IMMUNE PROTECTION POTENTIAL OF MEMBRANE-BOUND PROTEINS FROM THE MID-GUT OF *AMBLYOMMA VARIEGATUM*, FABRICIUS 1794 (ACARINA:IXODIDAE).

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DECLARATION

I Henry Kimathi Kiara, hereby declare to the Senate of the Rivers State University of Science and Technology that this thesis is the result of my own original work except where it is acknowledged in the text. It has not been submitted nor is it concurrently being submitted for a similar qualification in any other University

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DEDICATION

This work is dedicated to my father the late Gideon M'Ikiara Kariungi who was my inspiration throughout my school life but did not live to see me graduate

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ABSTRACT

The need for an alternative to chemical control of ticks is an urgent and a continuing one. Immunological approaches through vaccination of hosts with tick derived antigens has shown great promise. As a critical first step towards the development of a commercial anti-tick vaccine, the antigens inducing immunity to the host must be purified and characterised.

Mid-gut extracts from partially fed *Amblyomma variegatum* females were solubilised with the non-ionic detergent Triton X-114 and partitioned into the peripheral and integral membrane proteins. Rabbits were immunised with both fractions and later challenged with all the instars of *A. variegatum*. Only the integral membrane proteins had any effect on the feeding and development of the ticks. Adults had reduced engorgement weight, reduced egg mass and reduced egg conversion efficiency. The larvae and nymphs feeding on immunised rabbits fed for longer duration and the yield was significantly lower.

In order to assess whether the antigens were species specific or were cross-reactive with ticks of other genera , rabbits immunised with both protein fractions were challenged with all the instars of *Rhipicephalus appendiculatus*. Again, only the integral membrane proteins had any effect on the feeding ticks. The egg conversion efficiency was reduced in adults and in the immatures yield was reduced while the feeding duration was longer. In all cases these effects were, however, lower than those observed in *A. variegatum* ticks.

Immunoblot tests using sera from immunised rabbits indicated that most of the reactive polypeptides were in the molecular weight range above 50kDa.

Fractionation of the integral membrane proteins by gel permeation chromatography yielded four peaks. Immunisation and challenge experiments with the peaks showed activity was spread almost evenly throughout the peaks. Fraction one containing the high molecular weight proteins affected mainly the adults mortality while the other fractions had effects mainly on the feeding duration and the engorgement weight of the immatures stages. Because of this spread the effects were considerably reduced.

Cross-protection experiments by challenge with R. *appendiculatus* ticks showed that the proteins and especially fraction two had effects on the adults and the nymphs. In adults the egg conversion efficiency was reduced while in nymphs the feeding duration was shortened significantly. The antigen had no effects on larvae. Like, the integral proteins before fractionation, these effects were lower than those in *A. variegatum* ticks.

Preparative SDS-PAGE gels of the integral membrane proteins were divided into high, medium and low molecular weight sections and the protein polypeptides eluted from them. Successful protection of rabbits against *A. variegatum* challenge was achieved by immunisation with the high molecular weight fraction. These polypeptides affected the engorgement weight of the adults and the feeding duration. In the larvae and nymphs the feeding duration was increased and the yield significantly lowered. These antigens did not only induce effects in most of the parameters assessed but their level of protection was much higher.

There was some activity in the low molecular weight polypeptides and more specifically on the immature stages.

The study confirms the presence of protective antigens in the mid gut of *A. variegatum* females. These antigens are integral membrane proteins with an approximate molecular weight above 67 kDa. The antigens are fairly stable and their activity is dependent on sequential antigenic determinants rather than conformational ones. They are unlikely to be enzymes. Evidence from this study indicates, immunity to ticks may be the result of more than one antigen working in concert. Cross-protection against ticks of other species is at best partial or absent and an anti tick vaccine may have to be a multi-component vaccine with antigens from several tick species.

CHAPTER ONE

INTRODUCTION

Ticks and tick-borne diseases are believed to be responsible for substantial losses and a major constraint to livestock production in many parts of the world (Bram, 1975). Losses attributable to tick infestation include, their role as vectors of diseases, reduction in live-weight gains through reduced feeding and injection of toxins leading to various toxicoses. Additionally, ticks also cause cutaneous lesions at their attachment sites often leading to hide damage and secondary bacterial infections.

Despite the absence of accurate records, current estimates of production losses and the cost of control of ticks world wide have been estimated to be approximately US$ 7,000M (MaCosker, 1979). Africa's share of this loss is believed to be considerable.

Since the beginning of this century tick control has largely depended on a rigorous, short interval dipping of cattle in chemical acaricides. This approach was so successful that in Africa for instance, East Coast fever (ECF) was eradicated in South Africa in the 1950's and in the rest of the continent, it was possible to establish viable livestock industries in ECF endemic areas (Norval, 1983).

Until recently, there was no serious scrutiny of this form of control as to whether it was suitable or even necessary, owing in part to the success of the method. During the last decade, however, the development of the concept of integrated pest management (IPM) has changed all that. The concept advocates the development of control strategies, that make optimal use of each of the control methods available without putting too much reliance on any one method (FAO,

1990). The other reason for the shift from total reliance on chemical control of ticks has been the escalating cost of acaricides together with other infrastructural costs. This has proved impossible to support economically, especially for the resource poor farmers.

The rapid development of resistance to acaricides by ticks (Wharton, 1976) is perhaps, the most important reason for the shift in attitudes from intensive use of chemicals for tick control. Furthermore, the development of new acaricides is both costly and risky because of the rapidity with which resistance has been shown to develop (Durand, 1976). The continued supply of new acaricides is, therefore, not guaranteed.

Chemical control has also been shown to destroy enzootic stability (Callow, 1983), rendering the cattle totally susceptible to both ticks and tick-borne diseases with catastrophic consequences should control fail for any reason (Lawrence *et al.*, 1980). Concern for environmental pollution by pesticides and the reduction of non-target populations are the other undesirable characteristics associated with chemical control (Wall and strong, 1987). Residues in meat and other animal products (McDougal *et al.,* 1979) has also contributed to the need to avoid or at least reduce the dependence on chemicals as the sole means of tick control.

In view of the above, there is, an urgent need to search for alternative tick control strategies that are not based on the unrelenting use of chemicals. Ecological, biological control, acquired host resistance and immunisation have all been suggested as possible alternative methods.

1.1 Immunisation against ticks

Trager (1939) was the first to demonstrate that resistance to *Dermacentor variabilis* by guinea pigs could be artificially induced by immunisation with whole tick homogenates. Since then, resistance to ticks has been induced in both cattle and laboratory animals using a variety of tick derived antigens, including salivary glands, mid-guts, whole tick extracts and reproductive organs (Brown, 1988).

An important attraction to immunisation, especially with molecular vaccines, compared to other methods of pest control, is its safety and low cost (Murray, 1989). It has also been suggested that vaccination against the vector might also interfere with the transmission of the parasites (Wikel, 1980)

Unfortunately, studies on the purification and characterisation of the tick antigens responsible for inducing immunity to ticks are few (Willadsen and Kemp, 1988), especially for African ticks. This would have to be done before a tick vaccine can be developed. This is because the only practical way of producing the vaccine commercially and in a relatively cheap way would be through recombinant DNA technology.

The most detailed study todate, to isolate, purify and characterise tick antigens inducing immunity is that of Willadsen *et al.,* (1989). They isolated a membrane associated glycoprotein with molecular weight of 89 kDa that was able to induce resistance to *B. microplus* in cattle. A commercial vaccine has since been made and is on the market (Willadsen *et al.,* 1995).

1.2 Economic importance of *A.variegatum*

A.variegatum is a tropical and subtropical tick. In Africa it occupies the Savannah belt bordering the Sahara in the North and Zimbabwe and Mozambique to

the South. In East Africa it is found at altitudes from sea level to 2,500 m and a rainfall range of 500-2000 ml (Walker, 1974). It is, therefore, to be found in Ecoclimatic zones II and III described by Pratt *et al.,* (1966) with extensions into zone IV in some areas. Whereas *A.variegatum* and *R. appendiculatus* are sympatric (Hoogstral, 1956), *A.variegatum* can tolerate drier conditions and, therefore, has a slightly wider distribution than the latter species.

A.variegatum was introduced to the West Indies and the Caribbean Islands in the middle of the 19th century when cattle were imported from West Africa into the French possessions (Uilenberg, 1986). With it, heart-water ,was also introduced, and now poses a danger to most of Latin American cattle and those in some parts of Southern United States (Rawlings and Mansingh, 1987). *A.variegatum* is a tick of great economic importance. It is the most important vector for the rickettsial parasite *Cowdria ruminantum*, the causative agent of heart-water in ruminants (Uilenberg *et al.,* 1984). It also transmits *Theileria mutans* and *T. verifera* (Uilenberg, *et al.,* 1974). It has also been implicated in cutaneous streptothricosis caused by *Dermatophilus congolensis.* The females of *Amblyomma* species are regarded as the most damaging of the African Ixodidae (Tatchell, 1988) largely because of their large size and long mouth parts.

1.3 Objectives of the study

Some progress has been made on research on the development of an immunological approach to the control of *R. appendiculatus.* With regard to *Amblyomma* species in Africa, very little has been done towards the characterisation of the antigens responsible for the induction of resistance in hosts.

Heller-Haupt *et al.,* (1987), induced resistance in rabbits with extracts from unfed larvae. Serum antibodies were also demonstrated by double immunodiffusion tests. No attempts were made to characterise the antigens responsible. Jongejan *et al.,* (1989) successfully induced resistance in goats with salivary gland extracts of partially fed *A.variegatum* but failed to demonstrate any resistance in goats with mid-gut extracts. Analysis of the gut extracts on SDS-PAGE revealed about 29 polypeptides several of which were reactive with immune sera in immunoblotting experiments.

For the full utilisation of immunological methods for tick control in East Africa, it would be necessary to develop control strategies aimed at both *R. appendiculatus*, and *A.variegatum*, as they are the most economically important tick species and occur in the same ecoclimatic regions. For such a strategy to be developed, the first step has to be the characterisation of the antigen(s) responsible.

Therefore, this study proposes to identify, isolate and characterise antigens from *A.variegatum* that play a role in the induction of resistance to ticks in rabbits. It also proposes to study the cross-resistance between this species and *R. appendiculatus*. It is envisaged that at a later stage the information from this study could be used to clone the genes responsible for the production of these proteins into a suitable vector system to effect their expression. Eventually, production of the antigens will be scaled up to produce the antigens in sufficient quantities for further study and for the production of a vaccine.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of ticks

Ticks belong to the Phylum Arthropoda, Class Arachnida and the Order Acarina. There are two well defined families, Ixodidae (hard ticks) and Argasidae (soft ticks) and a third, Nuttalliellidae, that has features resembling the other two families (Hoogstral, 1973).

Ixodidae are characterised by possession of a highly sclerotised dorsal scutum covering the entire dorsal surface of the males and a small portion behind the capitulum of females. They have an anteriorly placed capitulum and a reduced fourth segment which arises from a pit on the second segment.

Argasidae on the other hand have no scutum on their dorsal surfaces. They have an apical fourth segment and a ventrally placed nymphal and adult capitulum.

Nuttallielidae are rare and poorly known primitive ticks. Like the ixodids, they posses a dorsal scutum covering a part of the female and the entire body of the male. However, unlike ixodids their scutal and integumental skin are indistinguishable. They have an apical fourth segment just like argasid ticks. The only species known is *Nuttalliella namaqua* described in South Africa (Hoogstral, 1973).

From a medical and veterinary point of view, Ixodid ticks are by far the most important (Walker, 1970). Argasidae are mostly parasites of reptiles but a few species are important parasites of man and animals.

2.2 Ixodid life cycles

Ticks are obligate ectoparasites of both warm and cold blooded animals. Typically, Ixodid adult females drop off the host after engorgement to lay a single egg batch (10,000-20,000 eggs) and dies. After a period of time depending on the tick species and climatic conditions, the eggs hatch into six legged larvae which after a period of quiescence followed by hardening climb on top of the grass or other vegetation to await a passing host. After feeding the larvae moult into eight legged nymphs which in turn feed and moult into adults which start another cycle. Ixodid ticks, therefore, have two moults in their lifecycle, larval/nymphal and nymphal adult.

Depending on where moulting takes place, Ixodid ticks are categorised as either one-host ticks, which have all their moults on the host, two-host ticks, which have the larval/nymphal moult on the host while the nypmhal/adult moult takes place on the ground and three-host ticks, which have all their moults on the ground. Most Ixodid ticks have a three-host type of life cycle.

Apart from this standard life cycle a few species undergo a parthenogenetic life cycle. However, it must be emphasised that parthenogenesis is rare and biologically unimportant (Oliver,1971).

2.3 Economic importance of ticks

The deleterious effects of ticks on their hosts have been known for a long time. However, it was the discovery by Smith & Kilborne in 1893 that *Boophilus* ticks were the vectors of *Babesia bigemina*, the causative agent of Texas fever, that

the magnitude of the problem caused by ticks was appreciated. Later discoveries that they also transmit Filaria, Protozoa, Rickettsia and many viruses underscored their importance. It is now known that about 80% of the world's cattle are tick infested (Snelson, 1975). Ticks are widely distributed throughout the world. In Africa, unlike other continents, they infest livestock in all climatic & vegetation zones (Dipeolu, 1989).

2.3.1 Loss of production

The most often quoted effect of tick infestation on their hosts is the loss in live weight gains (LWG). This results from loss of grazing time due to tick worry as well as due to specific effects caused by tick feeding (Surtherst *et al.,* 1983). There is no doubt that ticks lead to reduced growth rates, but what is controversial is the extent of this reduction. There are varied and wide differences in the literature with regard to loss of live weight gains due to tick infestation (Seifert, 1971; Johnston *et al.,* 1981; De Castro, 1985; Tatchell, 1987).

Australian workers have studied the effect of *B. microplus* infestation on livestock production more extensively than any other tick species (Surtherst *et al.,* 1983). They estimated that a single female *B. microplus* causes a reduction in live weight gain of 0.7g upon engorgement (Surtherst *et al.,* 1983). Taylor & Plumb (1981) quoted differences of up to 48 kg mean weight gain between tick infested and tick free herds. Tatchell *et al* (1986) reported that there were no significant differences in LWG between Boran cattle under various acaricidal regimens inspite of fairly large differences in tick burdens among treatment groups. They explained that Zebu cattle are able to develop resistance to ticks and intensive acaricide

application prevented this from occurring. In another study Tatchell (1988) reported that treated Kenana breed (*Bos indicus*) of cattle infested with *A. lepidum* gained 109 g per day compared to 98 g gained by untreated animals. The difference was, however, not significant. De Castro (1985) reported that dipped Boran cattle had significant differences in their weight gains equivalent to 0.55 Kg per animal per week over undipped cattle. Norval *et al.,* (1987) compared losses in local Sangas and European breeds when artificially infested with both *R. appendiculatus* and *A. hebraeum*. He reported that *R.. appendiculatus* adults caused significant reductions in LWG in European but not in the Sanga breeds. Adults of *A. hebraeum* had no effect on either breed. He also found immature stages had no effect on LWG in both breeds.

An estimate of the production losses due to ticks is necessary because the central point of integrated pest management (IPM) is the maintenance of the pest population at levels below those that cause economic loss. However, it is not the actual weight differences caused by tick infestations that is of importance to livestock farmers. Rather, it is the economic thresholds. For *B. microplus* this was estimated at 159 engorging females per day (Surtherst, *et al.,* 1983). The most detailed work on economic thresholds for African ticks is that by Pegram *et al.,* (1991). Using a system analysis approach, which means all production traits and their interactions were monitored simultaneously, they reported that the overall productivity improved by 25% in acaricide treated group over the untreated controls. This increase, however, was not enough to cover the cost of control.

The conclusion to be drawn from these studies is that in some years or some areas with low tick challenge where Zebu (*Bos indicus*) cattle are kept, there is little

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The conclusion to be drawn from these studies is that in some years or some areas with low tick challenge where Zebu (*Bos indicus*) cattle are kept, there is little

concerned (Surtherst, 1987). For the accurate estimation of losses from tick-borne diseases an accurate diagnostic test and survey to determine the overall prevalence of tick-borne diseases is necessary. This should be followed by specific experimentation in representative areas with emphasis on interactions between tick-borne diseases and other diseases.

2.3.3 Hide damage, toxicoses and secondary bacterial infections

Other losses associated with tick infestation include, damage to the hide, toxicoses and secondary bacterial infection at the sites of attachment of ticks. It has been estimated that lesions caused by tick feeding reduce the value of the hide by about 10% (Surtherst, 1987). However, this would largely depend on the tick species involved. This is because damage depends on the type of lesion caused and area of the body the tick feeds on. Presently there are no accurate estimates of losses due to hide damage for the majority of tick species.

Many species of ticks inject toxins into their hosts while feeding, leading to paralysis (*D. andersoni*), sweating sickness (*H. truncutum*, *R. evertsi evertsi*) or generalised toxicoses (*I. holocyclus*). The damage resulting from these effects depends on whether the hosts are killed or incapacitated (Drummond *et al.*, 1988). The extent of damage may also depend on the level of tick infestation (Njau and Nyindo, 1987).

Injury resulting from tick bites often leads to secondary infections by other opportunistic organisms. A positive correlation between the number of *Amblyomma* ticks feeding and the incidence of streptothricosis has been reported (Hadrill and Walker,1994). This damage is more difficult to quantify.

2.4 Chemical control of ticks

The use of chemicals for tick control has been reviewed by Drummond (1985). The first record of the use of chemicals to control ticks was in 1893 in South Africa (Keating, 1983). This was in the form of arsenical solution. Its use quickly spread to Australia in 1895 and to the United states to control Texas fever. New chemical were introduced, including organochlorines, organophosphorous compounds, carbamates, amidines and synthetic pyrethroids. Most of these chemicals have been used in dipping vats and spray races and to a lesser extent for hand dressing (Drummond *et al.,* 1988).

One of the main advantages of chemical control of pests is the ease with which pesticides are available for the control of most pests (Miller, 1986). The availability of acaricides played a major role in their widespread use during the last century. It only cost one or two cents to dip an animal in the early days of the introduction of arsenic, today, it costs nearly a dollar to dip an animal in synthetic pyrethroids (Tatchell, 1984). Several studies have shown that the intensive use of acaricides is not economically justifiable (Surtherst *et al.,* 1983, Tatchell *et al.,* 1986; Norval *et al.,* 1987; Pegram *et al.,* 1991,).

2.4.1 Acaricide resistance

Acaricide resistance is perhaps the single most important reason for the need to look for alternative tick control methods. The subject has been reviewed by Drummond (1977). Although different classes of acaricides have different modes of action, ticks have developed resistance to nearly all known classes of acaricides

(Nolan and Roulston, 1979). The mechanism by which this occurs has been reviewed by Nolan (1985). The genetic basis of resistance is attributed to a single gene mutation transmitted within the population through the normal Mendelian fashion (Stone,1981). Under normal circumstances, resistant mutants pre-occur in very low frequencies in field populations because resistance confers no advantage to them. However, upon application of acaricides, these mutants become advantaged and their numbers begin to increase.

The rate at which resistance to a particular chemical develops depends on a number of factors, including the initial frequency of the resistant genes and the intensity of selection (Nolan, 1985). It is, therefore, impossible to determine with certainty how soon resistance would develop to a particular acaricide. However, experience has shown that resistance is expected in five to ten years of the introduction of the chemical (Powel and Reid, 1982).

It is known both from practice and genetics that the prospects of the re-use of an acaricide once resistance has developed are very poor (Wharton and Roulston, 1970). However, possibilities do exist of prolonging the useful life of an acaricide by suppressing the mechanisms of resistance when this is clearly known (Nolan, 1985).

2.4.2 Environmental pollution

The other main reason for the desire to limit or eliminate the continued use of broad spectrum chemical pesticides is their indiscriminate effect on non-target organisms (Wall and Strong, 1987). The situation is made worse by the residual effect of acaricides which is a necessary characteristic of a good acaricide.

However, there are possibilities for the development of chemical pesticides specifically targeted to particular species thus reducing their environmental pollution (Briggs *et al,* 1984). Additionally, acaricides have also been reported to leave residues in meat and other animal products (McDougal *et al.,* 1979).

The realisation that there may not be an alternative to chemical control in the near future, has led to the development of novel ways of their delivery, in order to overcome some of the problems associated with chemical control.

The use of ear-tags impregnated with chemical pesticides has successfully controlled the gulfcoast ear tick *Amblyomma maculatum* (Gladney, 1976). Young *et al.,* (1985) also reported excellent control of *R. appendiculatus* for about 160 days using eartags impregnated with several different acaricides. The method significantly reduces environmental pollution by limiting the chemicals to only the target pests. Unfortunately the method does not work as well for other species of ticks like *Boophilus*, that feed all over the body, or *Amblyomma* species that feed on restricted areas like the neck, dewlap, and the udder (Young *et al.,* 1985). The other problem with ear-tag acaricides is the fact that they might encourage the development of resistance because of the long exposure to persistently high selection pressure (Miller, 1986). However, this is a debatable issue because it is not universally agreed whether it is exposure to sublethal doses or very high doses to a toxicant that encourage the development of resistance.

`Pouron' formulations of acaricides have been developed to overcome the problem of water scarcity and to reduce environmental pollution. They have been reported to provide excellent control of most ticks in cattle, sheep and goats (Rinkanya and Tatchell, 1990).

Systemic pesticides in the form of slow release boluses given with food or water have limited use for the control of ectoparasites because they are required in large doses (Miller and Miller, 1978). However, good control of six species of three host ticks was reported with Ivermectin in cattle (Drummond *et al.,* 1981). `Fumpur', an organophosphorus compound, was also effectively delivered in bolus to control ticks (Teel *et al.,* 1979).

While it is recognised that acaricides have played an invaluable role in the past, it is abundantly clear that they have failed in their primary objective of providing a stable and economical long-term solution to tick control. It is now accepted that the best option for tick and tick-borne disease control lies in integrated systems, based on host immunity, vaccines, chemical control, biological control and ecological options. While the search for alternative strategies continues, it must be realised that chemical control is still the most widely used, and in many cases the only method available, and that the situation is not likely to change dramatically in the near future.

2.5 Biological control of ticks

2.5.1 Parasites, predators and pathogens

Although several species of parasitoids have been reported to infest a number of tick species (Howard, 1907; Fiedler, 1953; Cooley and Kohls, 1934), their importance in the regulation of tick populations is only now being assessed (Mathewson, 1984). Mwangi (1990), reported that nearly 50% of the nymphs of *A.variegatum* were parasitised by the hymenopteran parasitoid *Hunterelus hookeri*

in one region of Kenya. Several other workers have reported the presence of the parasitoid in more than ten species of ticks (Cole, 1965). Ticks have many predators like, rodents, birds, ants and lizards (Mwangi, 1990). Hassan (1990), reported the possibility of using domestic chicken as a component of IPM for controlling ticks.

Many species of bacteria including *Klebsiella pneumoniae*, *Pseudomonas mirabilis*, *Proteus mirabilis* and *Serratia marcescens* have been isolated from dead or sick ticks and have been thought to be pathogenic (Calberg, 1986; Hendry and Rechav, 1981; Mwangi, 1990).

These strategies are useful components of an integrated tick control package. Further investigations are, however, necessary before they can be incorporated into tick control strategies. The general lack of enthusiasm in the study of parasites, pathogens and predators, for tick control could reflect the uncertainty with which their delivery is viewed. While there is no doubt that these biological agents play a role in the regulations of natural tick populations, the difficulty on how to manipulate them to blend with other tick control methods, is still problematic.

2.5.2 Modification of the habitat

Ticks spend a good proportion of their life, the free living phase, on the ground questing or quiescent and are totally dependent on the environmental conditions for their survival. The environment can be modified to make it inimical to their survival and development.

Clearing of bushes to remove moist patches has been used to control *Ixodes ricinus* in the United Kingdom and *A. americanum* in the United States (Milne, 1944). Pasture spelling or rotational grazing on two paddocks with strategic

acaricidal treatment of cattle in between the two paddocks forms part of control *B. microplus* in Queensland, Australia (Powell and Reid, 1982). However, this requires good fencing and the absence of alternative hosts for the ticks . It must also be balanced with the deterioration of the pastures that is likely to occur (FAO, 1984). The method is impractical in Africa as these conditions are unlikely to be fulfilled. Besides, where two and three-host ticks occur, with the nymphs and the unfed adults lasting up to two years, the situation becomes more complicated (Newson *et al,* 1984). Controlled burning combined with control of stock movement are expected to considerably reduce tick burdens in the environment (FAO, 1984). Other agricultural practices like ploughing, zero grazing and pasture improvement have a major impact on tick populations in the environment (Tatchell, 1987). Unfortunately these practices are currently occasional and are not integrated into conventional tick control practices.

A development that may have a major impact on tick control is the inclusion of pastures with anti-tick properties. Surtherst *et al.,* (1982) reported that the perennial legume *Stylosanthes* spp has glandular trichomes that immobilise the larvae of *B.microplus* and are subsequently killed by the volatile toxic compounds released by the grass. Several species of the legume have been shown to possess this property. However, the report by Norval *et al.,* (1983), that the larvae of *B. decoloratus* and the nymphs of *R. appendiculatus* preferred wheat straw to several species of *Stylosanthes* could limit their usefulness for tick control.

2.5.3 Interference with host finding

The aim of this strategy is to make hosts unavailable to the ticks when they require them thus leading to their death. The alternative strategy is that the available hosts are made unsuitable for tick feeding. Attempts have been made to graze sheep which are known to be unsuitable to a number of tick species before cattle. Grazing of adults before calves reduces the number of ticks likely to attach on calves because adults are generally more resistant to ticks than calves (FAO, 1984).

2.5.4 Other biological control options

Other novel ways for controlling ticks that are categorised as biological include the use of insect growth regulators like juvenile hormones. Miller *et al,* (1981) reported that diflobenzuron, a chitin inhibitor prevented the development of the immatures of horn fly and face fly in manure of treated cattle. Recent immunisation studies with ecdyson, a hormone responsible for eclosion, failed to interfere with moulting in *R. appendiculatus* although other developmental parameters like engorgement weight and egg production, were affected (Mbogo, 1995)

2.6 Immunological control of ticks

Among the various alternative tick control strategies, immunological control has become increasingly important. There are two approaches for the use of immunological responses in tick control. The first involves the exploitation of acquired resistance by hosts through selection of a breed or individuals of a breed

that are known to be resistant to ticks. This has found practical use in Australia, where it forms the basis in the control of *B. microplus* (Wharton and Norris, 1980). The second approach involves the use of isolated tick antigens to induce immunity to hosts by direct immunisation (Willadsen, 1980). This has yet to be applied in a practical way but it has great potential.

2.6.1 Acquired resistance

Johnson and Bancroft (1918) were the first to demonstrate host resistance to ticks in cattle. This was received with scepticism, partly because of the large number of ticks that cattle are capable of carrying (Bennet and Wharton, 1968). Wilkinson (1955) confirmed these early observations and further elucidated the nature of the resistance.

Lush (1924) proposed the concept of `innate' resistance to explain the differences observed in different breeds of cattle. Riek (1962) described the resistance as comprising both an innate and acquired components, with the acquired resistance becoming expressed only if the hosts were exposed to ticks. However, recent studies have disputed the concept of innate resistance, arguing that all resistance is acquired (Wagland, 1975; Rechav and Zeederberg, 1986). These workers suggest that resistance is acquired and that *Bos indicus* cattle are capable of acquiring resistance faster and to a higher degree than *Bos taurus*. Brown (1985) has also suggested the two types of animals might have different mechanisms for the development of resistance.

Although most of the earlier work demonstrated bovine resistance to *B. microplus* , acquired resistance has been demonstrated in a wide of range of host-tick

associations. Acquired resistance to ticks has been demonstrated in most cases as a reduction in the number of attaching ticks, reduced engorgement weight, longer feeding duration and reduced fecundity and/or fertility (Brown, 1985). Others have reported reduced hatchability, reduced moulting capacity of immatures into the next instar and increased mortality (Hewetson and Nolan, 1968).

In the two and three-host ticks, resistance occurs to all the instars of the particular species, although less effective so to adults than larvae (Walker *et al.*, 1990). Cross resistance between a number of tick species has also been demonstrated (McTier *et al.*, 1981; Brown and Askenase, 1981; Wagland, 1975). It was reported by Labarthe (1985) that antibodies induced against *B.microplus* ticks, cross-reacted with extracts of *Stomoxys calcitrans*. These reports suggest that identical epitopes may be conserved in different haematophagous parasites, a situation which may facilitate the development of an anti-tick vaccine.

Some other workers have reported cross-resistance as either absent or partial among other species of ticks (McTier *et al.*, 1981; Heller-Haupt *et al.*, 1981 and Wagland *et al.*, 1985).

2.6.1.1 Mechanism of acquired resistance

Although all the mechanisms involved in acquired resistance are not well understood, it is now known that acquired resistance to ticks is an immune-mediated phenomenon (Brown, 1985; Wikel and Allen, 1982 ; Willadsen, 1980). Riek (1962) suggested that acquired resistance response might be an immediate hypersensitivity response.

Histological examinations sites for attachments of ticks, has given clues to the mechanism of acquired resistance. Intense infiltrations of basophils on the dermis of resistant hosts has been reported (Allen, 1973; Walker and Fletcher, 1986). It was also reported by Schleger *et al,* (1976) that there was an accumulation of eosinophils, polymorphonuclear cells and lympocytes at tick feeding sites. Tatchell and Moorehouse (1970) reported an aggregation of neutrophils at tick attachment sites. Mast cell degranulation was also observed by Pavlovski and Alfeeva (1941); the more resistant the host, the greater the degranulation.

Allen *et al.,* (1977) suggested that acquired resistance might be a form of cutaneous basophil hypersensitivity, a type of delayed hypersensitivity (Richerson *et al.,* (1969).

The role of basophil in resistance may be in the mediation of the release of tissue histamine which in turn leads to vasopermeability. Tick feeding might then be inhibited by making the skin unsuitable for tick feeding through development of oedema. It was reported that tick resistant cattle have twice the level of histamine as susceptible ones (Willadsen *et al.,* 1979). Kemp and Bourne (1980) reported that high histamine levels caused ticks to detach and die. It was also shown by Tatchell and Bennet (1969) that cattle treated with histamine inhibitors had reduced resistance to ticks. These cattle had also far reduced skin reactivity to tick infestation compared to the controls (Willadsen *et al.,* 1979). Basophils might also inhibit tick feeding by acting through complement which has been shown to play a role possibly through the alternate pathway (Wikel and Allen, 1982).

Trager (1939) was the first to propose a role for humoral factors in acquired resistance to ticks, when he conferred some resistance by serum transfer from tick

resistant to susceptible guinea pigs. This was confirmed by Roberts and Kerr (1976), when they administered plasma from resistant cows to tick naive calves and conferred low-level resistance. Many other workers have reported the presence of specific antibodies as a result of tick feeding (Brossard, 1976; Njau *et al.,* 1988). Jackson and Opdebeeck, (1990) showed a positive correlation between the level of antibody titres specific for the IgG1 with the level of protection. This has led to suggestions that IgG1 is the antibody isotype that is involved in the protective response. The following series of events has been suggested by Brown, (1985) as to what most likely leads to the development of acquired resistance. Antigens from salivary glands are processed by the local antigen-processing cells such as the Langerhans cells. They are then presented to specifically sensitised T-cells. The T-cells are activated to produce chemotactic factors that recruit circulating basophils. The basophils in turn degranulate to release histamine through antibody and T-cell mediation. Histamine has been shown to be directly involved in the immune resistance response (Willadsen *et al.,*1979).

2.6.1.2 Application of host resistance to tick control

Acquired resistance to ticks may have a significant impact on tick control, if a number of management practices that maximise the benefits from the use of resistant hosts are adopted. Animals within the herd that show low resistance may be culled (FAO, 1984). Measurement of individual resistance by ranking of cattle according to their tick burdens is dependable (De castro *et al.,* 1991). Where culling is not a practical option, separation of the cattle into two herds, one of low and the other of high resistance may be applied. Strategic chemical control may then be

instituted to the susceptible herd. This approach has been successfully applied in Australia in the control of *B. microplus* (Ralph, 1989). Studies on three-host ticks in Africa, have shown that this approach may be successfully employed (Norval *et al.*, 1987; Tatchell *et al.*, 1986 ; Pegram *et al.*, 1991. These studies indicate that low or moderate tick burdens cause no damage when *Bos indicus* cattle are kept in areas free of tick-borne diseases.

The fact that resistance has been shown to be a hereditary character (Wharton *et al.*, 1970) means that selection for tick resistance is a viable option especially with the advances made in embryo transfer. Embryo transfer can increase the resistant offspring many fold within a relatively short time. Although this technology is currently expensive, it will be cheaper and more widely available in the future. It is also possible, that with developments in molecular biology the manipulation of individuals to incorporate resistant genes for the induction of immunity will be a viable option in the near future.

2.6.2 Immunisation against ticks

While acquired resistance has great potential in tick control, artificial induction of resistance through immunisation would have the advantage of not exposing the hosts to diseases or the other loses associated with tick infestations. Furthermore, naturally acquired resistance and artificially induced host resistance to ticks have immunologically different mechanisms (Willadsen, 1986) ; their combined effects may be exploited for a more effective immunological control of ticks (Essuman *et al.*, 1991).

Artificial induction of immunity to ticks was first demonstrated by Trager (1939) when he induced immunity to guinea pigs with whole larval extracts of *D. variabilis*. The subject has been reviewed by Willadsen (1980) and more recently by Brown (1988). Until the last decade, most of the work on artificial induction of immunity was with salivary glands in an attempt to imitate acquired immunity. The work of Allen and Humphreys (1979) had a major impact; for the first time they showed extracts of internal organs could be used to induce immunity to *D. andersoni*. They used both mid-gut and reproductive organs to induce immunity in both cattle and guinea pigs, leading to a drastic reduction in the engorgement weight and a reduction in the number of eggs laid.

Since then, immunity has been induced with a wide variety of tick tissues including, whole tick extracts (McGowan *et al.*, 1980); larval and nymphal extracts (Heller-Haupt *et al.*, 1981); midguts (Opdebeeck *et al.*,1988a; Essuman *et al.*, 1991) salivary glands (Brown, 1984 *et al.*, Nyindo *et al*, 1989) and reproductive organs(Allen and Humpreys,1979). Other tissues that have been used to induce immunity are cement substance (Shapiro *et al.*, 1989); antigen-antibody complexes (Mongi *et al.*, 1986) and haemolymph (Ben-Yikir and Baker, 1987).

Immunity has been demonstrated in both cattle and laboratory animals including rat (*Rattus rattus*), rabbit (*Oryctolagus cuniculus*) and guinea pig (*Cavia cobaya*) (Brown, 1988). On account of cost and convenience laboratory animals have been used extensively as models in most experiments.

As with acquired resistance, reduction in the number of feeding ticks, reduced engorgement weights and egg laying capacity have been used to demonstrate artificially induced resistance.

2.6.2.1 Immunisation with `concealed' antigens

Ticks have a large number of potential targets that could be prone to the host's immunological response (Galun, 1975). This is because a large number of the tick tissues come into contact with host blood during normal feeding. Although this idea was not immediately appreciated in tick research, it had been exploited in other parasites. Alger and Cabrera (1972) demonstrated that vaccination of rabbits with mid-gut homogenates of *Anopheles stephensi* led to increased mortality of the mosquitoes feeding on immunised rabbits. More importantly, however, was the finding by Schlein and Lewis (1976) that when rabbits were immunised with thoracic muscles, abdominal tissue and wing buds they induced a variety of effects including paralysis of legs and wings in *Stomoxys calcitran* flies feeding on them. This observation suggested that the immunological response was directed against internal organs.

In a related development, it was reported that host blood components can cross the tick gut into the haemolymph. Ackerman *et al.,* (1980) showed the presence of specific IgG antibodies against salivary gland antigens and ovaries in the haemolymph of *D.variabilis* ticks which had fed on rabbits immunised with these organs. Other workers also demonstrated the crossing of intact immunoglobulins in the haemolymph of both Ixodid and argasid ticks (Ben-Yikir, 1989) and Minoura *et al.,* (1985) respectively. Brossard and Rais (1981) demonstrated the presence of intact rabbit anti-haemolysin antibodies in the haemolymph of *Ixodes ricinus* ticks fed on rabbits immunised with sheep red blood cells. Furthermore, Fujisaki *et al.,*(1984) demonstrated the presence of bovine IgG against *Theileria sergenti* in the

haemolymph of *O. moubata* and *H. longicornis* ticks that fed on a calf immunised with the parasite.

Galun (1975) was the first to make a specific proposal for the use of `concealed' antigens, (antigens that are not normally exposed to the host immune responses during normal feeding), for immunisation. The author suggested that parasites could be controlled if they ingested antibodies from the host, directed against a vital developmental hormone. Evidence for the success of this approach was provided by many workers (Ackerman *et al.,* 1981; Opdebeeck *et al.,* 1988b; Kemp *et al.,* 1986 and Willadsen *et al.,* 1987).

The observation by Kohler *et al.,* (1967) that antibodies from rabbits infested with *H. anatolicum excavatum* could react with both salivary gland antigens and mid-gut extracts of the same tick, cast some doubt on the concept of concealed antigens. This could mean either the two organs shared common antigenic determinants or mid-gut antigens were exposed to the host immune responses during feeding. In another study, Opdebeeck *et al.,* (1990) compared the responses of cattle to infestation and immunisation with *B. microplus* antigens. Infested cattle were found to have significant antibodies against larval, mid-gut and salivary gland membrane antigens. Furthermore, they reported that cattle vaccinated with gut membrane antigens had positive cellular responses to both gut and salivary gland antigens. These results tend to support the view that the concept of concealed antigens does not exist.

However, the work of Johnson *et al.,* (1986); Kemp *et al.,* (1986) and Willadsen, (1987) showed that immunisation with gut antigens produced effects that were different from those produced by acquired resistance. An interesting feature of

this study was the finding of coloured ticks on immunised cattle as a result of the leakage of bovine erythrocytes into the tick haemolymph (Agbede and Kemp, 1986). They also showed that ticks that fed on immunised cattle had evidence of the disruption of the integrity of the gut wall.

Willadsen and McKenna (1991) concluded that the demonstration of antibodies by infested cattle to intrinsic membrane glycoproteins, is due to the presence of carbohydrate determinants on many tick glycoproteins. Credence is given to this view by the fact that sera from infested cattle reacted with the native Bm86 molecule but not with the non-glycosylated recombinant version of the same molecule (Willadsen and McKenna, 1991).

2.6.2.2 Characterisation of tick antigens

Identification and characterisation of tick antigens must be achieved first before the development of an anti-tick vaccine. Attempts to characterise resistance inducing tick antigens in hosts by a variety of immunochemical techniques have been reported (Reviewed by Brown, 1988). Unfortunately there are few reports of fully-characterised tick antigens, inducing resistance in their hosts except perhaps in *B. microplus* (Willadsen *et al.*, 1989)

Geczy *et al.*, (1971) isolated a protein of 30 kDa by ion exchange and isoelectric focusing techniques that evoked a strong immediate skin responses. It was shown to have esterase-like activity. Its role in the induction of resistance is not known. Willadsen and Wiliams (1976) isolated from adult *B. microplus* , a protein of about 60 kDa by gel permeation chromatography that was shown to have carbohydrate activity. Reich and Zorzopulos (1980) isolated 60 kDa protein with

strong skin reactivity from larval *B. microplus* by gel permeation on Sephadex G-200. The protein was shown to resemble phosphomonoesterase, an enzyme associated with the adsorptive surface of the gut. Brown and Askenase (1986) used immune sera from guinea pigs resistant to *A.americanum* in an affinity column to purify a 20 kDa protein that they suggested was responsible for the induction of the immune resistance response. Ruti *et al.,* 1989) isolated a 43 kDa protein from the mid-guts of adult *R. appendiculatus* that showed strong skin reactivity. Its specific role in the induction of resistance is not well understood. Willadsen *et al.,* (1989), using a combination of several immunochemical procedures, isolated a glycoprotein (Bm86) from partially fed *B. microplus* females with a molecular weight of 86 kDa. The protein could protect cattle against tick infestation by nearly 90%. Part of the amino acid sequence has been determined (Rand, *et al.,* 1989) and studies on its specific function are underway.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Use of membrane-bound proteins from the midgut of *A. variegatum* to induce immunity in rabbits

3.1.1 Tick rearing

A pathogen free laboratory strain of *A.variegatum* was used. The parasitic stages of all the instars were reared on rabbit ears according to Bailey (1960). The adult ticks were found not to attach easily thus leading to high mortalities. In order to overcome this problem, several rearing procedures were tried and one in which a glass tube measuring 2.5 cm x 7.5 cm was retained in the ear bag for about 24 h, was found to give higher yields. Briefly, a cotton cloth ear bag was secured at the base of the rabbit ear using leukoplast adhesive tape (BDF, Beiersdorf AG, Hamburg, Germany). The tube was held in place by fastening the cotton cloth firmly on top. This confined the ticks close to the ear but leaving enough space for the ticks to move freely. The glass tube was removed after 24 h by which time most of the ticks had attached, leaving the cotton cloth in place.

The non-parasitic stages were kept in Hotpack refrigerated incubators (Philadelphia, PA, USA) maintained at 27^0 C and 85-90% relative humidity (RH). Humidity was maintained with a 40% saturated potassium chloride solution (Hogman, 1948). Unfed adult stages were stored in glass tubes kept in aluminium cans filled with sterile moist sand and kept at 18^0 C.

3.1.2 Vertebrate hosts

New-Zealand white rabbits (6-8 months old), of both sexes weighing 3.5-4 Kg, of both sexes were used in all the experiments. They were kept singly in galvanised steel cages 50 cm x 1 m x 1 m, maintained on a commercial rabbit pellet feed. The feed and water, were provided *ad libitum*. There was a 12h day length during most of the period of the experiment. The mean minimum and maximum temperatures were 10.8 and 25.4 ^{0}C respectively during the experimental period

3.1.3 Preparation of membrane-bound proteins of the mid-gut

3.1.3.1 Dissection of mid-guts

Midguts were dissected from partially fed *A.variegatum* females by a modification of the method of Purnel and Joiner (1968). Ticks were embedded in their dorsal position on paraffin wax in Petri dishes. The dishes were filled with phosphate-buffered saline (PBS: 0.01 M NaH2PO4, 0.01 M Na2HPO4 and 0.15 M NaCl) containing 0.01 M EDTA and 0.001 % (v/v) Phenylmethyl sulphonyl fluoride (PMSF) as protease inhibitors. Using number 11 scalpels, the integument was removed by making an incision along the side of the body. Mid-guts were removed with number 5 fine forceps and dropped in stainless steel Dewar flasks (Cole Parmer Instrument Company, Chicago, IL. USA) filled with liquid nitrogen. The tissues were then stored at -70^0 C.

3.1.3.2 Homogenisation and solubilisation of the mid-guts

Mid-guts (5g) were homogenised and solubilised by a modification of the Willadsen method (1989). Briefly, the material was washed several times with PBS to get rid of the host material by centrifugation in Labofuge (Haraeus Sepatech GmbH, Germany) centrifuge (3000 x g, 10 min, 4^0 C). This was continued until the supernatant solution became clear. The pellet from the final washing step was resuspended in 5 ml of PBS and homogenised in a polytron tissue homogeniser (Kinematic gmbH, Switzerland) for 3 min in cycles of 30 sec, with 30 sec pauses at setting 5 in an ice bath. The homogenate was then centrifuged (600 x g, 10 min, 4^0 C). The supernatant solution was centrifuged in a Beckman preparative ultracentrifuge (Beckman Instruments Inc, Palo Alto, CA. USA) (100 000 x g, 1 h, 4^0 C) and the pellet obtained was re-suspended in 5 ml PBS containing 1 % (v/v) Triton X-114 (Sigma Chemical Corporation, St. Louis, MO.,USA) pre-condensed according to Bordier (1981). The sample was homogenised as earlier stated followed by mild sonication at an amplitude of 14 in a Soniprep 150 sonicator (MSE Ltd, Crawley, Sussex, England) for 3 min in cycles of 30 sec with 30 sec pauses. The sample was allowed to stand for 1 hour on ice before centrifugation in Sorvall RC-5 centrifuge (Sorvall Instruments, Du Pont Company, Newtown, CT., USA) (20 000 x g, 30 min, 4^0 C).

3.1.3.3 Phase-partition of membrane-bound proteins of the mid-gut

The supernatant solution obtained after ultracentrifugation was phase-partitioned according to Bordier (1981). The supernatant solution (600 μl) was

overlaid on 200 µl of a 6% (w/v) sucrose cushion in 1.5 ml Eppendorf micro test-tube (Eppendorf-Netheler, Hinz GmbH, Hamburg, Germany), incubated in a water bath (3 min, 30^0 C) and centrifuged in an Eppendorf Microfuge (3 min, 10,000 x g). The material partitioned into a clear aqueous phase on top and a viscous detergent phase at the bottom.

The aqueous phase was removed and pooled and in order to remove any contaminants from the detergent phase, Triton X-114 was added to a concentration of 0.5 % v/v and re-dissolved and kept on ice for 30 min. This was overlaid on fresh cushion of sucrose and the procedure repeated as described above. The new detergent phase was collected and mixed with the previous one. The aqueous phase was again pooled and fresh Triton X-114 was added to a final concentration of 2 % v/v and the procedure repeated as described. However, the detergent phase was discarded and the aqueous phase pooled and concentrated by ultrafiltration in a dialysis cell using an Amicon membrane (Amicon Corporation, Danvers, MA, USA) with a cut off of 5,000 daltons at a pressure of 2 psi. The samples were stored at -20 ^{0}C

3.1.4 Estimation of protein concentration

Determination of the protein content of the detergent and the aqueous phases was conducted by the Pierce Bicinchonic acid (BCA) protein determination method (Pierce, Rockford, IL., USA). Bovine serum albumin (BSA) fraction V (Pierce) was used as the standard. Optical density was read at 562 nm in a Du-50 Spectrophotometer (Beckman).

3.1.5 Immunisation of rabbits with membrane-bound proteins

Twenty four rabbits were randomly divided into three groups of eight rabbits. Each rabbit in the two experimental groups was injected intramuscularly using 1 x 19 Gauge Sterijekt needles (Japan R.I Irradiation Industry, Tochigi, Japan) at the posterior end of the thigh muscles of the hind legs. Each rabbit received 250 μg of the protein, emulsified in the ratio of 1:2 in Freund's complete adjuvant (Sigma) . The dose was divided and given at two sites. Two weeks later the rabbits were injected with a booster injection with a similar amount of protein but emulsified in Freund's incomplete adjuvant. A second booster injection was given after a further two weeks. Rabbits in the control group were injected with PBS and the adjuvant.

3.1.6 Collection of serum

Rabbits were bled through the central ear vein into universal bottles before the start of the experiment and weekly afterwards. Serum was prepared by allowing the blood to clot in a water bath at 37^0 C. The clot adhering on the wall of the container was freed after one hour in order to enhance its retraction. Serum was collected after 3 hours and loose erythrocytes removed by centrifugation (3000 x g, 30 min, 27^0 C) and stored at -20^0 C.

3.1.7 Tick challenge

Ten days after the last booster injection, four of the eight rabbits in each group were challenged with 100 larvae, 100 nymphs, 40 males and 20 females of *A.variegatum* as described in section 3.1., while the other four were challenged with

a similar number of *R.appendiculatus* ticks. Adults were used in the ratio of 2:1 males to females to ensure mating was not a limitation to the full engorgement of the females. Larvae and nymphs were placed in one ear while the adults were kept in the other.

3.1.8 Data collection

For each tick species the following parameters were assessed.

(1) Feeding duration (in days) of the larvae, nymphs and adults. This is the time from when ticks were placed into the ear bags to when they were collected as engorged ticks from the bags.

(2) Percentage yield : This is the proportion of ticks that fed to engorgement out of the total applied that were applied expressed as a percentage.

(3) Engorgement weight: Engorged larvae, nymphs and adults were weighed immediately after collection using a Mettler AC balance (Mettler instruments AG,Greifensee, Zurich Switzerland.

(4) Egg mass: Engorged females were placed in glass tubes containing Whartman no 1filter papers on which to lay eggs. After completion of oviposition all the eggs were removed from this filter paper and weighed.

(5) Egg conversion efficiency (ECE): This is the ratio of the engorgement weight of the female to the weight of the egg mass.

(6) Moultability: All the larvae and nymphs that did not moult into nymphs and adults respectively were counted and expressed as a percentage of the total collected.

(7) Mortality: All engorged ticks in the bag that were collected dead were counted and expressed as a percentage of the total collected.

(8) Hatchability: An estimate of the number of eggs that hatched into larvae was done visually and expressed as a percentage of the eggs laid .

3.1.9 Assessment and quantification of specific antibodies production

3.1.9.1 Double-immunodiffusion test

The presence of serum antibodies was assessed before tick challenge by the Ouchterlony test (1949). The test was carried out on glass plates using 1 % agarose gels in PBS (pH 7.4), 0.1% sodium azide and 3 % w/v polyethylene glycol (Sigma) was included into the gels so as to enhance precipitation. The samples were allowed to diffuse for 24 h at room temperature in a humidified chamber. Excess antibody and antigen were washed with several changes of PBS pH 7.4. To visualise, the precipitin lines, the gels were stained in Coomassie Brilliant Blue and destained in 50 % methanol and 9 % acetic acid.

3.1.9.2 Enzyme linked immunosorbent assay (ELIZA)

Serum antibodies were quantified by the ELIZA technique. Optimal reaction concentrations of antigens and conjugates were pre-determined by titration. The detergent or aqueous phase proteins, 10 μg in 100 μl of 5 mM carbonate-bicarbonate buffer pH 9.6, was added to each well of a Microtitre immuno-plates (Nunc Intermed, Denmark). Plates were left to coat overnight at 4^0 C and then washed with

PBS that contained Tween-20 to block non-specific binding sites. The test sera (100 µl) diluted 1:100 in PBS, pH 7.2 were added to each well and incubated for 1 hr at 37^0 C. The plates were then washed 5 times with distilled water and a final wash with PBS that contained Tween-20 to remove any unbound antibodies. Goat anti-rabbit IgG, conjugated to alkaline phosphatase (Sigma) was diluted 1:2000 and 100 µl, added to each well and incubated for 1 hr at 37^0 C. Plates were washed 3 times before adding 100µl of the substrate, p-Nitrophenyl phosphate (Sigma) in citrate buffer pH 5.0.

The optical density was read after 30 min of colour development at 505 nm in a Titertek Multiscan MC (Flow Laboratories Ltd, Irvine, Scotland, UK).

3.1.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (Laemmli, 1970). Gradients were cast using a gradient maker (BRL, Gaithersburg, MD, USA). Molecular weight markers were obtained from Pharmacia (Uppsala, Sweden). Samples with sample buffer (125 mM Tris-HCl, pH 6.8, 20% sucrose, 4% SDS and 5% 2-mercaptoethanol) were boiled in a water bath for five minutes and separated by electrophoresis on 3% stacking and 12.5% separation gels or 5-20% gradient with Bis-acrylamide in a continuous buffer system. The current was set at 25 mA until the Bromophenol blue migrated to the interphase between the stacking and the separation gels. Electrophoresis was stopped when the tracking dye had reached the end of the separation gel.

After electrophoresis the gels were either stained in Coomassie Brilliant Blue R250 in methanol and destained in 50 % methanol, 9% acetic acid or with silver reagent according to Merril et al., (1981).

3.1.11 Characterisation of separated proteins by Western Blot

Electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose paper (NCP) and the immunological detection of the of proteins was performed according to Townbin et al., (1979) with minor modifications. Nitrocellulose paper (Bio-Rad, Richmond, CA, USA) was of 0.45 um pore size. Transfer was carried for 3 h, at 90 volts in a Trans-blot cell (Bio-Rad). After the transfer, non-specific binding sites were blocked at room temperature by incubation with PBS, pH 7.5, containing 0.3% v/v Tween-20. The strips of NPC were then separately incubated for 4 h with a 1:200 dilution of sera from rabbits immunised with either the detergent or the aqueous phase proteins. Antisera from naive rabbits were used as the control. The NCP strips were then incubated with goat anti-rabbit IgG (Heavy and light chain), conjugated to alkaline phosphatase (Sigma) at a dilution of 1:1000 (v/v) in PBS-Tween 20. The bands were visualised by adding 20 mM Nitroblue tetrazolium (NBT), 20 mM 5-bromo-4 chloro-3 indolyl phosphate (BCIP), (Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA) in 0.1 M Tris buffer pH 9.5 at room temperature.

3.2 Purification of detergent-phase proteins by gel permeation chromatography

Gel permeation chromatography was undertaken after Porath and Flodin (1959) with few modifications. Detergent phase proteins were extensively dialysed against PBS (10 mM Na2HPO4, 10 mM NaH2PO4, O.1 M NaCl), pH 7.0 containing 10 mM EDTA and 0.02% (w/v) sodium azide and 0.001% (v/v) Triton X-114. The Protein concentration was adjusted to 6.0 mg/ml and the sample filtered through a 0.22 µm pore Millex-GS filter unit (Millipore Corporation, Bedford, MA. USA).

Sephadex G75 dextran was washed with several changes of the buffer and degassed before packing a 2 x 120 cm Econo column (Bio-Rad). Calibration of the column was performed according to the instruction of the manufacturer. Both low and high molecular weight standards (Pharmacia) were used. Dextran 2000 (I mg) (Pharmacia) was added to the column to determine the exclusion volume. Samples (1 ml) were applied onto the column and 3 ml fractions were collected using Frac 100 automatic fraction collector (Pharmacia) at a flow rate of 15 ml per hour. Fractionation of proteins was monitored at 280 nm using a 2100 UVICORD (Pharmacia) and recorded directly by a chart recorder (Pharmacia). The different peaks were separately pooled and concentrated to about 1 ml by ultrafiltration as described above, then dialysed against PBS pH 7.4. All activities were carried out at 4^0 C and the final product stored at -20^0 C.

Protein concentration of the various peaks was determined as described above. Protein composition of the proteins eluted from Sephadex G75 column was

analysed by SDS-PAGE as described above and the immunological specificity of the sera raised against the detergent phase proteins was assessed by immunoblotting experiments as described above.

Immunisation and challenge experiments to determine the biological activity of the proteins obtained from gel permeation chromatography were undertaken as described above (3.1.5), except that in this case each rabbit was immunised with 300 μg of protein.

The feeding and the development parameters of the ticks feeding on both immunised and control rabbits was assessed as described above (3.1.8).

3.3 Preparative polyacrylamide gel electrophoresis and elution of proteins from the gel

Detergent phase proteins were resolved in a 12.5 % preparative SDS-PAGE gels. Pre-stained low molecular weight standards (BRL) were resolved alongside the sample. After completion of the elecrophoresis, the gels were quickly rinsed in PBS and cut along the 68 kDa and at 24 kDa molecular weight markers. The three strips were separately cut into smaller pieces (5 mm x 2 cm) and placed into the glass tubes of model 422 electroeluter (Bio-Rad). The tubes were filled with the elution buffer (Tris-HCl, pH 8.8) and eluted at a constant current of 10 mA per tube. Elution lasted for at least 4 h or overnight at 4^0 C. The eluted proteins were collected in about 600 μl per tube, pooled and concentrated as described above.

In order to assess the efficiency of elution, gel slices were stained with silver reagent after each run and a comparison of the protein content of the gels before and after elution was visually assessed.

The eluted detergent phase proteins were analysed by polyacrylamide gel electrophoresis and their immunological specificities for the already produced antibodies assessed by immunoblotting test as described above.

The design and protocol for immunisation of rabbits was similar to the ones described above, except that cross protection experiments were not done in this case. Each rabbit was injected intramuscularly with 50 µg of the protein of the appropriate fraction. Rabbits were bled for sera as described above and the sera stored at -20^0 C.

The parameters described in section 3.1.8 were used to assess the effect of the eluted proteins on the feeding performance and development of ticks.

3.4 Statistical analyses

The statistical analysis system (SAS), computer programme was used for the analysis of variance by the general linear model (GLM) and Duncan's multiple range test and Tukey's Studentized Range (HSD) test was used for the determination of the differences between means. For all the tests, significance level was 0.05. Where percentages were involved the data was first normalised with arcsin transformation before analysis.

CHAPTER FOUR

RESULTS

4.1 Induction of immunity to rabbits with gut membrane proteins

4.1.1 Analysis of the membrane-bound mid-gut proteins by SDS-PAGE

Examination of silver stained SDS-PAGE gels of solubilised whole gut extract, detergent and the aqueous phase proteins revealed numerous polypeptide bands (Figure 1) with their approximate molecular weights (Table 1).

Most of the polypeptide bands in the whole gut extract could be accounted for either in the detergent or the aqueous phases. Although the two phases were markedly different, 12 polypeptides were common to both phases (Table 1). This finding meant either some proteins from one phase contaminated the other phase or the proteins were actually different but had similar relative mobilities.

Since the electrophoresis took place under reducing conditions, most of these polypeptides are probably sub-units of the same protein. Attempts to run the material in the native form were unsuccessful.

Therefore, phase-partitioning was able to separate gut extract into two distinct fractions, integral membrane proteins in the detergent phase and peripheral membrane proteins in the aqueous phase with minimum contamination.

4.1.2 Double-immunodiffusion test

Figure 2 shows the results of an immunodiffusion test of the detergent-phase proteins tested with anti-sera from rabbits immunised with the detergent and aqueous-phase proteins. The detergent-phase formed three precipitin lines with rabbit antisera against the detergent phase proteins and five precipitin lines with the antisera against the aqueous phase proteins. One major precipitin line had the reaction of identity, suggesting that proteins from both phases cross-reacted but that there were also some unique proteins in each phase.

Aqueous phase proteins also reacted with antisera against proteins from both the aqueous and the detergent phases to form three and four precipitin lines, respectively (Figure 3).

There was greater intensity in the reaction of the aqueous phase proteins compared to that of the detergent-phase proteins suggesting that these proteins are more immunogenic than those of the detergent phase.

4.1.3 Immunoblot test

The immunobloting experiments revealed that both fractions contained several polypeptide bands that were reactive with antisera against both the detergent and aqueous-phases. There were 12 polypeptide bands in the detergent phase and 8 in the aqueous phase that were reactive with antisera raised against the detergent phase proteins (Figure 4). Antisera raised against the aqueous phase proteins reacted with 13 and 11 polypeptides of the aqueous-phase proteins and detergent-phase,

respectively (Figure 4)., There was extensive serological cross-reactivity between the two fractions (Table 2, figure 4).

There were even four polypeptide bands in the aqueous phase that were reactive with control antisera. None of the polypeptides in the detergent phase reacted with control antisera (Figure 4). In all cases most of the reactive bands had molecular weight in excess of 50 kDa except four bands in the detergent phase and five in the aqueous phase.

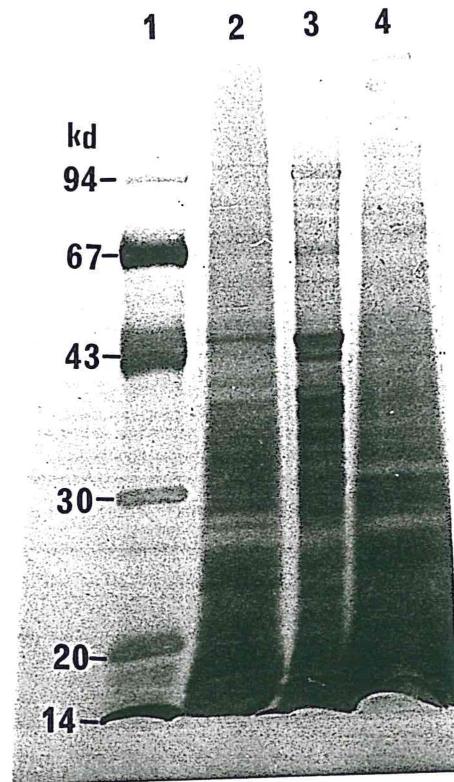

Figure 1. Analysis of whole gut extract (Lane 2), aqueous-phase (Lane 3) and detergent-phase (Lane 4) of *A.variegatum* by SDS-PAGE. Molecular weight markers are on lane 1

Table 1. Approximate molecular weight (kDa) of *A.variegatum* whole gut extract, detergent and aqueous-phases fractionated by SDS-PAGE and stained with silver reagent

Molecular weight in kilodaltons		
Whole gut	Detergent	Aqueous
233.0	154.0	112.0
104.0	94.0	84.0
85.0	81.0	74.0
81.0	77.0	68.0a
69.0	68.0a	66.0a
66.0	66.0a	64.0a
63.0	64.0a	57.0a
58.0	62.0	52.0
54.0	57.0a	47.0a
52.0	54.0	44.0
50.0	51.0	43.0a
45.0	50.0	39.5
44.0	47.0a	39.0a
41.0	43.0a	37.5
39.0	42.5	37.0
37.0	39.0a	35.5
36.0	36.0	34.0
35.0	34.0	33.5
34.5	33.0	32.0
33.0	32.0	31.0a
32.0	31.5	27.5
31.0	31.0a	26.5
28.0	30.5	26.0
27.0	29.0	25.0a
25.0	28.0	24.0a
24.0	25.0a	19.0
23.0	24.0a	17.0a
21.0	21.0	16.5
20.0	20.0	16.0
19.0	17.0a	14.0a
17.0	15.5	
16.0	15.0	
13.5	14.0a	
13.5	13.5	
13.0	13.0	
	12.5	

a____Polypeptide bands with similar molecular weights found in both the detergent and the aqueous phases.

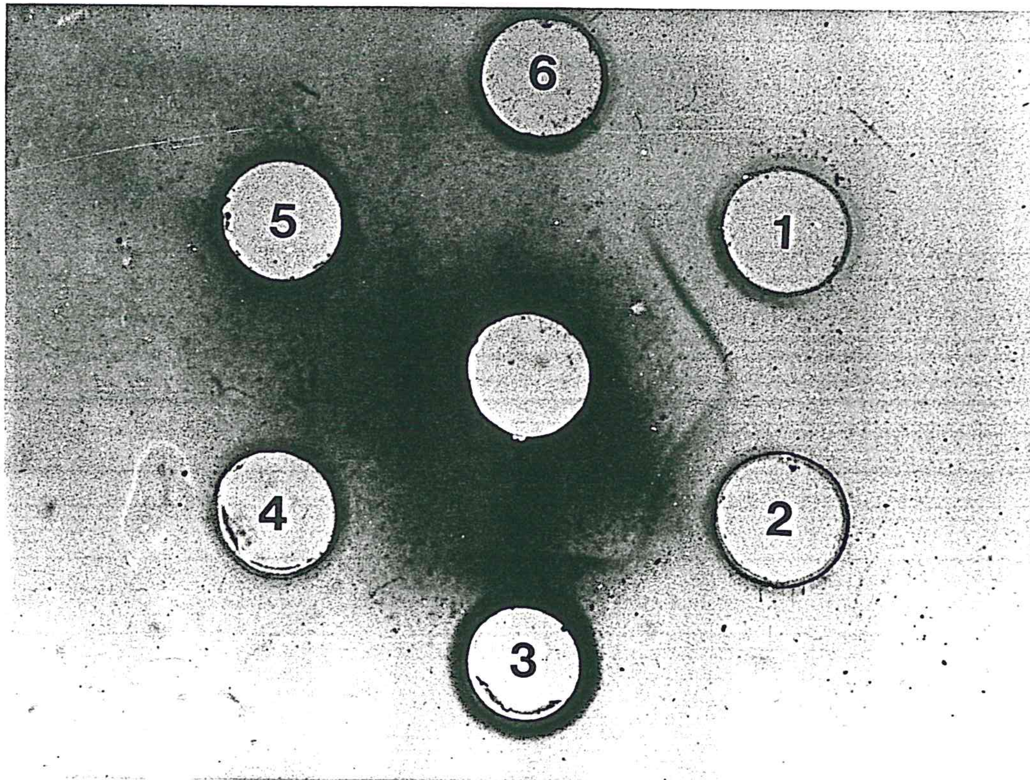

Figure 2. Immunodifusion pattern of detergent-phase (well 1),and aqueous-phase (well 3) proteins reacted against rabbit antisera to the detergent-phase proteins (central well). Well 4 contained bovine serum albumin (BSA) while well 5 contained PBS.

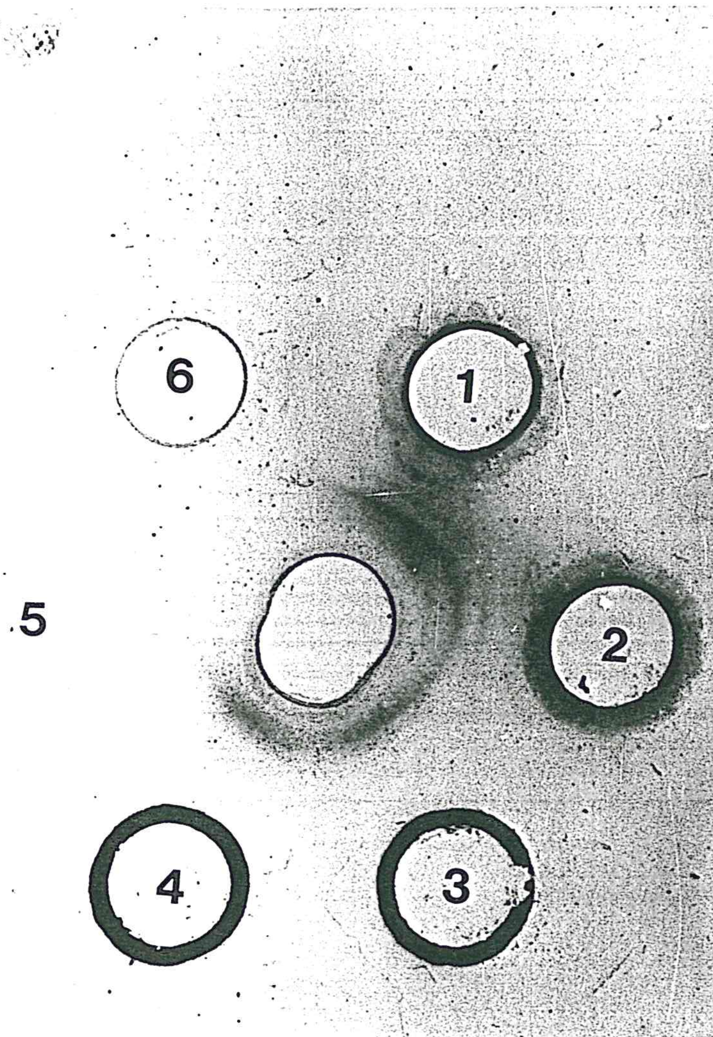

Figure 3. Immunodifusion pattern of aqueous-phase (wells 1 and 2), detergent phase (wells 3 and 4) proteins reacted against rabbit antisera to the aqueous-phase proteins (central well). Well 5 contained bovine serum albumin (BSA) while well 6 contained PBS.

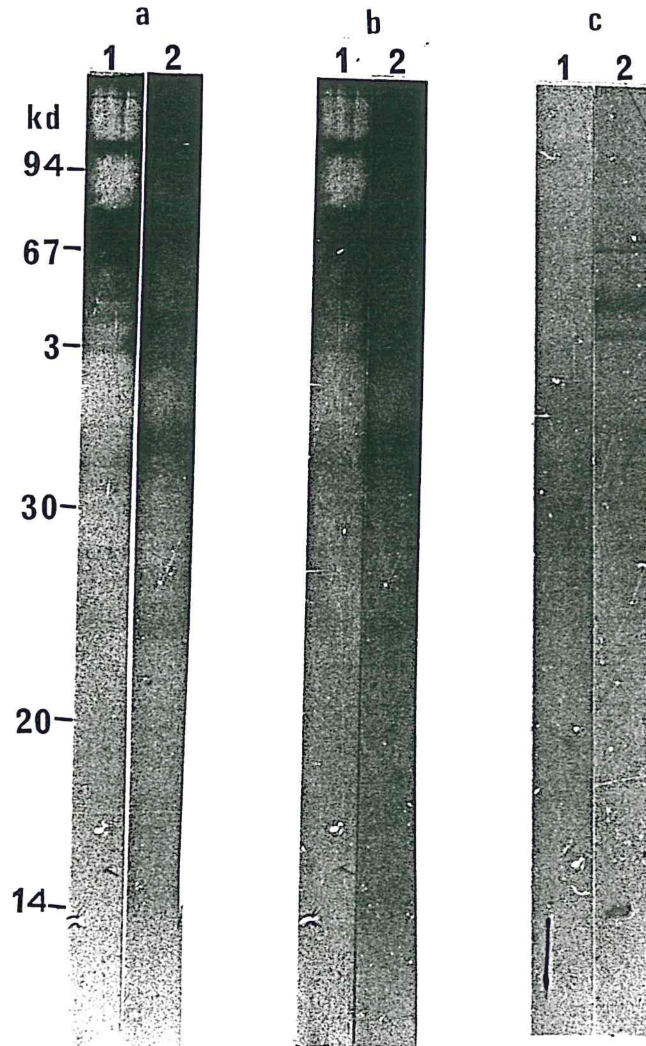

Figure 4. Immunoblot analyses of the polypeptides in the detergent (lane 1) and aqueous phases reacting with rabbit antisera to (a) detergent, (b) aqueous phase proteins (c) control sera. Molecular weight markers are shown on the left

Table 2. Approximate molecular weight of the protein polypeptides in the detergent and the aqueous-phases reacting with sera from immunised rabbits

	ANTIGEN FRACTION			
ANTISERA	Detergent phase		Aqueous phase	
	195.0	56.02	199.0	41.0
Rabbits immunised	166.0	51.0	158.0	33.0
with Detergent.	112.0	42.0	128.0	31.0
phase proteins	77.0	39.0	108.0	30.0
	70.0	34.0	96.0	23.0
	63.0	30.0	88.0	
	184	68.0	179.0	72.0
Rabbits immunised	158.0	66.0	123.0	39.0
with aqueous-phase	77.0	60.0	102.0	28.0
proteins	74.0	58.0	85.0	14.5
			63.0	
Control sera			54.0	
		——	48.0	
			39.0	
			32.0	

4.1.4 Enzyme linked immunosorbent assay of the detergent and aqueous-phase proteins

Figure 5 shows the level of titres of serum antibodies in an ELISA test. In both groups of rabbits the titres rose to a maximum after the third booster injection. Higher titres were detected in serum of rabbits immunised with the aqueous phase proteins than with detergent phase. This is similar to the immunodiffusion test where the intensity of the reaction was higher with the aqueous than with the detergent-phase proteins. Control rabbits had detectable titres against the aqueous phase proteins especially after the second booster injection.

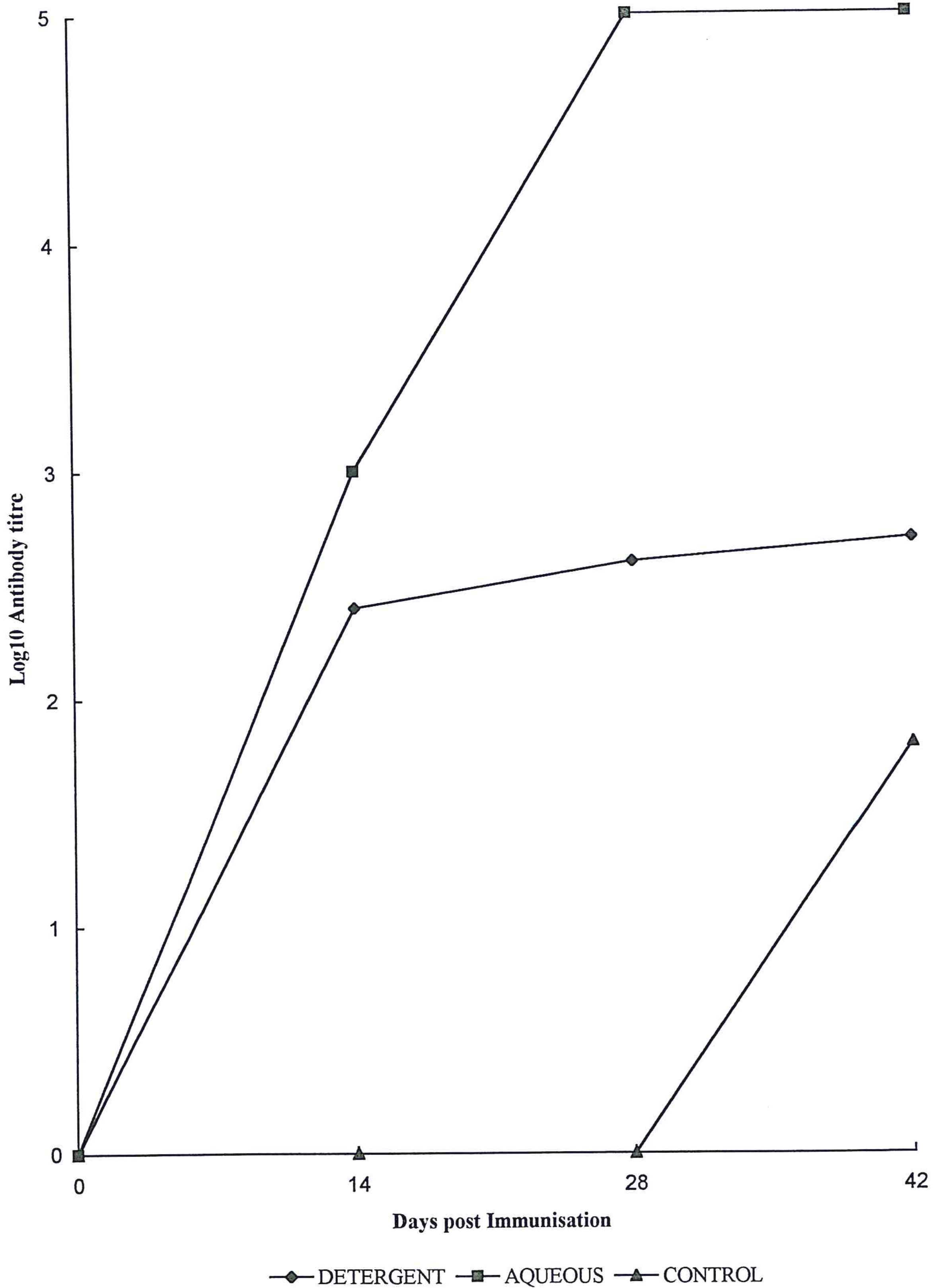

Figure 5. Mean ELISA absorbance values of antisera from three groups of rabbits before and after immunisation with (□) detergent, (+) aqueous phase proteins and (◊) non-immunised controls.

4.1.5 Effect of detergent-phase proteins on the feeding and development performance of *A.variegatum*

Tables 5, 6 and 7 summarises the effects of feeding ticks on rabbits immunised with detergent-phase proteins

4.1.5.1 Adults

Adult ticks feeding on rabbits immunised with the detergent-phase antigens had significantly reduced engorgement weight (30%); reduced weight of the egg mass (45%) lower egg conversion efficiency (20%) and reduced hatchability of the eggs (Table 5). There was no effect on the yield (number of ticks feeding successfully to engorgement) or the duration of their feeding.

Egg conversion efficiency is of particular interest in that it indicates that there was an effect on the process of oogenesis other than the normal reduction of egg mass as a result of the reduced engorgement weight (Egg mass and engorgement weight are positively correlated with the former which is normally about 50% of the latter).

4.1.5.2 Nymphs

Both the engorgement weight and the feeding duration of the ticks feeding on immunised rabbits were significantly affected. Engorgement weight was reduced while the feeding duration was increased. There was a slight reduction in the number of the nymphs feeding to engorgement but this was not statistically

significant at 5% level. There was no effect on the moulting capacity or on the mortality of the engorged nymphs which was about 3% (Table 6).

4.1.5.3 Larvae

Larvae were the least affected of the three instars. Only the mean feeding duration was significantly increased (Table 7). There was also a slight but not statistically significant reduction in the number of larvae feeding to engorgement.

The engorgement weight, moulting and the mortality of the engorged larvae were all unaffected.

4.1.6 Effect of aqueous-phase proteins on the feeding and development performance of *A.variegatum* feeding on immunised rabbits

The results of feeding and development of *Amblyomma variegatum* ticks fed on rabbits immunised with aqueous phase proteins are summarised in Tables, 5, 6 and 7. In adults both the engorgement weight and the egg mass were significantly reduced (16% and 21.5 %), respectively (Table 3). The egg conversion efficiency and the hatchability of the eggs were also slightly reduced but the differences were not significant at the 5% level. Only the feeding duration of the larvae was significantly increased and no parameter was affected in nymphs.

Table 3. Feeding and development parameters of *A. variegatum* females fed on rabbits immunised with detergent and aqueous phase proteins

	ANTIGEN		
PARAMETER	DET[2]	AC[3]	CONTROL
Mean[1] Yield (Number±SE)	8.50 ± 2.66a	9.33 ± 2.40a	8.00 ± 2.51a
Mean engorgement period (days±SE)	18.85 ± 0.40a	18.46 ± 0.54a	18.58 ± 0.59a
Mean engorgement weight(mg±SE)	1480.05 ± 142.22b	1691.62 ± 62.18b	2035.19 ± 166.1a
Mean egg mass (mg±SE)	470.35 ± 69.34c	680.20 ± 99.19b	866.78 ± 91.34a
Mean egg conversion efficiency	31.67 ± 3.30b	36.03 ± 2.43a	39.86 ± 095a
Mean hatchability (%)	15.52 ± 4.73b	32.16 ± 6.42a	36.21 ± 7.47a

[1] Means of four rabbits each challenged with 20 females and 40 males of *A. variegatum*

[2] DET____Detergent phase proteins

[3] AC_____Aqueous phase proteins

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

Table 4. Feeding and development parameters of nymphs of *A.variegatum* fed on rabbits immunised with detergent and aqueous phase proteins

A

		NTIGEN	
PARAMETER	DET[1]	AC[2]	CONTROL
Mean[1] yield (number ±SE)	83.25 ± 3.64a	93.00 ± 3.51a	84.00 ± 6.73a
Mean engorgement period (days ±SE)	8.82 ± 0.09c	8.09 ± 0.08b	8.51 ± 0.08c
Mean engorgement weight (mg±SE)	44.79 ± 0.78b	47.72 ± 0.82a	47.85 ± 078a
Mean moultability(%)	93.07 ± 3.10a	91.13 ± 2.28a	88.27 ± 6.56a
Mean mortality(%)	3.57 ± 1.56a	1.76 ± 033a	4.57 ± 1.37a

[1] ____ Means of four rabbits each challenged with 100 *A.variegatum* nymphs

[2] DET____Detergent phase proteins

[3] AC_____Aqueous phase proteins

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$

Table 5. Feeding and development parameters of *A.variegatum* larvae fed on rabbits immunised with detergent and aqueous-phase proteins

	ANTIGEN		
PARAMETER	DET[2]	AC[3]	CONTROL
Mean[1] yield (number ±SE)	51.75 ± 2.49a	62.66 ± 5.54a	58.00 ± 7.34a
Mean engorgement period (days±SE)	7.33 ± 0.09b	7.54 ± 0.12b	7.08 ± 0.08a
Mean engorgement weight (mg±SE)	2.33 ± 0.05a	2.31 ± 0.04a	2.30 ± 0.04a
Mean moultability(%)	84.50 ± 5.20a	86.16 ± 0.52a	76.95 ± 2.9a
Mean mortality(%)	9.95 ± 2.72a	10.43 ± 1.86a	10.62 ± 3.2 a

[1]______Means of four rabbits each challenged with 100 A. variegatum larvae

[2]DET___Detergent phase proteins

[3]AC____Aqueous phase proteins

Similar letters in the same row indicate no significant difference at $p<0.05$

4.1.7 Assessment of cross-protection of detergent-phase proteins on *R.appendiculatus*

In view of the extensive cross-reactivity in serological tests, it was necessary to establish whether the antigens are species specific or they are cross protective to ticks of other genera by challenge experiments. The results of immunisation and challenge experiments are summarised in tables 8, 9 and 10.

4.1.7.1 Adults

Both the mean engorgement period and the egg conversion efficiency were significantly affected (5% and 7%), respectively.

The number of ticks feeding to engorgement, the weight of the egg mass and hatchability were also slightly reduced although the reductions were not significant at the 5% level.

Interestingly, other than the lower level of the effects, these parameters were similar to those affected in *Amblyomma variegatum*.

4.1.7.2 Nymphs

The yield of nymphs was reduced by nearly 14%, while the feeding duration was increased both of which were significant.

There was no effect on the engorgement weight or the ability to moult to adults.

4.1.7.3 Larvae

The larvae of *R..appendiculatus* were more affected than those of *A.variegatum*. The feeding duration was lengthened and the mean engorgement weight reduced by 7% each. The mean yield and the percentage of the successful feeders that moulted into nymphs were also lower although the decreases were not statistically significant at 5% level.

4.1.8 Assessment of cross-protection of aqueous-phase proteins against *R. appendiculatus*

The aqueous-phase proteins had no effect on any parameter in all three instars of *R. appendiculatus* (Tables 6, 7,8).

On the whole, the effects of the antigens on *R. appendiculatus* were much less than those on *A.variegatum*. Cross protection between *A.variegatum* and *R. appendiculatus* can at best be described as partial.

Table 6. Feeding and development parameters of females of *R.appendiculatus* fed on rabbits immunised with detergent and aqueous-phase proteins

	ANTIGEN		
PARAMETER	DET[2]	AC[3]	CONTROL
Mean[1] yield (number ± SE)	15.75 ± 1.37a	17.25 ± 0.75a	18.33 ± 1.66a
Mean engorgement period (days ± SE)	7.90 ± 1.61b	7.13 ± 0.08a	7.49 ± 0.12a
Mean engorgement weight (mg±SE)	426.27 ± 13.21a	426.21 ± 8.81a	404.37 ± 11.67a
Mean egg mass (mg±SE)	184.72 ± 10.35a	205.19 ± 5.69a	194.16 ± 8.02a
Mean egg conversion efficiency	40.27 ± 2.00b	44.04 ± 0.48a	43.16 ± 0.75a
Mean hatchability(%)	67.25 ± 5.86a	77.34 ± 4.29a	77.50 ± 4.12a

[1]_____Means of four experimental rabbits each challenged with 20 females and 40 males of *R.appendiculatus*.

[2]DET__Detergent phase proteins

[3]AC___Acqueous phase proteins

Similar letters on the same horizontal row indicate no significance at $p<0.05\%$.

Table 7. Feeding and development parameters of *R.appendiculatus* nymphs fed on rabbits immunised with detergent and aqueous-phase proteins

	ANTIGEN		
PARAMETER	DET[2]	AC[3]	CONTROL
[1]Mean yield (number ± SE)	74.75 ± 3.19b	85.00 ± 4.88a	87.00 ± 4.58a
Mean engorgement period (days ± SE)	5.49 ± 0.05b	5.31 ± 0.04a	5.34 ± 0.04a
Mean engorgement weight (mg±SE)	10.37 ± 0.38a	9.94 ± 0.14a	10.32 ± 0.11a
Moultability(%)	98.62 ± 0.94a	98.27 ± 0.76a	100a

[1] ___Means of four rabbits each challenged with 100 *R.appendiculatus* nymphs

[2] DET___Detergent-phase proteins

[3] AC____Aqueous-phase proteins

Similar letters on the same horizontal row indicate no significance at $p<0.05$.

Table 8. Feeding and development parameters of *R.appendiculatus* larvae fed on rabbits immunised with detergent and aqueous phase proteins

	ANTIGEN		
PARAMETER	DET[2]	AC[3]	CONTROL
Mean[1] yield (number±SE)	44.50 ± 8.91a	48.12 ± 5.99a	60.25 ± 7.0a
Mean engorgement period (days±SE)	4.44 ± 0.05b	4.18 ± 0.02a	4.15 ± 0.06a
Mean engorgement weight (mg±SE)	0.53 ± 0.015b	0.61 ± 0.013b	0.57 ± 0.017a
Moultability (mean ± SE%)	69.32 ± 8.88a	82.45 ± 5.85a	83.75 ± 5.69a

[1]___Means of four rabbits each challenged with 100 *R.appendiculatus* larvae

[2] DET_____Detergent phase proteins

[3] AC______Aqueous phase proteins

Similar letters on the same horizontal row indicate no significance at $p<0.05$

4.2 Purification of detergent-phase proteins by Gel permeation Chromatography

4.2.1 Gel permeation

Figure 6 shows the elution profile of detergent-phase proteins eluted from Sephadex G75 column. In all four peaks were yielded. The resolution of the first three (Peaks 1,2 and 3) was definite while that of the peak 4 was indistinct (Figure 6).

4.2.2 Analysis of the proteins eluted from Sephadex G-75 gel permeation chromatography by SDS-PAGE

Table 9 shows the approximate molecular weight of the polypeptide bands in (Figure 7). It is clear from this table that fraction 1 contained bands with molecular weight below 80 kDa which was the exclusion limit of Sephadex G75 column (Range 3,000-80,000). This fact could be explained by the fact that some of the proteins in their native form would be much larger as they move through the column but separate into their different polypeptides when run under reducing conditions. However, it is evident that none of the polypeptides in the other fractions had molecular weight above 80 kDa indicating that they were all included in the column. Since most of the reactive polypeptides in the Western blot were in the higher molecular weight region, it is possible that Sephadex G75 with an inclusion range of M_r 3000 to 80, 000 concentrated all the reactive polypeptides into fraction 1.

4.2.3 Analysis of protein fractions eluted from Sephadex G-75 column by Western blot

Figure 8 is the immunoblot analysis of the protein fractions eluted from Sephadex G-75 column and reacted with sera from rabbits immunised with the three fractions. The approximate molecular weights of the reactive polypeptides is shown in table 10. Sera from rabbits immunised with fraction 1 proteins reacted with 8 polypeptide proteins in the fraction 1, two polypeptides in fraction 2 and three polypeptides in fraction 4.

Sera from rabbits immunised with fraction 2 reacted with two polypeptides in all the three fractions while the sera from rabbits immunised with fraction 4 reacted with what seems like the same polypeptides in the three fractions. This serum had a much weaker reaction with fraction 4 proteins than either fraction 1 or 2.

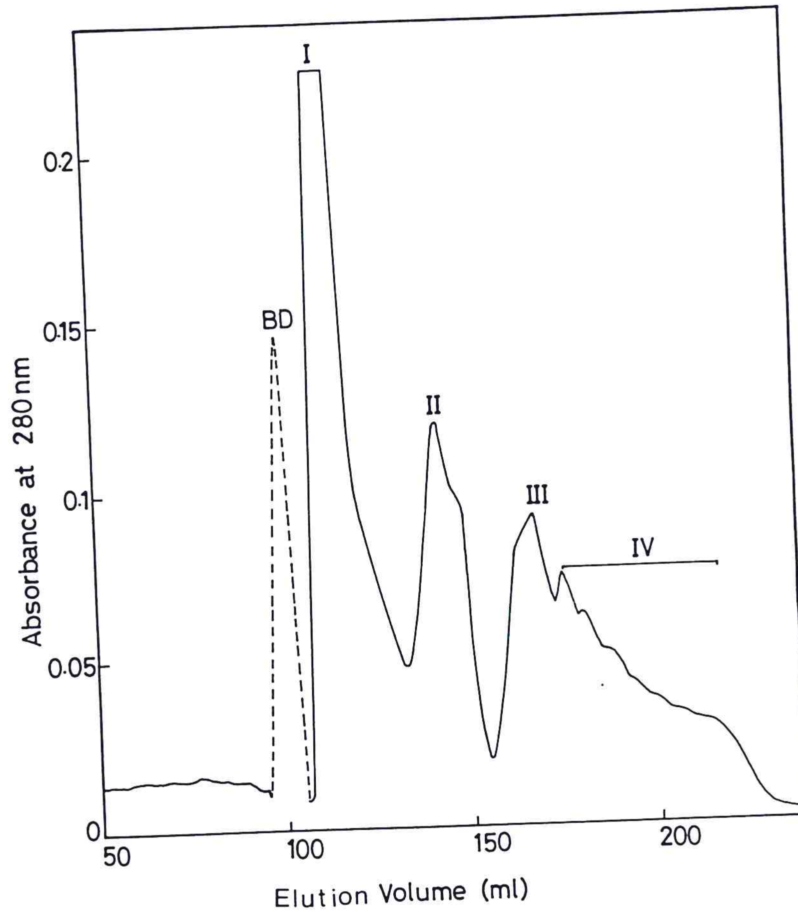

Figure 6. Elution profile of detergent-phase proteins of *A.variegatum* from Sephadex G75 column

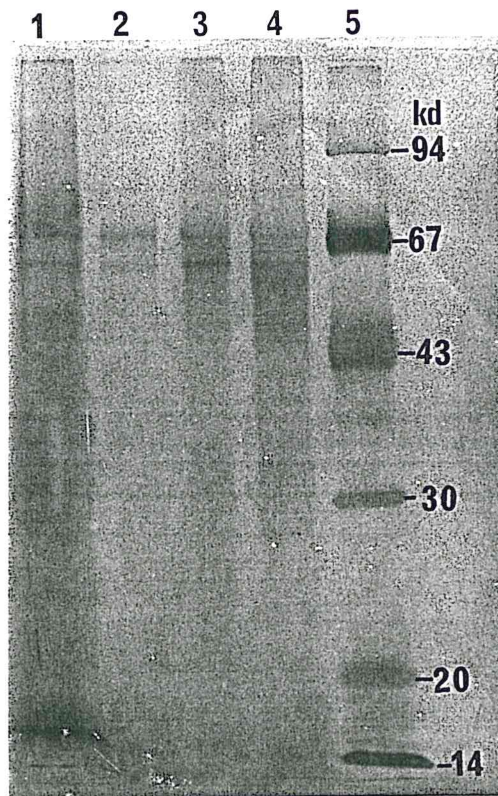

Figure 7. SDS-PAGE of protein fractions from Sephadex G75 gel permeation chromatography stained with silver reagent. Fraction one (lane 1), fraction two (lane 2), fraction three (lane 3) and fraction four (lane 4). Molecular weight markers are showm on lane 5

Table 9. Approximate molecular weight (kDa) of detergent-phase proteins eluted from Sephadex G75 column and fractionated by SDS-PAGE (12.5%)

Eluted fractions			
F1	F2	F3	F4
148.0	72.0	60.0	63.0
100.0	62.0	58.0	55.5
85.0	58.0	55.5	50.0
62.0	55.5	54.0	48.0
56.0	54.0	50.0	39.0
55.0	52.0	47.0	29.0
51.0	50.0		12.0
50.0	48.0		
48.5	47.0		
47.0	30.0		
32.0	17.0		
30.0	16.0		
19.5			
17.5			
16.0			
14.5			

Table 10. Immunoblot analysis of protein fractions eluted from Sephadex G75 and reacted with sera from immunised rabbits

	ELUTED FRACTION		
ANTISERA	F1	F2	F4
	240.0	65.0	18.0
Rabbits immunised	171.0		14.0
with F1 fraction	133.0		
	109.0		
	94.0		
	65.0		
	60.0		
Rabbits immunised	65.0	65.0	65.0
with F2 fraction	60.0	60.0	60.0
Rabbits immunised	65.0	65.0	65.0
with F4 fraction	60.0	60.0	60.0

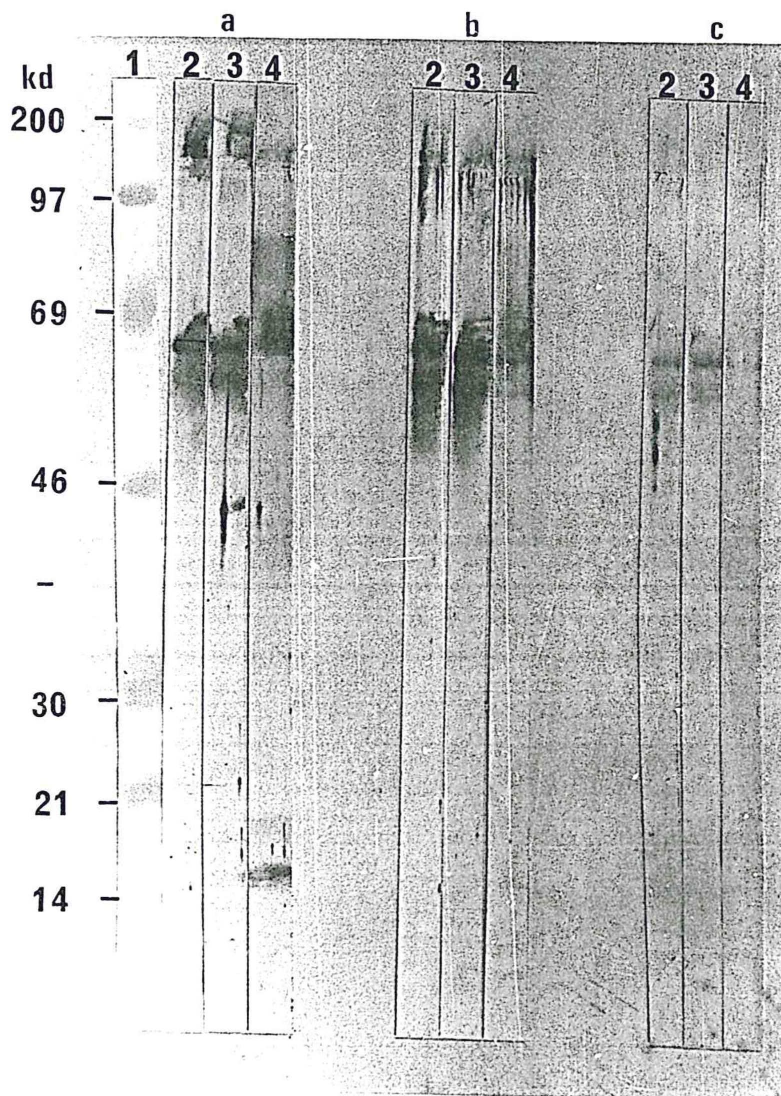

Figure 8. Immunoblot analysis of fraction 1 (lane 2) fraction 2 (lane 3) and fraction 4 (lane 4) eluted from Sephadex G75 column and reacted against rabbit antisera to (a) Fraction 1 (b) Fraction 2 and (c) Fraction 4 protein polypeptides. Molecular weight markers are shown on fraction 1

4.2.4 Feeding and development performance of *A.variegatum* feeding on rabbits immunised with proteins eluted from Sephadex G-75 column

Tables 11, 12 and 13 show the feeding performance of *A.variegatum* fed on rabbits immunised with fractions of Sephadex G-75 column chromatography.

4.2.4.1 Adults

The mean mortality of engorged ticks that fed on rabbits immunised with F1 fraction was significantly higher compared to the control (13.5% and 0%) respectively (Table 11). The cause of the mortality was not clear as no histological analysis was carried out. Ticks appeared to be in good condition but then they turned black and died. Most of these died before they could lay eggs. The other parameters had no significant differences but as seen in Table 11 the mean number of ticks feeding to engorgement, the engorgement weight, the egg mass and the egg conversion efficiency were all reduced although these differences was not statistically significant at 5% level. These are the same parameters that were affected in ticks feeding on rabbits immunised with the detergent-phase proteins before fractionation. This indicates that the antigens causing the effects were in the different ticks.

The feeding duration of the ticks and the hatchability of the eggs were not significantly affected.

4.2.4.2 Nymphs

Both fraction 1 and 2 had significant effects on the feeding performance of the nymphs of *A.variegatum* (Table 12). Both the feeding duration and the engorgement weight were affected in ticks feeding on rabbits immunised with fraction 1 proteins extracts, while the engorgement weight and mortality were affected on those that fed on rabbits immunised with fraction 2. The number of ticks that fed successfully and their ability to moult into adults was not affected.

4.2.4.3 Larvae

The effects on feeding parameters of larvae fed on immunised rabbits were spread between fraction 2 and fraction 4 protein extracts. Ticks fed on rabbits immunised with fraction 2 had significantly lower engorgement weight; a higher mortality was recorded (Table 13). There was no effect on the other parameters assessed. On the other hand ticks that fed on rabbits immunised with fraction 4 fed for a significantly shorter period of time and had fewer ticks feeding to engorgement although this difference was not statistically significant at 5%.

Table 11. Feeding and developmental parameters of *A.variegatum* adults fed on rabbits immunised with proteins eluted from Sephadex G-75 column

	ELUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	9.75 ± 6.80a	7.25 ± 4.8a	10.0 ± 7.9a	84.0 ± 16.2a
Mean engorgement period (days±SE)	13.5 ± 0.46a	13.9 ± 0.6a	12.8 ± 0.52a	14.0a ± 0.5a
Survival (%)	86.5 ± 3.60b	93.6 ± 3.2ab	100a	100a
Mean engorgement weight (mg±SE)	1475.2 ± 142.20a	1408.6 ± 62.1a	1261.2 ± 166.1a	1369.0 ±158.2a
Mean egg mass (mg±SE)	522.3 ± 69.30a	584.1 ± 99.1a	542.1 ± 91.3a	698.6 ± 84.6a
Mean egg conversion efficiency	38.5 ± 2.30a	41.7 ± 2.6a	41.8 ± 2.1a	45.9 ± 2.4a
Hatchability (%)	57.4 ± 6.14a	55.1 ± 6.05a	53.8 ± 8.4a	48.1 ± 5.4a

[1] ___Means of five experimental rabbits each challenged with 20 females and 40 males.

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

Table 12. Feeding and developmental parameters of *A.variegatum* nymphs fed on rabbits immunised with proteins eluted from Sephadex G-75 column

	E LUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	87.2 ± 5.70a	86.6 ± 11.9a	87.0 ± 13.3a	84.0 ± 16.20a
Mean engorgement period (days±SE)	6.6 ± 0.04b	6.8 ± 0.03a	6.9 ± 0.01a	6.7 ± 0.04a
Mean engorgement weight (mg±SE)	49.3 ± 0.78b	49.4 ± 0.8b	52.01 ± 78a	53.65 ± a
Mean moutability(%)	95.7 ± 2.69a	97.9 ± 2.03a	94.4 ± 1.97a	98.5 ± 3.0a
Mean survival (%)	98.3 ± 0.40a	90.7 ± 1.2b	98.3 ± 0.40a	99.4 ± 0.2a

1_____Means of five rabbits each challenged with 100 *A.variegatum* nymphs

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

Table 13. Feeding and developmental parameters of *A.variegatum* larvae fed on rabbits immunised with proteins eluted from Sephadex G-75 column

	ELUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	57.7 ± 5.90a	63.2 ± 12.01a	48.0 ± 7.70b	70.7 ± 8.10a
Mean engorgement period (days)	7.8 ± 0.10ab	8.0 ± 0.12a	7.5 ± 0.08b	7.9 ± 0.03a
Mean engorgement weight (mg±SE)	2.10 ± 0.05a	1.98 ± 0.04b	2.14 ± 0.04a	2.18 ± 0.02a
Mean moutability(%)	80.7 ± 3.62a	70.9 ± 2.67a	76.8 ± 4.34a	77.0 ± 4.40a
Mean survival (%)	89.9 ± 1.20b	76.7 ± 1.50c	93.9 ± 1.20a	89.7 ± 1.20b

[1] Means of five experimental rabbits each challenged with 100 larvae

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$

4.2.5 Assessment of the cross-protection of the protein fractions eluted from Sephadex G75 to *R. appendiculatus*

4.2.5.1 Adults

Only the egg conversion efficiency of the adults that fed on rabbits immunised with fraction 2 was slightly but significantly reduced (Table 14). The engorgement weight and the egg mass were also slightly reduced but not significantly so. These are the same parameters affected in *A.variegatum* adult females although at higher level. The other parameters were not significantly affected.

4.2.5.2 Nymphs

The feeding duration was significantly shorter for rabbits feeding on F2 immunised rabbits than the control and so was the number of those feeding to engorgement although this particular difference was not significant at 5% level (Table 15). Non of the other parameters were affected. Additionally non of the fractions had any effects on any of the parameters.

4.2.5.3 Larvae

None of the fractions had any effect on any of the feeding parameters of the larvae of *R..appendiculatus*.

Table 14. Feeding and development parameters of *R.appendiculatus* females ticks fed on rabbits immunised with proteins eluted from Sephadex G-75 column

	ELUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	18.3 ± 2.8a	15.5 ± 4.0a	15.7 ± 3.2a	15.5 ± 1.0a
Mean engorgement period (days±SE)	10.3 ± 1.60a	9.7 ± 0.20ab	10.2 ± 0.4a	9.1 ± 0.2b
Mean engorgement weight (mg±SE)	375.5 ± 14.50a	332 ± 19.70a	360.9 ± 19.6a	347.3 ± 12.9a
Mean egg mass (mg±SE)	184.1 ± 9.90a	163.6 ± 12.90a	179.6 ± 11.8a	182.7 ± 8.2a
Egg conversion efficiency	51.4 ± 0.90a	46.8 ± 2.20a	48.8 ± 1.6a	40.2 ± 0.9a
Mean hatchability (%)	69.7 ± 4.03a	69.6 ± 4.44a	68.4 ± 6.6a	67.3 ± 26.1a

[1] ____Means of five rabbits each challenged with 20 *R.appendiculatus* females and 40 males.

Similar letters on the same horizontal row indicate no significance at $p<0.05$.

Table 15. Feeding and development parameters of *R.appendiculatus* nymphs fed on rabbits immunized with proteins eluted from Sephadex G-75 column

	ELUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	89.0 ± 31.50a	76.7 ± 32.20a	88.7 ± 9.70a	83.3 ± 15.90a
Mean engorgement period (days±SE)	4.9 ± 0.05b	4.7 ± 0.05c	5.3 ± 0.07a	4.8 ± 0.05b
Mean engorgement weight (mg±SE)	9.4 ± 0.11a	9.8 ± 0.29a	9.5 ± 0.11a	9.7 ± 0.11a
Mean moultability (%)	91.5 ± 1.73a	93.4 ± 2.99a	89.9 ± 1.16a	91.4 ± 1.23a

[1]_____ Means of five rabbits each challenged with 100 *R. appendiculatus* nymphs

Similar letters on the same row indicate no significance at $p<0.05$.

Table 16. Feeding and development parameters of *R..appendiculatus* larvae fed on rabbits immunised with proteins eluted from Sephadex G-75 column

	ELUTED FRACTIONS			
PARAMETER	F1	F2	F4	CONTROL
Mean[1] yield (number±SE)	53.7 ± 4.69a	59.5 ± 7.12a	68.0 ± 6.65a	46.3 ± 4.21a
Mean engorgement period (days±SE)	3.8 ± 0.05b	3.8 ± 0.02b	3.8 ± 0.06b	3.9 ± 0.03a
Mean engorgement weight (mg±SE)	0.52 ± 0.01ab	0.52 ± 0.01ab	0.54 ± 0.02a	0.50 ±0.01b
Mean moultability (%)	69.3 ± 9.63a	68.7 ± 2.94a	69.3 ± 6.64a	70.0 ± 4.93a

[1]_____ Means of five rabbits each challenged with 100 *R.appendiculatus* larvae

Similar letters on the same horizontal row indicate no significance at $p<0.05$

4.3 Purification of detergent-phase proteins by preparative SDS-PAGE

4.3.1 Eletrophoretic analysis of the proteins eluted from SDS-PAGE gels

Figure 9 shows the analysis of the three fractions by SDS-PAGE, with the approximate molecular weight of the polypeptide bands in table 17. Sixteen, ten and six polypeptide bands were eluted from the high medium and low molecular weight fractions respectively. A surprising observation was that the fractions did not behave in the manner expected in that the high molecular weight fraction had polypeptides as low as 10 kDa whereas the low molecular weight fraction had a polypeptide in the region of 66 kDa. These findings could be as a result of the rearrangement of some of the polypeptides in solution some dissociating and others forming some weak bonds among themselves. It may be that sodium dodecyl sulphate does not completely reduce the proteins into their constituent polypeptides.

4.3.2 Immunoblot analysis of the eluted proteins from SDS-PAGE gels

Very few proteins reacted with sera from rabbits immunised with the three fractions apart from a group of proteins with an approximate molecular weight of about 65 kDa. Sera from rabbits immunised with the high molecular weight fraction reacted with these proteins in the three fractions. Sera from rabbits immunised with the medium range proteins reacted with these proteins in both the medium and the high molecular weight fractions whereas sera from rabbits immunised with the low molecular weight fraction reacted with the fractions in the low molecular weight fraction. The only explanation for these results is that these proteins may not be similar although they appear to be son. For some unexplained reason they aggregate

around this region when run under reducing conditions. There was also extensive cross-reactions between the fractions which was not at all surprising.

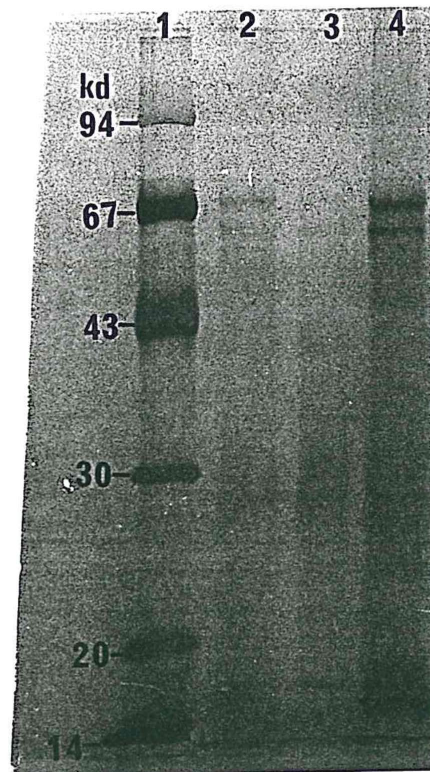

Figure 9.Electrophoretic analysis of the high molecular weight fraction (lane 2), Medium range molecular weight fraction (lane 3) and the low molecular weight fraction (lane 4) eluted from SDS-PAGE gels. Molecular weight markers are on lane 1.

Table 17 Approximate molecular weight (kDa) of the detergent-phase proteins fractionated and eluted from SDS-PAGE gels

ELUTED FRACTION		
HIGH (>67kDa)	MEDIUM(23-67kDa)	LOW(<23kDa)
121.0	66.0	66.0
107.0	64.5	62.0
67.0	64.0	53.5
65.0	62.0	47.0
64.0	55.0	14.0
62.0	50.0	10.0
58.0	42.0	
57.0	39.0	
55.0	25.0	
50.0	24.0	
47.0		
40.0		
39.0		
13.0		
10.0		
9.0		

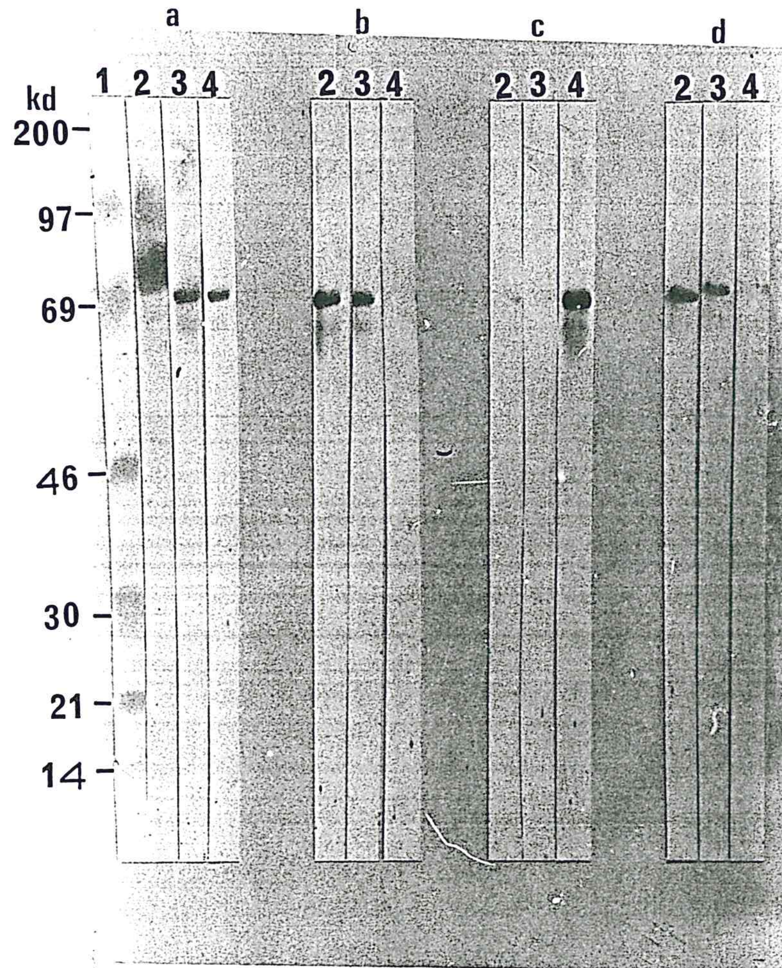

Figure 10. Immunoblot analysis of the High molecular weight fraction (lane2) Medium range molecular weight fraction (lane 3) and the low molecular weight fraction eluted from SDS-PAGE gels reacted with rabbit antisera to (a) high molecular weight fraction (b) medium range fraction (c) low molecular weight fraction (lane 4) and (d) non-immunised rabbits. Molecular weight markers are on lane 1

Table 18 Approximate molecular weight (kDa) of proteins eluted from SDS - PAGE gels, reacted with sera from immunised rabbits

	ELUTED FRACTION		
ANTISERA	HIGH	MEDIUM	LOW
	105.0	70.0	70.0
Rabbits immunised	94.0	65.0	
with the high molecular	75.0		
weight fraction	65.0		
	70.0	70.0	—
Rabbits immunised	65.0	65.0	
with the medium			
molecular weight			
Rabbits immunised	—	—	70.0
with low molecular			
weight fraction	65.0		

4.3.3. Feeding and development performance of *A.variegatum* fed on rabbits immunised with proteins eluted from SDS-PAGE gels

Tables 19, 20, and 21 shows the feeding performance of *A.variegatum* ticks fed on rabbits immunised with detergent-phase protein fractions eluted from SDS-PAGE gels. From these tables it is clear the high molecular weight fraction not only had effects on all the parameters assessed but to a much higher level, including engorgement weight, yield, egg mass, egg conversion efficiency and mortality.

4.3.3.1 Adults

The high molecular weight fraction had the greatest significant impact on the feeding and development of the adults. The mean engorgement weight was reduced by as much as 30% and the yield reduced by nearly 10% (Table 19). Egg mass was slightly reduced but the major impact was on the egg conversion efficiency which was only 36% compared to 61 % for the control rabbits. High molecular weight fraction also induced a 22% mortality of the adult ticks. Only the feeding duration was not affected. The low molecular weight fraction significantly affected the yield of adults by a reduction of about 10%. They also induced a significant mortality of 12%. The egg conversion efficiency was also reduced but this was not statistically significant. Medium range molecular weight fraction had no effect on any feeding parameter.

4.3.3.2 Nymphs

The high molecular weight fraction had effects on both the yield and the feeding duration (Table.20). Yield was reduced by about 15% while the feeding

duration was increased by as much as a day. There was a significant reduction in the moulting capacity of nearly 10%. While there was a slight reduction in the mean engorgement weight, this decrease was not significant.

The low molecular weight proteins caused a significant reduction of more than 20% on the yield and an increase of more than one day in the feeding duration. There was a slight but significant reduction in the moulting of about 2%. There was no effect on the engorgement weight.

Medium range proteins caused a significant reduction on the yield and moulting but not on any other parameter.

4.3.3.3 Larvae

The high molecular weight fraction caused a significant reduction in the yield (9%) and a slight but significant decrease in the feeding duration. The medium range molecular weight fraction had significant effects on the yield, feeding duration and moulting while the low molecular weight fraction had an effect on only the yield (Table 21).

None of the protein fraction had any effect on the engorgement weight of the larvae.

Table 19 Feeding and developmental parameters of *A.variegatum* females fed on rabbits immunised with proteins eluted from SDS-PAGE gels

	ELUTED FRACTIONS			
PARAMETER	HIGH	MEDIUM	LOW	CONTROL
Mean[1] yield (number±SE	11.52 ± 0.29b	13.32 ± 0.53ab	10.36 ± 0.48b	12.40 ± 0.56a
Mean engorgement period (days ± SE)	19.55 ± 0.47a	18.48 ± 0.44a	19.7 6 ± 0.59a	19.57a ± 0.47a
Mean engorgement weight (mg±SE)	909.08 ± 57.44b	1051.64 ± 72.68a	1225.20 ± 93.92a	1281.95 ±68.21a
Mean egg mass (mg±SE)	411.05 ± 34.42a	448.25 ± 41.23a	544.47 ± 62.34a	542.32 ± 43.95a
Mean egg conversion efficiency (%)	36.95 ± 1.44b	48.11 ± 8.89ab	40.08 ± 1.76ab	61.08 ± 0.48a
Mean mortality(%)	22.53 ± 3.10b	10.72 ± 0.55b	12.61 ± 2.03b	5.53 ± 1.27a

[1] ____Means of six experimental rabbits each challenged with 20 females and 40 males.

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

Table 20. Feeding and developmental parameters of *A.variegatum* nymphs fed on rabbits immunised with proteins fractionated and eluted from SDS-PAGE gels

	ELUTED FRACTIONS			
PARAMETER	HIGH	MEDIUM	LOW	CONTROL
Mean[1] yield (number±SE)	30.07 ± 0.90b	28.95 ± 0.5b	27.47 ± 1.03b	35.70 ± 0.62a
Mean engorgement period (days±SE)	7.47 ± 0.15b	6.86 ± 0.13a	7.90 ± 0.25b	6.80 ± 0.13a
Mean engorgement weight (mg±SE)	40.48 ± 1.33a	38.56 ± 1.82a	42.39 ± 1.90a	42.24 ± 1.20a
Percentage moult (mean±SE)	93.67 ± 0.36c	97.14 ± 0.35b	98.64 ± 0.34b	100.00a

[1] Means of six rabbits each challenged with 50 *A.variegatum* nymphs

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

Table 21.
Feeding and developmental parameters of larvae of *A.variegatum* ticks fed on rabbits immunised with proteins fractionated and eluted from SDS-PAGE gels

	ELUTED FRACTIONS			
PARAMETER	HIGH	MEDIUM	LOW	CONTROL
Mean[1] yield (number±SE)	63.77 ± 0.38b	64.15 ± 0.87b	63.77 ± 0.38b	70.33 ± 0.55a
Mean engorgement period(days±SE)	8.11 ± 0.08b	8.28 ± 0.08b	9.03 ± 0.09a	8.92 ± 0.10a
Mean engorgement weight(mg±SE)	2.32 ± 0.03a	2.32 ± 0.03a	2.28 ± 0.08a	2.41 ± 0.03a
Percentage moult (%±SE)	3.73 ± 0.16	91.81 ± 0.35	93.14 ± 0.22	93.69 ± .24a

[1]——Means of six rabbits each challenged with 100 *A.variegatum* larvae

Similar letters in the same horizontal row indicates no significant difference at $p<0.05$.

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protective antigens in the gut extract were in the membrane component of the gut and not the soluble fraction (Opdebeeck *et al*.,1988; Lee and Opdebeeck 1991). Ackerman (1981), found protection from both the soluble and the membrane component of the gut of *D.variabilis*, although greater protection was obtained from the membrane fraction.

Therefore, from the available evidence, the gut seems to be the better source of immunogens. This is partly because gut antigens are said to be `concealed'; they are not normally exposed to the hosts immunological mechanisms during feeding (Willadsen and Kemp, 1988). Secondly if the antigens damage the integrity of the gut wall, the likely hood of harmful substances entering the haemolymph and leading to deleterious effects is increased. There is evidence that this occurs in *Boophilus microplus* and probably in other tick species as well.(Agdede *et al.*, 1986).

Many ectoparasites, including ticks are efficient in evading the hosts immunological mechanisms (Wikel, 1984). *Amblyomma americanum* is known to cause immunodepression in their hosts (Wikel, 1984). It is therefore, reasoned that a successful vaccine would be one which encourages early tick rejection and death. It has been observed that antigens of salivary origin lead to tick rejection whereas those of gut origin mainly affects fecundity of the tick (Wikel, 1884). This led some workers to suggest that salivary glands are a better source of immunogens than other tick tissues including gut. Unfortunately, unlike gut antigens, salivary gland antigens are more likely to be avoided by the host, because they come into contact with the host's immunological response during normal feeding. (Willadsen and Kemp, 1988).

This was the overriding reason for this study to concentrate on gut antigens. However, it appears as if protective antigens are located in various organs of the tick, as several workers have reported the successful immunisation of hosts against tick feeding using a variety of tick tissues (Fujisaki *et al.,* 1982). The use of a multi-component vaccine consisting of different antigens from various sources to induce certain desired effects is possible.

The choice of rabbits as models was based on cost consideration. With laboratory animals, it is possible to undertake quantitative experiments with large numbers of animals which is a necessary step in the early screening of antigens. However, Wildness and Keep (1988) urged caution in the interpretation of immunological results obtained from laboratory animals as directly applicable to the cattle. There are important differences in the immunological responses of hosts to ticks which are not specific parasites of that particular host. In the case of *A.variegatum*, however, the choice of rabbits as a model was not entirely misplaced because the immatures of the tick normally feed on small rodents (Walker, 1977).

Solubilisation of membrane proteins has been used extensively as interest in membrane biochemistry has increased. Different membranes have been solubilised by various non-ionic detergents. Solubilisation takes place by the replacement of the lipid bilayer of the membrane by the detergent molecules through their apolar (hydrophobic) domains (Helenius, and Simons, 1975). Non ionic detergents do not interfere with the protein:protein interactions of the solubilised membranes. They also have no effect on biological activities such as immune action of the proteins (Bjerrum and Bhakti, 1982). This is because detergent binding is confined to the apolar surfaces of the membrane proteins and does not interfere with the hydrophilic

molecular regions of the molecule, which contain the antigenic sites (Roit, 1984). However, it is expected that proteins that depend on the presence of lipids for their immunogenicity may be interfered with as lipids are replaced with detergent molecules (Bjerrum and Bhakti, 1982). Solubilisation, therefore, results into the separation of membranes into three fractions, an integral component composed of apolar proteins, a hydrophilic fraction consisting of hydrophilic proteins and a detergent:lipid micelle mixture.

The non-ionic detergents in the Triton X series separate into aqueous detergent phases when their temperature is raised above the cloud point (Bordier, 1981). Cloud point is the temperature at which the detergent micelles aggregate and are no more soluble. (Helenius and Simons, 1975). The temperature of the cloud point depends on the number of the hydrophilic oxyethylene units condensed on the hydrophobic octylphenyl residue of the detergent molecule (Helenius and Simons, 1975). If there are proteins in solution in these detergents, they are also separated into a detergent phase containing most of the apolar (amphiphillic) proteins and an aqueous phase containing most of the hydrophilic proteins (Bordier, 1981). For membrane proteins, hydrophilic proteins are mainly the peripheral (extrinsic) membrane proteins, while the hydrophobic proteins are the integral (intrinsic) membrane proteins (Radolf *et al.*, 1988)). The cloud point of Triton X-114 is 23 ^{0}C, a convenient working temperature for biological substances including proteins. This is the reason for selecting Triton X-114 in the solubilisation of the gut membranes.

The first objective of the study was, therefore, to establish whether the proteins responsible for the induction of immunity to ticks are located in the peripheral or integral components of the membrane.

Most of the protective activity was found in the detergent phase ,which means that most of the protective antigens are integral membrane proteins. This is similar to the finding of Kemp *et al.,* 1986) that *B. microplus* ticks feeding on immunised cattle had damaged midguts. This damage is expected if the immunological response is directed against a structural component of membrane. Furthermore, Rand *et al.,* (1989) reported the presence of a 20 amino acid hydrophobic region adjacent to the carboxyl terminal on Bm86. This is a purified gut glycoprotein inducing a protective immune response to *B.microplus*.

All the instars were affected but , at different levels. This is similar to the finding of Heller-Haupt (1987). The effect on immatures was generally lower than on the adults. This was not entirely surprising because the antigens were derived from the adults of *A. variegatum* and any effect would most likely be directed against the homologous stage. The reduced effect on the immature stages might also be a reflection of the smaller quantity of blood and consequently less gut-damaging immunoglubulins ingested by the larvae and nymphs. It might also be associated with the shorter feeding time of the immature stages and, therefore, less time for the immunoglobulins directed against the gut antigens to cause damage. Willadsen, (1987) also reported that artificially induced immune response has less effect on the immature stages than the adults.

The aqueous phase (peripheral) membrane proteins induced very little protective effect on all the instars of *A.variegatum*. This was surprising in view of the fact that it induced higher antibody titres against the aqueous phase and the detergent phase proteins than that induced by the detergent phase proteins. It is possible that these proteins (antigens) do not induce any protective immunity and

that the slight effect was probably due to the contamination of the aqueous phase by proteins from the detergent phase. Pryde (1986) reported that it is possible to recover hydrophobic proteins in the aqueous phase.

In general, the hydrophilic regions of proteins tend to be more variable than the hydrophobic domains and are thought to be the sites for antibody production. Evidence from foot and mouth disease virus and hepatitis B virus confirm that it is these sites that are responsible for the production of neutralising antibodies (Harris, 1984). If this is the case with membrane proteins in *A.variegatum*, the aqueous phase proteins would have been more protective than the detergent phase proteins. However, the reverse occurred. This situation explains why the aqueous phase proteins produced higher titres in immunised rabbits, as aqueous phase proteins have proportionately more antigenic determinants that are responsible for antibody production. The implication of this finding is that cellular responses play a greater role than humoral responses in the protective response against *A.variegatum*.

The immunoblotting experiments indicated that most of the reactive polypeptides were found in the region 50-180 kDa. This finding was comparable to that of Wikel (1988) with *A.americanum*. There were many other polypeptide bands that were not reactive ranging in molecular weight from as low as 14 kDa. This may be as result of larger proteins having relatively more potential antigenic determinants. Although all the reactive bands may not be important in the induction of a protective response, it is probable that the important antigen(s) are among the ones that reacted with sera from the rabbits that showed immunity against tick feeding.

Affinity columns made with antibodies from hyper-immune sera could have been used as a possible purification procedure. However, this approach was not followed because as noted above, selection of antigens on the basis of humoral responses alone may be defective as this does not take account of the equally important cellular responses. Furthermore, IgG1 which is important in the protective response against ticks (Brown *et al* ., 1984) does not have strong affinity for antigens (Sela, 1986). Another limitation to this approach, was that with a crude extract, more antibodies would be produced to the most immunodominant antigen which might not necessarily be a protective antigen as evidence from *B. microplus* suggests (Willadsen *et al.,* 1989). Additionally, with extensive cross reactivity observed, polyclonal sera would have been unsuitable as this would have bound many different antigens including irrelevant ones.

Gel filtration chromatography separates proteins according to their molecular sizes. The next objective of the study was to separate the integral membrane proteins according to their molecular sizes with the prospect of enriching the protective proteins into one fraction. Sephadex G-75 has an inclusion range of M_r 3000-80000 . It is, therefore, possible to obtain all the proteins with molecular weight in excess of 80 Kda in the void volume and hopefully enrich the fraction with the polypeptides reacting with immune sera.

This approach was not very successful. Although most of the activity was found in fraction 1, which should have contained the fraction with the high molecular weight proteins, some activity was observed in all the other fractions. Besides, the level of protection was lower than that provided by the detergent-phase proteins before fractionation by gel permeation chromatography. This is probably

an indication that the protective response is due to several antigens acting synergistially. The interpretation of this is that as purification proceeds protective effect of the proteins may increase as irrelevant antigens are removed or it may decrease as some of the immuno-potent proteins are fractionated out (Willadsen, 1986). It was clear from the SDS-PAGE gels that whereas the other fraction had no polypeptides exceeding 80kDa, the exclusion range of Sephadex G75, fraction 1 had protein polypeptides well below the exclusion limit. It was probably because in gel permeation chromatography , proteins are separated in their native form, but separate into smaller constituent polypeptides when separated in SDS-PAGE gels under reducing conditions.. It was therefore difficulty to compare directly the results of immunoblot experiments with those carried out with native proteins.

In order to improve the identification of the polypeptides inducing immunity, it was decided to elute proteins directly from SDS-PAGE gels for immunisation experiments. Immunisation and immunoblot experiments would deal with polypeptides rather than native proteins.

It has been suggested, however, that there is a loss of up to 80-90% of the potential antigenic determinants as a result of conformational changes to the native protein molecule during the reduction of native proteins into polypeptides (Sela, 1978). Conformational antigenic determinants require intact macromolecules and are probably lost when polypeptides are used as antigens.

There is, however, evidence that almost all the surface of a molecule is antigenic (Berzofskey, 1885),; the more exposed a molecule is, the more likely it is to expose a useful epitope. This implies that a polypeptide is more likely to be exposed to the host's immune system than the native protein.

As expected, this purification procedure was found to be the most effective than all the previous steps. The method concentrated most of the protective polypeptides into the fraction containing the high molecular weight proteins (>67kDa). This fraction not only provided the highest level of protection, but also affected most of the parameters assessed. However, there was some activity in the low molecular weight proteins and most importantly affecting tick rejection. This was similar to the finding with gel permeation chromatography where mild activity was recorded from the fraction with the low molecular weight proteins. Brown (1987) suggested that low molecular weight proteins are responsible for cell-mediated responses. This hypothesis is consistent with the present results because cell mediated responses normally lead to tick rejection (Kemp and Bourne, 1980).

A question of considerable interest in much of Africa, where more than one tick species is of economic importance, is whether the antigens are cross-protective to ticks in other genera.. This was the reason for the desire to select antigens that were protective to both *A. variegatum* and *R.. appendiculatus*. Contrary to the findings of Heller-Haupt *et al.,* (1987), that there is no cross protection between *A. variegatum* and *R.. appendiculatus* , our results indicate cross protection between the two species exists. However, the effect on *R.appendiculatus* was generally lower than on *A. variegatum*. This was demonstrated serologically and more importantly by biological assays. Jongejan *et al .,* (1989) also demonstrated cross-protection between the two species with salivary gland extracts. The failure by Heller-Haupt (1987) to demonstrate cross-protection could be attributed to the use of immunodiffusion which is less sensitive than enzyme immunoassays. However,

even with cross-reacting antibodies, it does not necessarily mean there would be cross protection between the two species without challenge experiments.

Although serological tests were useful in demonstrating the antigenicity and the effectiveness of the immunisation process, they were not reliable in identifying the usefulness or otherwise of the material as a protective antigen. Although several workers have reported a positive correlation between antibody titres and the level of protection (Brown, 1987) and more specifically between the level of IgG and IgG1 (Jackson *et al.,* 1990; Johnston and Opdebeeck, 1991), others have also reported no correlation (Willadsen, 1978). A possible explanation for this difference could be the different immunological responses of various tick-host associations. It could also be due to the use of crude proteins which contained numerous proteins. As in the Bm86 antigen, if the protective antigen is a minor component, (Willadsen and McKenna, 1991) then the antibody titres of the crude material may not show a positive correlation with the level of protection. Willadsen and McKenna (1991) explained that the presence of cross-reacting antibodies in many tick tissues is due to non-specific reactions with carbohydrate determinants on tick glycoproteins. However, Brown and Askenase (1983) noted the presence of shared antigens among both different tick species and different haematophagous arthropods. It is also entirely possible that the partial protection observed, was incidental. It could be the result of non-specific immunological responses affecting ticks generally.

A pertinent question is whether the same antigen(s) are involved in cross-protection or whether one or more antigens are responsible for causing the various effects observed in different instars. This study suggests that there may be more than one antigen involved. The observation of some effects from fractions with high

and low molecular weight proteins from both the gel permeation chromatography and eluted proteins from SDS-PAGE gels tend to support this view. Willadesn (1986) suggested that all the varied effects found in immunised hosts might be due to a single pathological effect caused by a single antigen.

The levels of protection obtained in these studies was considerably lower than those reported for one host ticks (Opdebeeck *et al.,* 1988) especially with regard to tick rejection. This is probably because one-host ticks all the instars feed on the same host and the results obtained are the cumulative effects of the larvae nymphs and the adults feeding on an immunised host. The same explanation accounts for the absence of obvious tick damage in this study. The time spent on an immune host by one-host tick is considerably longer; there is, therefore, ample time for the pathological effects in the gut to take effect.

It is possible that the efficacy of these antigens maybe significantly increased if basic information on adjuvants, route of administration, antigen dose and immunisation protocol are optimised. However, these could not be effected , because of time constraint.

In conclusion, the question as to whether ticks in general and *A.variegatum* in particular can be controlled by through immunisation is no longer in doubt. this has been amply demonstrated experimentally. However, several questions remain unanswered. Novel methods of accurately identifying and isolating the useful antigens are needed as conventional protein purification methods may never identify and isolate the useful antigens. If immunity is the result of several antigens working synergistically, as seems likely, then this process becomes more complicated.

Questions regarding the duration of immunity, shelf life of the vaccines and the cost-effectiveness of the vaccination vis a vis other methods have to be addressed.

In Africa, the presence of several tick species on the same host and the feeding of different instars of the same species on different hosts make the problem complex. Technically it may not be difficult to incorporate genes from different tick species into the same vector to co-produce a multi-component vaccine.

However, the greatest hindrance to the practical use of an anti-tick vaccine in Africa, is the slow progress in the development of a vaccine against East coast fever and heartwater. Without a vaccine against these diseases acaricides would continue to be applied diligently because control of these diseases requires animals to be nearly tick free. Vaccination may never attain these goals.

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APPENDICES

APPENDIX 1

ANALYSIS OF VARIANCE

General linear model procedure for table 3

Dependent variable	DF	SS	MS	F value	p
Feeding duration	85	601.5	7.2	0.17	0.84
Eng. weight	69	44638991.67	614882.30	2.80	0.068
Egg mass	69	13446605.89	174036.15	5.13	0.008
Egg conversion efficiency	69	11969.49	167.25	2.28	0.110
Hatchability	65	57556.35	829.08	3.21	0.047

Duncans Multiple Range Test for table 3

Duncan's grouping		Mean feeding duration	N	Antigen
	A	18.85	34	DET
	A	18.58	24	CONTROL
	A	18.46	28	AC

Duncan's grouping		Mean Eng. weight	N	Antigen
	A	2035.2	19	CONTROL
B	A	1691.6	24	AC
B		1480.	27	DET

Duncan's grouping		Mean egg mass	N	Antigen
	A	866.8	19	CONTROL
B	A	680.2	24	AC
B		470.4	27	DET

Duncan's grouping		Mean egg conversion efficiency	N	Antigen
	A	39.86	19	CONTROL
B	A	36.03	24	AC
B		31.67	27	DET

Duncan's grouping		Mean hatchability	N	Antigen
	A	36.21	18	CONTROL
B	A	32.16	24	AC
B		15.52	24	DET

APENDIX 2

General linear model procedure for table 4

Dependent variable	DF	SS	MS	F value	p
Feeding duration	949	2337.49	2.33	9.53	0.0001
Eng. weight	912	176061.16	190.47	3.06	0.0057

Duncans Multiple Range Test for table 4

Duncan's grouping	Mean feeding duration	N	Antigen
A	8.82	333	DET
B	8.52	337	CONTROL
C	8.09	280	AC
Duncan's grouping	**Mean Eng. weight**	**N**	**Antigen**
A	47.85	321	CONTROL
A	47.72	277	AC
A	44.79	315	DET

APPENDIX 3

ANALYSIS OF VARIANCE

General linear model procedure for table 5

Dependent variable	DF	SS	MS	F value	p
Feeding duration	628	1309.39	2.05	5.32	0.0051
Engorgement weight	571	199.19	0.35	0.10	0.90

Duncan's Multiple Range Test for table 5

Duncan's grouping		Mean feeding duration	N	Antigen
	A	7.54	188	AC
B	A	7.33	209	DET
	B	7.08	232	CONTROL

Duncan's grouping	Mean Eng. weight	N	Antigen
A	2.33	178	DET
A	2.31	180	AC
A	2.30	214	CONTROL

Means with the same letter are not significantly different

APPENDIX 4

ANALYSIS OF VARIANCE

General linear model procedure for table 6

Dependent variable	DF	SS	MS	F value	p
Feeding duration	85	601.5	7.2	0.17	0.84
Eng. weight	69	44638991.67	614882.30	2.80	0.068
Egg mass	69	13446605.89	174036.15	5.13	0.008
Egg conversion efficiency	69	11969.49	167.25	2.28	0.110
Hatchability	65	57556.35	829.08	3.21	0.047

Duncan's Multiple Range Test for table 6

Duncan's grouping	Mean feeding duration	N	Antigen
A	18.85	34	DET
A	18.58	24	CONTROL
A	18.46	28	AC

Duncan's grouping		Mean Eng. weight	N	Antigen
	A	2035.2	19	CONTROL
B	A	1691.6	24	AC
B		1480.	27	DET

APPENDIX 4

Duncan's Multiple Range Test for table 6

Duncan's grouping		Mean egg mass	N	Antigen
	A	866.8	19	CONTROL
B	A	680.2	26	AC
B		470.4	27	DET

Duncan's grouping		Mean egg conversion efficiency	N	Antigen
	A	39.86	19	CONTROL
B	A	36.03	24	AC
B		31.67	27	DET

Duncan's grouping		Mean hatchability	N	Antigen
	A	36.21	18	CONTROL
B	A	32.16	24	AC
B		15.52	24	DET

Means with the same letter are not significantly different

APPENDIX 5

ANALYSIS OF VARIANCE

General linear model procedure for table 7

Dependent variable	DF	SS	MS	F value	p
Feeding duration	899	592.7	0.6	6.8	0.0001
Engorgement weight	858	15542.1	18.2	0.5	0.73

Duncan's Multiple Range Test for table 7

Duncan's grouping	Mean feeding duration	N	Antigen
A	5.49	299	DET
B	5.34	261	CONTROL
B	5.31	340	AC

Duncan's grouping	Mean Eng. weight	N	Antigen
A	10.38	295	DET
A	10.33	242	CONTROL
A	9.94.	322	AC

Means with the same letter are not significantly different

APPENDIX 6

ANALYSIS OF VARIANCE

General linear model procedure for table 8

Dependent variable	DF	SS	MS	F value	p
Feeding duration	769	400.9	0.48	14.4	0.0001
Engorgement weight	715	39.1	0.05	2.83	0.02

Duncan's Multiple Range Test for table 8

Duncan's grouping		Mean feeding duration	N	Antigen
	A	4.44	176	DET
	B	4.18	353	AC
	B	4.15	241	CONTROL

Duncan's grouping		Mean Eng. weight	N	Antigen
	A	0.61	340	AC
B	A	0.57	209	CONTROL
B		0.54	167	DET

Means with the same letter are not significantly different

APPENDIX 7

ANALYSIS OF VARIANCE

General linear model procedure for table 11

Dependent variable	DF	SS	MS	F value	p
Feeding duration	130	1147.7	8.8	0.97	0.40
Engorgement weight	98	41005798.1	427766.0	0.29	0.83
Egg mass	87	11838697.7	136192.7	0.98	0.40
Egg conversion efficiency	85	10828.6	124.9	1.55	0.20

Duncans Multiple Range Test for table 11

Duncan's grouping	Mean feeding duration	N	Antigen
A	14.03	33	CONTROL
A	13.93	29	F2
A	13.51	39	F1
A	12.87	30	F4

Duncan's grouping	Mean Eng. weight	N	Antigen
A	1408.7	23	F2
A	1369.3	25	CONTROL
A	1275.2	31	F1
A	1261.2	20	F4

APPENDIX 7

Duncan's Multiple Range Test for table 11

Duncan's grouping		Mean egg mass	N	Antigen
	A	698.6	20	CONTROL
	A	584.1	22	F2
	A	542.1	19	F4
	A	522.3	27	F1

Duncan's grouping		Mean egg conversion efficiency	N	Antigen
	A	45.9	20	CONTROL
	A	41.8	18	F4
	A	41.7	21	F2
	A	38.8	27	F1

Duncan's grouping		Mean hatchability	N	Antigen
	A	36.21	18	CONTROL
B	A	32.16	24	AC
B		15.52	24	DET

Means with the same letter are not significantly different

APPENDIX 8

ANALYSIS OF VARIANCE

General linear model procedure for table 12

Dependent variable	DF	SS	MS	F value	p
Feeding duration	1294	1205.4	0.91	7.45	0.0001
Engorgement weight	1269	211469.7	163.6	8.78	0.0001
Mean mortality 1294	178154.4	127.4	35.74		0.0001

Duncan's Multiple Range Test for table 12

Duncan's grouping	Mean feeding duration	N	Antigen
A	6.91	348	F4
A	6.84	263	F2
A	6.75	336	CONTROL
B	6.58	348	F1

Duncan's grouping	Mean Eng. weight	N	Antigen
A	53.65	334	CONTROL
A	52.01	345	F4
B	49.39	249	F2
B	49.27	342	F1

APPENDIX 8

Duncan's Multiple Range Test for table 12

Duncan's grouping	Mean survival	N	Antigen
A	99.41	336	CONTROL
A	98.30	348	F1
A	98.28	348	F4
A	90.67	263	F2

Means with the same letter are not significantly different

APPENDIX 9

ANALYSIS OF VARIANCE

General linear model procedure for table 13

Dependent variable	DF	SS	MS	F value	p
Feeding duration	960	2012.74	2.07	4.65	0.003
Engorgement weight	854	194.80	0.22	7.09	0.0001
Mortality	960	442234.78	420.25	31.77	0.0001

Duncans Multiple Range Test for table 13

Duncan's grouping		Mean feeding duration	N	Antigen
	A	8.09	253	F2
	A	7.96	282	CONTROL
B	A	7.82	234	F1
B		7.60	192	F4

Duncan's grouping	Mean Eng. weight	N	Antigen
A	2.18	266	CONTROL
A	2.14	187	F4
A	2.10	219	F1
B	1.98	183	F2

Means with the same letter are not significantly different

APPENDIX 10

ANALYSIS OF VARIANCE

General linear model procedure for table 14

Dependent variable	DF	SS	MS	F value	p
Feeding duration	224	986.24	4.21	4.38	0.005
Engorgement weight	191	2498667.67	13008.2	1.36	0.25
Egg mass	174	871500.87	5030.5	0.75	0.52
Egg conversion efficiency	169	15878.68	92.65	1.79	0.15

Duncan's Multiple Range Test for table 14

Duncan's grouping			Mean feeding duration	N	Antigen
	A		10.35	59	F1
		A	10.24	47	F4
B	A		9.70	57	F2
B			9.13	62	CONTROL

Duncan's grouping	Mean Eng. weight	N	Antigen
A	375.6	52	F1
A	360.9	44	F4
A	347.4	53	CONTROL
A	330.1	43	F2

APPENDIX 10

Duncan's Multiple Range Test for table 14

Duncan's grouping		Mean egg mass	N	Antigen
	A	184.0	52	F1
	A	182.7	43	CONTROL
	A	179.6	40	F4
	A	163.6	40	F2

Duncan's grouping		Mean egg conversion efficiency	N	Antigen
	A	51.5	48	F1
B	A	50.3	43	CONTROL
B	A	48.8	40	F4
B		46.8	39	F2

Means with the same letter are not significantly different

APPENDIX 11

ANALYSIS OF VARIANCE

General linear model procedure for table 15

Dependent variable	DF	SS	MS	F value	p
Feeding duration	1168	1186.9	0.97	18.35	0.0001
Engorgement weight	1164	11260.4	9.66	1.28	0.27

Duncan's Multiple Range Test for table 15

Duncan's grouping	Mean feeding duration	N	Antigen
A	5.28	266	F4
B	4.97	266	F1
B	4.87	332	CONTROL
B	4.68	305	F2
Duncan's grouping	**Mean Eng. weight**	**N**	**Antigen**
A	9.84	302	F2
A	9.77	331	CONTROL
A	9.45.	266	F4
A	9.44	266	F1

Means with the same letter are not significantly different

APPENDIX 12

ANALYSIS OF VARIANCE

General linear model procedure for table 16

Dependent variable	DF	SS	MS	F value	p
Feeding duration	792	257.8	0.32	1.53	0.20
Engorgement weight	656	17.8	0.02	1.69	0.16

Duncan's Multiple Range Test for table 16

Duncan's grouping		Mean feeding duration	N	Antigen
	A	3.93	185	CONTROL
B	A	3.86	238	F2
B	A	3.85	204	F4
B		3.80	166	F1

Duncan's grouping		Mean Eng. weight	N	Antigen
	A	0.54	171	F4
B	A	0.52	140	F1
B	A	0.52	181	F2
B		0.50	165	CONTROL

Means with the same letter are not significantly different

APPENDIX 13

ANALYSIS OF VARIANCE

General linear model procedure for table 19

Dependent variable	DF	SS	MS	F value	p
Yield	209	24772.9	109.7	6.62	0.0003
Feeding duration	209	6.3	0.03	1.46	0.22
Engorgement weight	209	59.7	0.26	7.12	0.0001
Egg mass	176	72.0	0.40	2.27	0.08
Egg conversion efficiency	174	7780.6	44.7	0.95	0.41
Mortality	209	47684.7	206.5	8.32	0.0001

Tukey's Studentized Range(HSD) Test for variable:Yield of Table 19

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-12.2	-6.5	-0.7 ***
MEDIUM-CONTROL	-2.6	2.5	7.7
HIGH-CONTROL	-8.3	-3.3	1.8
MEDIUM-LOW	3.3	9.0	14.7 ***
MEDIUM-HIGH	0.8	5.8	10.8 ***
HIGH-LOW	-2.3	3.2	8.8

APPENDIX 13

Tukey's Studentized Range(HSD) Test for variable: Feeding duration of Table 19

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-0.08	-0.007	0.10
MEDIUM-CONTROL	-0.1	-0.05	0.03
HIGH-CONTROL	-0.08	-0.003	0.08
MEDIUM-LOW	-0.15	-0.06	0.03
MEDIUM-HIGH	-0.13	-0.05	0.03
HIGH-LOW	-0.10	-0.01	0.08

Tukey's Studentized Range(HSD) Test for variable:Engorgement weight of Table 19

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-0.36	-0.08	-0.20
MEDIUM-CONTROL	-0.50	-0.25	0.008
HIGH-CONTROL	-0.66	-0.41	-0.16 ***
MEDIUM-LOW	-0.45	-0.16	0.11
MEDIUM-HIGH	-0.08	0.16	0.41
HIGH-LOW	-0.60	-0.33	-0.06 ***

APPENDIX 13

Tukey's Studentized Range (HSD) Test for variable: Percent mortality of Table 19

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	0.9	8.8	16.7 ***
MEDIUM-CONTROL	2.6	9.7	16.8 ***
HIGH-CONTROL	6.0	12.9	19.8 ***
MEDIUM-LOW	-6.9	0.90	8.7
MEDIUM-HIGH	-10.0	-3.2	3.6
HIGH-LOW	-3.5	4.1	11.8

Tukey's Studentized Range(HSD) Test for variable: Egg mass of Table 19

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-4.2	-0.21	0.38
MEDIUM-CONTROL	-4.2	-0.65	2.8
HIGH-CONTROL	-5.7	-2.1	1.4
MEDIUM-LOW	-4.5	-0.45	3.5
MEDIUM-HIGH	-2.0	1.5	5.0
HIGH-LOW	-5.9	-1.9	2.0

APPENDIX 13

Tukey's Studentized Range(HSD) Test for variable: Egg conversion efficiency of Table 19

Antigen comparison	**Simultaneous lower confidence limit**	**difference between means**	**Simultaneous upper confidence limit**
LOW-CONTROL	-4.2	-0.21	3.8
MEDIUM-CONTROL	-4.2	-0.66	2.8
HIGH-CONTROL	-5.7	-2.1	1.4
MEDIUM-LOW	-4.5	-0.45	3.5
MEDIUM-HIGH	-2.0	1.5	5.0
HIGH-LOW	-5.9	-1.9	2.0

Comparisions significant at the 0.05 level are indicated by ***

APPENDIX 14

ANALYSIS OF VARIANCE

General linear model procedure for table 20

Dependent variable	DF	SS	MS	F value	p
Yield	500	59677.3	102.2	28.9	0.0001
Feeding duration	500	26.8	0.05	9.8	0.0001
Engorgement weight	500	100.0	0.20	2.0	0.10

Tukey's Studentized Range(HSD) Test for variable:Yield for Table 20

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-14.2	-10.5	-6.8 ***
MEDIUM-CONTROL	-11.8	-8.8	-5.8 ***
HIGH-CONTROL	-10.5	-7.4	-4.3 ***
MEDIUM-LOW	-2.0	1.7	5.5
MEDIUM-HIGH	-4.6	-1.4	1.8
HIGH-LOW	-0.7	3.1	6.9

APPENDIX 14

Tukey's Studentized Range(HSD) Test for variable: Feeding duration for Table 20

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-0.06	0.14	0.22 ***
MEDIUM-CONTROL	-0.05	0.01	0.08
HIGH-CONTROL	0.03	0.09	0.16 ***
MEDIUM-LOW	-0.21	-0.13	-0.04 ***
MEDIUM-HIGH	-0.15	-0.08	0.01 ***
HIGH-LOW	-0.13	-0.05	0.04

Tukey's Studentized Range(HSD) Test for variable:Engorgement weight of Table 20

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-1.56	0.003	0.16
MEDIUM-CONTROL	-0.25	-0.11	0.01
HIGH-CONTROL	-0.17	-0.04	0.09
MEDIUM-LOW	-0.29	-0.12	0.04
MEDIUM-HIGH	-0.08	0.16	0.41
HIGH-LOW	-0.22	-0.08	0.06

Comparisions significant at the 0.05 level are indicated by ***

APPENDIX 15

ANALYSIS OF VARIANCE

General linear model procedure for table 21

Dependent variable	DF	SS	MS	F value	p
Yield	1291	55209.9	39.3	39.3	0.0001
Feeding duration	1291	46.5	0.03	26.8	0.0001
Engorgement weight	1291	79.3	0.06	2.9	0.03

Tukey's Studentized Range(HSD) Test for variable:Yield of Table 21

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-1.8	-0.5	0.77
MEDIUM-CONTROL	-4.9	-3.7	-2.4 ***
HIGH-CONTROL	-5.4	-4.2	-3.0 ***
MEDIUM-LOW	-4.5	-3.2	-1.8 ***
MEDIUM-HIGH	-0.8	0.50	1.75
HIGH-LOW	-4.9	-3.6	-2.4 ***

Tukey's Studentized Range (HSD) Test for variable: Feeding duration of Table 21

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-0.02	0.02	0.06
MEDIUM-CONTROL	-0.10	-0.07	-0.03
HIGH-CONTROL	-0.12	-0.09	0.05
MEDIUM-LOW	-0.13	-0.08	-0.05
MEDIUM-HIGH	-0.01	-0.02	0.05
HIGH-LOW	-0.14	-0.10	-0.07

Tukey's Studentized Range (HSD) Test for variable:Engorgement weight of Table 21

Antigen comparison	Simultaneous lower confidence limit	difference between means	Simultaneous upper confidence limit
LOW-CONTROL	-0.10	-0.05	0.001
MEDIUM-CONTROL	-0.09	-0.04	0.004
HIGH-CONTROL	-0.09	-0.04	0.006
MEDIUM-LOW	-0.05	-0.005	0.06
MEDIUM-HIGH	-0.05	-0.004	0.04
HIGH-LOW	-0.04	-0.009	0.06

Comparisions significant at the 0.05 level are indicated by ***