinuous buffer system (Laemmli, 1970). The stacking gel contained 6%

acrylamide (Whelen et al., 1984) prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N-bis-mythylene acrylamide in 0.5M Tris-HCl buffer of pH 6.8 containing 0.1% sodium dodecyl sulphate (SDS). The separating gel was 5% to 20% acrylamide gradient gel in 1.5M Tris-HCl of pH 8.8 in the presence of 0.1% SDS. The polymerization reaction was quickened by adding TEMED (N,N,N'-N'-tetramethylethylenediamine) as the catalyst and ammonium persulphate as initiator. Samples and the molecular weight markers (lactalbumin, 14,400 Da; trypsin-inhibitor, 20,100 Da; carbonic-anhydrase, 30,000 Da; ovalbumin, 43,000 Da; albumin, 67,000 Da; and phosphorylase-b 94,000 Da) (Pharmacia LKB Biotechnology, Sweden) were mixed in equal volumes with the sample buffer; 630mM Tris-HCl of pH 6.8, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.001% Bromophenol blue as tracking dye (Jongejan et al., 1989) and heated at 100°C for 5 min. The samples were then loaded into the slots on the stacking gel and the SDS-PAGE slab gel was electrophoresed at 10mA until Bromophenol blue tracking dye reached the stacking gel separating gel interface, at which point the current was increased to 20mA. The resolved proteins in the separating gel were stained with 0.1% Coomassie Brilliant Blue R-250 in 35% methanol and 7% acetic acid for 1hr (Jongejan et al., 1989), and destained in methanol:acetic acid:water

(5:2:13 by volume). The molecular weights of the resolved proteins were determined from the molecular weight standard graph.

2.6:   Experimental design

2.6.1: Immunization of rabbits

Three groups of rabbits were immunized as shown in Table 1. Group A1 was immunized with R. appendiculatus GMBP antigen with A2 as control. Group B1 was immunized with R. evertsi evertsi GMBP antigen with B2 as control, while group C1 was immunized with A variegatum GMBP antigen with group C2 as control.

Rabbits were injected intramuscularly in both shoulder and thigh muscles. The antigen diluted in sterile PBS was emulsified in Freunds Complete Adjuvant (FCA; Difco, Detroit, Michigan, USA) in a 1:2 ratio. A total of 1 mg/ml of emulsified antigen was administered per rabbit as a primary dose. The control groups received sterile PBS emulsified in FCA in a 1:2 ratio. Booster injections were administered three times at 2 weeks intervals in Freunds incomplete adjuvant (FIA; Dico, Detroit, Michigan, USA.) containing 1mg/ml of the antigen and sterile PBS for the experimental and control groups respectively.

Table 1. Immunization regimen of rabbits with GMBP antigens from partially engorged
R. appendiculatus, _R. evertsi evertsi_ and _A. variegatum_ female ticks.

Group	No. of rabbits	Antigen Administered
A1 Immunized	15	_R. appendiculatus_ GMBP + FCA
A2 Control	6	Sterile PBS + FCA
B1 Immunized	15	_R. evertsi evertsi_ GMBP + FCA
B2 Control	6	Sterile PBS + FCA
C1 Immunized	15	_A. variegatum_ GMBP + FCA
C2 Control	6	Sterile PBS + FCA

NB. 1. FCA = Freund's Complete Adjuvant

2. PBS = Phosphate-buffered saline

2.7:   Serology

2.7.1: Collection of sera from unimmunized and immunized rabbits

Rabbits were bled two weeks prior to immunization and two weeks after every inoculation. The blood was collected from the central ear vein in universal bottles. The blood was allowed to clot at room temperature for 1hr, followed by 18 hours incubation at 4^0C. Serum was collected by centrifugation at 8,000 x g and stored at -20^0C in 1 ml aliquots until required.

2.7.2: Double Immunodiffusion test

Ouchterlony double immunodiffusion tests were performed in 1% agarose gels (Difco, Detroit, Michigan, USA.) in PBS according to the method described by Ouchterlony (1958; 1964). To enhance precipitation, 3% polyethylene glycol 6000 was added in the agarose (Harrington et al., 1971). The precipitin reaction was allowed to develop for 48 hours in a humid box at room temperature. Then, the slides were washed in 3% trisodium citrate in PBS and 0.1% sodium azide for 18 hours to remove the nonspecific and unprecipitated proteins. The slides were pressed overnight on a flat surface, air dried and then stained with 0.1% Coomassie Brilliant blue R-250 (Weeke, 1973). The slides were then placed in the destaining solution (methanol:acetic acid:distilled water in 5:2:13 ratio) followed by air

drying. The dry slides were labelled and photographed with Panatomic-X film (Eastman Kodak Co., Rochester, N.Y., USA.).

2.7.3: Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA technique was used to determine the antibody titres in immunized rabbits specific for R. appendiculatus, R. evertsi evertsi and A. variegatum GMBP antigens. Optimal reactant concentrations of the antigens and conjugates were standardized by titrations. The procedure followed is a modification from that described by Jongejan et al. (1989). The polyvinyl chloride microtitre plates (Cooke Microtitre plates MZa AR) were coated with 4 ug/ml GMBP antigen in carbonate/bicarbonate buffer, pH.9.6 per well for 2 hours at 37°C and afterwards incubated at room temperature in a humid box for 18 hours. The plates were blocked with 5% fat free milk (FFM) in PBS and 0.05% Tween-20 (Sigma Chemical Co., St. Louis, Missouri, USA.) for 1 h followed by washing 5 times with TPBS. 100ul of antibody samples raised against R. appendiculatus, R. evertsi evertsi and A. variegatum diluted 1:100 in TPBS were added to each well in duplicate and incubated for 2 h at 37°C. The plates were washed 5 times with TPBS. The serum antibody in the microtitre plates was localised by goat anti-rabbit

IgG (Miles Laboratories) conjugated to horse-radish peroxidase (HRP; Nordic Immunological Laboratories, Tilburg, The Netherlands), diluted 1:2000 with TPBS. The plates were incubated for 30 min at 37°C. The plates were then washed 5 times with TPBS. 100ul of 4-chloro-naphthol and hydrogen peroxide (30% w/v) were added to all the wells and mixed on a shaker platform for 30 seconds. The reactions were stopped with 3M sodium hydroxide. The plates were kept in the dark for 30 min after which the optical densities (OD) were read at 449nm on an automatic ELISA Titertek Multiskan M-C plate reader (Flow Laboratories, U.K.). The presence or absence of antibodies was determined as described by Voller et al. (1974, 1976); De-Savigny and Voller (1980).

2.7.4: Western blotting

Rhipicephalus appendiculatus, R. eversti evertsi and A. variegatum GMBP antigens resolved by SDS-PAGE were transferred to nitrocellulose membrane by a method modified from Burnette (1981). Gels were prepared for transfer by soaking them in cold tansfer buffer (25mM Tris, 192mM glycine in 20% methanol, pH 8.3). Nitrocellulose membranes (0.45mm, Schleidner and Schuell, Dassel, West Germany), the Scotch Brite pads and the Whatmann 3MM papers cut to size of the gels were soaked in cold transfer buffer for 30 min before

use. Electrophoretic transfer was carried out at 90 volts for 3 h. At the end of the transfer, the nitrocellulose membranes were stained with Ponceau 2R (100ml of 3% Ticholoroacetic acid; Helena Laboratories Beaumont, Texas, USA.). Thereafter, the blots were cut into the appropriate strips and the ponceau washed off in Tris-buffered saline (TBS). The nonspecific reactive sites on the strips were blocked in quenching buffer (TPBS-FFM 5%) for 1 h at room temperature (25°C) on a rocking platform. The strips were then washed 2 times with distilled water at 5 min intervals followed by 2 times with TPBS at 5 min intervals. The blots were placed in long test-tubes (separately for each serum) and incubated with rabbit-anti-tick serum, diluted 1:500 in TPBS-1% FFM for 4 h on a rocking platform. The blots were again washed 4 times with TPBS at 20 min intervals, followed by 2 times for 5 min each. Subsequently, the binding of antibody to proteins was localised by goat-anti-rabbit IgG conjugated to horse-radish peroxidase (HRP) diluted 1:3000 in TPBS for 2 h, followed by washing 2 times at 5 min intervals, then once for 20 min, and finally, with distilled water for 20 min. Binding of conjugate was visualized by immersing the blots for 5 to 10 min in (0.03%) 4-chloro-1-naphthol (Bio-Rad Laboratories, USA.) substrate diluted 1:10 cold methanol followed by 1.5ul/ml of hydrogen peroxide in TBS. The blots were

finally rinsed in distilled water, air dried and
photographed with Panatomic-X film.

2.8: Challenge infestation of immunized and control rabbits

Ten days after the last booster dose, both the
immunized and control rabbits were infested with all
instars of R. appendiculatus, R. evertsi evertsi and A.
variegatum of homologous and heterologous tick species
(Table 2). In the group immunized with R.
appendiculatus GMBP antigens, 7 rabbits were challenged
with all instars of R. appendiculatus, 7 rabbits with
Rhipicephalus evertsi evertsi and 7 rabbits with A.
variegatum. The same procedure was repeated for the
groups of rabbits immunized with R. evertsi evertsi and
A. variegatum GMBP antigens. 25 female and 25 male
ticks (except for A. variegatum where 6 females and 6
males were used because Amblyomma species take alot of
blood and would kill the rabbits if the same numbers
like R. appendiculatus or R. evertsi evertsi were used)
were applied on the right ear and 50 nymphs plus 100
larvae were applied on the left ear of enclosed in
cloth ear bags. The ear bags were checked every day
(starting from day 1) for the dead, dropped fed, and
unattached ticks. The dead and unattached ticks were
recorded while the engorged ticks were counted and
weighed. The larvae that dropped off the rabbits on a

Table 2. Challenge infestation of rabbits with all instars of R. appendiculatus, R. evertsi evertsi and A. variegatum tick species.

	Tick species used in challenge				
Groups immunized	R. app.	R. eve.	A.var.	No/left ear	No/right ear
R. appendiculatus				25F, 25M	50NN, 100LL
Immunized	5	5	5		
Control	2	2	2		
R. e. evertsi				25F, 25M	50NN, 100LL
Immunized	5	5	5		
Control	2	2	2		
A. variegatum				25F, 25M	50NN, 100LL
Immunized	5	5	5		
Control	2	2	2		

NB. LL=Larvae, NN=Nymphs, F=Females, M=Males, R. app.=R. appendiculatus, R. eve.=R. evertsi evertsi and A. var=A. variegatum.

particular day were all weighed in mass and their mean
weights determined. The nymphs and adults were weighed
individually. Each engorged female tick was placed in
a vial and allowed to oviposit. The weighed ticks were
then placed in a desiccator over saturated potassium
chloride, relative humidity, 85%, and kept in a Hotpack
at 28°C. The larvae and nymphs were allowed to moult
and later the moulting were recorded as percentages
recorded. The egg mass weights of the batches of eggs
laid by each female were recorded and the eggs were
allowed to hatch. The percentage hatchability of the
eggs was determined by counting the larvae that hatched
and, the unhatched eggs, by microscopic examination.

2.9: Manifestation of acquired resistance

The parameters assessed for manifestation of the
acquired resistance during the challenge infestation
experiments included: mortality rates of all instars,
feeding durations, engorgement weights, egg mass
weights, percentage of larvae and nymphs successfully
moulting and percentage hatchability of the eggs
oviposited.

2.10: Statistical analysis of data

Significant differences was tested using student's
t-test and analysis of variance (ANOVA).

CHAPTER 3

RESULTS

3.1: Feeding performance of the three stages of R. appendiculatus, R. evertsi evertsi and A. variegatum ticks fed on rabbits immunized with R. appendiculatus GMBP antigens.

3.1.1: Larval

Rhipicephalus appendiculatus, R. eversti evertsi and A. variegatum larvae infesting rabbits immunized with R. appendiculatus GMBP and the control rabbits were attached by 2 days after infestation and remained attached until they dropped off the host as engorged larvae. The mean feeding durations of homologous R. appendiculatus and heterologous A. variegatum larvae fed on immunized rabbits were not significantly different (P > 0.05) from that of controls.

The mean engorged weights of both the homologous R. appendiculatus and heterologous A. variegatum larvae fed on immunized rabbits were significantly lower (P < 0.05) than that from control rabbits (Table 3). The mean percentage moulting of homologous R. appendiculatus and heterologous A. variegatum larvae fed on immunized rabbits were significantly lower (P < 0.01 and P < 0.05 respectively), than that from control rabbits (Table 3). The mean percentage mortality of homologous R. appendiculatus and heterologous A. variegatum larvae during and after feeding on immunized rabbits were significantly higher (P< 0.001) than that

Table 3. Feeding performance of homologous R. appendiculatus and heterologous A. variegatum challenge larvae fed on rabbits immunized with R. appendiculatus GMBP (mean +SE).

	Parameters Assessed			
Challenge Tick species	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	$4.22^a \pm 0.17$	$0.4^a \pm 0.05$	$69.9^a \pm 1.53$	$61.3^a \pm 10.9$
Control	$3.66^a \pm 0.28$	$0.6^b \pm 0.005$	$96.1^b \pm 1.88$	$30.5^b \pm 17.5$
% Protection	13.2	33	29.7	50.6
Pr>F	0.138	0.0335^*	0.003^{**}	0.0001^{***}
A. variegatum				
Immunized	$6.55^a \pm 0.23$	$2.38^a \pm 0.78$	$75.1^a \pm 2.74$	$69.2^a \pm 8.24$
Control	$6.13^a \pm nd$	$3.01^b \pm nd$	$96.6^b \pm nd$	$15.0^b \pm nd$
% Protection	5.7	21	23.2	78.3
Pr>F	0.5010	0.0310^*	0.032^*	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = No data
4. Percent protection against tick challenge was calculated by comparing the parameter assessed for the ticks dropped from immunized rabbits to the same mean value for the ticks dropped from control rabbits in the same experiment (this applies for all the tables below).

from control rabbits (Table 3). The affected larvae were bright red in colour instead of the normal dark red colour of the larvae fed on control rabbits.

3.1.2: Nymphal

The mean feeding durations of homologous R. appendiculatus and heterologous R. evertsi evertsi nymphs fed on immunized rabbits were significantly longer (P < 0.01 and P < 0.05, respectively), than from control rabbits (Table 4). The mean feeding duration of heterologous A. variegatum fed on immunized rabbits was not significantly different (P > 0.05) from that of control rabbits (see Table 4). The mean engorged weights of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum nymphs fed on immunized rabbits were significantly lower (P < 0.001) than that from control rabbits (Table 4). The mean percentage moulting of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum nymphs fed on immunized rabbits were not significantly different (P > 0.05) from that of control rabbits (Table 4). The mean percentage mortality of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum nymphs fed on immunized rabbits was significantly higher (P < 0.001, P < 0.05 and P < 0.001, respectively), than that from control rabbits (Table 4).

Table 4. Feeding performance of homologous R. appendiculatus and heterologous
R. evertsi evertsi and A. variegatum challenge nymphs fed on rabbits
immunized with R. appendiculatus GMBP (mean +SE)

	Parameters Assessed			
Challenge Tick species	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	5.22^a + 0.011	9.33^a + 0.27	88.7^a + 4.28	39.5^a + 8.8
Control	4.83^b + 0.1	10.5^b + 0.19	100^a + nd	13.0^b + 5.0
% Protection	8.4	11.4	11.3	67.1
Pr>F	0.0273^*	0.0001^{***}	0.1530	0.0001^{***}
R. evertsi evertsi				
Immunized	20.6^a + 0.33	13.7^a + 0.49	74.9^a + 8.33	9.6^a + 6.6
Control	19.4^b + 0.26	17.5^b + 0.34	98.5^a + nd	32.0^b + nd
% Protection	5.8	21.7	23.9	63.2
Pr>F	0.0019^{**}	0.0001^{***}	0.2959	0.0
A. variegatum				
Immunized	5.8^a + 0.006	47.3^a +0.93	85.8^a + 0.54	12.0^a + 3.26
Control	5.6^a + 0.11	68.7^b +1.7	100^a + nd	11.2^a + nd
% Protection	4.1	31.2	14.7	45.1
Pr>F	0.226	0.0001^{***}	0.3500	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly
different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = no data

In addition to the parameters considered above, the affected nymphs were whitish-grey instead of the normal dark grey colour of those nymphs fed on control rabbits.

3.1.3: Adults

The mean feeding periods of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum female ticks fed on immunized rabbits were significantly longer ($P < 0.05$, $P < 0.01$ and 0.001, respectively), than that from control rabbits (Table 5). The mean engorged weights of homologous R. appendiculatus, and heterologous R. evertsi evertsi and A. variegatum female ticks fed on immunized rabbits were significantly lower ($P < 0.001$) than that from control rabbits. The mean percentage reduction in engorged weight was 33%, 30.8% and 31%, respectively (Table 5). The mean egg mass weights laid by homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum female ticks which dropped from immunized rabbits were significantly lower ($P < 0.001$) than that from control rabbits (Table 5). The mean percentage hatchability of eggs laid by homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum female ticks fed on immunized rabbits were significantly lower ($P < 0.001$) than that from control rabbits (Table 5).

Table 5. Feeding performance and fecundity of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum challenge female ticks fed on rabbits immunized with R. appendiculatus GMBP (mean +SE).

	Parameter Assessed				
Challenge Tick species	Feeding duration (days)	Engorgement weight (mg)	Egg mass weight (mg)	%Hatchability	%Mortality
R. appendiculatus					
Immunized	8.38^a + 0.15	288.2^a + 0.01	31.7^a + 0.006	48.9^a + 2.92	10^a + 0.29
Control	7.58^b + 0.15	431.8^b + 0.01	280.3^b + 0.008	85.9^b + 1.59	4^b + 0.14
% Protection	9.6	33	53.5	43.1	60
Pr>F	0.0130^*	0.0001^{***}	0.0001^{***}	0.0001^{**}	0.0001^{***}
R. evertsi evertsi					
Immunized	9.52^a + 0.232	566.4^a + 0.017	254.4^a + 0.013	57.9^a + 2.94	22.7^a + 0.49
Control	8.05^b + 0.143	818^b + 0.018	380.7^b + 0.01	90^b + 1.82	0^b + nd
% Protection	15.4	30.8	33	35.6	–
Pr>F	0.0041^{**}	0.0001^{***}	0.0001^{***}	0.0001^{***}	0.0001^{***}
A. variegatum					
Immunized	20.0^a + 0.86	2362.3^a + 0.018	1044^a + 0.328	45.2^a + 4.56	0
Control	15.4^b + 0.08	3397.2^b + 0.02	2082^b + 0.26	77.5^b + 4.96	0
% Protection	22.9	31	45.5	41.9	0
Pr>F	0.0006^{***}	0.0004^{***}	0.0001^{***}	0.0002^{***}	–

NB. 1. Means in the same column followed by a different letter are significantly different thus, *=at 5%; **=at 1% and ***=at 0.1% level.

2. SE = Standard error of the mean

3. nd = No data

Finally the effect of immunization on ultimate mortality of the female ticks was examined. In both the homologous R. appendiculatus and heterologous R. evertsi evertsi female ticks, mortality of the adult female ticks fed on immunized rabbits was significantly higher (P < 0.001) compared to that from control rabbits . None of the heterologous A. variegatum female ticks fed on both the immunized and control rabbits were reported dead (Table 5). The dead engorged female ticks were black, rather than the usual grey colour of the fully-engorged R. evertsi evertsi and R. appendiculatus females. The appearance of the dead ticks could be interpreted to mean that gut damage and leakage of host blood into the haemocoel had occurred. Some of the female ticks that engorged on immunized rabbits failed to lay eggs in both the homologous and heterologous systems.

3.2: Feeding performance of all instars of R. appendiculatus, R. evertsi evertsi and A. variegatum ticks fed on rabbits immunized with R. evertsi evertsi GMBP antigens.

3.2.1: Larval

Homologous R. evertsi evertsi and heterologous R. appendiculatus and A. variegatum larvae fed on rabbits immunized with R. evertsi evertsi GMBP antigens and the control group were all attached by 2 days after

Table 6. Feeding performance of heterologous R. appendiculatus and A. variegatum
challenge larvae fed on rabbits immunized with R. evertsi evertsi GMBP
(mean +SE).

Challenge Tick species	Parameters Assessed			
	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	4.12^a + 0.32	0.43^a + 0.009	62.9^a + 8.8	49.7^a + 7.6
Control	3.39^a + 0.13	0.62^b + 0.03	98.1^b + 0.3	9.5^b + 13.0
% Protection	18	30.6	33.9	80.8
Pr>F	0.1806	0.0430*	0.0078**	0.0001***
A. variegatum				
Immunized	6.69^a + 0.25	2.49^a + 0.05	58.7^a + 5.2	67.0^a + 9.7
Control	33^a + 0.05	3.81^b + 1.61	95.9^b + 2.9	15.0^b + 2.5
% Protection	5.2	35.0	39.9	76.1
Pr>F	0.2284	0.0001***	0.0062**	0.0001***

NB. 1. Means in the same column followed by a different letter are significantly
different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = No data

challenge infestation. The mean feeding duration of
heterologous R. appendiculatus and A. variegatum
larvaeinfesting immunized rabbits were not
significantly different (P > 0.05) from that of control
rabbits although the values were above control values
as shown in Table 6.

There were high significant differences
(P < 0.001) in the mean engorged weights percentage
moulting and percentage mortality of heterologous
larvae fed on immunized and control rabbits. The mean
engorged weights of heterologous R. appendiculatus and
A. variegatum larvae fed on immunized rabbits were
significantly lower (P < 0.05 and P < 0.001,
respectively), than that from control rabbits (Table
6).

More engorged heterologous R. appendiculatus and
A. variegatum larvae fed on control rabbits moulted to
active nymphs than those from immunized rabbits (Table
4). The mean percentage mortality of the heterologous
R. appendiculatus and A. variegatum larvae fed on
immunized rabbits were significantly higher (P < 0.001)
than that from control rabbits (Table 6). The affected
larvae were bright red in colour, suggesting leakage of
erythrocytes into the tick's haemocoel.

3.2.2: Nymphal

The mean feeding duration of homologous R. evertsi
evertsi and heterologous R. appendiculatus challenge

infestation nymphs fed on immunized rabbits were
significantly longer (P < 0.001 and P < 0.01,
respectively), compared to that of control rabbits
(Table 7). As shown in table 7, the mean feeding
duration of heterologous A. variegatum nymphs fed on
immunized rabbits was not significantly different (P >
0.05) from that of control rabbits. The mean engorged
weights of homologous R. evertsi evertsi and
heterologous R. appendiculatus and A. variegatum nymphs
fed on immunized rabbits were significantly lower (P
<0.001) than that from control rabbits (Table 7).

Reduction in moulting of nymphs into adults
occurred in both the homologous and heterologous
challenge infestation systems. Mean percentage
moulting of homologous R. evertsi evertsi, and
heterologous R. appendiculatus and A. variegatum nymphs
fed on immunized rabbits were significantly lower
(P < 0.001, P < 0.05 and P < 0.05, respectively), than
that from control rabbits (Table 7). The percentage
reduction in mean percentage moulting is presented in
Table 7. The mean percentage mortality of homologous
R. evertsi evertsi and heterologous R. appendiculatus
and A. variegatum nymphs fed on immunized rabbits were
significantly higher (P < 0.01, P < 0.001 and P <
0.001, respectively), than that from control rabbits
(Table 7).

Table 7. Feeding performance of homologous R. evertsi evertsi and heterologous
R. appendiculatus and A variegatum challenge nymphs fed on rabbits
immunized with R. evertsi evertsi GMBP (mean +SE)

Challenge Tick species	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	5.56^a + 0.13	8.3^a + 0.18	67.2^a + 0.13	38^a + nd
Control	4.83^b + 0.07	12.3^b + 0.26	100^b + nd	4^b + nd
% Protection	7.7	33	33	89.5
Pr>F	0.0078^{**}	0.0001^{***}	0.0290^*	0.0001^{***}
R. evertsi evertsi				
Immunized	19.9^a + 0.39	10.7^a + 0.45	59.1^a + 8.14	88.7^a + 6.04
Control	17.5^b + 0.15	16.8^b + 0.33	92.9^b + 0.49	50.0^b + 4.0
% Protection	12.3	36.4	36.6	43.6
Pr>F	0.0001^{***}	0.0001^{***}	0.0001^{***}	0.0018^{**}
A. variegatum				
Immunized	6.61^a + 0.14	50.4^a + 1.22	75.2^a + 5.2	43^a +10.4
Control	6.39^a + 0.11	64.3^b + 1.21	98.9^b + 1.11	9^a +4.0
% Protection	3.2	22	24	79
Pr>F	0.2490	0.0001^{***}	0.0400^*	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly
different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = no data

3.2.3: Adults

The mean feeding duration of homologous R. evertsi evertsi and heterologous R. appendiculatus female ticks fed on immunized rabbits were significantly longer (P < 0.001 and P < 0.05, respectively), than that from control rabbits (Table 8). The mean feeding duration of heterologous A. variegatum female ticks fed on immunized rabbits was not significantly different (P >0.05) from that of control rabbits (Table 8). The mean engorged weights of both homologous R. evertsi evertsi and heterologous R. appendiculatus and A. variegatum female ticks fed on immunized rabbits were significantly lower (P < 0.001) than that from control rabbits (Table 8). There were significant reductions (P < 0.001) in the mean weights of egg batches laid by both homologous and heterologous female ticks which engorged on the immunized animals compared to the controls (Table 8). The mean percentage egg weights laid by homologous R. evertsi evertsi and heterologous R. appendiculatus and A. variegatum female ticks fed on immunized rabbits were significantly lower (P < 0.001) than that from control rabbits (Table 8). The mean percentage hatchability of eggs laid by homologous R. evertsi eversti and heterologous R. appendiculatus and A. variegatum female tick fed on immunized rabbits were significantly lower (P < 0.001) than that from control rabbits (Table 8). The mean percentage mortality of

Table 8. Feeding performance and fecundity of homologous R. evertsi evertsi and heterologous R. appendiculatus and A. variegatum challenge female ticks fed on rabbits immunized with R. evertsi evertsi GMBP (mean +SE).

Challenge Tick species	Feeding duration (days)	Parameters Assessed			
		Engorgement weight (mg)	Egg mass weight (mg)	%Hatchability	%Mortality
R. appendiculatus					
Immunized	7.42^a + 0.16	295.0^a + 11.02	137.8^a + 5.31	35.3^a + 2.71	29.3^a + 0.26
Control	6.92^b + 0.14	395.9^b + 13.6	203.5^b + 5.63	78.7^b + 2.23	8.0^b + 0.15
% Protection	6.7	25.5	32.5	55.4	72.7
Pr>F	0.0421^*	0.0001^{***}	0.0001^{***}	0.0001^{***}	0.0001^{***}
R. evertsi evertsi					
Immunized	9.87^a + 0.183	592.6^a + 20.7	259.0^a + 12.3	28.5^a + 3.19	22.0^a + 0.47
Control	8.84^b + 0.184	864.5^b + 18.6	401.9^b + 14.5	80.3^b + 2.13	12.0^b + nd
% Protection	10.5	31.5	35.4	64.5	45.5
Pr>F	0.0004^{***}	0.0001^{***}	0.0001	0.0001^{***}	0.0001^{***}
A. variegatum					
Immunized	20.0^a + 1.56	1257.2^a + 69.9	800.1^a + 97.3	35.8^a + 2.71	0
Control	19.7^b + 0.08	3240.6^b + 218	1823.1^b + 138	83.4^b + 2.03	0
% Protection	1.6	61.2	56.1	57.1	0
Pr>F	0.8454	0.0001^{***}	0.0001^{***}	0.0001^{***}	-

NB. 1. Means in the same column followed by a different letter are significantly different thus,
*=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = No data

homologous R. evertsi evertsi and heterologous R.
appendiculatus female ticks fed on immunized rabbtis
were significantly lower (P < 0.001) than that from
control rabbits (Table 8). The adult female ticks
which died after engorgement were black, indicating
that the gut had ruptured with leakage of rabbit
erythrocytes into the tick haemolymph. None of
heterologous A. variegatum female ticks fed on both
immunized and control rabbits died.

3.3: Feeding performance of all instars of R. appendiculatus, R. evertsi evertsi and A. variegatum fed on rabbits immunized with A. variegatum GMBP antigens.

3.3.1: Larval

Both the homologous A. variegatum and heterologous
R. appendiculatus and R. evertsi evertsi larvae fed on
immunized and control rabbits were attached by 2 days
after infestation. The mean feeding periods of the
homologous A. variegatum and heterologous R.
appendiculatus larvae fed on immunized rabbits were not
significantly different (P > 0.05) from the control
(Table 9). However, the mean feeding duration values
for the larvae fed on immunized rabbits were above
those of the control (Table 9).

The mean engorged weights of homologous A.
variegatum and heterologous R. appendiculatus larvae

Table 9. Feeding performance of homologous A. variegatum and heterologous
R. appendiculatus challenge larvae fed on rabbits immunized with
A. variegatum GMBP (mean +SE).

Challenge Tick species	Parameters Assessed			
	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	4.25^a + 0.26	0.42^a + 0.003	62.6^a + 7.14	71.5^a + 9.12
Control	4.03^a + nd	0.61^b + nd	97.9^b + nd	4.0^b + nd
% Protection	5.0	30.4	33	94.4
Pr>F	0.7375	0.0521^*	0.0144^*	0.0001^{***}
A. variegatum				
Immunized	6.67^a + 0.09	1.83^a + 0.127	56.7^a + 3.18	74.2^a + 5.46
Control	6.34^a + nd	2.67^b + nd	99.7^b + nd	15^b + nd
% Protection	4.9	58.9	42	79.83
Pr>F	0.3633	0.0001^{***}	0.0125^*	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly
   different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
   2. SE = Standard error of the mean
   3 nd = No data

fed on immunized rabbits were significantly lower (P <
0.001) than that from control rabbits.

The mean percentage moulting of homologous
A. variegatum and heterologous R. appendiculatus larvae
fed on immunized rabbits were significantly lower (P <
0.05) than that from control rabbits (Table 9). The
mean percent mortality of homologous A. variegatum and
heterologous R. appendiculatus larvae fed on immunized
rabbits were significantly higher (P < 0.001) than that
from control rabbits (Table 9).

3.3.2: Nymphal

The mean feeding duration of homologous A.
variegatum and heterologous R. evertsi evertsi nymphs
fed on immunized rabbits were significantly longer (P <
0.001 and P < 0.05, respectively), than that from
control rabbits (Table 10). The mean engorged weights
of both homologous A. variegatum and heterologous R.
appendiculatus and R. evertsi evertsi nymphs fed on
immunized rabbits were significantly lower (P < 0.01, P
< 0.001 and P < 0.01, respectively) than that from
controls (Table 10). The percent reduction in mean
engorgement weights are shown in Table 10. The mean
percentage moulting of homologous A. variegatum and
heterologous R. evertsi evertsi and nymphs fed on
immunized rabbits were significantly lower (P < 0.01
and P < 0.05, respectively), than that from control

Table 10. Feeding performance of homologous A. variegatum and heterologous
R. appendiculatus and R. evertsi evertsi challenge nymphs fed on
rabbits immunized with A. variegatum GMBP (mean +SE)

Challenge Tick species	Parameters Assessed			
	Feeding duration (days)	Engorgement weight (mg)	%Moult	%Mortality
R. appendiculatus				
Immunized	$5.33^a \pm 0.08$	$9.65^a \pm 0.31$	$63.7^a \pm 6.9$	$43^a \pm 7.8$
Control	$5.08^a \pm 0.13$	$14.16^b \pm 0.32$	$97.9^a \pm$ nd	$4^b \pm$ nd
% Protection	4.6	31.4	34.9	66
Pr>F	0.1342	0.0001^{***}	0.1134	0.0001^{***}
R. evertsi evertsi				
Immunized	$21.8^a \pm 0.32$	$14.7^a \pm 1.22$	$46.2^a \pm 5.3$	$89.3^a \pm 14.8$
Control	$20.2^b \pm 0.34$	$20.1^b \pm 0.43$	$97.8^b \pm$ nd	$38^b \pm$ nd
% Protection	7.6	27	52	57.5
Pr>F	0.0198^*	0.0030^{**}	0.0404^*	0.0001^{***}
A. variegatum				
Immunized	$6.32^a \pm 0.07$	$46.8^a \pm 0.97$	$45.7^a \pm 3.1$	$61.2^a \pm 6.49$
Control	$5.82^b \pm 0.14$	$68.4^b \pm 1.82$	$100^a \pm$ nd	$2.0^b \pm$ nd
% Protection	18	32	54.3	96.7
Pr<F	0.0001^{***}	0.0015^{**}	0.0020^{**}	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly
different thus, *=at 5%; **=at 1% and ***=at 0.1% level.
2. SE = Standard error of the mean
3. nd = no data

groups. The protection was more pronounced in the homologous A. variegatum than the heterologous challenge infestation system (Table 10). The mean percentage moulting of heterologous R. appendiculatus nymphs fed on immunized rabbits was not significantly different (P > 0.05) from that of control rabbits (Table 10).

The mean percent mortality of homologous A. variegatum and heterologous R. appendiculatus R. evertsi evertsi infestation nymphs fed on immunized rabbits were significantly higher (P < 0.001) than that from control rabbits (Table 10).

3.3.3: Adults

The mean feeding duration of homologous A. variegatum and heterologous R. appendiculatus female ticks fed on immunized rabbits were significantly longer (P < 0.05 and P < 0.01, respectively), than that from control rabbits (Table 11). The mean feeding duration of heterologous R. evertsi eversti female ticks fed on the immunized rabbits was not significantly different (P > 0.05) from control rabbits (Table 11). The mean engorged weights of homologous A. variegatum and heterologous R. appendiculatus and R. evertsi evertsi female ticks fed on immunized rabbits were significantly lower (P < 0.01, P < 0.001 and P < 0.001, respectively), than that from control rabbits

Table 11. Feeding performance and fecundity of homologous A. variegatum and heterologous R. appendiculatus and R. evertsi evertsi challenge female ticks fed on rabbits immunized with A. variegatum GMBP (mean +SE).

Challenge Tick species	Feeding duration (days)	Engorgement weight (mg)	Egg mass weight (mg)	%Hatchability	%Mortality
R. appendiculatus					
Immunized	$9.49^a \pm 0.169$	$272.7^a \pm 0.011$	$152.4^a \pm 0.006$	$38.6^a \pm 3.18$	$30^a \pm 0.446$
Control	$8.08^b \pm 0.179$	$424.2^b \pm 0.014$	$236.0^b \pm 0.008$	$86.8^b \pm 1.58$	$4^b \pm 0.14$
% Protection	14.8	35.7	35.4	55.6	66.7
Pr>F	0.0010^{***}	0.0001^{***}	0.0001^{***}	0.0001^{***}	0.0001^{***}
R. evertsi evertsi					
Immunized	$10.3^a \pm 0.26$	$660.9^a \pm 0.017$	$336.8^a \pm 0.013$	$40.9^a \pm 3.41$	$30.7^a \pm 0.6$
Control	$10.1^a \pm 0.25$	$926.3^b \pm 0.021$	$471.5^b \pm 0.019$	$85.9^b \pm 1.77$	$4.0^b \pm nd$
% Protection	1.5	28.7	28.6	52.4	86.7
Pr>F	0.714	0.0001^{***}	0.0001^{***}	0.0001^{***}	0.0001^{***}
A. variegatum					
Immunized	$18.1^a \pm 0.70$	$1837.0^a \pm 0.07$	$617.8^a \pm 0.102$	$12.8^a \pm 4.17$	$8^a +nd$
Control	$14.8^b \pm 0.086$	$3028.3^b \pm 0.13$	$1633.4^b \pm 0.034$	$88.4^b \pm 4.88$	$0^b +nd$
% Protection	17.9	39.3	62.2	85.5	–
Pr> F	0.0421^*	0.0024^{**}	0.0001^{***}	0.0001^{***}	0.0001^{***}

NB. 1. Means in the same column followed by a different letter are significantly different thus,
   *=at 5%; **=at 1% and ***=at 0.1% level.
   2. SE = Standard error of the mean
   3. nd = No data

(Table 11).   The mean egg mass weights laid by
homologous A. variegatum and heterologous R.
appendiculatus and R. evertsi evertsi female ticks fed
on immunized rabbits were significantly lower (P <
0.001) than that from control rabbits (Table 11).   The
mean percent hatchability of eggs laid by homologous R.
evertsi evertsi and heterologous R. appendiculatus and
A. variegatum fed on immunized rabbits were
significantly reduced (P < 0.001) (Table 11).   The mean
percent mortality of homologous A. variegatum and
heterologous R. appendiculatus and R. evertsi evertsi
female ticks fed on immunized rabbits were
significantly higher (P <0.001) than that from controls
(Table 11).

3.4: Sodium dodecyl sulphate-Polyacrylamide gel
     electrophoresis (SDS-PAGE).

     Protein components in the GMBP antigen
preparations from R. appendiculatus. R. evertsi evertsi
and A. variegatum were analysed by SDS-PAGE.
Approximately thirty-seven protein bands were
fractionated from R. appendiculatus GMBP antigen
preparation with molecular weghts ranging from 14,400
to 140,000 Daltons (Figure 3).   Approximately forty-
five protein bands were resolved from R. evertsi
evertsi GMBP antigen preparation with molecular weights
ranging from 14,400 to 140,000 Daltons (Figure 3).
Approximately thirty-nine protein bands were resolved

Figure 3. A photograph showing SDS-PAGE

gradient gel stained in Coomassie

Brilliant Blue. Lane 1= Molecular weight

markers; Lane 2= R. appendiculatus GMBP

Lane 3= R. evertsi evertsi GMBP and

Lane 4= A. variegatum GMBP.

from A. variegatum GMBP antigen preparation with molecular weights ranging from 14,400 to 180,000 Daltons (Figure 3). Rhipicephalus appendiculatus and R. evertsi evertsi were found to share 30 common protein bands. Rhipicephalus appendiculatus and A. variegatum had 23 protein bands. Rhipicephalus evertsi evertsi and A. variegatum shared 24 protein bands. Rhipicephalus appendiculatus, R. evertsi evertsi and A. variegatum shared 22 protein bands (Figure 3).

3.5: SEROLOGY

3.5.1: Ouchterlony double immunodiffusion tests.

3.5.1.1: Rhipicephalus appendiculatus GMBP antigen

Ouchterlony double immunodiffusion reactions with homologous sera against R. appendiculatus GMBP antigen produced 3 to 4 precipitin lines. There were no lines formed with control sera (Figure 4). Anti-sera against R. evertsi evertsi and A. variegatum GMBP antigens each formed a single and bold precipitin line of partial identity among the three tick species (Figure 5).

3.5.1.2: Rhipicephalus evertsi evertsi GMBP antigen

Two to three precipitin lines were formed with homologous sera (Figure 6) while a single and bold precipitin line of partial identity was formed with heterologous anti-sera against R. appendiculatus and A. variegatum. There was no precipitin line observed with the control sera (Figure 7).

Figure 4. A photograph showing Ouchterlony of

antigen-antibody precipitation of R.

appendiculatus GMBP against homologous

immune serum. Where a= R. appendiculatus

GMBP; 1= R. appendiculatus immune serum

and 2= control sera. 3-4 precipitin lines

were formed with immune serum and no line

was formed with control serum.
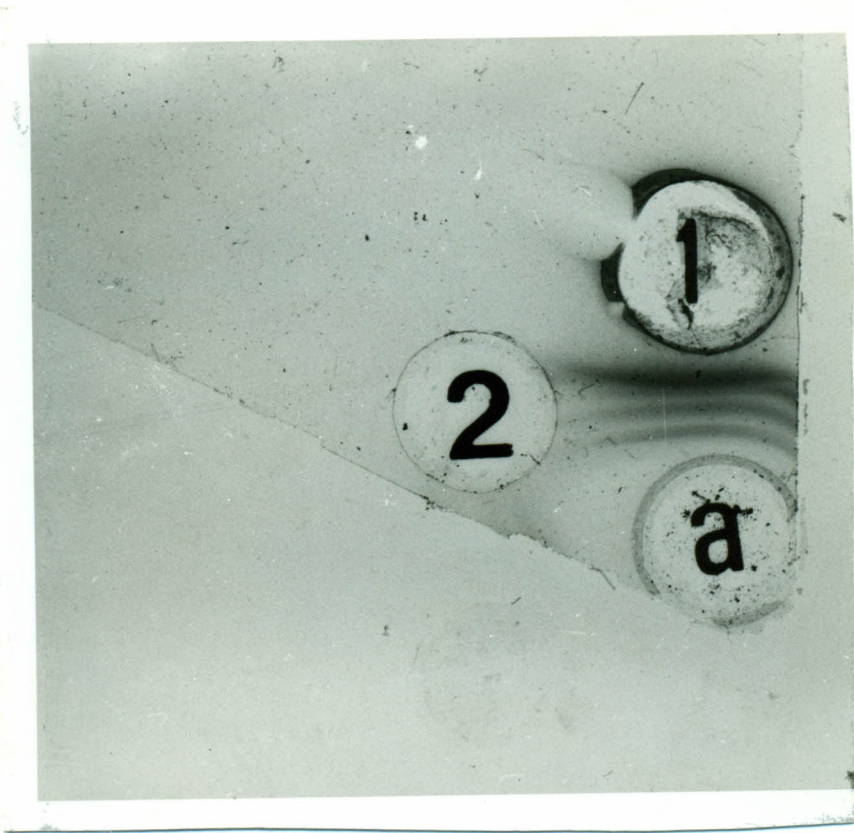
Figure 5. A photograph showing Ouchterlony of

antigen-antibody precipitation of R.

appendiculatus GMBP against homologous

R. appendiculatus and heterologous

R. evertsi evertsi and A. variegatum

immune sera.   Where a= R. appendiculatus

GMBP; 1= R. appendiculatus immune serum;

2= R. evertsi evertsiimmune serum; 3 and

6= A. variegatum and 4 and 5= control sera.

3-4 precipitin lines were formed with

homologous serum, 1-2 with each heterologous

sera and no line with control sera.
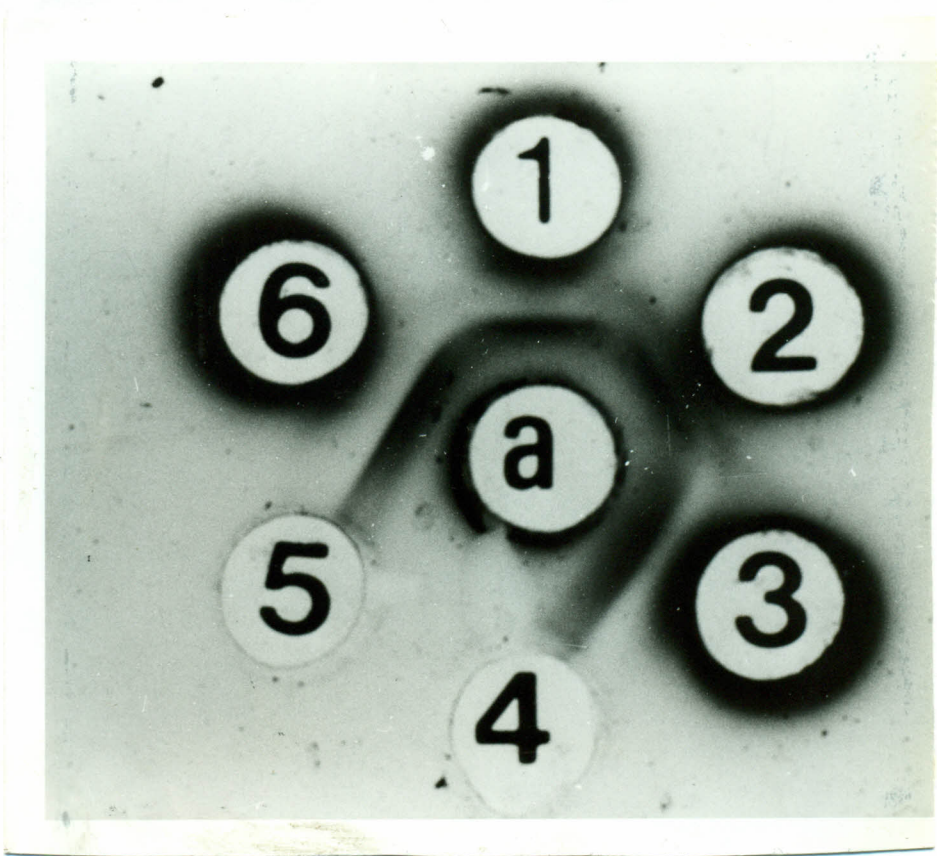
Figure 6. A photograph showing Ouchterlony of

antigen-antibody precipitation of R.

evertsi evertsi GMBP against homologous

serum . Where a= R. evertsi evertsi

GMBP; 1= R. evertsi evertsi immune serum

and 2= control serum. 3-4 precipitin lines

were formed with immune serum and no line

was formed with control serum.
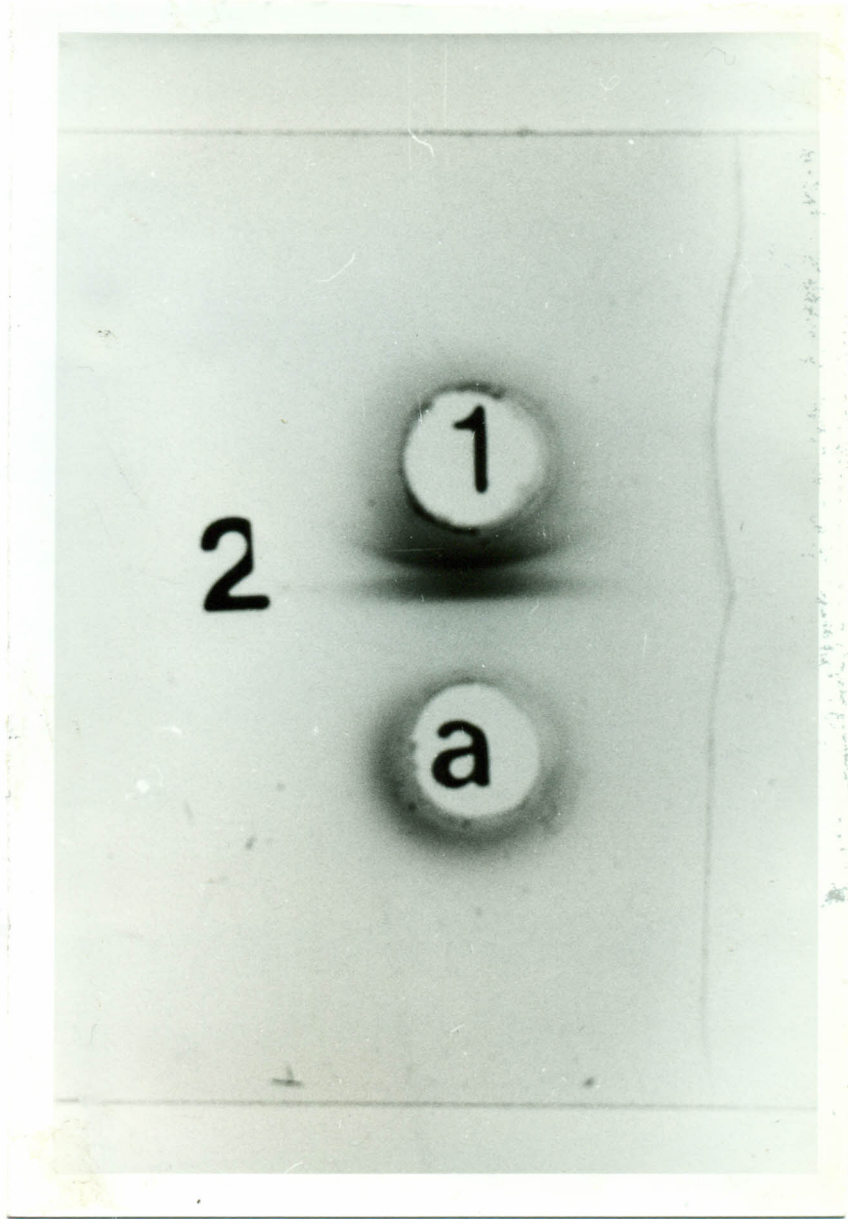
Figure 7. A photograph showing Ouchterlony of

antigen-antibody precipitation of R.

evertsi evertsi GMBP against homologous R.

evertsi evertsi and heterologous

R. appendiculatus and A. variegatum

sera. Where a= R. evertsi

evertsi GMBP; = R. appendiculatus immune

serum; 2= R. evertsi evertsi immune serum;

3 and 6= anti-A. variegatum and 4 and

5= control serum. 3-4 precipitin lines

were formed with homologous serum,

1-2 with each heterologous sera and

no line with control sera.
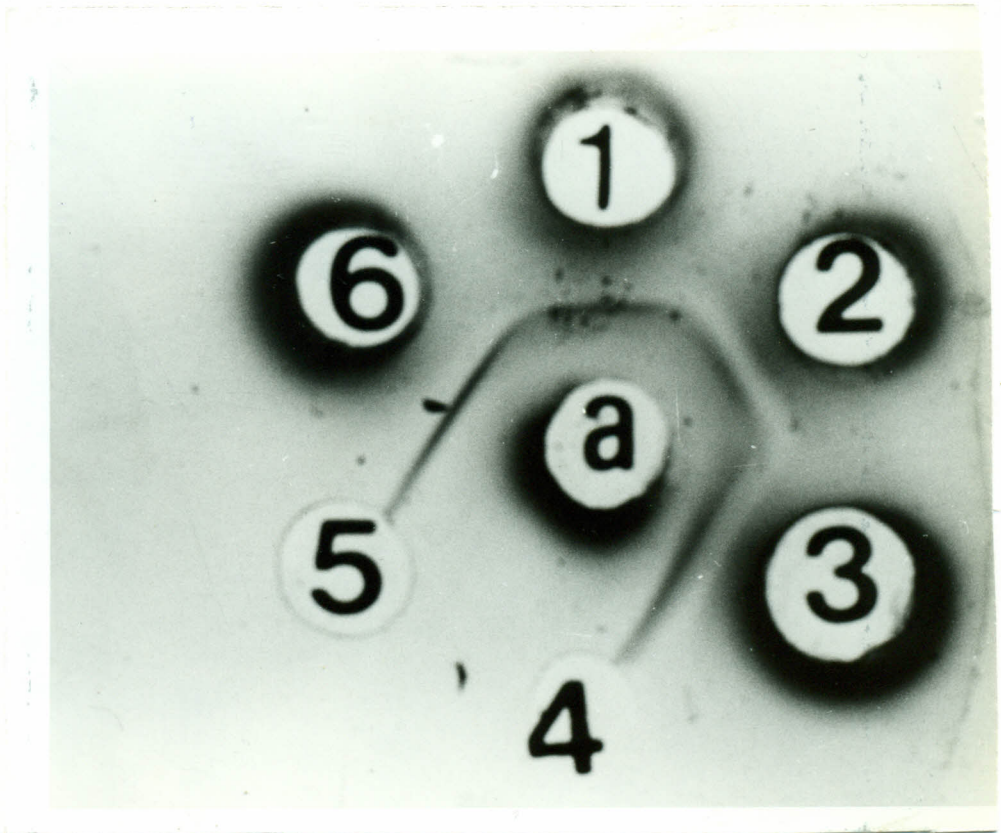
Figure 8. A photograph showing Ouchterlony of
antigen-antibody precipitation of A.
variegatum GMBP against homologous
immune serum. Where a= A. variegatum
GMBP; 1= A. variegatum immune serum and
2= control sera. 3-4 precipitin lines
were formed with immune serum and no line
was formed with control serum.
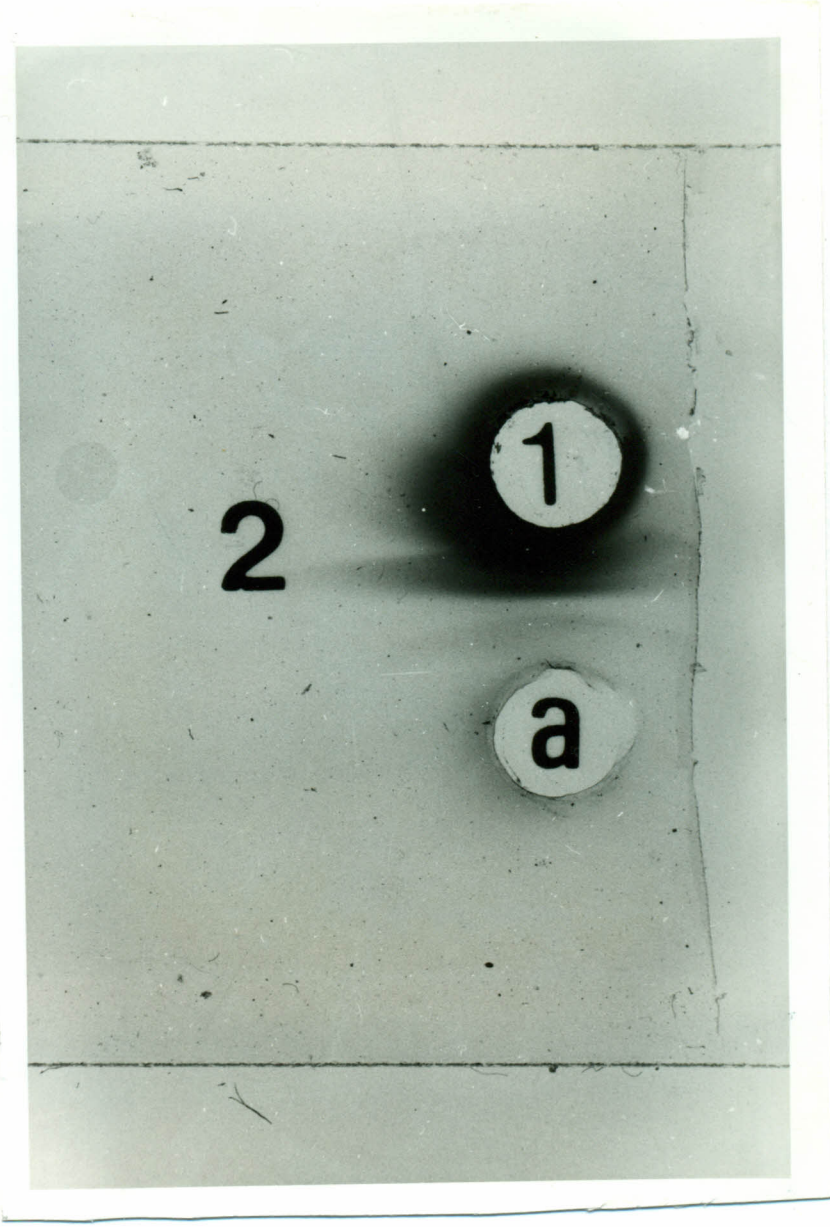
Figure 9. A photograph showing Ouchterlony of

antigen-antibody precipitation of A.

variegatum GMBP against homologous A.

variegatum and heterologous R.

appendiculatus R. evertsi evertsi

immune sera. Where a= A. variegatum

GMBP; 1= R. appendiculatus immune serum;

2= R. evertsi evertsi serum; 3 and

6= A. variegatum immune sera and 4 and

5= control serum. 3-4 precipitin lines

were formed with homologous serum,

1-2 with each heterologous sera and no

line with control sera.

3.5.1.3: Amblyomma variegatum GMBP antigen

Three to four precipitin lines were observed with homologous sera (Figure 8) while a single precipitin line (Figure 9) showing partial identity was formed with heterologous anti-sera to R. appendiculatus and R. evertsi evertsi tick species.

3.5.1.3: Amblyomma variegatum GMBP antigen

Three to four precipitin lines were observed with homologous sera (Figure 8) while a single precipitin line (Figure 9) showing partial identity was formed with heterologous anti-sera to R. appendiculatus and R. evertsi evertsi tick species.

3.5.2: Enzyme-Linked Immunosorbent Assay (ELISA)

3.5.2.1: Rhipicephalus appendiculatus GMBP antigen

ELISA technique detected circulating antibodies specific to R. appendiculatus GMBP antigen in the homologous anti-sera one week after the primary dose. The antibody titres increased progressively (from OD 0.241 to 0.783) following each booster dose. Similar results were observed for the heterologous R. evertsi evertsi and A. variegatum GMBP antigens (Table 12).

3.5.2.2: Rhipicephalus evertsi evertsi GMBP antigen

Antibody titres were demonstrated in the homologous sera against R. evertsi evertsi GMBP one week after the primary dose. The titres increased progressively with successive immunizations (from OD 0.287 to 0.742). There were no antibodies detected in the control serum. Similar results were obtained by the heterologous R. appendiculatus and A. variegatum GMBP antigens (Table 13).

Table 12. Antibody levels measured by ELISA in sera of rabbits to R. appendiculatus GMBP antigens

| Bleed | Antigen used in ELISA | | | |
	R. appendiculatus	R. evertsi evertsi	A. variegatum	Control
14 days before vaccination	0.050 +0.011	0.052 +0.010	0.055 +0.010	0.055 +0.010
7 days after primary dose	0.241 +0.034*	0.217 +0.031*	0.237 +0.029*	0.055 +0.010
14 days after primary dose	0.395 +0.042*	0.356 +0.045*	0.383 +0.041*	0.050 +0.013
14 days after first booster	0.596 +0.014*	0.562 +0.015*	0.568 +0.013*	0.051 +0.010
14 days after second booster	0.724 +0.012*	0.691 +0.017*	0.703 +0.022*	0.050 +0.008
14 days after third booster	0.783 +0.018*	0.725 +0.016*	0.736 +0.015*	0.052 +0.007

Results are mean absorbance values at 492nm +SE.
Superscripts indicate values significantly higher than for controls within rows at P<0.01.

Table 13. Antibody levels measured by ELISA in sera of rabbits to R. evertsi evertsi GMBP antigens

	Antigen used in ELISA			
Bleed	R. appendiculatus	R. evertsi evertsi	A. variegatum	Control
14 days before vaccination	0.051 +0.010	0.057 +0.009	0.052 +0.010	0.055 +0.010
7 days after primary dose	0.232 +0.032*	0.287 +0.030*	0.228 +0.031*	0.053 +0.010
14 days after primary dose	0.396 +0.043*	0.399 +0.044*	0.391 +0.046*	0.050 +0.011
14 days after first booster	0.582 +0.013*	0.586 +0.012*	0.580 +0.017*	0.051 +0.012
14 days after second booster	0.698 +0.014*	0.702 +0.011*	0.761 +0.008*	0.056 +0.009
14 day after third booster	0.724 +0.015*	0.742 +0.010*	0.718 +0.011*	0.053 +0.017

Results are mean absorbance values at 492nm +SE.
Superscripts indicate values significantly higher than for controls within rows at P<0.01.

Table 14. Antibody levels measured by ELISA in sera of rabbits to A. variegatum GMBP antigens

Bleed	Antigen used in ELISA			
	R. appendiculatus	R. evertsi evertsi	A. variegatum	Control
14 days before vaccination	0.050 +0.013	0.051 +0.009	0.055 +0.007	0.052 +0.011
7 days after primary dose	0.211 +0.027*	0.205 +0.010*	0.317 +0.019*	0.054 +0.010
14 days after primary dose	0.325 +0.046*	0.316 +0.039*	0.399 +0.034*	0.055 +0.009
14 days after first booster	0.552 +0.011*	0.528 +0.010*	0.583 +0.011*	0.055 +0.008
14 days after second booster	0.621 +0.010*	0.601 +0.012*	0.710 +0.017*	0.054 +0.010
14 day after third booster	0.704 +0.012*	0.695 +0.023*	0.762 +0.010*	0.055 +0.010

Results are mean absorbance values at 492nm +SE.
Superscripts indicate values significantly higher than for controls within rows at P<0.01.

3.5.2.3: Amblyomma variegatum GMBP antigen

One week after the primary dose, antibody titres were detected in the homologous sera against A. variegatum GMBP antigens. Antibody titres increased progressively with subsequent immunizations (from OD 0.317 to 0.762). Similarly, antibodies were detected heterologous by R. appendiculatus and R. evertsi evertsi GMBP antigens. There were no antibodies demonstrated in the control sera (Table 14).

3.5.3: Western blotting

3.5.3.1: Rhipicephalus appendiculatus GMBP antigen

In GMBP of homologous R. appendiculatus 25 bands with molecular weights ranging from 23,000 to 130,000 Daltons were detected. Two of these bands with molecular weights 58,000, 54,000 and were also detected by pre-immune srerum. Sixteen bands with molecular weights of 24,000 to 14,0000 Daltons were detected by serum from R. evertsi evertsi immunized rabbits (Figure 10). Sera raised in rabbits to A. variegatum GMBP detected thirteen R. appendiculatus GMBP antigens with molecular weights of 21,000 to 130,000 (Figure 28). Three of these bands with molecular weights of 50,000, 54,000, 57,000, were also detected by pre-immune serum.

3.5.3.2: Rhipicephalus evertsi evertsi GMBP antigen

Immunoblot reactions established that there were antibodies in both the homologous R. evertsi evertsi

Figure 10.   A photograph showing Western blot

analysis of R. appendiculatus GMBP antigen

against homologous R. appendiculatus

(Lane 2) and heterologous R. evertsi

evertsi (Lane 3) and A. variegatum

(Lane 4) sera. Lanes 5,6,7,and 8 are

antigen + normal rabbit serum + conjugate

control; antigen + conjugate control;

conjugate control and substrate control

respectively. Lane 1= Molecular weight

markers.
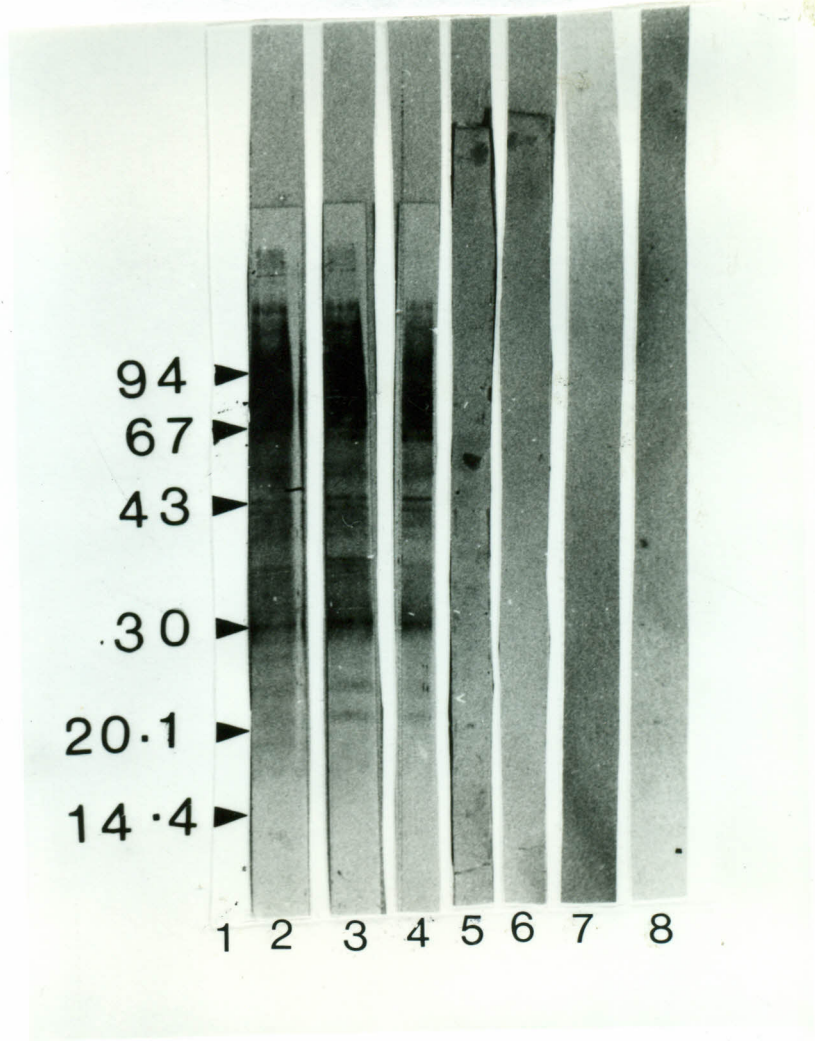
Figure 11. A photograph showing Western blot

analysis of R. evertsi eversti GMBP **antigen**

against homologous R. evertsi evertsi

(Lane 3) and heterologous R. appendiculatus

(Lane 2) and A. variegatum (Lane 4) serum.

Lanes 5,6,7,and 8 are antigen + normal

rabbit serum + conjugate control; antigen

+ conjugate control; conjugate control and

substrate control respectively.

Lane 1= Molecular weight markers.
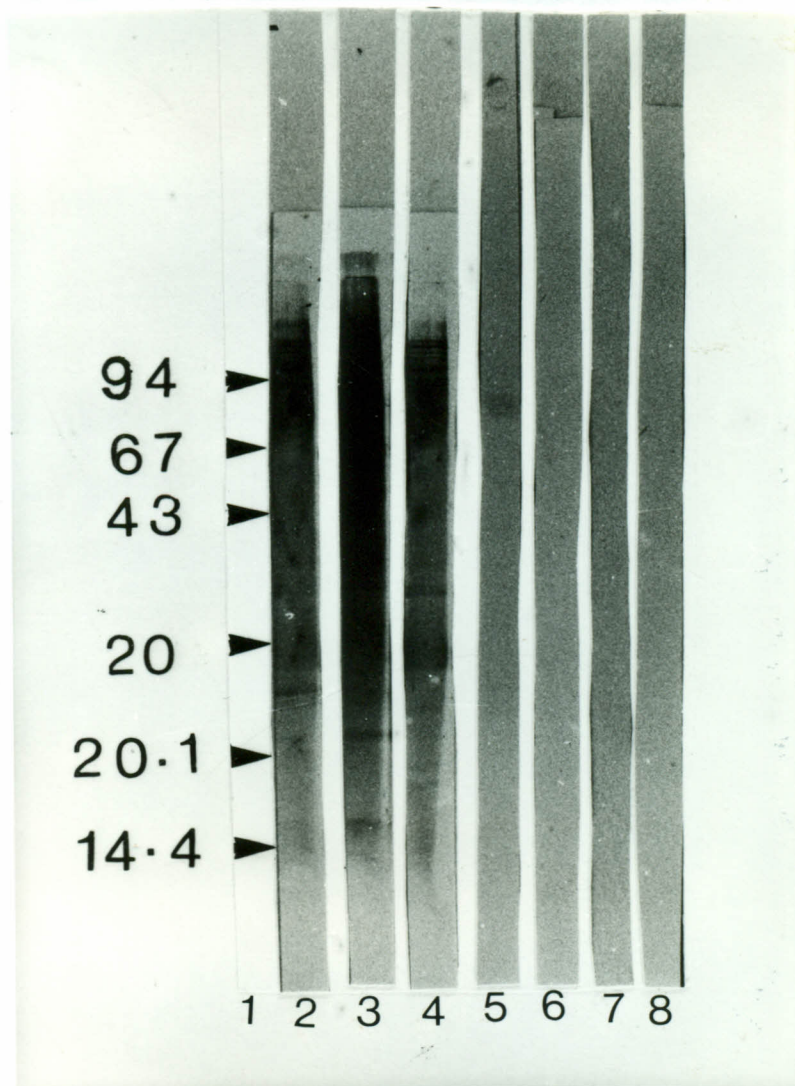
Figure 12. A photograph showing Western blot

analysis of A. variegatum GMBP antigen

against homologous A. variegatum (Lane 4)

and heterologous R. appendiculatus (Lane 2)

and R. evertsi evertsi (Lane 3) sera. Lanes

5,6,7,and 8 are antigen + normal rabbit

serum + conjugate control; antigen +

conjugate control; conjugate control and

substrate control respectively.

Lane 1= Molecular weight markers.

and heterologous R. appendiculatus and A. variegatum
anti-sera specific to R. evertsi evertsi CMBP antigens
(Figure 11). The homologous R. evertsi evertsi anti-
sera obtained recognised at least fourteen
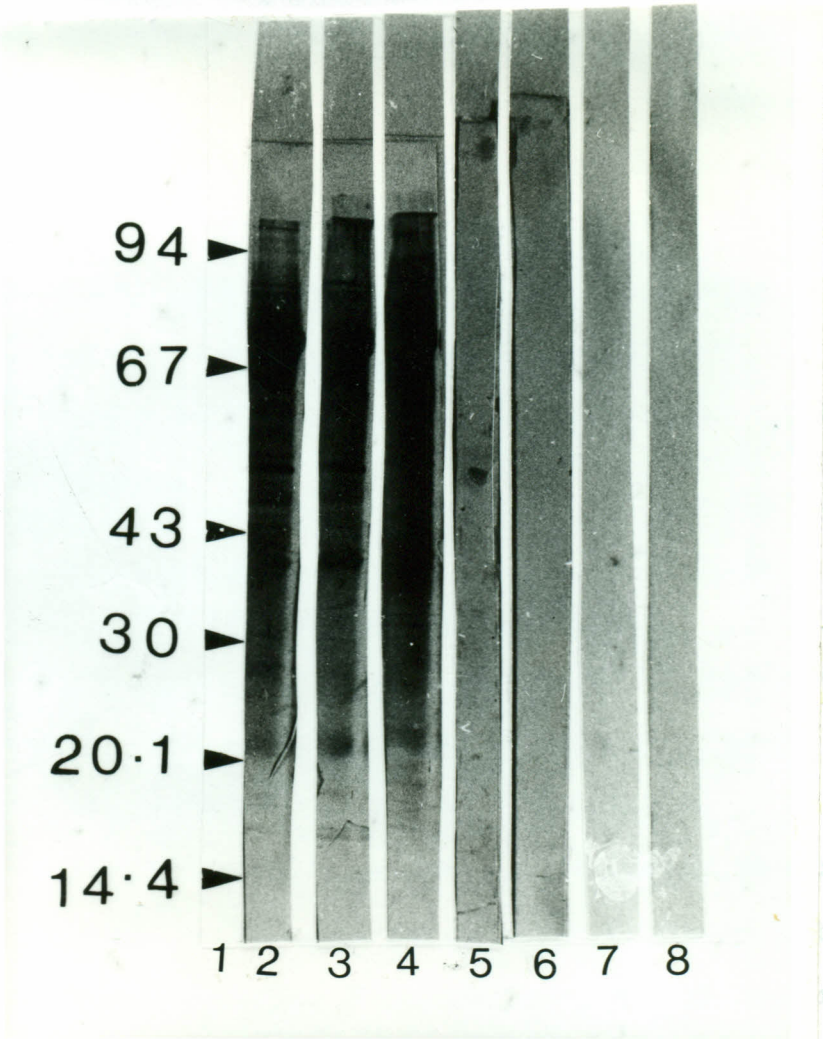
94 ▶

67 ▶

43 ▶

30 ▶

20·1 ▶

14·4 ▶

1 2   3   4   5   6   7   8

and heterologous R. appendiculatus and A. variegatum
anti-sera specific to R. evertsi evertsi GMBP antigens
(Figure 11). The homologous R. evertsi evertsi anti-
sera detected immunospecifically at least fourteen
bands with molecular weights ranging from 16,000 to
140,000 Daltons. The heterologous R. appendiculatus
anti-sera detected fourteen R. evertsi evertsi GMBP
antigens with molecular weights ranging from 16,000 to
130,000 Daltons. The heterologous A. variegatum anti-
sera detected thirteen R. evertsi evertsi GMBP
antigens. Their molecular weights ranged from 16,000
to 130,000 Daltons.

3.5.3.3: Amblyomma variegatum GMBP antigen

Anti-sera to A. variegatum detected 23 bands in
the homologous GMBP whose molecular weights ranged from
16,000 130,000 Daltons. Anti-sera against R.
appendiculatus GMBP antigens immunzed rabbits detected
seventeen bands in A. variegatum GMBP antigens with
molecular weights ranging from 16,000 130,000 Daltons.
Sera against R. evertsi evertsi GMBP antigens detected
eighteen bands in A. variegatum GMBP antigens and their
molecular weights ranged from 16,000 to 130,000 Daltons
(Figure 12).

CHAPTER 4

DISCUSSION, CONLUSIONS AND RECOMMENDATIONS

Animals are able to acquire a significant degree of immunity following artificial immunization with tick extracts (Willadsen, 1987; Maranga, 1988; Wishitemi, 1988; Opdebeeck et al., 1988a, b; Wikel, 1988; Jongejan et al., 1989; Wong and Opdebeeck, 1990). The results of studies reported in this thesis have shown that immunization of rabbits with tick midgut membrane-bound proteins can confer protection which has adverse effects on the feeding success of the challenge infestation larvae, nymphs and adults. This observation was evidenced by prolongation of feeding periods, reduction in engorgement weights, egg mass weights, percentage hatchability, the number of all instars successfully completing engorgement and the number of larvae and nymphs moulting into nymphs and adults, respectively.

The use of tick midgut as a potential vaccine to induce resistance to ixodid tick challenge infestation has been described by previous investigators (Kohler et al., 1967; Allen and Humphreys, 1979; Johnston et al., 1986; Agbede and Kemp, 1986; Willadsen and Kemp, 1988; Opdebeeck et al., 1988a,b, 1989; Willadsen and Kemp, 1988;

Willadsen et al., 1988; 1989; Jongejan et al.,
1989).

Cross-protection against homologous and
heterologous tick instars has been described (De
Casrto et al., 1989; Shapiro et al., 1989; Wong and
Opdebeeck, 1990). Immunization of rabbits with GMBP
conferred protection against homologous species as
well as heterologous species. It was shown, in
these studies, that vaccination of rabbits with
R. appendiculatus GMBP had no effect on the mean
feeding period of homologous R. appendiculatus and
heterologous A. variegatum larvae. However, it was
shown that, immunization reduced mean engorgement
weights, mean percentage of larvae moulting to
nymphs and the number of A. variegatum and
R. appendiculatus larvae which successfully
completed their blood meal on immunized rabbits
(Table 3). These results reveal the presence of
common antigens in instars of homologous and
heterologous ticks (De Castro et al, 1989; Shapiro
et al., 1989; Jongejan et al., 1989). These
results, therefore, suggest the importance of cross-
reactive antigens in the control of ticks. The
results of the present study show that the effect of
immunization was more pronounced for the homologous
than the heterologous challenge ticks (Table 3).

The acquired resistance by immunization of rabbits with R. appendiculatus GMBP effectively prolonged the feeding period of homologous R. appendiculatus nymphs by 8.4% and of heterologous R. evertsi evertsi nymphs by 5.8%. However, vaccination of rabbits with R. appendiculatus GMBP had no effect on the feeding duration of heterologous A. variegatum nymphs (Table 4). The engorgement weights of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum were also reduced by 11.4%, 21.7% and 31.2%, respectively. Similar results were reported by De Castro et al. (1989). These results showed high percentage mortalities among the nymphs fed on immunized rabbits. The highest mortalities were recorded in R. evertsi eversti nymphs, probably because they were applied on the rabbits as larvae and dropped off as engorged nymphs (Rechav and Dauth, 1987). However, vaccination of rabbits had no effect on the moulting success of nymphs fed on immunized rabbits compared to control rabbits.

The feeding performance and fecundity of homologous and heterologous female ticks were also determined. Immunization of rabbits with R. appendiculatus GMBP was effectively prolonged the feeding period of homologous R. appendiculatus and heterologous R. evertsi evertsi and A. variegatum

females (Table 5). These results are in conformity
with those reported earlier by Njau and Nyindo
(1987). The engorged weights, egg mass weights,
percentage hatchability and number of females which
survived to full engorgement were all reduced (Table
5). These findings suggest the presence of common
antigenic components to more than one instar and
species or even genus of ticks (McTier et al., 1981;
Brown and Askenase, 1981; Shapiro et al., 1989;
Opdebeeck et al., 1989; Jongejan et al., 1989; Wong
and Opdebeeck, 1990). None of the heterologous A.
variegatum female ticks fed on both immunized and
control rabbits were found dead.

Immunization of rabbits with adult R. evertsi
evertsi GMBP conferred protection against both
homologous and heterologous challenge infestation
instars. The feeding periods of heterologous R.
appendiculatus and A. variegatum challenge larvae
were prolonged and the mean engorged weights,
moulting and the number of larvae that engorged
successfully on immunized rabbits were reduced
(Table 6). The results obtained in this part of the
study demonstrated the presence of cross-protection
between species and genera. These results agree
with the findings of previous investigators
(Njau,1985; Latif, 1985; Rechav, 1987; Heller-Haupt
et al., 1981, 1987; Fivaz and Norval, 1989; Jongejan

et al., 1989; De Castro et al., 1989) who showed
that rabbits immunized against one tick species
conferred immunity which was cross-protective to
challenge infestation with other tick species in the
same or different genus. Cross-protection to
homologous tick instars has recently been described
by Wong and Opdebeeck (1990).

Immunization of rabbits with R. evertsi evertsi
GMBP prolonged the feeding duration of both
homologous and heterologous nymphs (Njau, 1985; De
Castro et al., 1989; Jongejan et al., 1989) and
reduced engorgement weights by 33%, 36.4% and 22%,
respectively. Fewer nymphs fed on immunized rabbits
successfully completed engorgement and survived
moulting compared to the control groups (Table 7).
Percentage reduction in engorged weight was higher
for homologous R. evertsi evertsi than for either
heterologous A. variegatum or R. appendiculatus
nymphs. Similar results were reported by Njau
(1985).

Vaccination of rabbits with R. evertsi evertsi
GMBP increased the feeding duration, while engorged
weights, egg mass weights and percent hathability
were reduced (Table 8) for homologous R. evertsi
evertsi and heterologous A. variegatum and R.
appendiculatus female ticks. The results in this
part of the present study agree with results

reported by Rechav (1987) that ticks which feed
longer on resistant hosts are more adversely
affected hence are correspondingly lighter. The
number of the homologous R. evertsi evertsi and
heterologous R. appendiculatus females which fed to
full engorgement on immunized rabbits was reduced.

The mean feeding duration of homologous
A. variegatum and heterologous R. appendiculatus
larvae was not affected by immunization of hosts
with A. variegatum GMBP. However, the mean engorged
weights, moulting and the mean number of
heterologous R. appendiculatus and homologous
A. variegatum larvae were reduced (Table 9). These
results indicate that immunization of rabbits with
A. variegatum GMBP has more severe effect on the
homologous challenge than the heterologous challenge
instars. These findings are similar to the results
of previous workers (Njau, 1985; Rechav, 1987; Allen
and Humphreys 1979; Johnston et al., 1986; Opdebeeck
et al., 1988a,b; Wikel, 1988; Fivaz and Norval,
1989; De Castro et al., 1989).

Immunization of rabbits with A. variegatum GMBP
prolonged the feeding duration of both homologous
and heterologous R. appendiculatus and R. evertsi
evertsi and homologous A. variegatum nymphs. The
prolongation of feeding period for R. appendiculatus

was not different from the control. In contrast,
Ackerman et al. (1980), McGowan et al. (1980),
De Castro et al. (1985) and Chiera and Newson (1989)
showed that the feeding periods of challenge ticks
was shortened. Previous studies have indicated that
artificial immunization of hosts decrease the number
of challenge ticks which finally complete
engorgement, and that, the ticks which feed longer
on resistant animals are lighter (Clarke et al.,
1989). Similar findings have been shown in this
study. Immunization of rabbits with A. variegatum
GMBP reduced mean engorged weights, moulting of
nymphs into adults (Jongejan et al., 1989). The
number of heterologous R. appendiculatus and R.
evertsi evertsi and homologous A. variegatum nymphs
fed A. variegatum GMBP immunized rabbits were
reduced (Table 10) (Njau, 1985).

Brown (1985) showed that rabbits made resistant
to A. americanum gave significant protection
resulting in 39% rejection, 27% decrease in engorged
weights, and 63% decrease in egg mass weights of
engorged females. Similar results, but using
different antigens and hosts were reported by
several other investigators (Allen and Humphreys,
1979; Njau, 1985; Johnston et.al., 1986; Kemp
et.al., 1986; Shapiro et.al.,1989; Opdebeeck, 1988a,
b; Wikel, 1988; Wong and Opdebeeck, 1989; 1990).

Rabbits immunized with A. variegatum GMBP from partially engorged female ticks effectively prolonged the feeding period of homologous A. variegatum and heterologous R. appendiculatus females. However, immunization of rabbits with A. variegatum GMBP had no significant effect on the feeding duration of heterologous R. evertsi evertsi female ticks fed on immunized rabbits compared to control rabbits. The acquired resistance reduced engorged weights, egg mass weights, percentage hatchability and the number of homologous A. variegatum and heterologous R. appendiculatus and R. evertsi evertsi females surviving engorgement on immunized rabbits was reduced compared to the controls (Table 11). These results agree with the findings by Brown (1988) and Njau (1985) which showed that there exists common antigens to both closely and distantly related ticks. These common antigens result in cross-protection. Similar results described by Brown and Askenase (1984), Heller-Haupt et al. (1981) and McTier et al. (1981) show that immunity to one species of ticks is induced by the feeding of another species or even genus of ticks.

Examination of partially engorged R. appendiculatus, R. evertsi evertsi, and

A. variegatum GMBP antigens by SDS-PAGE followed by
Coomasie Brilliant Blue R-250 staining revealed 37,
45 and 39 protein bands, respectively. Twenty-two
bands were common to the three tick species. More
protein bands were common between R. appendiculatus
and R. evertsi evertsi than either between
R. appendiculatus and A. variegatum or R. evertsi
evertsi and A. variegatum. Similar findings by Njau
(1985) showed that cross protection was more
pronounced between closely related ticks,
R. appendiculatus and R. evertsi evertsi than
between R. evertsi evertsi and A. variegatum. This
shows that more closely related ticks shared more
common protective antigens than distantly related
ticks. The presence of cross-reactive antigens is
reponsible for the cross-protection confirmed in
this study. These findings constitute an important
aspect in the control of ticks. Opdebeeck et al.
(1988a, b,; 1989) reported that protective antigens
may also be common to all stages of the parasite.
These common antigenic components to the live
stages, species and genus are attractive candidates
for vaccines because damage to more than one stage
in the life cycle, species and genus may be achieved
(Rajasekariah et al., 1980). Kemp et al. (1986)
reported that cattle immunized with crude extract of
whole ticks had adverse effects on adult ticks but

not larvae during feeding. In the present study, it has been shown that the effect of immunization of rabbits with midgut antigens damaged all the instars of both homologous and heterologous challenge infestation ticks.

Wishitemi (1988) demonstrated that anti-sera against solubilized midgut membrane antigens from partially fed R. appendiculatus female ticks precipitated 26 proteins by Western blot. Their molecular weights ranged from 14,500 to 105,000 Daltons. In the the present study, Western blot analysis of R. appendiculatus GMBP reacted with anti-sera precipitated 24 band with molecular weights ranging from 14,400 to 140,000 Daltons. These results are in conformity with those of Wishitemi (1988) in sheep. Anti-sera in other studies precipitated tick antigens of 20,000 Daltons (Brown et al., 1984), between 30 and 110,000 Daltons (Whelen et al., 1984), between 16,000 and 120,000 Daltons (Shapiro et al., 1986) 90,000 Daltons (Shapiro et al., 1987). Similarly, Mongi et al. (1986) identified protein bands between 82,000 and 180,000 Daltons from R. appendiculatus whole tick homogenate. Western blot in these studies illustrated common antigens among R. appendiculatus, R. evertsi evertsi and A. variegatum. This was demonstrated by high degree of cross-reactivity

between anti-R. appendiculatus GMBP, anti-R. evertsi evertsi GMBP and anti-A. variegatum GMBP against R. appendiculatus antigens. Similar results by Shapiro et al. (1989) showed that serum from guinea pigs made resistant to R. appendiculatus SGA identified cross-reactive antigens from Rhipicephalus. pulchellus, R. evertsi evertsi, A. variegatum and A. gemma SGA.

    Western blot analysis established that there were cross-reacting antibodies in both the homologous R. evertsi evertsi and heterologous R. appendiculatus and A. variegatum anti-sera specific to R. evertsi evertsi GMBP antigens. Anti-sera from R. evertsi evertsi GMBP immunized rabbits detected 15 bands specific to it with molecular weights ranging from 16,000 to 140,000 Daltons. Anti-sera from R. appendiculatus GMBP immunized rabbits detected 14 R. evertsi evertsi GMBP bands with molecular weights ranging from 16,000 to 130,000 Daltons. Similarly, anti-sera to A. variegatum GMBP detected R. evertsi evertsi bands with molecular weights ranging from 16,000 to 130,000 Ddaltons. These results suggest the presence of common antigens among R. appendiculatus, R. evertsi evertsi and A. variegatum which conferred cross-protection. Similar findings by Njau (1985) demonstrated that sensitization of rabbits with

adult R. evertsi evertsi resulted in strong protection against the homologous R. evertsi evertsi challenge infestation and significant cross-protection against challenge to all instars of R. appendiculatus and larvae and adults of A. variegatum heterologous ticks. In a similar study, Latif (1985) showed that R. evertsi evertsi were prevented from feeding on cattle made resistant to H. anatolicum anatolicum.

Western blot analysis of larval tick extract illustrated considerable cross-reactivity between A. americanum SGA-induced antibodies and larval A. americanum antigens (Brown, 1988). Amblyomma americanum antibodies were able to recognize a number of proteins from D. variabilis and B. microplus ticks (Brown, 1988). These results demonstrated the presence of common antigens as inferred in resistance studies using one species of ticks to sensitize hosts against subsequent challenge infestations by a different species (Trager, 1939a; Brown and Askenase, 1981; McTier et al., 1981; Latif, 1985; Njau, 1985). Heller-Haupt et al., (1981) showed that there was low level cross-resistance between A. variegatum and A. hebraeum.

Anti-sera to A. variegatum GMBP detected approximately 23 specific to it with molecular weights ranging from 16,000 to 130,000 Daltons

(Figure 12). Anti-sera against R. appendiculatus
GMBP detectedapprximately 17 bands from A.
variegatum GMBP with molecular weights ranging from
16,000 to 130,000 Daltons. Similarly, anti-sera to
R. evertsi evertsi detected 18 bands from A.
variegatum GMBP with molecular weights ranging from
16,000 to 130,000 Daltons.

In all cases, Ouchterlony double
immunodiffusion tests revealed 3 to 4 precipitin
lines with homologous sera (Mongi et al., 1986;
Wishitemi, 1988; Maranga, 1988) and one precipitin
line with each heterologous sera (Njau, 1985). These
results suggest that specificity due to antigen-
antibody reactions was more pronounced with rabbit
anti-sera raised against homologous than
heterologous tick antigens (Njau, 1985).

ELISA detected significant levels of
circulating antibodies in immunized rabbits to GMBP
of R. appendiculatus, R. evertsi evertsi and
A. variegatum. The results of these studies are in
confirmity with the findings described by Voller et
al. (1976) and Wishitemi (1988).

In conclusion, rabbits immunized with R.
appendiculatus, R. evertsi evertsi and A. variegatum
in three separate experiments acquired strong
resistance to challenge infestation with all instars
of both homologous and heterologous ticks. These

results showed that the challenge infestation ticks whose feeding period was prolonged were most adversely affected. These results strongly suggest that the potential candidate antigen for tick control lies in the tick midgut membrane. The larvae fed on immunized rabbits were bright red in colour, the nymphs were grey in colour and the female ticks which died either partially fed or fully engorged on immunized rabbits were black in colour instead of the normal grey colour. These results confirm that immunization of rabbits with midgut membrane bound protein results in rupture of the digestive tract of the tick instars leading to leakage of the gut contents into the haemolymph (Johnston et al., 1986; Agbede and Kemp, 1986; Wikel, 1988; Opdebeeck et al., 1988a, b; Willadsen and Kemp, 1988; Willadsen et al., 1988,1989). Antisera to R. appendiculatus, R. evertsi evertsi and A. variegatum GMBP antigens recognized several polypeptide antigens from both homologous and heterologous antigens. These results suggest presence of common antigens in both closely related as well as distantly related tick species.

Challenge of immunized rabbits showed cross-protection against all instars of homologous tick species and against all instars of the heterologous tick species. The presence of common antigens

appeared to be responsible for cross-protection
against R. appendiculatus. R. evertsi evertsi and
A. variegatum. There was a strong protection
against the feeding of homologous tick challenge
while there was significant cross-protection against
heterologous tick challenge. Although considerable
cross-protection is reported in this thesis, further
research is needed, particularly on the
charaterization and purification of the cross-
reacting antigens which conferred cross-protection.

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APPENDIX 1

A photograph showing R. appendiculatus female ticks fed on immunized and control rabbits. Row 1 shows the female ticks fed on controls and Row 2 shows the female ticks fed on immunized rabbits.

APPENDIX 2

A photograph showing R. _evertsi_ _evertsi_ female
ticks fed on immunized and control rabbits. Row 1
shows the female ticks fed on controls and Row 2
shows the female ticks fed on immunized rabbits.

APPENDIX 3

A photograph showing A. variegatum female ticks
fed on immunized and control rabbits. Row 1 shows
the female ticks fed on controls and Row 2 shows
the female ticks fed on immunized rabbits.