STUDIES ON THE IMMUNIZATION OF SAVANNA BROWN GOATS AGAINST HEARTWATER USING THE GROUND UP TICK SUPERNATANT (GUIS)

BY

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DECLARATION

This is certify that the work reported in this thesis was carried out in The Protozoology Research Laboratory of the Department of Veterinary Parasitology and Entomology under the supervision of Prof. R.I.S. Agbede, Prof. K.A.N. Esievo and partial supervision of late Dr. V.C. Ogbogu.

The work of other investigators, in this field is duly acknowledged and referred to appropriately.

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DEDICATION

This thesis is dedicated to my family for their total support.

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All thanks go to Almighty God for seeing me through the rigours of trying to obtain a Doctorate degree. The hunter says "He cannot recount all the difficulties he goes through in the bush before he succeeds in killing an animal". I agree with him in toto.

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My learned major supervisor once said that research is continuous researching and researching. I pray to Almighty God to provide me the powers and fortitude to re-search more into what other scientists and I have searched, so that the war against ticks and tick-borne diseases will continue unabated in Nigeria.

ABSTRACT

The fatal side effect of heartwater vaccine derived from *Amblyomrna hebraeum* infected *Cowdria ruminantium* has reduced its use in Southern Africa despite its numerous advantages over the infected sheep blood vaccine. The work reported here aims at eliminating the side effect to enable its use within this environment. Poor technology and logistic problems may not allow effective use of inactivated of molecular vaccine locally. . Moreover, there is the emerging fear of antigenic diversity among *Cowdria* isolates which could be worsened by the use of a sub unit vaccine.

In preparing the crude vaccine (ground up tick supernatant). *Amblyomrna variegatum* ticks were collected from resident domestic ruminants in Zaria. Fully and partially engorged females of these ticks were incubated at 27^{o}C and 80% relative humidity, Giemsa stained haemolymph smears were made, examined and found negative for rickettsia bodies. Fully engorged ticks oviposited 7 to 10 days post incubation while the partially engorged ones oviposited on the 9th day.

The larvae progeny were fed using earbags, on a goal identified as NNI infected with cryo-preserved *Cowdria* ***ruminantium*** (Jaji stock). Engorged larvae were harvested from the earbags when the goat was reacting to infection (indicated by a rise in temperature of 40°C - 41.3°C). The lavae were expected to have been infected by feeding on the goat, were subsequently incubated as described earlier and moulted to nymphae. The nymphae were pre-fed on a susceptible goat which reacted 9 days post infestation and the brain was positive for *Cowdria* colonies confirming that the nymphae were infected and infective.

Crude vacciene was prepared by grinding them up in morta and pestle with pnosphate buffered saline (PBS pH 7.4) added as diluent. Two mls of the supernatant (vaccine) was inoculated into a parasite free susceptible goat which had fever (temperature 4l°C) 10 days later showing that the vaccine was potent.

Reaction-free minimum infective/dose (mid) was determined by inoculating different levels of dilutions of the crude vaccine into 25 savanna brown goats that had been kept in arthropod-free pen. The coneentration of each dilution was determined based on the calculated number of nymphae per ml of the vaccine (N/ml). Two levels of concentrated forms (2 N/ml; 3.6 N/ml) were used to determine the threshold level. Other concentrations used were 0.025, 0.0125, 0.03125,

N/DOSE. Inoculation of the concentrated forms (3.6 N/m; 7 N/ml) elicited side reactions classified as violent. These included staggering, urination, constant bleating, profuse salivation. The animal inoculated with 3.6 N/ml recovered while 7 N/ml caused death of the animal. The side reactions decreased with decreasing concentrations. Side reactions were completely eliminated at 0.03125, 0.025 and 0.0125 N/dose. However, 0.0125 N/dose did not give effective result as an animal which failed to react at this concentration reacted at a higher concentration of 0.10 N/dosc. Incubation period ranged from 0 to 14 days and were affected by the concentration of the inoculum, being shorter at higher and longer at lower concentration. Duration of febrile reactions were longer in younger than older animals. There was no clinical reactions within the three weeks observation period following homologous challenge using a uniform concentration of 0.10 N/dose.

Immunity and cross immunity studies were done using *Cowdria ruminantium* isolates from Akure (Forest) and Shika (Savanna). Differential white blood counts were done. Mice, rats and guinea pigs were inoculated.

Isolation of C. *ruminantium* from Akure (forest zone) was done by collecting *Amblyomma variegatum* ticks from resident cattle in some farms in Akure. The ticks were brought to Zaria and ground up using morta

and pestle with broken glass slides to facilitate grinding. Phosphate buffered saline (P.B.S pH.7.4) was added as diluent at l.8ml/tick. The mixture was allowed to settle on iee at room temperature and 2mls of the supernatant was inoculated into a goat No. 5256, while the remaining supernantanl was preserved as stabilate (CR4) alter dimethyl sulphoxide (DMSO) was added at 1ml per 9 mls of the supernatant. The animal showed a febrile reaction at day 13 post inoculation with maximum febrile temperature of 40.5°C and stabilate CR7 was prepared and cryo-preserved in liquid nitrogen. The brain of the goat was heavily colonised by *Cowdria* granules.

Isolate from Shika was similarly obtained from *A. variegatum* ticks (collected from a sedentary herd in Shika) which were ground up as described above and 2mls of the supernatant injected into a goat identified as 5253 while the remaining supernantant was cryo-preserved as stabilate (CR 6). The animal reacted 3 days post inoculation (pi) with a maximum temperature of 40°C. The animal died and the brain was positive tor *Cowdria* colonies which were scanty.

The forest isolate was used to immunize goats Nos. 5255. 271 and NNII. Reactions to primary infection were followed by secondary (homologous) challenges. There was no reaction to secondary infection. None of the animals was treated. The savanna isolate was also used to

immunize animal Nos. 5253, 5254, 5257 which reacted between clays 2 to 4 clays post inoculation. Two of the animals (5252, 5257) that were treated during pyrexia survived while those not treated died within two weeks of infection. While none of the animals immunized with Shika isolate reacted when cross challenged with forest isolate, one of the animals, No. 0271, which was immunized against forest isolate died and *Cowdria* colonies typical of the Shika isolate were observed in the brain. Although no clinical reactions were observed after the heterologous challenge.

The three isolates used in these studies were compared and found to have differing characteristics. The forest isolate resembled closely another savanna isolate (Jap stock) in terms of characteristic of the colonies, length of incubatuion period and infectivity for the savanna goats. Akcie and Jaji isolates showed extensive colonization of brain capillaries while Shika isolate is scanty. However none of the isolates is murinotrophic.

No definite pattern could be established as regards the percentage circulating white blood cells in the infected animals. There is a wide variation in % lymphocytes in Akure isolate infected animals.

Animals infected with Shika isolate showed a decrease in the percentage of neutrophils and an increase in lymphocyte during pyrexia

although the mean pre- and post infection values did not vary much. Monocytes remained within the normal range while a total absence of eosinophils was observed during reactions.

The highlights of these studies are as follows

i) Eggs of fully engorged female *Amblyomma variegatum* are better for preparing crude infected lick-derived *Cowdria* vaccine than those from partially engorged ticks

ii) Concentrations of 0.03125 and 0.025 of *A. variegatum* nymphae in 2 mls inoculum are both infective, immunizing and do not produce side effects when inoculated intravenously

iii) An isolate from the forest zone (Akure) is immunogenic but produces mild reaction in savanna brown goats

iv) Akure and Shika isolates are cross-protective but a goal that was immunized against the Akure isolate reacted and died when cross-challenged with the Shika isolate.

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ABBREVIATIONS

et al	=	and others
FAO	=	Food and Agricultural Organization
WHO	=	World Health Organization
FDLPCS	=	Federal Department of Livestock and Pest Control Services
um	=	Micrometer
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
ug	=	Microgram
ml	=	Millimeter
HCL	=	Hydrochloric acid
°C	=	Degree Celsuis (centigrade)
IFN	=	Interfereone
ng	=	Nanogram
KDa	=	Kilodalton
ELISA	=	Enzyme linked immunosorbent assay
gm	=	Gram
MAP	=	Major antigenic protein

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CHAPTER ONE

INTRODUCTION

Heartwater, also known as *Cowdriosis* is a septicaemic tick-borne disease of ruminants caused by a rickettsial organism called *Cowdria ruminantium* (Cowdry, 1925). It is transmitted transtadially by three - host ticks belonging to the Genus *Amblyomma*. The causative organism is commonly found in the endothelial cells of the brain capillaries, although it can also be found in the intima of large blood vessels (Ilemobade, 1976).

The disease may either be peracute, acute, subacute or chronic in susceptible animals. Characteristically, the signs of the acute form; which is commonest, are fever, diarrhoea, and various forms of nervous signs like hyperesthesia, intermittent convulsion, high stepping gait, opisthotonus and pedaling of limbs especially at the terminal stage and death.

Hyperaemia of the intestinal mucosa, hydrothorax and hydropericardium from where the name heartwater was derived are the main postmortem signs.

The disease was first recognised in South Africa (Hutcheon, 1900) and was first named heartwater in Dutch/Afrikan which was later translated into English and German as heartwater and heartwater respectively. However the origin of heartwater has also been traced to the East, especially Persia (Iran) (Lounsbury, 1904). It was said that the disease spread through the east coast of Africa to most of the countries in Africa south of the Sahara and Madagascar (Uilenberg, 1983) through direct importation of infected animals, movement of diseased or tick infested animals across the borders or through migratory birds especially the cattle egrets or wild animals. Perreau *et al.* (1980) first reported the presence of heartwater in the Western hemisphere. This generated a lot of world wide interest in a disease that was hitherto considered a peculiar problem to Africa. The tropical bont tick, *Amblyomma variegatium*, was first introduced into the Caribbean in 1928 from Africa (Giersabeck, 1993). Presently about thirteen Caribbean islands are infested with the ticks while heartwater has been confirmed in three of them, viz: Maria Galante, Antigua and Guadeloupe (Camus and Barre, 1982). There has been an increasing fear that the disease may spread into the American mainland (Uilenberg *et al.* 1984). This fear is further heightened by Sutherst and Mayward (1985) who reported that the Southern, Central America and large part of South America are climatically suitable for the

survival of *A. variegatum* using the climate matching model (climex). This invariably means that the disease can easily establish once it spreads into these areas. In another report, sera from white tailed deer (*Odocoileus virginianus)* tested positive with the recombinant *C. ruminantium* antigens in South eastern United States although negative results were obtained with cattle sera (Katz *et al;* 1996).

However, Clark and Doten (1995) reported that 46% of the 39,934 animals imported into United States were infested with a wide range of ticks which include *A. hebraeum, A. variegatum, A. lepidum, A. gemma, A. Sparsum* (natural vectors), *A. mormoreum and A. dissimile* (experimental vector). It has been estimated that the spread of heartwater into the American mainland would cost livestock producers in the United States $761 million annually, $224 million annually in Brazil and $144 million annually in Mexico (Gersaberk, 1993). An eradication campaign therefore started in 1994 in the Caribbeans, sponsored by several donor agencies.

Africa has 175 million heads of cattle which are at risk due to heartwater (FAO/WHO/OIE, 1990). These cattle are either in countries where the disease has been confirmed (Provost and Bezuidenhout, 1987)

or where the vectors and the disease are likely to occur (Walker and Olwage, 1987). Ironically heartwater receives less attention in Africa when compared to enormous control effort in the Caribbean despite the relatively low cattle population on the islands (the lowest is about 7000 heads on St. Lucia and Nevis and the highest is 74,000 heads on Guadeloupe, FOA/WHO/OIE, 1990). The presence of *A. variegatum* and heartwater have been sources of concern on these islands because of their economic importance.

Reviewing the economic impact of the disease Bezuidenhout (1993) classified them into lethal losses due to mortality resulting from movement of susceptible animals into endemic areas, the disease outbreaks, failure of tick control and raising of susceptible animals in endemic areas. The non-lethal losses are due to production losses caused by subclinical infection, cost of disease and vector control and treatment of clinical cases and production losses caused by infestation of vectors and inability to upgrade local livestock by cross-breeding with exotic breeds causing loss of production potential (Uilenberg, 1982). Although, the non-lethal losses are not easily quantified, Maltezer (1990) estimated that some large scale livestock farmers in Zimbabwe pay from US$1 to 17 US dollars per head/year to dip their animals against ticks.

In Nigeria, heartwater was first reported in Vom stock farm and later in various parts of northern and southern provinces (Hall, 1931 as cited by Ilemobade, 1976). It causes losses in local and exotic animals (Isoun *et al.* 1974; Lawal *et al;* 1992). Nigeria maintains a dynamic small ruminant population over the years. In 1981, the population of sheep and goats was 24 million and 11.7 million respectively (FDLPCS, 1981). This increased to 32.1 million sheep and 34.5 million goats in 1991 (FDLPCS, 1991). Small ruminants especially goats contribute immensely to individual and national economy in Nigeria. Their small sizes allow them to reach maturity earlier than cattle. They have short reproductive cycles, high fecundity, adapt better to hostile environment and resist draught more than cattle. Goats are well distributed in Nigeria ranging from 3 - 5 goats per household in the South to 10 per household in the northern parts of the country (Wosu and Ibeke, 1990).

Tick-borne diseases are serious impediments to small ruminant production in Nigeria and heartwater is the most important of the tick borne haemoparasitic diseases. The Savanna brown goats are the predominant breeds in the northern parts of Nigeria. The high susceptibility of these breeds to heartwater has been widely reported (Ilemobade & Blotkamp, 1978b; Audu *et al;* 1995; Ulayi *et al;* 1992).

Recently, the West African dwarf goats were also reported to be highly susceptible to the disease although the mortality varied (Okayeito, 1995). The most feasible method of controlling the disease is by immunising susceptible stocks (Van der Merwe, 1979). Consequently trial vaccinations have been done in some parts of Nigeria (Anon, 1989). As the blood vaccines possess several inherent problems especially the likelihood of transmitting other diseases, the ground up tick supernatant (GUTS) vaccine has been used during these trials (Anon, 1989). The GUTS vaccines do elicit side reactions which can be fatal atimes when intravenously inoculated. Consequently, administration of the vaccine is usually limited to few experienced hands. This has affected the use of the vaccine in South Africa (Pipano, 1995). It is pertinent to mention at this point that several advancements have been made in vaccine production and vaccination against heartwater disease. These include the use of attenuated vaccines (Jongejan *et al.* 1993c); inactivated vaccines (Martinez *et al;* 1994) and the use of molecular vaccines or recombinant vaccines (Mahan, 1993). However, the problems of these vaccines are numerous. These include high cost of production, induction of shorter immunity than that caused by live material and problems of antigenic diversity (Uilenberg, 1995). Therefore, the use of live material, by

infection and treatment technique is still the main method of controlling heartwater today despite its shortcomings.

There is a serious gap of knowledge in the epidemiology of heartwater in Nigeria. Most studies have been limited largely to the northern parts (Ilemobade, 1976; Audu *et al;* 1995; El.Imairi, 1991; Lawal *et al;* 1992). There is paucity of information on the status of the disease in the southern parts, a situation which calls for concern because of the widespread nature of the tick vector (Anon, 1989).

These studies were therefore undertaken with the following specific objectives:

1) Determination of reaction-free minimum infective dose (MID) of ground up tick supernatant (GUTS) heartwater vaccine in brown savanna goats.
2) Isolation of *C. ruminantium* from the forest vegetation (Southern part) zone and savanna zone (Northern part) of Nigeria.
3) Immunity/Cross immunity studies of the forest and savanna isolates.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Historical development

Heartwater has been recognised for many years and known by different local names in several African countries (Hemobade, 1976). It was stated by Neitz (1947) that a Voortreker or South African Pioneer Louis Trichardt mentioned it in his dairy dated 9th March 1888 that a disease "nintas" was noticed among his sheep that had massive tick infestation on February 17, 1888 approximately three weeks previously. In another version, Hutcheon (1900) said that heartwater was first observed in 1860, the time that bont tick *Amblyomma hebraeum* was also recognised. Almost 50 years later after the record of Louis Trichardt, a farmer, John Webb reported to the cattle and sheep commission at their meeting in Grahamstown in February, 1937 that a disease "boschsickness" which has by now been generally known as heartwater caused heavy mortality among sheep in eastern Cape (Alexander, 1931). According to Henning (1956) the disease was subsequently reported in different parts of South Africa. However, Lounsbury and Robertson (1904) believed that heartwater was brought into South Africa by imported Persian sheep in 1872. They felt that the strong resistance shown by these animals must have been due to previous exposure at the place of their origin which was

thought to be in eastern countries. One of the outstanding break through in the early research on heartwater was by Dixon (1898 as cited by Provost and Bezuidenhout,1987). He showed that susceptible animals could be infected artificially by intravenous inoculation of blood from sick animals and concluded that heartwater was caused by a living microorganism which was neither demonstratable in the blood nor tissues of dead animals. Hutcheon (1900) speculated that the causative microorganism was an unltravisible virus. Later, Spreull (1904) and Lounsbury (1902) simultaneously confirmed its vector to be *A. hebraeum*.

The work of Dixon allowed the disease to be reproduced in susceptible animals. This paved way for studies on its control.

Sir Arnold Theiler a renowned parasitologist had speculated that heartwater might be caused by rickettsia, thus Cowdry, a visiting Scientist from Rockfeller Institute in New York in various experiments confirmed it by demonstrating the organisms in tissues of affected animals and infected ticks (Cowdry, 1925). He named the agent *Rickettsia ruminantium* but Moshkeviski (1947) changed the name to *Cowdria ruminantium* in honour of Cowdry. The discovery by Cowdry stimulated other workers to develop a method of its diagnosis (Jackson 1931; Purchase, 1945). The brain squash technique of Purchase (1945) is still

the most acceptable method for diagnosing heartwater. It has long been discovered that wild ruminants and laboratory animals are susceptible to heartwater. Neitz (1944) first demonstrated the susceptibility of the blesbok (*Damaliscu albifrons)*. The black wild beast (*Choanochaetes gnu*) and springbok (*Antidorcase masurpialis*) to heartwater. Heartwater spreads from South Africa through the eastern coast to Kenya and other areas, through several ways especially movement of animals across borders.

In Kenya, Daubney (1930) confirmed the disease by demonstrating the organism in brain and kidney sections and showed that the disease was transmitted by *A. variegatum*. In Nigeria, the disease was first confirmed in a stock farm in Vom in a sheep according to Ilemobade (1976). The Fulani cattle rearers also recognised heartwater as "Kaboa" which means trembles (Anon, 1934 cited by Ilemobade, 1976). It was also stated that by 1943, heartwater was known to occur in all the provinces of the former northern Nigeria and one of the Agricultural farms in southern Provinces.

2.2 Distribution of heartwater

The distribution of heartwater is confined to areas favourable to the development of its vectors (Haig, 1955). According to Provost and Bezuidenhout (1987), Camus and Barre (1982), heartwater has been reported in almost all the African countries South of the Sahara apart from Guinea, Sierra Leone, Burkina Faso, Togo, Equitorial Guinea and Namibia where there are presently no available records of the disease. Heartwater was recently reported in Gambia (Mattoli *et al;* 1993). It has been reported in some Indian Ocean Islands of Madagascar (Buck, 1965), Mauritius (Perraeu *et al*, 1980), Reunion (Perraeu *et al;* 1980) and SaoTome (Uilenberg *et al*; 1982). At the Caribbean Islands, there are reports of its existence in Antigua (Birnie *et al;* 1985), Guadeloupe (Perraeu *et al*; 1980) and Marie Galante (Camus *et al;* 1984). The report of heartwater in Guadeloupe was the first case outside the African continent apart from the accidental case in Brazil (Alves *et al;* 1937 cited by Camus and Barre *et al* (1987). There is high possibility that heartwater may be recorded in some countries infested by *A. variegatum* in future. *A. variegatum* adapts easily to domestic livestock and is the only natural vector known to have established itself outside the African continent (Walker and Olwage, 1987). It has been predicted that it may

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find suitable ecological environment for establishment if it eventually gets into the American mainland. The distribution of heartwater in Africa and the Caribbean is shown in Figs. 1 and 3.

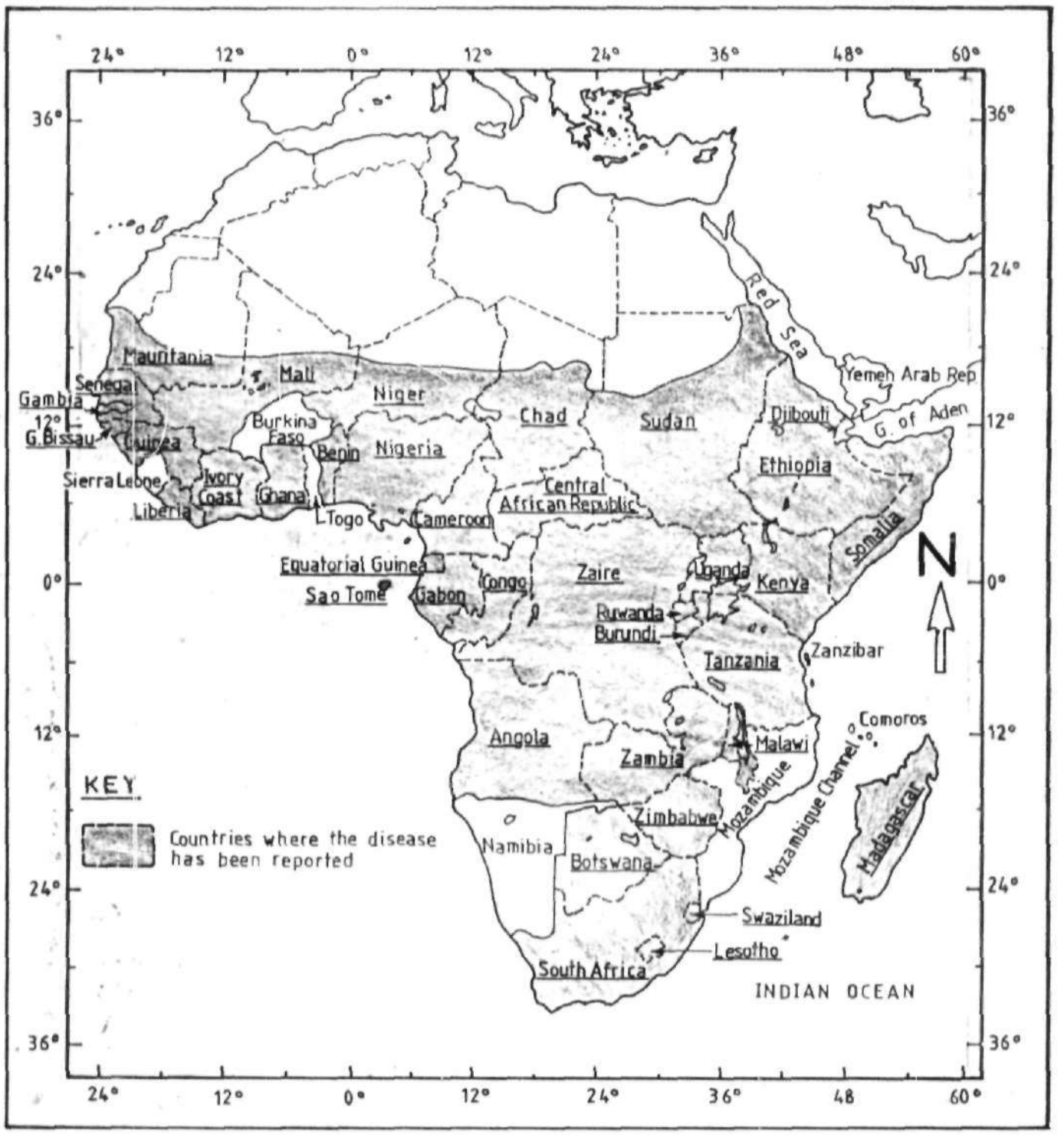


Fig. 1. The distribution of heartwater in Africa (Provost and Bezuidenhout, 1987

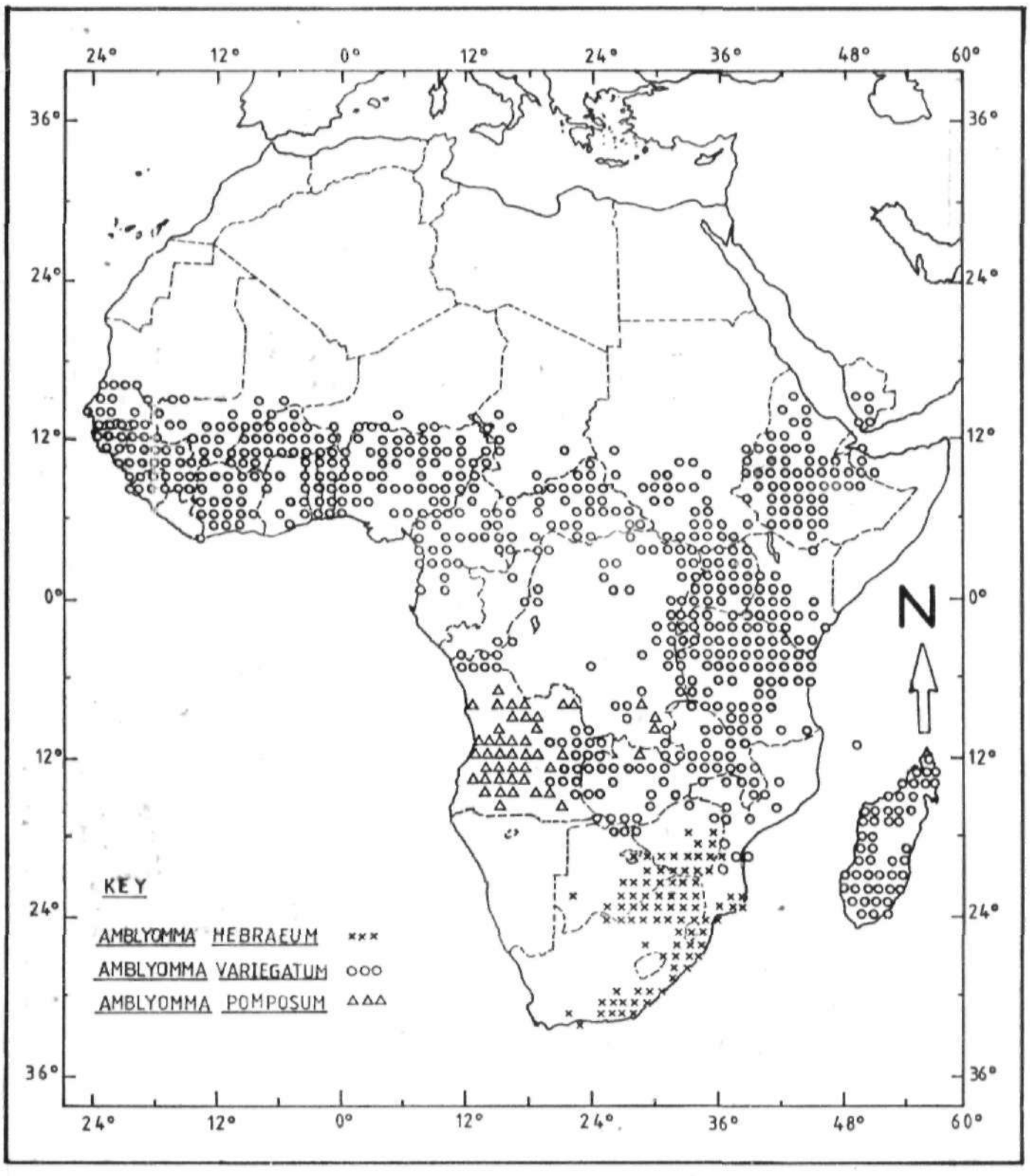


Fig. 2. Distribution of Amblyomma variegatum, A. hebraeum and A. Pomposum in the Afrotropical region, the Yemen Arab Republic and Madagascar (Walker and Olwage, 1987)

2.3 Host range of *Cowdria ruminantium*

2.3.1 The invertebrate host/Natural Vectors of *C. ruminantium*

Heartwater is transmitted exclusively by three host ticks of the genus *Amblyomma*. These belong to the order *Acarina*, suborder Ixodoidea; *family* Ixodidae (Soulsby, 1982). The disease is transtadially transmitted (Hemobade and Leeflang, 1978; Soulsby 1982; Losos, 1986). A report of transovarian transmission by larvae, nymphs and adults of *A. hebraeum* has been made (Bezuidenhout and Jacobsz; 1986). *Amblyomma* ticks which can transmit the disease either experimentally or naturally are many. The known natural vectors are *A. variegatum, A. hebraeum; A. lepidum; A. pomposum and A. gemma* (Karrar, 1965). *A. variegatum* is the only natural vector that has successfully established outisde the African continent.

It is also called "The variegated tick" or "The tropical bont tick". It is the most widely distributed of all the *Amblyomma* species (Walker and Olwage, 1987). It occurs in West Africa from Sahel downwards, Southern Africa to far south as Zambezi River and central Mozambique (Fig. 2).

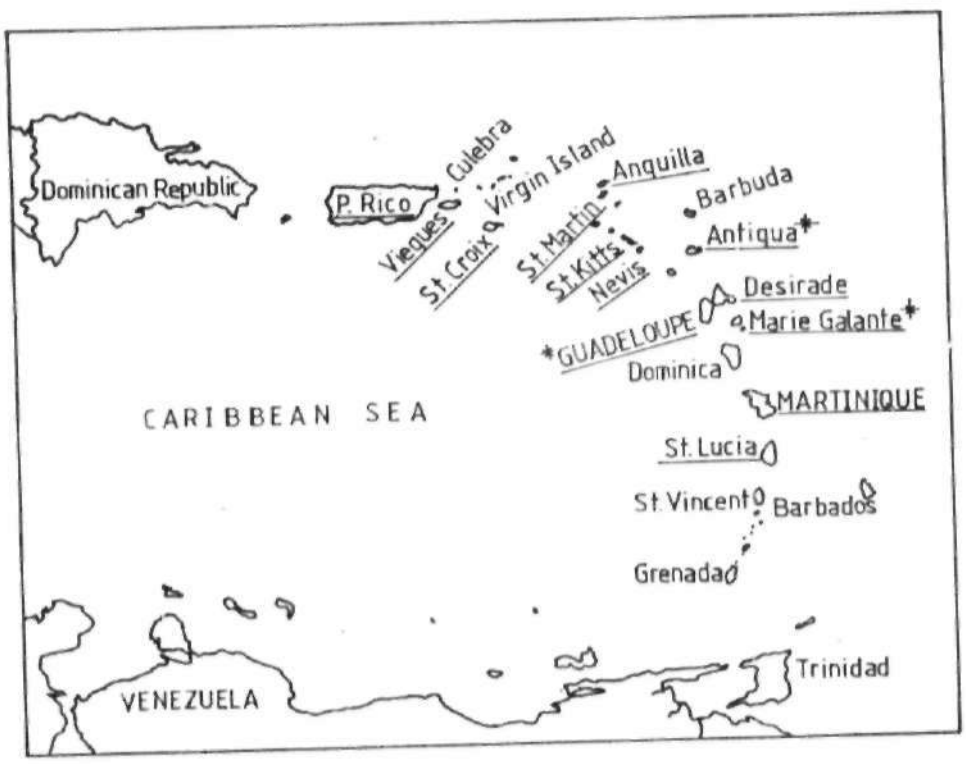


Fig. 3. Distribution of Amblyomma variegatum and heartwater in the Caribbean. The islands where A. variegatum has been reported are underlined and asterics show where the disease was reported (Uilenberg et al, 1984).

It is also the most widely distributed ticks in Nigeria (Anon, 1989). Apart from the African continent, *A. variegatum* is found in Yemen, Indian Ocean Islands of Reunion, Madagascar, Grande Comoro and Mauritius. Atlantic Ocean Islands of Cape Verde, Caribbean Islands of Guadeloupe, Maria Galante, Martinique, Antigua, St. Lucia, St. Kitts, Nevis, St. Maarten, La Desirable, MontSerrat and Dominica (Walker and Olwage, 1987). *A. variegatum* also occurs in Vesques, Culebra, Anguilla and St. Croix (Alderink and McCauley, 1988). Some other Islands of the Antillean chain of Barbados, Puerto Rico, Saba, St. Eustatius, St. Vincent and the US Virgin Islands are also reportedly infested with *A. variegatum* (Alderink and MacCauley, 1988). The ticks were first introduced in 1830 into the Caribbean with African zebu cattle from Senegal (Curason, 1943) and has since then been spreading to many Islands (Fig. 3). Daubney (1930) showed that heartwater is transmitted by *A. variegatum*. Apart from heartwater, it also transmits Nairobi sheep disease, Q fever (Soulsby, 1982), dermatophilosis (Bida, 1973; Barre *et al;* 1987). Cattle and sheep are the principal hosts of all stages of the tick (Strickland, 1961). The ecology of the tick in the Caribbean Islands seems to differ from what obtains in Africa. In Guadeloupe for example, 97% of Nymphs and nearly 100% of adults feed on cattle and goats while 4.5% of immature stages feed on wild life and birds. Only 1 to 4% of adults

are infected and only a small proportion of these are actually infective (Camus and Barre *et al;* 1987). *A. variegatum* in the Caribbeans are found mainly on domestic livestock (Barre *et al;* 1987) while a large number of secondary hosts are usually involved in Africa (Strickland *et al;* 1976). Thus control of the ticks by application of acaricide to livestock is more likely to succeed in the Caribbean than Africa.

A. *hebraeum* also called "The bont tick"

It occurs in warmer parts of South and Central Africa parasitizing all domestic and many wild animals (Fig. 2). However, the young stages attack birds (Soulsby, 1982). Males of *A. hebraeum* are known to remain on hosts for several months (Norval, 1974) and to mate with multiple females. Unlike many other three-host Ixodid ticks that rest on vegetation awaiting contact with a suitable host, post-larval bont ticks actively look out for their hosts. They emerge from sub-surface resting places and run rapidly to hosts on sensing their presence (Norval *et al;* 1988). It has been reported that losses in domestic ruminants caused by heartwater in Zimbabwe and Mozambique are greater when the vector is *A. hebraeum* than when it is *A. variegatum* (Norval *et al;* 1992). This is thought to be associated with the rates at which unfed adults of these two species attach to uninfested hosts. The males of *A. hebraeum* attach more

rapidly than *A. variegatum*. Unfed adults of *A. hebraeum* are usually stimulated by odors of cattle and sheep and high concentration of carbon dioxide (Norval *et al;* 1987). The behaviour of this tick must have contributed to its efficiency in the transmission of heartwater.

A. pomposum are found in highland areas of northern Angola. Gomes (1993) reported that it is the most widely distributed and most important of all the six *Amblyomma* species identified in Angola (Fig. 2).

2.3.1.1 Experimental vectors of *C. ruminantium*

These include *A. asterian* (Uilenberg and Nieuwold, 1981); *A. cohaerens* (Walker and Olwage, 1987); *A. tholloni* and *A. marmoreum* (Fivaz *et al;* 1990); *A. maculatum*, "The Gulf coast ticks" and *A. cajennese* "The cayene ticks" (Uilenberg *et al; 1984)*. Jongejan (1992) showed that *A. dissimile* recovered from snakes imported from Suriname into the Netherlands transmsitted heartwater experimentally.

2.3.1.2 Natural or Field vectors of heartwater

Natural or field vectors are ticks that are capable of transmitting heartwater from asymptomatic carriers to susceptible hosts. Presently the capability to transmit from carriers has been demonstrated only for

nymphs of *A. hebraeum* and *A. variegatum* (Andrew and Norval, 1989; Jongejan *et al;* 1988) respectively. Experiments have shown that all proven vectors of heartwater belong to *Amblyomma* species (Bezuidenhout, 1987). Alexander (1931) and Hemobade (1976) reported that male can transmit heartwater but are poor vectors.

2.3.2 The vertebrate hosts of heartwater

2.3.2.1 Susceptibility of domestic ruminants to heartwater

All domestic ruminants are susceptible to heartwater although with some variations (Neitz, 1939). The Merino sheep are highly susceptible while the Persian sheep have high resistance (Alexander, 1931). Local domestic animals raised in endemic areas of Madagascar also show high resistance (Uilenberg, 1971a). Age resistance noticed in lambs and calves ranges from 1 to 3 weeks and upto 6 weeks in kids (Neitz and Alexander, 1941). Local breeds are more resistant than the exotic breeds. Bonsma (1944) observed a 5% mortality in native African deer compared to 60% in European calves imported into South Africa. In Guadeloupe a mortality of 0.8% in native Creole zebu was recorded as against the 13% in half bred limousine and Zebu cross. The local small ruminants however varied in their susceptibility. While some authors reported high resistance of local sheep and goats to heartwater (Uilenberg, 1971a),

others observed high susceptibility (Karrar, 1968; Ilemobade, 1976; Aklaku, 1980; Audu *et al;* 1995; Okaiyeto 1995). The resistance rate of different populations of the same breeds of Creole goats was reported to be depended on the previous exposure (Matheron *et al;* 1987). Ilemobade and Blotkamp (1978b) observed that the Nigerian brown savanna goats that originated from Zaria and areas north of it had high uniform susceptibility to heartwater whereas goats from areas south of Zaria and sheep from different parts of northern Nigeria showed variable susceptibility. The West African Dwarf goats have high susceptibility but variable mortality (Okaiyeto, 1995).

2.3.2.2 Susceptibility of wild animals to heartwater

Clinical and subclinical reactions to heartwater have been observed in wild animals (Oberem and Bezuidenhout, 1987). These include Black wild beast (Neitz, 1933); Sitatunga (Okoh *et al;* 1986) and Bushbuck (Young and Basson, 1973).

2.2.2.3 Susceptibility of Laboratory Animals to heartwater

The search for suitable laboratory animals for heartwater research started several years ago. Earlier attempts to infect Guinea pigs and rats only resulted in the survival of the organisms for only short periods

(Manson and Alexander 1940). Hudson and Henderson (1941) reported the survival of *C. ruminantium* for two weeks in infected albino rats fed on Vitamin B_2 deficient diet and *Rhabdomys pumilo* kept on normal diet. Haig (1952) observed that the Kaasplaas stock of *C. ruminantium* could be reclaimed from the spleen of 6 week old albino mice 90 days post infection and the Mora stock upto 60 days.

The first isolate of *C. ruminantium* (Kumm stock) that was highly pathogenic to mice was reported by Du Plessis and Kumm (1971). Mackenzie and McHardy (1984) reviewed the mouse infective stock of *C. ruminantium* and classified them into three groups based on their level of pathogenicity. The pathogenic stocks: These kill infected mice within a given range of time. They include the Kumm stock (Du Plessis and Kumm 1971) the Kwanyanga stock (Mackenzie and Van Rooyen, 1981) Nonile stock (Mackenzie and McHardy, 1984), the Welgevonden and Mali stocks (Du Plessis 1985).

The non-pathogenic but infective stock: They produce non-noticeable clinical signs in laboratory animals. They can also be successfully passaged and still retain their pathogenicity for sheep, goats and cattle. Examples are the mara stock (Haig, 1952), Kaaplass stock

(Haig, 1952); Ball 3 stock (Haig, 1952); K2 Malagasy stock (Ramisse and Uilenberg, 1971) Zeerust stock (Uilenberg, 1983) and Senegal stock. The non-infective stock: These are stocks that are neither lethal to mice nor successfully passaged in mice. They simply do not survive in laboratory mice. They include the Umme Banein stock from Sudan (Uilenberg, 1983); SaoTome stock (Uilenberg, 1983); D225 stock from Nigeria (Uilenberg, 1983); Gardel stock from Guadeloupe (Logan *et al;* 1985) and Moribabougou stock from Mali (Logan *et al;* 1985).

Pathogenicity of *C. ruminantium* mice is also affected by route of infection. The Kumm stock is highly pathogenic to mice when inoculated by either intravenous or intraperitoneal routes. On the contrary, the Kwanyanga and the Nonile stocks are only pathogenic when intravenous route is used (Mackenzie and Van Rooyen, 1981). The variation in susceptibility was believed to be related to histocompatibility factors (McHardy and Mackenzie, 1984).

The susceptibility of domestic ruminants to murinotropic stocks of *C. ruminantium* varies. The Kwanyanga stock is mild and produces no nervous signs in Dutch goats (Wassink *et al*; 1986) but it results in high mortality in sheep (Mackenzie and Van Rooyen, 1981). The Kumm stock

is not pathogenic for cattle (Du Plessis, 1981) but it is highly virulent in Dutch goats but produces no nervous symptoms in them before death (Wassink *et al;* 1986). Mouse pathogenic strain of *C. ruminantiumi* has not been isolated in Nigeria although efforts have been made by several workers (Anon, 1989). There will be less dependence on domestic ruminants for in vivo studies of heartwater when a murinotrophic isolate is obtained.

2.3 Classification of *C. ruminantium*

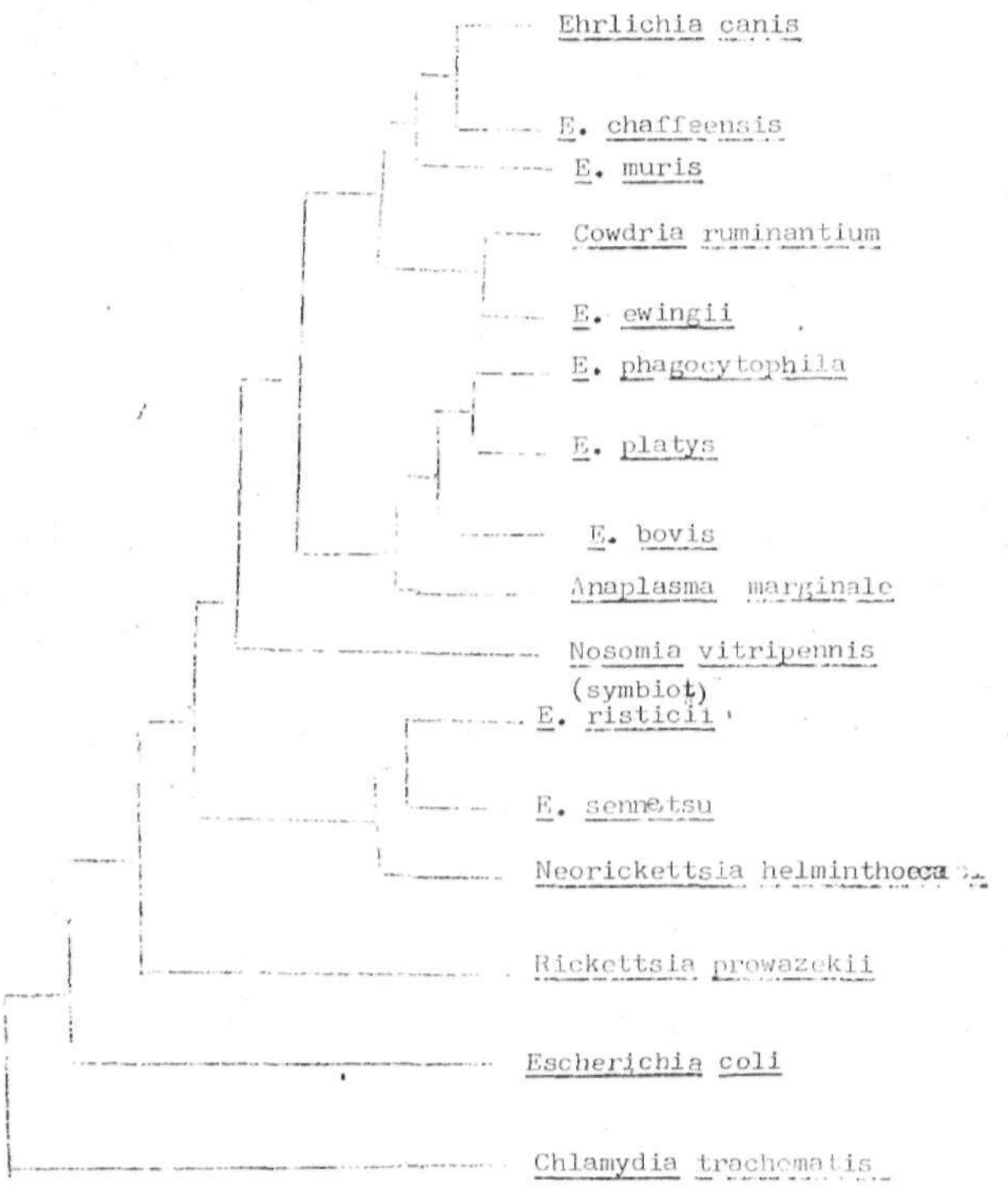
The classification of *C. ruminantiumi* has been an area of controversy. New classification keep on emerging as more facts and advances are made. Several records on this subjects are therefore available (Cowdry, 1925a; Moshkoviski, 1947; Pienaar, 1970; Hemobade, 1976).

A new classification based on the findings from the molecular studies has been proposed. Jongeja *et al;* (1989) noticed numerous similarities between *Cowdria* and *Chlamydia* but failed to agree with earlier suggestions that they are related and that *Cowdria* might occupy an intermediary position between *Chlamydia* and *Ehrlichia*. They reported that Chlamydias and rickettsiales are not closely related. In another study

Van Vliet *et al.* (1992) showed through the analysis of the 16 S ribosomal DNA sequence that *Cowdria* forms a tight phylogenetic cluster with *E. canis* and human *E. chaffeensis* despite their differences in host preferences and target cells. The analysis also showed that *E. ewingii* (agent of granulocytic canine ehrlichiosis) and *E. muris* from wild mouse are closely related to *Cowdria*. The studies showed that *Anaplasma marginale*, *E. phagocytophila E. equi*, *E. risticii* and *E. sennetsu* were distantly related.

The evolutional distance of Cowdria and other rickettsia (Van Vliet *et al.* 1992) is shown on page 26.

The evolutional distance of Cowdria and other rickettsia (Van Vliet et al, 1992).



2.5 Morphology of *C. ruminantium*

2.5.1 In the Vertebrate hosts

The morphology of *C. ruminantium* has been widely studied in the vertebrate hosts and the vectors using the light and electron microscopes. Cowdry (1925a) reported that the organisms consisted of small comma or coccus shaped, pleomorphic, spherical or ellipsoidal and rod shaped occasionally and vary in size from 0.2μm to 0.5μm. Diploid forms were occasionally observed. Jackson (1931) also described various forms, shapes and sizes in smears which varied from 0.2μm to 2.5μm. Ring forms, horse show shaped, bacillary forms, comma forms, coccoid form and single large bodies were also described by Jackson and Neitz (1932). They classified the organisms as small (coccoid) medium and large forms noting that small organisms in one group were fairly uniform although the large forms varied or are pleomorphic. It has also been reported that although the organisms may vary in size, they are generally less than 0.5μm.

Through the examinations of Giemsa stained brain squash smears of a Nigerian isolate, Ilemobade (1976) recognised four types based on the sizes of colonies and the organisms. These are type I (0.60 - 1.32μm) containing loose colonies and the organisms staining blue to reddish

purple. Type 2 (0.69 - 1.72μm) small to medium size colonies with the organism staining purplish to deep blue. Type 3 (0.72 - 2.16μm) small to medium size colonies with large size ring shaped parasites and staining pale to purplish blue. Type 4 (1.2 - 2.39μm) small sized colonies. They contain giant sized organism which stain purplish.

2.5.2 In the tick vectors:

The midgut epithelial cells of both nymph and adult of *A. hebraeum* infected as larvae and nymphs and adult *A. variegatum* infected as nymphs showed various sizes of the organisms using light microscopy. It ranged from 3 to 45μm in *A. hebraeum* and 1 to 60μm in *A. variegatum* (Kocan *et al;* 1986). Ultrastructurally electron dense and reticulate forms were identified in midgut epithelial cells and salivary gland acini cells of *A. hebraeum* nymphae infected as larvae (Kocan *et al;* 1987). Colonies were formed only when the ticks had fed to repletion on infected animals. The organisms were usually found within an inclusion membrane and were more of reticulated than the electron dense forms in the gut cells and were usually with inclusions to which the organisms were adhered. The morphology and the staining qualities of these inclusions were similar to haemoglobin inclusions usually seen in tick gut cells. It seems from these

studies that the organisms found in the vectors are larger than those seen in the vertebrate hosts.

2.5.3 **In Culture:**

Using the calf endothelial cell line, Bezuidenhout *et al* (1985) reported that the morphology of *Cowdria* colonies were similar to the type observed in brain smears (Jackson 1931, Pienaar, 1970) and infected ticks (Cowdry, 1925a). He observed various intracytoplasmic colonies in Giemsa stained smears. Many cells had multiple colonies and some had as many 15 in a cell. Individual parasites were difficult to distinguish using the direct fluorescent antibody test. Mixed colonies have also been observed in culture (Prozesky, 1987).

Ultrastructurally, reticulate bodies and smaller intermediate bodies characterised by electron dense core were identified. Prozesky *et al.* (1993) observed some extracellular reticulate bodies with peptidoglycan-like layer. Hart *et al.* (1992) using the transmission microscopy noticed various forms of colonies or organisms which also included single large reticulated organisms or in colonies, small reticulated organisms, electron dense forms and pleomorphic reticulated forms in endothelial cell cultures.

2.5.4 **In vitro Cultivation of *C. ruminantium***

The successful cultivation of *C. ruminantium* in endothelial cell line is a major stride in heartwater research (Bezuidenhout *et al;* 1985). The cultured organisms have been used as antigens for serological tests such as the direct and indirect fluorescent antibody (FA) tests (Bezuidenhout *et al;* 1985; Yunker *et al*; 1988; Martinez *et al*; 1990) and ELISA, test (Viljoen, et al 1988). The competitive ELISA test (Jongejan 1990) was also based on the monoclonal antibody, from culture. It has also been employed in the isolation of *Cowdria*, DNA for the purposes of establishing DNA genomic libraries and DNA probes (Jongejan, 1990; Yunker, 1991) and in biochemical studies leading to the isolation of immunodorminant surface proteins (Jongejan and Theilemans, 1989; Rossow *et al;* 1990). Attenuation of the Senegal stock of *C. ruminantium* has been achieved using the culture technique (Jongejan *et al;* 1993c) and consequently an attempt to replace the present vaccine, which uses in vivo derived antigens with live tissue cultured materials. Presently no laboratory in Nigeria is involved in vitro cultivation of the organism. Consequently all the studies were based on in in vivo experiments using domestic ruminants.

2.5.5 Growth and Life Cycle of *Cowdria* in cultures

Most of the information on the growth stages of *Cowdria* are mainly based on the observations and studies in cultures using the light and electron microscopes. The endothelial cells (EC) take up infected materials injected into cultures. The organisms divide by binary fission in these cells forming the reticulate colonies. These develop into smaller intermediate bodies which have electron dense core after 3 to 4 days. The intermediate bodies condensed further into electron dense elementary bodies which rupture from the cells and are released into culture medium as initial bodies to initiate a new infectious cycle lasting between 4 - 6 days (Jongejan *et al;* 1993c). Colonies can be detected as early as 2 days after inoculation (Bryom and Yunker, 1990). Cytopathic effect (CPE) usually observed from day 5 after infection is more noticeable when cultures are kept in constant movement on a rocker or in roller bottles. CPE is seen as streaks in the monolayer where affected cells are almost detaching (Bryom *et al;* 1991). The reticulate bodies were observed to be less infective than the elementary bodies (Jongejan *et al;* 1991b). Similar pattern of life cycle was noticed by Hart *et al.* (1992).

Bezuidenhout (1993) noticed that the hazard, of *Cowdria* cultivation included sporadic contamination of cultures and poor

performance. The slow rate of cell division especially the bovine endothelial cells are also part of problem areas. The ovine endothelial cell line SBE 189 supports growth well and may possibly replace the bovine umbilical endothelial cell (BUE) as culture medium. Yunker *et al.*(1988) also observed that poorly rinsed glasswares could create problems in growing *C. ruminantium* in vitro. He also stated that since the nutritional requirements and metabolic processes of *C. ruminantium* are poorly known, researchers are therefore never specific when culture media are selected. Consequently culture failures usually result in some cases. From the foregoing, initiation of in vitro culture for *C. ruminantium* using endothelial cell line requires a standard well equipped laboratory which most faboratories in developing countries can not afford.

2.5.6 **Staining Properties**

Brain is the most convenient and reliable site for the diagnosis of heartwater in infected animals (Purchase, 1945). In addition, histological sections of tissues such as the lungs, heartmuscles, bronchial, prescapular and mesenteric lymph nodes, kidneys, spleen, adrenal glands, abomasum, choroid plexus, small intestine, and spinal cord could also be examined for the parasite but with difficulty (Ilemobade 1976) especiallty in their vascular endothelia. In these sections, Ilemobade (1976) observed that

Cowdria organisms stained light purplish blue with Haematoxylin and Eosin (H&E) and were located in the cytoplasm of the endothelial cells. The colonies were surrounded by chromophobic halo (Cowdry, 1926a; Pienaar, 1970 and Du Plessis, 1975). Other stains that have been successfully tried included Diff Quick (Awa, 1991) and Toluidine blue (Prozesky, 1987a).

C. ruminantium stains reddish purple to blue with these stains. The Romanowsky stains particularly the Giemsa stains have been used and the colour observed is usually influenced by the type of fixation used (Alexander, 1931).

The small forms of *C. ruminantium* appeared reddish purple and the large forms stains bluish in Giemsa stained brain squash smears (Uilenberg, 1983). He also reported that prolonged (15 minutes) washing of the stained smears in tapwater may improve the staining qualities.

In the tick vector, Yunker *et al.* (1987) used Mallory's Phyloxine methylene blue stain to differentiate colonies of *C. ruminantium* in *A. variegatum* gut epithelial cells in paraffin sections of tissues. *Cowdria* colonies were effectively differentiated from the tick cell nuclei and other

structures because of the bi-chromatic rendering of tick midgut by the stain.

Bezuidenhout (1984) and Kocan *et al.* (1987) used epoxy-embedded sections, varied the Mallory's stain which produced a monochromatic effect rendering colonies, tissue component and haemoglobin in various shades of blue. However, electron microscope was required to differentiate the parasite from pigmented granules and tick tissue inclusions. The use of Giemsa methyl green pyronin Y- and acridine orange stains in frozen tick sections gave unsatisfactory results (Bezuidenhout, 1984).

2.5.7 Localisation of *C. ruminantium* in blood and ticks

Several controversial results had been produced as to the portion of the blood in which the organism is usually located. Knowing the location is necessary for the understanding of the pathogenicity and cultivation of the parasite. Early reports claimed an even distribution and attachment of *C. ruminantium* to both white and red blood cells (Alexander, 1931) or lying free in plasma (Jackson and Neitz, 1932). They argued that since the organisms did not pass through Seitz filters, they could be seen using the light microscope. Fawi *et al.* (1977)

reported that the organisms were located only in the red blood cells. Contrary to this, Hemobade and Blotkamp (1978a) found that the red blood cell component of an infected blood separated in Ficoll-hypaque gradient failed to induce heartwater whereas the leucocyte fraction and to a larger extent the plasma induced heartwater in susceptible animals. Serum of an infected animals was not infective when intravenously inoculated. No organism could also be detected in stained blood smears (Cowdry 1926; Alexander, 1931; Hemobade, 1976). Cowdry (1926a) suggested that *Cowdria* probably occurred singly in blood smears and not as large masses making them difficult to detect in blood smears. Apart from the vascular endothelium and the circulating blood, some workers reported the occurrence of *Cowdria* in various other cells such as macrophages, monocytes, histiocytes, Kupfer cells, reticula cells of lymphnodes fibroblasts and bronchial cells (Du Plessis, 1970a, Hemobade, 1976). However, Uilenberg (1983) observed with certainty that *Cowdria* is located and multiplied in vascular endothelial cells and circulating blood as most of these reports were not convincing. *C. ruminantium* is located in tick epithelial cells and gut lumen as densely packed group. As no other organs were found to contain the organism, Cowdry (1926b) therefore speculated that infection probably occurred by regurgitation, an assumption supported by Uilenberg (1983). It has however been

confirmed that the organism can be found in salivary gland cells, haemocytes and malphigian tubules of infected ticks (Kocan *et al;* 1987; Du Plessis, 1984). Infection of gut and salivary glands was confirmed immunologically using ELISA (Viljoen et al, 1988) and DNA probe (Yunker *et al;* 1993).

In the tick vector, two forms of *C. ruminantium* have been confirmed. These are reticulated and dense forms (Kocan *et al*; 1987; Hart *et al;* 1991). The simple granular cells of Type II and Type III salivary gland acini are usually infected and not the Type I acini or complex granular cells of the Type II and II acini. This feature is also shared by *Anaplasma morginale*, *Theileria* and *Babesia* (Kocan, 1995). However *A. marginale* is the only haemoparasite of the four organisms that has been known to infect and develop in tick gut muscle cells (Kocan *et al;* 1995).

2.5.8 **Preservation** of *C. ruminantium*

Different methods have been employed to preserve the organism. Addition to and modifications of previous methods are being progressively made as the understanding of the parasite is increasingly gained. Initially the organism was thought to be fragile that it could hardly survive for

over 24 hours at room temperature because Alexander (1931) could only preserve it in blood for 38 hours at room temperature and at 4°C. This was later improved upon when the organism was preserved for 4 days at *room temperature* (Henning, 1956). Weiss *et al.* (1952) first stored the organism at low temperature using 10% phosphate buffered spleen homogenate at -74°C. The organism has also been transported in infected blood frozen on dry ice at -45°C over a long distance (Haig, 1955). Neitz (1968) preserved the organism in infected spleen and blood homogenate maintained at -76°C for 2 years. On the contrary however there were reports of futile attempts at preserving it at -32°C and -70°C (Rahim and Shommein, 1978). The use of Dimethyl sulphide (DMSO) and snap freezing as preservation technique was initially reported by Ramise and Uilenberg (1970). *C. ruminantium* was successfully preserved using this method at -75°C and -180°C for about 13 months using 10% DMSO as cryopreservant. Slow freezing of blood using glycerol as crypopreservant failed to preserve the organism so also rapid freezing without crypopreservant, slow freezing with 7.5% DMSO and rapid freezing with 20% DMSO. On the contrary Ilemobade *et al.* (1975) preserved the organism at -85°C and -196°C with and without using cryopreservant. He preserved the organism in whole blood at varying temperature range of 4°C and 35°C for 27 hours. The organism was

reportedly preservable for an indefinite period of time at -85°C and -196°C and could even undergo extra freeze thaw cycle. Ilemobade *et al.* (1975) also observed that since the organism survived rapid and not slow freezing, the use of a cryopreservant was not critical. He also succeeded in maintaining the organism in nymphs for 132 days and for 459 days in adult *A. variegatum*. Ilemobade *et al.* (1975) preserved the organism in brain homogenate in phosphate buffered saline (PBS) frozen with or without DMSO. Infected tick homogenate in buffered lactose phosphate and 5% DMSO snap frozen in liquid nitrogen has been employed to preserve the organism (Bezuidenhout, 1981).

Logan (1987) reported that fresh blood collected from febrile goats infected with the Kwanyanga stock and maintained for 72 hours at 4°C was infectious to mice so also were the neutrophils from the goats separated and maintained in modified RPMI medium at 37°C 68 hours and also 96 hours.

2.6 Transmission

2.6.1 Natural transmission

Heartwater is transmited naturally by a three host tick of the Genus *Amblyomma*. Lounsbury (1904) first proved that the bont tick *A.*

hebraeum was the vector of the disease. Of all the natural vectors *A. hebraeum* and *A. variegatum* exhibit complete transtadial transmission from larval through nymphal to adult stage (Bezuidenhout, 1987). Cowdry (1926b) reported that not all *Amblyomma* species transmit the disease as he did not detect the organism in gut smears of many *Amblyomma* and non *Amblyomma* tick.

The importance of proven vectors vary and this depends on the adaptation to domestic animals, the extent of distribution and its efficiency as vectors (Uilenberg 1983). Viewed in this context *A. variegatum* is the most important followed by *A. hebraeum* (Bezuidenhout, 1987). Adaptation of *C. ruminantium* strain to its local vectors does not seem to exist as strain of parasite can easily be transmitted by the *Amblyomma* species from a widely separated geographical areas (Uilenberg, 1983). Mahan *et al.* (1994) however, reported that *A. variegatum* was more susceptible to infection with Gardel (Guadeloupe) and Nigerian stock than with crystal spring (Zimbabwe) and South African Ball 3 stock whereas *A. hebraeum* was highly susceptible to all of them. *Cowdria ruminantium* usually retains its ability to be transmitted by its vectors after several mechanical passages and thus differ in this aspect from some parasites such as *Babesia* and *Trypanosomes*.

The organism is usually transmitted transtadially except for a report of transovarian transmission (Bezuidenhout and Jacobsz, 1986). Some vectors do not possess the ability for a complete transtadial transmission (Bezuidenhout, 1987). Lounsbury (1902) failed to transmit the disease intrastadially. Male ticks are also capable of transmitting the disease intranstadially by transfer of male ticks from infected to susceptible hosts (Kocan *et al;* 1993b).

Infection is acquired by ticks while feeding on a reacting animals (Lounsbury, 1902). Infection can also be acquired when ticks feed on subclinically infected animals or animals that have recovered from clinical attach (Barre *et al;* 1987) or perhaps immune animals after reinfection (Ilemobade and Leeflang, 1978). There is a marked increase in the infectivity of infected ticks during feeding but it decreases before and during moulting (Bezuidenhout, 1987). Homogenates from larvae during and after feeding on reacting sheep failed to initiate infection on intravenous inoculation (Du Plessis, 1982). Their homogenates after moulting to nymphae were only infective on high concentration of 5 nymphae. It has been shown that certain period of time is required for *C. ruminantium* to infect tick tissues and to be detectable. The initial tissues infected are the midgut epithelial cells *C. ruminantium* has been detected

in gut epithelial cells with electron microscope only after 15 days that *A. variegatum* had fed on susceptible animals (Kocan *et al;* 1986). Cowdry (1926b) however observed the organism in unfed *A. hebraeum* nymphae 40 days after being infected as larvae.

Hitherto it was thought transmission of the parasite to another host by ticks was by regurgitation or by exretion from the alimentary canal because the organism were only seen in gut epithelium and lumen (Cowdry, 1926b). However, *Cowdria* colonies have been demonstrated in salivary gland acini of *A. hebraeum* nymphae (Kocan *et al;* 1987). This suggests that infection probably takes place through the saliva after the organism have developed and multiplied in salivary glands (Kocan, 1995).

Neitz (1968) reported that the organisms are liberated within 24 hours after ticks have attached. Some authors also observed that it was within 38 hours for nymphae and 75 hours for the adult (Bezuidenhout and Oliver, 1987). Camus and Barre (1992) reported that *C. ruminantium* is transmitted from ticks to susceptible animals after a delay of 2 - 3 days of feeding by nymphae and 4 days by adults of *A. variegatum* in Guadeloupe. They also observed that the disease is more fatal when

transmitted by ticks than by needle. Infection and transmission rates are higher in nymphs than adult ticks (Gueye *et al;* 1993).

2.6.2 Artificial transmission

Heartwater was first transmitted artificially using infected blood (Dixon, 1898). Various infected tissues and even milk from reacting animals have been found infective. The intravenous route (iv) is more reliable than any other route. The size of inoculum is also important although 5 ml is usually used for small ruminants and 10 ml for cattle (Uilenberg, 1983).

Subcutaneous inoculation of brain homogenate has been used to transmit heartwater (Ilemobade and Blotkamp, 1978a). The route was however less reliable than intravwenous injection and the use of tick materials.

There were also futile attempts to use the intradermal and intravenous routes (Alexander, 1931). The intravenous route is also used to administer vaccine to control the disease making infection and treatment of vaccination cumbersome.

2.6.3 Vertical Transmission

Deem *et al.* (1996) demonstrated that the vertical transmission of heartwater does occur possibly through the colostrum. This assumption was based on the finding that the disease could be initiated in a goat by inoculating intravenously, colostral cells collected from dams resident in a heartwater endemic areas. This could be an advantage in endemic areas because animals could be exposed early in life and consequently develop immunity before the susceptible age.

2.6.4 Epidemiological factors of Heartwater in Nigeria

It is known that the incidence and severity of heartwater is commonly related to the presence and the prevalence of the vectors and in most cases their distribution and the density of some *Amblyomma* species. Epidemiology of the disease is affected by several factors which include variation in the *Cowdria* species *Amblyomma* species, response of the host and animal husbandry.

Presently only one species of *Cowdria* is known. However domestic, laboratory and ferral hosts differ in their susceptibility to the organism. Isolates in Nigeria such as D225 (Ilemobade, 1976) and Jaji (Anon, 1989) respond to common oxytetracycline chemotherapy and are

pathogenic to exotic breeds (Isoun *et al;* 1974) indigenous savanna goats and zebu cattle (Ilemobade, 1976). Okaiyeto (1995) experimented on a new uncharacterized isolate (Basawa stock) and noticed that it was less severe in the West Africa dwarf goats (Okeiyeto 1995). Also the susceptibility of Yankasa sheep and goats from areas South of Zaria (Ilemobade, 1976; El-imairi, 1991) vary. Immunity and cross immunity studies showed that solid immunity is conferred following homologous and heterologous challenges (Anon, 1989; Audu *et al;* 1995). Acute form of the disease are the most predominant in both natural and experimental infections (Ilemobade, 1976; Lawal *et al;* 1992). However, peracute form has been observed in some experimental infections in Savanna goats (Ulayi *et al;* 1992). Variation in the vector is also an important factor in the epidemiology of heartwater disease in Nigeria. It has been reported that *Amblyomma variegatum* is the most widespread tick in Nigeria (Anon, 1989). So far, only two species *A. variegatum* and *A. splendidum* is not considered important in the epidemiology of heartwater in Nigeria because of its restricted distribution (Ilemobade, 1976) have been identified.

The response of the host animal to *Cowdria ruminantium* infection also varies. This depends upon the species, breed, strain of the parasite,

sex, and age. Research findings in Nigeria showed that goats are more susceptible to heartwater than other species while the Savanna goats are more susceptible to the disease than the West Africa dwarf (Okaiyeto, 1995). Age resistance has also been noticed.

Kids exhibit some resistance (Audu *et al;* 1995) while the adults are highly susceptible (Lawal *et al;* 1992). Variation in sex related susceptibility has not been reported.

Animal husbandry system has also been reported to influence the incidence of heartwater (Ilemobade, 1976). Poor tick control and poor nutrition leading to stress are likely to contribute significantly to the incidence of the disease in Nigeria, although there is little or no report as regards this factor.

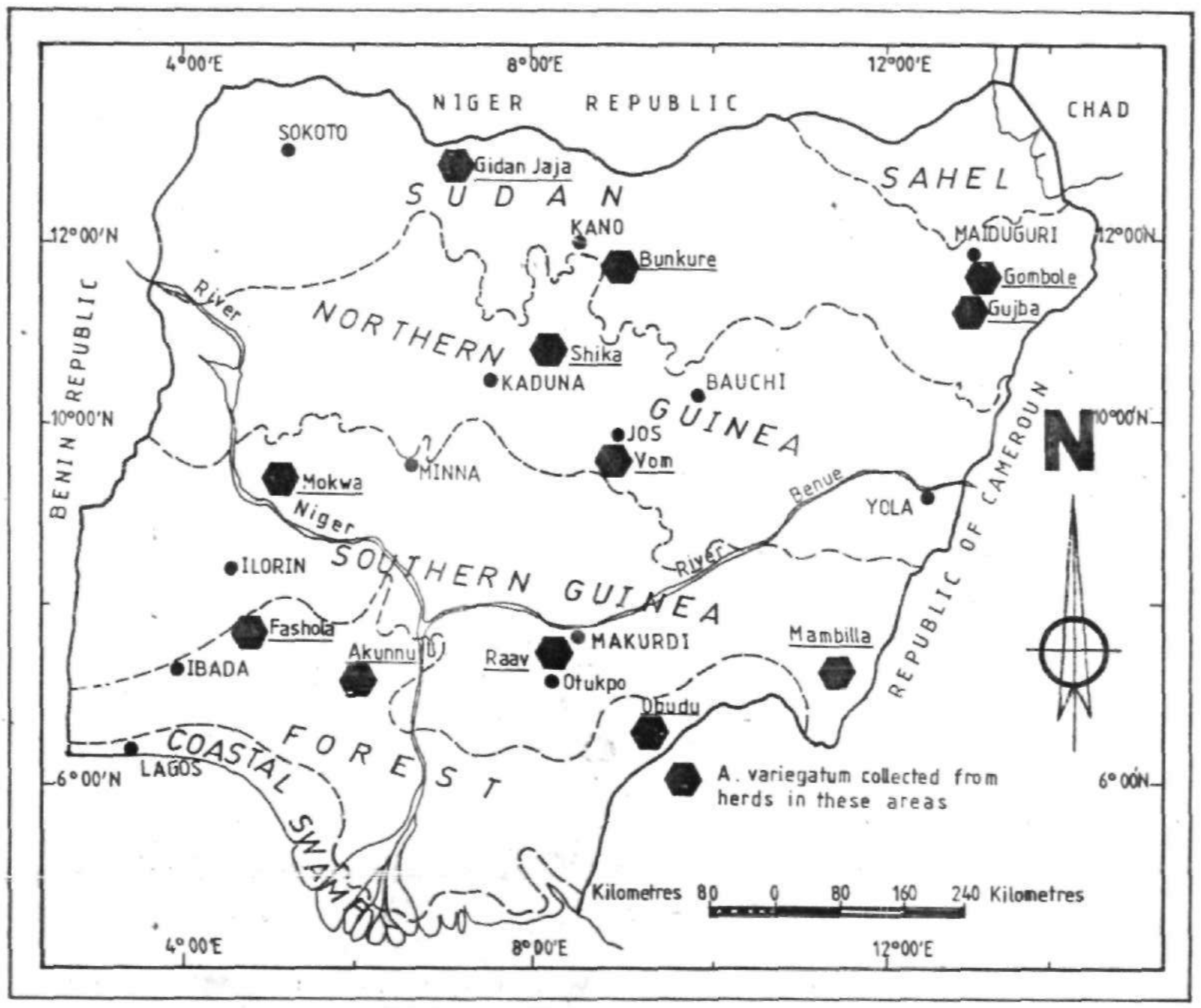


Fig. 4. Map of Nigeria showing the distribution of Amblyomma variegatum (Extracted from Anon, 1989).

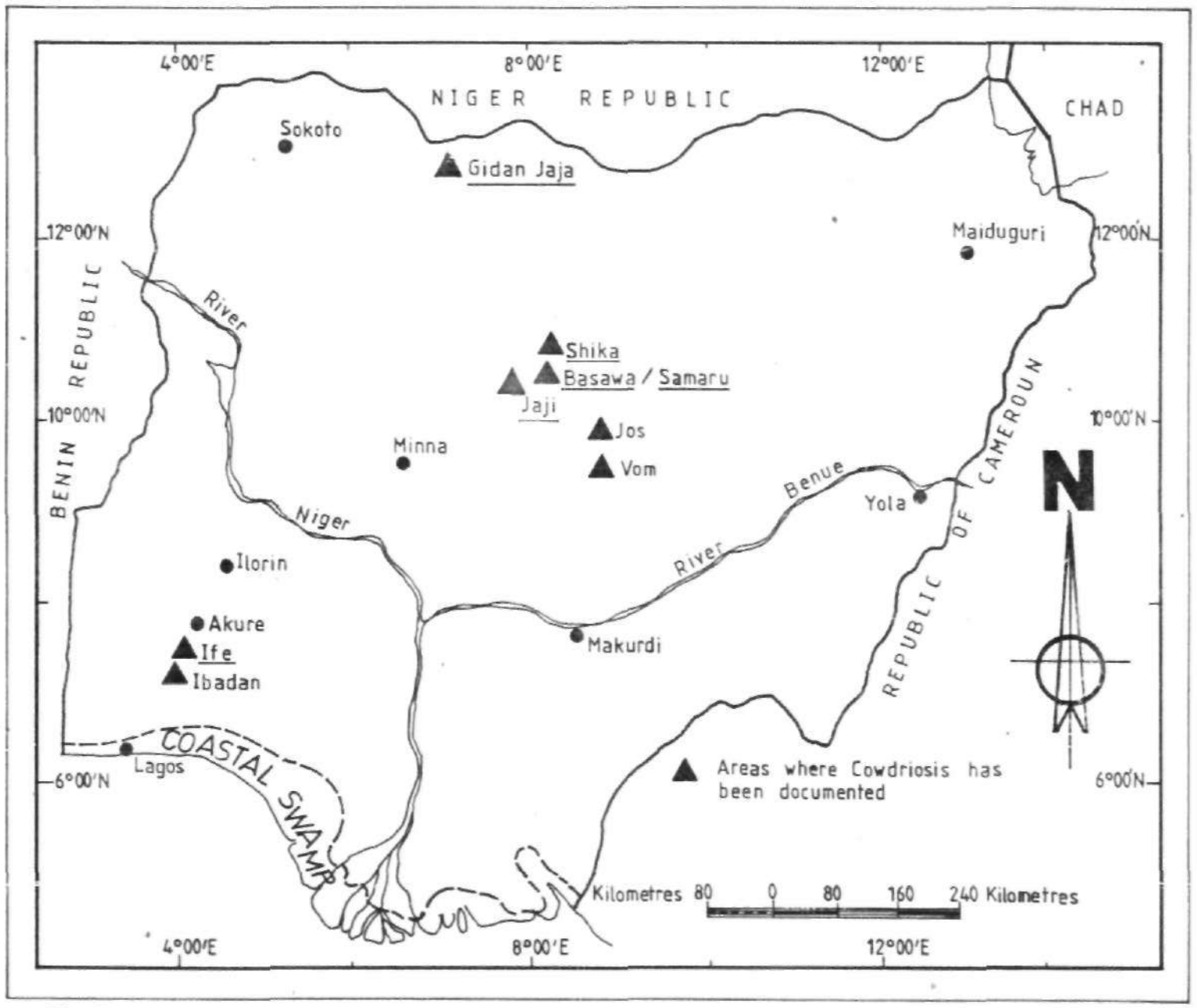


Fig. 5. Map of Nigeria showing places where Cowdriosis has been documented. Localities where isolates were made are underlined.

2.6.5 **Clinical Signs, Pathogenesis and Pathology of Heartwater**

2.6.5.1 **Clinical signs in ruminants**

Incubation period varies according to species and breeds of animals, the mode of transmission and size of inoculum. When transmitted by ticks, it varies from 1 to 5 weeks with an average of 9 to 10 days in small ruminants and an average of 12 days in cattle when it is *artificially transmitted through the intravenous route* (Uilenberg, 1983; Hemobade and Blotkamp, 1978b). However, incubation period as low as 3 days was reported after artificial infection (Gueye and Vassilides, 1985).

The clinical signs of heartwater in ruminants have been extensively reviewed by Alexander (1931), Henning (1956), Hemobade (1976), Uilenberg (1983) and Losos (1986). The information presented here is extracted mainly from the work of these authors. The intensity of clinical signs depends on the susceptibility of the animals, breeds and virulence of the organism. Four forms of heartwater have been recognized. These are peracute, acute, subacute or chronic and mild or abortive forms.

Peracute form

This form usually results in the death of the affected animals without any warning sign. This form is usually observed in exotic breeds

(Isoun *et al;* 1974). Peracute forms have also been noticed in indigenous brown Savanna goats experimentally infected with a Shika isolate of the parasite (Ulayi *et al;* 1992), in local calf (Bida and Adam, 1973) and also in West African dwarf goats (Okaiyeto, 1995). Animal often collapse suddenly with convulsion and die. Signs often last for few hours and usually begin with fever of 40°C or more.

Acute form

This is the commonest form of the disease in most susceptible animals. A sudden rise in temperature is usually the initial sign and usually reaching 41°C or more within the first or second day of fever and may drop to subnormal level at the terminal stage. Appetite is usually lost, rumination ceases, the mucous membrane becomes cyanotic followed by respiratory signs such as dyspnoea and moist rales on percussion of the thorax. The nervous signs which include rapid blinking of eyes, high stepping and unsteady gait with legs stretched apart for balance and leaning on the wall for support set in within a few days. The tongue is often protruded rapidly, and there is hypersentivity to touch. The animal may become vicious especially in cattle (Uilenberg, 1983) and charges at any object. The disease usually progresses rapidly when nervous signs set in, the animal can hardly recover at that stage and often several fits and

convulsions are observed. At this stage the animal usually goes on lateral recumbency, legs are stretched out and exhibit some pedaling movements of the legs, jewing movement of the jaws, nystagmus, opisthotonos and frothy from salivation from the mouth (Hemobade, 1977). Pregnant animals usually abort dead fetus (Okaiyeto, 1995). Recovery is usually rare but when it occurs it is usually prolonged or protracted (Uilenberg, 1983).

Chronic or Subacute form

The signs are similar to the acute form but milder and characterised by diarrhoea. It takes a longer course and could be upto 10 days (Hemobade,1977). Then the affected animals may just collapse and die from some other causes such as congestion of lungs causing pneumonia and rumen atony. Convalescence is usually slow when it occurs.

Mild or abortive or inapparent form:

The signs are so mild that the disease is seldom noticed under field conditions and the affected animal usually recovers. Animals with high natural resistance (lambs, kids, Persian sheep, Africander sheep, native cattle sheep and goats) in endemic areas and partially immune animals

usually contract this form of heartwater. The thermal response is mild lasting for only 2 - 3 days and infected animals usually act as reservoirs of infection for ticks.

Mortality and Morbidity rates:

In Angora goats a 90% mortality has been recorded (Uilenberg, 1983). In Nigeria 100% mortality have been recorded in the indigenous brown savanna goats (Hemobade, 1977) and 50% in experimentally infected West Africa dwarf goats (Okaiyeto, 1995). However, these two main breeds of Nigerian goats have shown a 100% morbidity (Hemobade, 1977; Okaiyeto, 1995). Uilenberg (1983) noticed that the mortality rate depended on the virulence of the strain of the organism and the morbidity rate which is also affected by tick control (Matheron *et al;* 1987), immunization and treatment. The age of the animals also affects mortality (Haig, 1955). The findings of Du Plessis, (1985) that animals with conglutinin (K) in the serum resist heartwater infection may also affect morbidity and mortality. He reported that animals with high concentration of conglutinin K were protected against fatal infection. The suckling - calf resistance which decreases as calves become older differs from K - associated resistance which in calves increases with advancing age thus one becomes stronger as the other wanes (Du Plessis, 1985). He also

observed that the levels of conglutinin were almost not detectable in new born calves and increased slightly during the few months of life.

2.6.5.2 **Clinical signs of heartwater in laboratory animals**

The clinical signs of heartwater described in mice are mainly in infected outbred Swiss albino mice experimentally infected with the Kwanyanga, Nonile and Welgevonden stocks. The signs are quite different from those observed in ruminants. An incubation period ranging from 9 - 14 days is usually observed and signs are most evident about 24 hours to death (Prozesky and Du Plessis, 1985). These include increase respiration, piloerection and lethargy. Incoordination may be seen occasionally in moribound mice (Mackenzie and Van Rooyen, 1981). Other signs such as lethargy, anorexia and ruffled coats may be observed (Prozesky and Du Plessis, 1985). Hypothemia within 12 - 72 hours to death is often a constant feature with the infected mice being cold to touch. A fall in the body temperature by as much as 10°C prior to death was recorded in mice infected with the Kwanyanga stock (McKenzie and Van Rooyen, 1981). There is always a complete absence of pyrexia in the infected mice.

2.6.5.3 **Gross Pathology in Ruminants**

The gross pathology of ruminants that died of heartwater was extensive discussed by Clark (1962); Hemobade and Blotkamp (1978b); Uilenberg (1983) and Prozesky and Du Plessis (1985). The external appearance of the carcass usually appears good but ticks may be found on the body (Hemobade, 1976). The major macroscopic lesions as described by Hemobade and Blotkamp (1978b); Uilenberg (1983) and Prozesky and Du Plessis (1985) are hydrothorax, lung edema characterised by dilatation of interlobular septa and presence of froth in the trachea and bronchi. Atelectasis of the ventral lung borders, hydropericardium, petechial haemorragges of endocardium, hyperaemia of the abomasum, enteritis and swelling of lymphnodes. Haemorrhages in central nervous system, vagina and urinary bladder have been reported (Camus and Barre, 1982; Prozesky, 1987). Splenomegaly and distension of bladder are also reported. Hydropericardium is more common in sheep and goats than cattle. There are usually no macroscopic brain lesion apart from congestion of meningeal blood vessel Uilenberg, 1983). In acute cases, the most striking feature is lung edema.

2.6.5.4 **Histopathology**

Several histopathological changes may be observed. These include marked alveolar congestion and edema, myocardial haemorrhages and

edema, dilation of portal vein, congestion of central, portal and sinusoidal vessels (Ilemobade, 1976). Spleen and lymphnodes are also congested with hyperplastic reticular cells. Renal vessels and glomeruli are congested. In the brain, there is leucostasis, striking proliferation of glial cells, congestion of chroid plexus and cerebellum.

2.6.5.5 **Haemogram of Heartwater**

There are various areas of disagreement as regards haemograms of heartwater, especially on the level of hemoglobin, haematocrit, total and differential white blood counts and eosinophil level as explained below:

Haemoglobin Level:

The level of haemoglobin usually decrease (Ilemobade and Blotkamp 1978; Owen *et al;* 1973; Rahim and Shommein 1978; Van Amstel *et al;* 1987a; and Okaiyeto, 1995). The decrease may occur just after the onset of pyrexia (Van Amstel *et al;* 1985b) or during pyrexia (Okaiyeto, 1995). The claim by Clark (1962) that there is terminal rise in haemoglobin was not confirmed by Ilemobade and Blotkamp (1976).

Haematocrit

A drop in haematocrit has been reported by several authors Hemobade and Blotkamp 1978b; Owen *et al;* 1973; Rahim and Shommein 1978; Van Amstel *et al;* 1994 and Okaiyeto, 1995).

Rahim and Shommein (1978) reported that the mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were significantly lowered in the course of heartwater and suggested that there was development of microcytic hypochromic anaemia. Van Amstel *et al.* (1985b) however reported that anaemia was more of nomocytic nomochromic type as no change in MCHC and MCV was observed. They suggested that such anaemia could result from acute blood loss, acute hemolysis, sequestration of red blood cells in spleen, haemodilution or bone marrow depression. Prozesky (1987) noticed that sequestration of red blood cells in the spleen could partly account for the drop in haemoglobin, haematocrit and red cell count. It has been confirmed that there is usually no blood loss.

Total and Differential White blood cell (WBC) count

Differing observations have been expressed as regards the Total and Differential (WBC) count.

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While Hemobade and Blotkamp (1978b) reported leucocytosis due to neutrophilia and lymphocytosis. The occurrence of neutrophilia was supported by Camus and Barre (1982). However, there is general agreement that the monocyte count remains the same (Van Amstel *et al;* 1987a). A terminal rise in the total leucocyte count has been reported (Van Amstel *et al;* 1994). Van Amstel *et al.* (1985) observed that neutropenia, lymphocytosis and eosinopenia were consistent changes in heartwater haemogram. He suggested that is could be due to bone marrow depression since they are produced there or due to elevation of cortisol but there is no lymphopenia which is also associated with this steroid.

Eosinophil level

The circulating oesinophils usually disappear either before the onset of fever (Clark, 1962) or during febrile reactions (Owen *et al;* 1973; Rahim and Shommein, 1978). The drop and probably the disappearance of the oesinophils has been attributed to stress (Hemobade, and Blotkamp 1978b).

2.6.6 Chemical Pathology in heartwater

Blood glucose:

A terminal rise in blood glucose was reported (Clark, 1962; Hemobade and Blotkamp, 1978b).

Blood proteins

Clark (1962) reported no change in serum protein. However a drop in gamma globulin and a rise in alphaglobulin have been reported (Hamobade and Blotkamp, 1978). A drop in gammaglobulin is generally a non specific response to injury observed in many conditions (Schalm *et al;* 1975).

A decline in albumin fraction has been reported (Van Amstel *et al;* 1987a). This is because of being smaller than globulin it could pass through the endothelium with ease.

Lactate and Pyruvate

There are increase in lactate and pyruvate. The rise in pyruvate was associated with the respiratory and cardiac insufficiency leading to less availability of oxygen to oxidise it (Hemobade and Blotkamp, 1978b). There is metabolic acidosis due to drop in blood pH and subsequent accumulation of lactic and pyruvate acids. The decrease in plasma

volume prior to death is due to a sudden increase in capillary permeability allowing escape of plasma proteins from vascular system (Clark 1962). The transudates in heartwater frequently show coagulation, indicating that proteins as large as fibrinogen can pass through the capillary wall.

Plasma colour

A darkening in colour (orange colour) of the plasma of heartwater infected Angora goat has been report (Gruss, 1981). The observation which was confirmed by Metelerkemp *et al.* (1986) was associated with elevations of total birilubin levels.

Blood urea nitrogen (BUN):

This usually rises while creatine remains normal (Clark 1962).

Owen *et al.* (1973) observed that the blood pressure usually changes in heartwater and this could explain the noticeable changes in blood urea nitrogen.

Serum electrolytes: Owen *et al.* (1973) reported a slight increase in the chloride and sodium levels while Clark (1962) observed no change. The blood potassium level shows a terminal drop because the level of effusion fluid is usually more than those in the plasma. This makes the

osmolarity in the effuse fluid greater than that of the plasma (Van Amstel *et al;* 1987).

A terminal hypocalcaemia has been reported while magnesium and inorganic phosphate remain constant (Owen *et al;* 1973). Van Amstel *et al.* (1994) noticed a progressive decline in total calcium while the ionized calcium also reported that thrombocyte count decline markedly but both prothrombin time (PT) and activated partial thromboplastin (APTT) increased including fibrinogen although there was no detectable increase in fibrinogen degradation products (FDP).

2.6.6.1 The central nervous system during heartwater

The electroencephalographic (EEG) changes showed a high voltage theta or delta rhythm without any discrete features and thus were non specific (Van Amstel, 1987). He associated these changes with brain edema and congestion. The changes are similar to those commonly observed in most central nervous disease (Klemm, 1969).

2.6.7 Pathology of heartwater in mice

The gross pathology of heartwater in mice varies greatly from that of ruminants. Brown and Skowronek (1990) noticed that the predominant

gross pathology were pulmonary edema, hydrothorax hydropericardium and splenomegaly. The lungs were congested, wet and failed to collapse normally. Microscopically, alveolar edema were the main findings. Prozesky and Du Plessis (1985) in addition to the above features, observed some small foci of emphysema, surrounded by areas of atelectasis distributed throughout the lungs of some mice.

In spite of the differences in the pathology of infected mice and ruminants, McHardy and Mackenzie (1987) showed that laboratory animals could provide models for screening chemotherapeutic agents. The results obtained using mouse model were comparable to those obtained in infected ruminants and in the fields.

The main shortcoming of using mice is the determinant factors such as time of death and lack of pyrexia which are rather poor. The organisms are usually difficult to observe in tissues and are hardly observed in the brain thus limiting its usefulness.

2.6.8 **Pathogenesis of heartwater**

There is no conclusive agreement by scientists as regards the pathogenesis of heartwater. The parasites are taken up by the free

mononuclear phagocytes of blood when the vertebrate hosts are infected either through ticks or by artificial inoculation of infected materials. The mononuclear phagocytes of either blood vessel lining or the lymph sinuses could also ingest the parasites. The parasites are transported to the regional lymphnodes where multiplication "primary replication" probably occurs (Losos, 1986). The parasites are released into blood circulation through the efferent vessels (Ilemobade, 1976). Parasitization of vasicular endothelium and other organs occur where another round of multiplication "secondary replication" occurs (Du Plessis, 1975). It was presumed that the endothelial cells are injured during this process and become permeable allowing plasma proteins to pass into perivasicular spaces. There is consequently an increase in osmotic pressure outside the vessels causing water to pass from plasma into tissues as edema and intocavities as hydropericardium, hydroperitoneum and hydrothorax. Reduction in plasma which could diminish by 70% in severe cases occur causing death from shock. The morphology of the parasite in the primary replication sites may differ from those of the secondary replication sites (Ilemobade, 1976).

Uilenberg (1983) noted however that the pathogenesis of heartwater is an area of controversy because the lesions observed, do not correlate

with the presence of the parasites. He stated that the parasitized endothelial cells rarely show morphological changes except that in some few occasions, growing colonies may push them aside or be flattened. Hydropericardium was associated with cardiac insufficiency and lung edema while death was thought to be due to asphyxiation (Uilenberg, 1983; Owen *et al;* 1973) rather than shock from decrease blood volume. Toxin (Neitz, 1968) rather than injury to endothelial cells was claimed to be responsible for increase capillary permeability. Also the nervous signs were also largely attributed to toxin rather than brain lesions. Uilenberg (1983) and Ilemobade (1976) also favoured the suggestion of toxin involvement in the pathogenesis of heartwater. The intense muscular activity was also suggested to be linked to toxin (Uilenberg, 1983). The increase activity in turn leads to the increased levels of blood lactate, pyruvate and glucose and a drop in blood pH. The rise in pyruvate was explained to be due to respiratory and cardiac insufficiency resulting in less availability of oxygen for immediate oxidation of pyruvate (Ilemobade, 1976). The drop in pH causes metabolic acidosis (Tasker, 1971), lactic and pyruvic acid accumulation.

Lymphopenia and subsequent decrease in gamma globulin result in suppression of humoral response. This tends to increase the severity of

the disease (Ilemobade 1976). The involvement of endotoxin and inflammatory products have also been suggested to explain the severity of the effusions found in the lungs and cavities which are less reflected in the histological and ultrastructural morphology of the infected cells (Camus and Barre, 1982; Du Plessis, 1985). Compliment and compliment binding proteins, conglutinin which are unique to ruminants were also speculated to partake in the pathogenesis (Du Plessis 1985; Du Plessis *et al;* 1987). Van Amstel *et al.* (1987b) demonstrated the presence of endotoxin in the blood of infected sheep. However, Van Amstel *et al.* (1994) reported that endotoxin was not too significant in the pathogenesis because the elevation of the endotoxin was observed to be of short duration and decrease in 24 hours follow-up samples. In the same experiment, the levels of leukotrienes (B_4, C_4 and D_4) in the blood or thoracic or pericardial fluids were not elevated. Leukotrienes are potent inflammatory products generated via the arachidonic acid from phospholipid membrane in response to cellular injury and causing effects like decrease in cardiac functions and decrease in vasicular permeability with plasma water leakage and edema (Bottoms and Adams, 1992). The non elevation of leukotrienes levels therefore questions their role in the pathogenesis. More studies are therefore required to resolve the role of endotoxin in heartwater.

Brown and Skowronek (1990) using immunochemical studies to support this arguement, suggested that the pathologic effects in the lungs and myocardium might be mediated from a distant site. This was because they found no organisms nor was there any immunochemical staining in these organs. He also disagreed with Prozesky and Du Plessis (1985) that the lung edema might be caused by gram negative endotoxemia because the histologic evidence of fibrin associated with endotoxaemia was lacking. Toxins, head trauma and rapid increase in cerebro spinal fluid (CSF) pressure were suggested as possible causes (Brown, 1990). It was noted that the multiplication of the parasites in cerebral endothelium could create enough local disturbance to increase CSF pressure and could cause neurologic myocardial degeneration and pulmonary edemia.

Totte *et al.* (1994) reported that some cytokines especially the interferons could play some "adjuvant" roles in the pathogenesis of heartwater by allowing the animal to resist the disease. They showed that alpha interferone (αIFM) possessed some limited anti *cowdria* activity in vivo, allowing infection to occur to induce a protective immune response. They also reported that animals that resist experimental infection produced a high level of circulating IFN - whereas animals that died did not. Daily injection of gamma interferone (IFN-y) for 2 weeks has been shown to

protect mice against *Cowdriosis* (Totte *et al;* 1994), but injection at lesser period only delayed the infection. Other cytokines such as interleukin-2 ($IL\text{-}^2$), interleukine -6($IL\text{-}^6$) and tumor necrosic factor (TNF) do not play a role in the pathogenesis of heartwater (Totte *et al;* 1994).

Mahan (1993) also reported that concanavalin agglutinin (Con A) stimulated bovine T-cell supernant but did not affect entry of *Cowdria* into cells. He suggested that it could be one of the mechanisms by which T-cells control the multiplication of the organisms and consequently the severity of the disease.

Totte *et al.* (1994) found that Human recombinant myeloperoxidase (rec. MPO) used in vitro at a concentration (18 units/mouse) protected mice against heartwater (Kumm stock).

2.7 Diagnosis of Heartwater in ruminants and mice

2.7.1 Antemortem diagnosis

The diagnosis of heartwater in five animals can either be tentative based on the clinical and epidemiological observation or confirmatory based on the brain biopsy (Synge, 1978) which is mainly used experimentally. Confirmation can also be done by subinoculation of blood

into susceptible animals (Hemobade and Blotkamp, 1978a) or subinoculation of homogenate of ticks which have fed on infected animals (Bezuidenhout, 1981).

Several haematological changes can also be used in tentative diagnosis of heartwater in live animals. These include considerable decrease in circulating eosinophils (Clark, 1962) leucopenia (Hemobade, 1976) neutrophilia (Perraeu, 1981), orange pigmentation of the blood plasma of Angora goats which is not due to bilirubinemia (Gruss, 1981). The electroencephalographic (EEC) changes in the clinical diagnosis of field and experimental cases were not specific and were attributed to brain edema Van Amstel, 1987). A DNA Probe which is a nucleic acid based test was recently developed to diagnose *C. ruminantium* in infected animals and ticks.

The DNA and RNA probes to detect rickettsia and other organisms in animals and arthropod vectors by nucleic acid hybridisation have been acclaimed to have high specificity and sensitivity (Barker et al, 1986).

Apart from the cloned genes from DNA of the Zimbabwean isolate (Crystal springs strains) genes fom DNA of Kiswani strain of Kenya have also been cloned PCr 9 (Waghela *et al;* 1991).

Using the nucelic acid based tests, it has been shown that *Cowdria* is closely related to *Anaplasma marginale* and *Ehrlichia canis* phylogenetically. Mahan *et al.* (1993) reported that the 32 KDa protein (MAPI) encoding gene (MAPL) has homology with MSP-4 protein of *A. marginale* (31 KDa) and cross react serologically with antigen containing cross reacting epitopes of *E. canis* (27 KDa). For these reasons, false positive in field sera from heartwater free areas in Zimbabwe were observed using the 32 KDa specific CELISA (De Vries *et al;* 1993) and immunoblotting assays (Mahan *et al;* 1993). It has been noticed that the sensitivity of the DNA probes is limited by the amount of the target DNA present in the sample. Posnett and Ambrosio (1991) observed that the sensitivity of DNA probes is within the range of microscopic detection (i.e 10^5 - 10^6) when used to detect parasites in blood. Conrad *et al.* (1992) reported that DNA probes based on the genes encoding for ribosomal RNA appeared to be more sensitive. The DNA is particularly useful in the identification of carrier animals or where specificity is required and also in post mortem diagnosis especially in decomposing animals (Jorgensen and Timms quoted by Dalgliesh, (1993). The DNA probe was meant to provide solutions to the non specific reactions in serological tests caused by cross-reactions with certain *Ehrlichia* species some of which can not be eliminated by dilution or the use of monoclonal antibodies

(Jongejan *et al;* 1993a). However, the DNA probes are expensive, slow, sophisticated, applicable in well established laboratory with facilities for handling radioactive substances (Camus, 1994) and lack sensitivity. It needs to be improved upon so that a non-radiolabelled indicator of hybridization can be used (Yunker and Allsopp, 1994).

Polymerase Chain Reaction (PCR) Techniques.

PCR primers of oligonucleotides are being developed from *C. ruminantium* small ribosomal RNA sequences, to identify carriers and active infections. The studies aim at finding a way of developing a non-radioactive detection methods for use with these probes to make the technology useful in most countries that have heartwater.

Where high sensitivity is required, the PCR supercedes the DNA probes. The PCR test repeatedly amplified a specific DNA sequence in the genome of the target organism using polymerase enzyme to produce products that are easily detectable. The test is so sensitive that as minute as 1 pg of DNA in a sample can be detected. Persing *et al.* (1992) observed a *Babesia* parasitaemia of 10^{9} can be detected making PCR 100 times more sensitive than the limit of microscopic detection. The PCR is particularly used in validating results from other diagnostic techniques

because of its sensitivity and specificity and also for export certification of livestock.

2.7.2 **Postmortem Diagnosis**

Van de Pypekamp and Prozesky (1987) and Prozesky (1987) suggested that an *anamnesis* of the animal should be taken before the post mortem is done. Information such as the signs before death, vaccination history, tick control programme species, breeds, age and origin of the animal are necessary.

Gross lesions

In peracute cases, gross lesions may not be readily seen. In acute and chronic cases, several non-pathognomonic macroscopic changes may be observed. These lesions are very similar in most domestic and wild ruminants and mice (Uilenberg, 1971a; Uilenberg, 1983; Ilemobade 1976; Prozesky 1987a). The main lesions include effusion of blood cavities, hydropericardium and lung edema. There are also mediastinum and brain edema, widespread petechiae haemorrhages and splenomegaly. Brain edema may be the only pathologic changes in animals that died of chronic infection (Prozesky, 1987b).

Microscopic lesions

Diagnosis of heartwater based on histological changes was said to be of limited diagnostic value (Prozesky,1987a).

The use of smears from brain cortex (Purchase, 1945) has been the most reliable method for confirming the disease. Thus the use of smears from intima of large blood vessels and tissue sections (Prozesky, 1987) for confirmation had to be abandoned. The difficulties often encountered in getting brain specimen under field situation often limit the diagnosis of heartwater (Uilenberg, 1983).

2.7.3 Diagnosis in treated animals

It has been reported that the organisms are difficult to identify in brain smears made 48 - 60 hours after animals are treated (Prozesky, 1987). It was explained that chemotherapy usually affected the small forms than the large forms making them to fuse and become poorly delineated and thus difficult to differentiate from phagosomes on endothelial cells, group of blood plateletes, the chromatin of capillary endothelial cell nuclei and mast cell granules.

2.7.4 Diagnosis of heartwater in mice

Using the Welgevonden isolate, the main gross pathology noticed were hudrothorax, hydropericardium, lung edema and splenomegaly (Du Plessis 1975; Prozesky and Du Plessis, 1985). Microscopically the organisms were reported to be highly concentrated in the lungs. Using the Kumm isolates, the organism were seen in many cells but they were most prominent in the peritoneal macrophages and reticulo endothelial cells in spleen and lymphnodes (Du Plesssis, 1975). In both cases, the authors reported the presence of the organisms in brain smears but in low concentration and not as frequent as observed in ruminants (Prozesky, 1987). For confirmatory diagnosis subinoculation of blood of infected mice into a susceptible ruminant is usually advocated (Prozesky, 1987b).

2.7.5 Serology

Several serodiagnostic tests have been developed to monitor heartwater infections in experimental and field infections. They include the followings:

i) Capaillary flocculation test (Hemobade and Blotkamp 1976);

ii) Indirect fluorescent antibody test using infected moused macrophages (Du Plessis, 1981);

iii) Indirect fluorescent antibody test using infected neutrophils (Logan,1987);

iv) a direct antibody test (Sahu, 1988);

v) an indirect fluorescent antibody test using an infected bovine endothelial cell antigen (Martinez et al., 1990; Semu *et* al., 1992);

vi) competitive enzyme linked immunosorbent assay (CELISA) based on an epitope of the major antigen protein (MAPI antigen) of C. *ruminantium* (Jongejan *et al;* 1991b);

vii) an immunoblotting/western blotting assay based on reactions to the MAPI antigen (Mahan *et al;* 1993);

viii) indirect ELISA based on *C. ruminantium* lysates (Martinez *et al;* 1993; Jongejan *et al;* 1991b);

ix) a recombinant indirect MAPIb ELISA based on partially expressed MAPI protein (Van Vliet *et al;* 1992);

x) MAPI - antigen (a baculovirus expressed) based immunoblotting assay (Katz *et al;* 1996);

xi) an MAP2 - based indirect and CELISA it also have been developed and evaluated for the diagnosis of heartwater (Jongejan *et al;* 1991b);

xii) a nuclei acid based test, the polymerase chain reaction

(PCR) has also been developed and evaluated for the diagnosis of heartwater (Mahan *et al;* 1993; Yunker and Allsopp, 1994).

The problems associated with these tests are many. They range from their variations in sensitivity and specificity, cross reactions with *Ehrlichia* species leading to false positive results and involvement of some sophisticated technology.

Immunofluorescent antibody (IFA) Test

IFA test was first developed for diagnosing heartwater by DU Plessis (1981). Infected peritoneal mouse macrophages was used as *Cowdria* antigens using the Kumm strain (Du Plessis and Kumm 1971). The test was mainly used in South Africa for seromonitoring of experimental animals (Du Plessis and Mahan, 1987).

There is cross reaction between *Cowdria* and *Ehrlichia* in IFA test based on infected neutrophil cultures (Logan *et al;* 1986) or infected mouse macrophages (Du Plessis, 1981). The cross-reactions usually cause problems in the interpretation of serological results.

A new dimension was created on the serology of heartwater with the successful in vitro cultivation of *Cowdria* on long term basis (Bezuidenhout *et al;* 1985). *Cowdria* antigens can therefore be produced on a large scale for serology. Various ovine and bovine endothelial cells were used (Yunker *et al;* 1988; Jongejan 1990; Byrom *et al;* 1991 and Mahan, 1993). The IFA test on cultured antigen is based on the brightly fluorescing free extracellular elementary bodies (EB) of *Cowdria* while the intracellularly located reticulate bodies which fluoresce only faintly are not considered.

Direct fluorescent antibody (DFA) Test

DFA test is not widely used. However, Sahu (1988) used it to detect *C. ruminantium* in tissues collected from goats, sheep and cattle that died of experimental heartwater. The test is for less superior to IFA test.

Monoclonal Antibody-mediated ELISA test

The direct ELISA test is usually found unsuitable for *Cowdria* serology because of the high background resulting from the unpurified *Cowdria* antigens harvested from endothelial cultures. The use of specific monoclonal antibody is therefore adopted to avoid this problem.

A monoclonal antibody-medicated competitive ELISA test (CELISA) was therefore developed based on the use of Mab to determine the localisation of Cr32 (MAPI) and the circulating antibody. Jongejan *et al.* (1991b) also showed that the serotype specifity does not play a role in the CELISA because Cr32 is a conserved protein present in most of the *C. ruminantium* that have been tested. It has been noticed that CELISA is better than IFA test but *Western blotting* appeared to be more sensitive than CELISA in detecting antibodies against *Cowdria* specific proteins. However Western blotting is not very practical for large scale serological test and is used as an additional method for evaluating previous exposure to *Cowdria* (Jongejan, 1993a).

The high background fluorescence in field sera which may probably be due to cross-reactions with *Ehrlichia* species poses another problem. This makes the determination of realistic cut-off point difficult especially in CELISA. The recombinant indirect MAPIb ELISA was developed to solve the problem of cross reactions with *Ehrlichia* species of CELISA.

The Indirect ELISA (IELISA) or Solid Phase ELISA

The IELISA has been used experimentally and in field surveys. It was used in Lesser Antilles for seroepidemiological surveys (Camus *et al;* 1993). The percentage of positive was higher in Antigua and Guadeloupe where the disease is known to occur and less in areas with no record of clinical disease. The positive test in these areas were attributed to cross-reactions with other organisms. The specificity of IELISA was reported to be lower for sheep than for goats and cattle (Martinez *et al;* 1993). They also recorded cross-reactivity with *Ehrlichia bovis* and *E. ovina* but not with *E. phagocytophila*.

Competitive ELISA (CELISA) has the advantage over the IELISA or indirect binding assays by having low background levels at high serum concentrations and by not requiring pure antigens because the specificity of monoclonal antibodies is used.

Oberem *et al.* (1984) and Neitz *et al.* (1986) had produced Cowdria antigens from infected sheep brain, *A. hebraeum nymphae and* sheep brain respectively using the percoll density gradient and Lecitin chromatography. The antigens produced were unsuitable for ELISA due to pleomorphism of the organism making it difficult to concentrate them

in a single band to give sufficient quantity antigens. In addition, the bands contained more impurities than the organism giving high background levels.

Cross reactions

The specificity of IFA test is greatly hampered by cross reactions between *Cowdria* and *Ehrlichia* species based on antigens from neutrophil cultures (Logan *et al;* 1986; Jongejan *et al;* 1989). Strong reactivities were recorded between *Cowdria* antigens and *E. equi* antisera but less with *E. canis* (Logan *et al;* 1986). *E. phagocytophila* showed bilateral cross reactions in neutrophil culture antigens while *E. bovis* had unilateral cross reactions using infected macrophages based IFA tests, so also were *E. phagocytophila* and *E. ovina* (Du Plessis *et al;* 1987). Cross reactions with *Ehrlichia* species usually hampers serological diagnosis of heartwater using CELISA, immunoblotting and IFAT. Consequently recombinant MAPIb ELISA was developed to solve the problems of cross reactions with *Ehrlichia* species. MAPIb ELISA involves using a specific fragment of the MAPI protein implemented as a possible specific alternative ELISA antigen. Cross reactions are not linked to cross immunity between organisms. *E. phagocytophila* did not cross react in CELISA and Western blots but cross reacted in IFA test (Jongejan *et al;* 1993a).

2.7.6 **Differential diagnosis**

Heartwater is characterised by many clinical and pathologic signs none of which is pathognomonic. It is therefore possible to confuse heartwater with several other diseases that share similar signs. The nervous involvement can make heartwater to be confused with rabies, cerebral babesiosis, tetanus, cerebral theileriosis and hyomagnesemic tetany (Ilemobade 1976; Uilenberg, 1983). Domenech *et al.* (1993) reported that the ovine nervous syndrome due to vitamin B1 deficiency can be confused with heartwater. However, its favourably response to Vit. B1 thereapy and absence of *Cowdria* granules in brain squash smears of infected sheep differentiates it from heartwater.

In peracute cases, heartwater can be confused with anthrax especially when there is high fever, blood stained faeces and sudden death (Uilenberg, 1983) but blood from natural orifices which often fails to clot on exposure to air and presence of *Bacillus anthracis* in pheripheral blood differentiates it from heartwater (Ilemobade, 1976).

Presence of haemorrhagic diarrhoea in heartwater can also make it to be confused with coccidiosis, arsenic poisoning and plant poisoning (Uilenberg, 1983). Haemorrhagic septicaemia can also be mistaken for heartwater because of pulmonary involvement.

2.8 Control of heartwater

The current control methods include vector control, immunization, chemotherapy, natural resistance of young animals and the use of indigenous breeds. Uilenberg (1990) observed that future control strategies would be in areas of immunology, epidemiology, pathogenesis, natural resistance to heartwater and diagnosis.

2.8.1 Vector control

The control of heartwater through the attack on the vectors began long ago when Lounsbury (1902) established that the disease is transmitted by *Amblyomma* species. Apart from heartwater, an engorged female *A. hebraeum* has been shown to cause reduced live weight per standard female tick per day of 10g (Norval *et al;* 1989) and 46 - 61g by *A. variegatum* (Pegram *et al;* 1989). The ticks are controlled by the use of appropriate acaricide and this has prevented the establishment of the ticks and the disease in some areas in Zimbabwe (Norval and Lawrence, 1979). Acaricidal tick control also enabled eradication of ticks in certain areas of South Africa and Kenya (Stampa, 1969). There are several problems associated with the use of acaricides (Uilenberg, 1983; Ilemobade, 1976; Bezuidenhout, 1993; Kocan, 1995). These include the cost of acaricide

especially the involvement of foreign exchange component and the application of the chemicals. Intense use of acaricides can create endemic instability for not only heartwater but other tick-borne diseases such as babesiosis and anaplasmosis (Norval and Lawrence, 1979).

The presence of alternative or wild hosts on which the *Amblyomma* species can easily feed on is a great impediment to this method of control as it is not possible to spray this category of hosts (Hemobade, 1976). In addition, ticks prefer to attach to animals that have not been treated with acaricides. The aggregate - attachment pheromones (AAP) emitted by attached males causes unfed nymphs and adults to attach to untreated hosts, domestic or wild complicating acaricidal control (Norval *et al;* 1989). Environmental pollution (water and vegetation) usually results when acaricides are continuously used (Kocan, 1995). Moreover when it is disrupted, disease outbreak is the likely consequence because of the increase in the population of susceptible animals (Young *et al;* 1988).

Several acaricides have been used but the best are usually those with long residual effects and with easy applicability (Barre *et al;* 1993). Pyrethroids are becoming very popular because of its effective activities at very low concentration and low toxicity for mammals and environment.

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Convenient formulation such as pour-on which are easy and rapid to apply requiring no withdrawal periods are available. There is also the spot-on method using a drench gun which is however less effective using the same flumethrin (Barre *et al;* 1993).

The slow release boluses and acaricide impregnated eartags although useful in reducing frequency of treatments, have short duration effects and therefore unsuitable for large scale eradication programme (Barre et al., 1993).

Recent progress in tick control involves the use of synthetic pheromones which is composed of methyl salicylate ornito-nitrophenol and non anoic acid (Pavis and Barre, 1993). It is combined with an acaricide which are incorporated into slow releasing plastic "tick decoy" and attached to the host. The advantages of the slow release decoy include less use of acaricides, longer effects, selective effects on *Amblyomma* only and the use of attachment aggregate pheromones (AAP) to attach unfed ticks making effective control of ticks possible even in the presence of alternative hosts. Wilson (1993) reported that ivermectin, an avermectin derived from a soil fungus is effective for tick control. The drug is also said to affect haemoparasites carried by ticks (Kocan, 1995).

2.8.2 Biological or Natural Control

Resistance to acaricides by ticks, environmental pollution and the cost are some of the factors that inform researchers that an alternative tick control measures be considered (Nolan, 1981). For these reason, the use of biological or natural tick control strategies such as the use of host resistance integrated with strategic dipping instead of pure intensive dipping, predators and pathogens, tick killing plants, habitat control and the development of resistant cattle have been considered (Kocan, 1995). However, Young *et al.* (1988) noted that these control measures could only be of limited success.

Biological or natural tick control has been classified under the use of host factors, vegetation and microclimate, direct use of parasites, pathogens, predators and genetics (Spickett, 1978). Pasture spelling could only have a very little impact because *Amblyomma* species have wide variety of hosts (Wilkinson 1957). Host availability such as high stocking density and presence of wild animals such as Kudu, impala and birds could contribute in increasing tick population (Horak, 1982). Some breeds of cattle are more resistant to tick infestation than others (Seifert, 1971).

Dossa *et al.* (1996) experimentally induced resistance to *A. variegatum* in Boran (*Bus indicus)* cattle after five consecutive infestations with nymphs and adults. The percentages of adults and nymph which died or moulted was the main indicator of resistance. Reduced susceptibility of the N'Dama breeds to adult *A. variegatum* and *Hyalomma* species has been reported (Morrow *et al;* 1996). Concurrent trypanosomosis did not also compromise tick resistance in this breed even though the disease is immunosuppressive.

Aposymbiosis is another area that has been mentioned for consideration (Spickett, 1978). This involves elimination of the symbiots in ticks using antisymbiotic antibody during feeding. This reduces the fecundity and longevity of the ticks, thereby reducing the population of the ticks.

Some authors have suggested the manipulation of vegetation and microclimate involving modification of tick habitats using bush burning. This is viewed as a poor technique because the burning of bushes may not coincide with the period of tick activity (Galum, 1978). However, clearing of bushes may reduce the population of such species like *A.*

hebraeum. *A. americanum* and *A. cajennense* that depend on tall grasses and tree shades (Norval, 1977; Barnard, 1981).

Planting of tick resistant or repellant grasses has also been suggested (Sutherst *et al;* 1982). Some plants have hooked trichomes forming a barrier to tick, for example the *Desmondium* species. There are plants that act also as tick deterrent (e.g *Melinis minutiflora*, molasses grasses) and Stylosanthes species (Spickett, 1978). *Stylosanthes viscosa* have granular trichomes that form viscous secretions which incorporate volatile chemicals that are toxic to tick larvae.

The direct effects on natural control involve the use of some hymnenopterus parasites known to affect a wide range of ticks (Mwangi *et al;* 1998). However, only one species of these is reported to have parasitized *A. tholloni* in Ivory Coast. The major problem identified in the use of this method is the low fecundity of these parasites which are available in such a small number that they cannot effectively control the high population of ticks especially during the period of seasonal activities (Sutherst *et al;* 1978). *Bacillus thuringiensis*, a bacterium has been shown to be highly lethal to ticks (Fieldler, 1969). Predators including mammals, reptiles, birds and insects are also known to feed on ticks. Ox

pecker (*Buphagus africanus africanus)* is a popular predators of ticks (Bezuidenhout and Stutterhein, 1980). Entomopathogenic fungi (*Beauveria bassiana* and *Metharhizium anisopliae)* have been used experimentally to control *R. appendiculatus* and *A. variegatum* (Kaaya *et al;* 1996; Bengaly, 1996). *Ixodiphagus hookeri,* a hymenopteran, has also been shown to be good tick parasitoid in the control of *A. variegatum* (Bengaly, 1996).

Genetical manipulations such as the use of sterile males, have also been seen as components of integrated control measures (Whitehead, 1969). *A. variegatum* has been sterilised using irradiation dosages of less than 8 Krad (Beutlner, 1975). The harmful effects of sterile male is still retained however and the principle that the species to be controlled should have small population can not be easily realised (Spickett, 1978).

2.8.3 **Immunization**

The initial attempts at immunization of ruminants was limited to cattle using the innate age related resistance of calves. Calves were inoculated intravenously with sheep blood containing *C. ruminantium* (Neitz and Alexander 1941; 1945). With the discovery of Uleron, a chemotherapeutic agent, infection and treatment method (MIT) of

vaccination was used (Netz, 1940) making it possible to immunize older cattle, sheep and goats (Uilenberg, 1983). Later, Uleron was replaced by tetracycline.

The main problem of infection and treatment method included possibility of losing vaccinated animals through shock or inability to successfully control the ensuing reactions, time constraints labour intensity, differences in virulence of strains, risk of transferring some other diseases and storage problems (Uilenberg 1983; Du Plessis and Letitia 1987). Storage problem could however be minimised with the use of vaccine produced from infected tick supernatant (Bezuidenhout, 1981). However, side reactions of this vaccine after intravenous inoculation limits its use.

Chemophylaxis a form of modification of infection and treatment method has been used (Purnel *et al;* 1984). Calves were successfully introduced into heartwater endemic areas by giving three injections of oxytracycline 7, 14 and 21 days after.

Alternative route of vaccination have been sought to alleviate the problems associated with the intravenous route of administration.

Bezuidenhout (1987) used subcutaneous route and achieved variable success of 0 - 100%.

Intraperitoneal route although proved unreliably for vaccination, was effective for administration of tetracycline to reacting animals (Hurtler, 1987).

Doxycline (Doximplant®) implanted subcutaneously under the skin was found to facilitate vaccination in South Africa as necessity for monitoring and treatment did not arise (Oliver *et al;* 1988). However, Doximplant was reported to interfere with immunity in Zebu cross in Malawi (Lawrence *et al;* 1993). Local reactions were also provoked by this drug in vaccinated cattle.

The use of block treatment of vaccination whereby vaccinated animals are given blanket treatment irrespective of whether they develop fever or not is to further reduce complication of infection and treatment methods (Poole, 1962a). It is particularly useful in situations where monitoring is impossible although its not advisable for valuable animals (Uilenberg, 1983). Early blocking is not usually accompanied by good immunity (Poole, 1962a). Immunity elicited seems to depend on the

severity of reaction that's immunogenicity parallels pathogenicity (Du Plessis and Letitia, 1987). This is contrary to the observation of some scientists (Uilenberg, 1983). Erasmus (1976) also noticed that the later the therapy the better the immunity. The immunity in block method is therefore inferior to infection and treatment method and the prolonged chemophylaxis method of Purnel (1984). A modification of block method has been suggested by taking temperatures of representative proportion (about 1%) of the animals and the whole group is treated between 1 - 4 days after the first distinct rise in temperature of one or more of the monitored animals (Du Plessis and Letitia, (1987).

The use of cultured attenuated vaccine (Senegal stock) in vaccination has been reported (Jongejan *et al;* 1983c, Gueye *et al;* 1994). Virulence of the parasite was lost after 21 passages while still retaining its immnogenicity. Fever which lasted for 2 days was the only sign observed in vaccinated animals. However, it does not give better protection and neither is the problem of antigenic diversity solved (Jongejan *et al;* 1993c). It was observed that there could also be possibility of reversal to virulence of the organism and be transmitted by ticks. Thus its use outside the area of origin has been discouraged. Attempts at attenuating

other *C. ruminantium* stocks has not been successful (Martinez *et al;* 1994).

Inactivated elementary bodies have been used as antigens for vaccination (Martinez *et al;* 1994). However, protection here was incomplete. An incubation period of 7 days was recorded as against 10 days with infective blood probably because the culture material contains higher concentration of parasites.

Du Plessis *et al.* (1990) attempted lyophilization (freeze drying) to facilitate storage and handling. He observed that infectivity decreased after 21 days at 4°C, 3 months at -18°C and 6 months at -28°C and concluded that lyophilized material could be used for vaccination.

Presently emphasis is directed towards the use of recombinant vaccine (Mahan *et al;* 1993). Maltezer (1990) estimated that a recombinant vaccine virus against heartwater could be produced for as little as US 10 cent per dose. Since the vaccine could easily be freeze dried, the establishment of continuous cold or frozen chain from the site of production to the farm site of use may not be necessary. This will make the production and distribution of the vaccine cheaper and can

therefore be easily undertaken in developing countries (Norval *et al;* 1992). However, inactivated and molecular vaccines often induce shorter immunity than that caused by live materials and the problems of antigenic diversity could be more pronounced (Uilenberg, 1995). Thus inactivated and molecular vaccines may not give effective protection in heartwater endemic area while different strains of the parasite may exist.

Another dimension to the research on immunization against heartwater is the use of Naked DNA immunization. A recombinant plasmid DNA encoding a protein of interest is injected directly into animals. This is taken up by cells (particularly muscle cells), and the relevant protein is expressed and immune response is induced. Protein purification is not required (Cohen, 1993). Contrary to the live delivery, the DNA used in naked DNA immunization does not replicate in animal host. However, it remains episomal for sometime and the gene expression can be detected for as long as 19 months after injection.

Immunization of cattle against ticks have been employed using crude antigen extracts from *Boophilus microplus* (Willadsen *et al;* 1979; Agbede and Kemp, 1986). The hidden tick antigens midgut has been used also to vaccinate cattle against *B. microplus* (Johnston *et al;* 1986). This technique has not been widely considered for heartwater.

2.8.4 **Chemotherapy**

Several drugs have been tested for their efficacies against heartwater. Uleron (4 - (4- aminobenzol sulphonamide benzol sulfone dimethyl amide) was the first drug known to be active against heartwater especially when administered early in the disease (Neitz, 1939). Later tetracyclines were found to be active. Chlortetracycline, Oxytetracycline and rolitetracycline were therefore used (Weiss *et al;* 1952, Haig *et al;* 1954; Poole 1961b).

Immelman and Dreyer (1982) found doxycline effective against heartwater. McHardy and Mackenzie (1987) showed that a series of d-4-methyl-thiosemicarbazones and gloxazone at 20 mg/kg subcutaneously were active against heartwater. However gloxazone was not developed because of its toxicity (McHardy *et al;* 1980).

Drugs that were reported to be ineffective against heartwater included Penicilin and Stretomycin (Bezuidenhout, 1981). Chloramphenicol and spiramycin (Uilenberg, 1971b) and imidocarb (McHardy and Mackenzie, 1987).

Tetracyclines are still the drug of choice presently and must be given early in the course of the disease. A second dose is usually given if fever persists (Uilenberg, 1983) but treatment is usually not effective when clinical signs have ensured (Ilemobade, 1976; Uilenberg, 1983). Tetracyclines do not interfere with immunity (Simpson and Wiley, 1951). The inclusion of supportive treatment and possibly the use of tranquilizers have been suggested (Uilenberg, 1983).

2.9 Immunity of animals to heartwater

The nature of immunity has not been clearly resolved. Differing opinions have been expressed on the role of humoral immunity, premunity and duration of immunity. It has been reported that immunity was still solid 6 months after clinical recovery (Neitz, 1939). However, it began to wane 2 months after recovery but immunized sheep were still protected against fatal attack after four years of recovery.

Immunity in goats is not well defined. Ilemobade (1976) found that goats that recovered from clinical attack were immune to challenge 2 - 8 weeks later.

In cattle, immunity that is not reinforced by repeated reinfection is short-lived (Uilenberg, 1983).

In mice duration of immunity has been shown to last for 18 months (Du Plessis, 1981). Existence of premunity in recovered animals has been proved by Hemobade (1976). He showed that *C. ruminantium* persisted for upto 40 days or more in recovered animals. The animals were immune on challenge and the blood was infective for 50 days. He also observed that the blood of goats that died 9 weeks after oxytetracycline treatment was not infective although *Cowdria* granules were found in the brain smears. Neitz (1939) had earlier reported that *C. ruminantium* was diagnosed 60 days after clinical recovery but added that immunity was snot based on premunity and neither was it solid.

2.9.1 **Cross Immunity**

Previously it has been the belief that *C. ruminantium* isolates were antigenically similar (Alexander, 1931). There were also no antigenic differences among five isolates from South Africa, Sudan, SaoTome and Nigeria (Uilenberg, 1983). The discovery of a mouse infective strain called the Kumm strain, was probably the first major indicator that antigenic differences might exist among *Cowdria* isolates (Du Plessis and

Kumm, 1971). This strain is not pathogenic to cattle but highly pathogenic to sheep (Du Plessis, 1981). It was suggested that they probably belonged to another species.

It has also been discovered that the South African strain (Ball 3) did not fully protect against an isolate from Senegal (Jongejan *et al;* 1988). Also, the Kumm, Kwanyanga and the Nonile stocks have limited or no cross protection among themselves and with Ball 3 from which they differ by being pathogenic to mice.

The Kwanyanga stock and the Caribbean Gardel stock are not cross protective (Logan, 1987). In addition the Gardel, Kwanyanga and the Mali stocks or their combination did not protect against the Kumm stock.

Du Plessis *et al.* (1989) tested 10 different stocks of *Cowdria ruminantium* and noticed that only the Ball 3, Germishuys and the Comoro (Comoro Islands) were closely related. The remaining stocks (6 from South Africa, 1 from Mali) differed in many respects. The Welgevonden, stock emerged as the stock that elicited an immunity against most of the African strains. Du Plessis *et al.* (1990) reported that sheep immuned to Ball 3, Kwanyanga stock or Mara stock (South Africa) or their

combinations were not protected against challenges by Mali stock. Jongenjan *et al.* (1988) equally observed the failure of Ball 3 to protect vaccinated sheep and goats against challenge by other stocks. It has therefore been suggested that the Ball 3 vaccine strain be replaced by another strain that has wider protection spectrum.

Genetic variations between isolates from similar geographical areas have been reported. Reddy *et al.* (1996) noticed that Caribbean isolates from Antigua and Guadeloupe deferred when their MAP1 gene sequences were compared. The Antigua isolates was reported to be closely related to West African Senegal isolate while the Gardel isolate from Gudeloupe was closely related to the isolate from Sudan in East Africa.

Also genetic variations among *A. variegatium* ticks have been reported through the analysis of cuticular hydrocarbon (Estrada-Pena *et al;* 1994). *A. variegatium* from St. Lucia differed from those from Guadeloupe which were more closely related to ticks from Ethiopia while the ticks from St. Lucia were more closely related to ticks from Senegal.

Apart from the reports from the Caribbean regions, differences in pathogenicity and growth rate in culture have been observed among isolates in Ghana, West Africa (Bell-Sakyi *et al;* 1996).

It is evident from the foregoing that there are variations among isolates of *C. ruminantium* and their vectors. However, such variations which have not been extensively investigated in Nigeria will be part of these studies.

CHAPTER THREE

3.0 STUDIES ON THE ESTABLISHMENT OF LARVAE OF *Amblyomma variegatum* AND EXPERIMENTAL INFECTION OF THE TICKS WITH *Cowdria ruminantium*

3.1 Summary

Forty eight female *A. variegatum* ticks (30 fully and 18 partially engorged) were collected from sedentary herds in Zaria. They were incubated at 28°C and 80% relative humidity. The ticks oviposited 7 to 10 days after incubation. The eggs were maintained under the same incubation conditions and hatched into larvae 40 to 59 days post oviposition. Giemsa stained hemolymph, egg and larval squash smears did not reveal rickettsia bodies. The larvae were infected by feeding them on a goat that was reacting to experimental heartwater disease. The resultant nymphae from the moulted larvae transmitted heartwater to susceptible goats.

3.2 Introduction

Cowdria ruminantium is transtadially transmitted and it is either the larval or the nymphal stage of *A. variegatum* that acquire the infection and is subsequently transmitted by the succeeding stages (Uilenberg, 1983).

In transmission sudies or tick-derived vaccine production such immature stages are required. They are usually reared in the laboratory to ascertain their cleanliness starting with the engorged female ticks (Hemobade, 1976) which are incubated at a given temperature and relative humidity for oviposition. Laboratory infection of larvae is done by feeding them on a heartwater infected experimental animal at the height of pyrexia of temperature $\geq 40^{0}C$ (Bezuidenhout *et al*: 1985). The work reported here involves the establishment of *Cowdria* - free larvae of *A. variegatum* and their subsequent infection with *C. ruminantium* (Jaji isolate).

3.3 Materials and Methods

Field trips were made to some livestock farms in Kaduna state. The farms visited, the types of livestock sampled, the members and state of engorgement of the ticks picked from the animals are recorded in Table 1. Fully and partially engorged female *A. variegatum* were carefully removed from the animals with hand forceps and put into MacCanthy bottles with holes bored on the cover for ventilation. They were transfered into a Petridish in the laboratory, cleaned with brush and water to remove surface dirt and dried with filter papers. They were then put into labelled glass tubes (one tick per tube) stoppered with moistoined cotton wool and incubated as described by Hemobade (1976).

At 7 days post incubation, the tips of their tarsals were carefully cut with a pair of scissors and haemolymph that oozed out was gently put on a labelled slide. Haemolymph coagulates quickly preventing the cut legs from infection and death of the ticks (Burgdorfer, 1970). Smears were made, allowed to dry, fixed in absolute methanol, stained with Giemsa and examined under the microscopes for rickettsial parasites (Burgdorfer, 1970).

The eggs were allowed to hatch and two weeks after hatching, the larvae were transfered to an incubator maintained at 18°C and 80% relative humidity to reduce their metabolism in order to keep them for a relatively longer time. Squash of some of the eggs were made fixed, stained with Giemsa and examined under the microscope for rickettsial bodies. Larval squash smears were also made from representative samples, fixed, stained and also examined for presence of rickettsial parasites.

A savanna brown buck identified as NNI was purchased from a market in Zaria, preinfection screening of blood showed a packed cell volume of 40%, negative haematocrit and phase contrast examinations, and no parasite was found in the Giemsa stained thin blood smears. No

parasite eggs were also seen in the faecal examination. Three weeks later, the goat was intravenously infected with 2ml of cryopreserved *Cowdria ruminantum* (Jaji, stock). The ears were shaved and washed with soap and water to clean them and facilitate tick attachment. Ear bags were fixed to the ears as described by Anon (1989). The larvae were introduced into the bags when they were 11 weeks old, on the days 4, 5, 6 and 8 post infection.

Engorged larvae that dropped into the bags when the goat reacted at a pyrexia of 40°C were cleaned and incubated at 28°C and 80% relative humidity (R.H.) to moult to nymphae. Larvae that detached prior to pyrexia are usually not infective (Bezuidenhout, 1987) and were therefore discarded.

The goat later developed signs of acute *Cowdriosis* and died. Brain squash smears were positive for *C. ruminantium* granules (Plate I).

3.4 **Results**

The preoviposition and the prelarval hatching periods of the incubated ticks are presented in Tables 1 and 2. Fully engorged ticks began oviposition between 7 and 10 days after incubation. The partially engorged ticks oviposited at 9 days post incubation.

The eggs of the fully engorged ticks hatched earlier than the eggs of the partially engorged ticks, the period of hatching ranging from 40 - 43 days and from 39.5 - 48.5 days respectively post oviposition. Examination of the egg, larval squash and haemolymph smears did not reveal rickettsial bodies in the haemocytes (Plates 1). The larvae that originated from the partially engorged ticks were not as strong as those from fully engorged ticks. Most of them died before they could be used for further experiments. In addition, survivors showed poor attachment ability during subsequent infestation experiments.

The harvest of detached engorged larvae began 7 days after being introduced into the the earbags (Table 3). Approximately four thousand, and two hundred engorged larvae were collected during pyrexia.

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Table 1: Layout of Procedure for collection of *Amblyomma variegatum* on livestock

Farms	Farm Management	Livestock Sampled	No. of Ticks Collected	Engorgement State of Ticks
Livestock Farm Kano Road	Traditional	Cattle	3 6	Full Partial
Danja Farm Zaria	Traditional	Cattle	6	Full
College of Agric. Farm, Samaru	Government	Cattle	3 3	Full Partial
Zaria City	Traditional	Goats Sheep Cattle	9 3 6	Full Full Full
Damau Cattle Ranch near Ikara	Traditional	Cattle	9	Partial

Table 2: Oviposition and hatchability of the field ticks incubated at 28°C and 80 per cent relative humidity

	Range (days)	No. of ticks incubated	State of engorgement
Preoviposition	7 - 10	30	Fully engorged
Preoviposition	9	18	Partially engorged
Oviposition to hatching (days)	40 - 43	30	Fully engorged
"	40 - 43	18	Partially engorged

Table 3: Experimental infection of *A. variegatum* larvae from goat (NN1) infected with *Cowdria ruminantium* (Jaji isolate)

Post inoculation (days)	Rectal Temperature(°C)	Remark
1	38.0	Animal infected with 2mls of stabilate
2	36.7	Ear bags attached to both ears
3	37.1	-
4	38.2	Larvae introduced into the right ear bag
5	38.2	Larvae introduced into the left ear bag
6	37.8	Another batch of larvae introduced into the right ear bag
7	38.0	-
8	38.0	Fourth batch into left ear
9	38.1	-
10	39.0	-
11	39.1	Engorged larvae harvested and discarded
12	39.2	"
13	40.0	Larvae harvested and retained
14	41.3	Engorged larvae harvested and retained and the animal treated

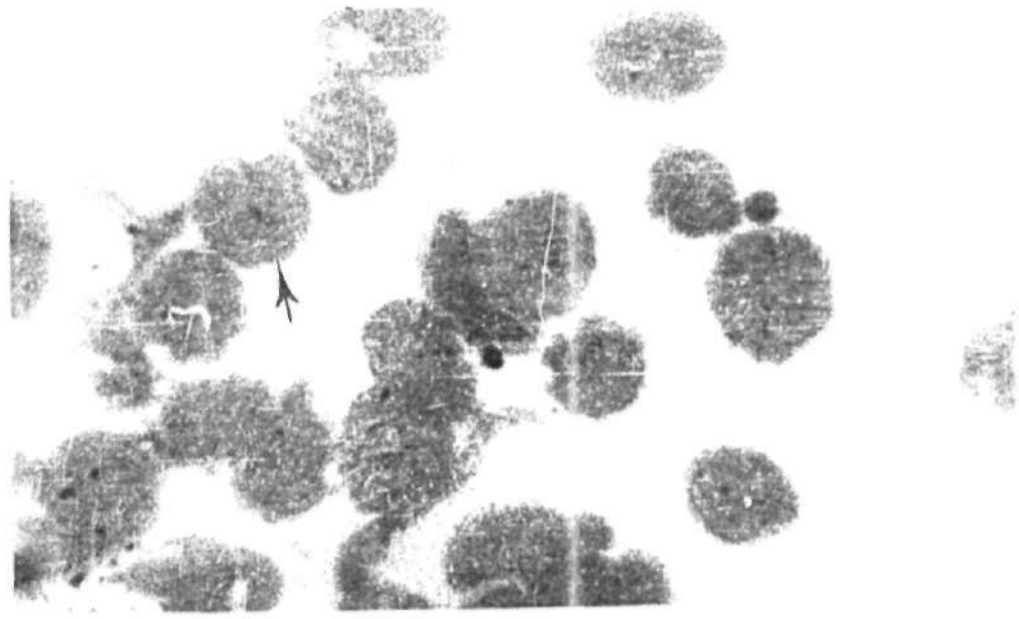


Plate I **Giemsa stained haemolymph smear of engorged *A. variegatum* ticks just before oviposition. No rickettsia bodies were found in the haemocytes (Arrowed)**

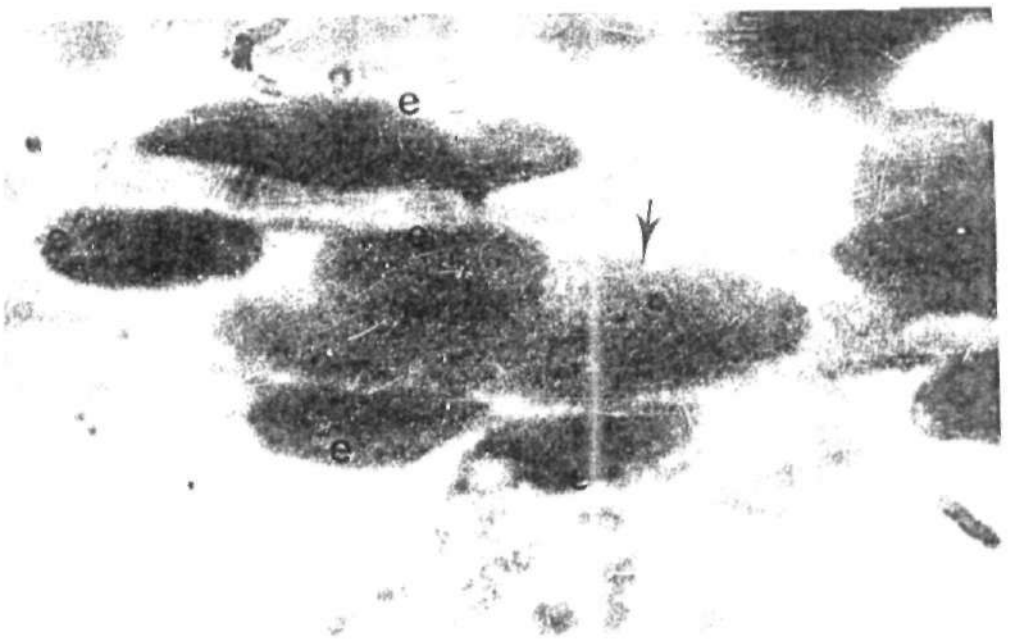


Plate . **II** Giemsa stained brain squash smear of donor goat (NNI) infected *C. ruminantium* Arrow shows a colony of the parasite sorrounded by endothelial cell nuclei.

3.5 Discussion

Oviposition seems to be affected by the level of feeding during collection rather than the variations in the localities or animal hosts of the ticks. The level of feeding might in turn be influenced by management practices of each farm. Thus the traditionally managed herds where acaricidal tick controls are not usually practiced might possess some measure of resistance to ticks due to repeated infestations and consequently reduced rate of feeding, oviposition and weak larva progeny. Latif *et al.* (1988) observed similar situations in laboratory studies. Most government farms due to management problems usually leave the animals undipped for months and creating a situation worse than the traditionally managed herds where although there is no acaridial application, adult ticks are regularly removed by hands.

The periods of oviposition and egg hatching conform with the observations of Hemobade (1976). Observations from this study showed that engorged female *A. variegatum* and their larval progeny are better for transmission experiments and probably than the partially engorged ticks because of shorter periods of oviposition, hatching and active larval progeny.

The hemolymph tests, larval and egg squash smears were used to screen the ticks prior to use. Other tests which could have been used were serology (Viljoen *et al;* 1988) and a DNA probe (Yunker *et al;* 1993). These tests were however not possible during this study because of none availability of equipment for them. Moreover, since *C. ruminantium* and most other haemoparasites that *A. variegatum* transmit are usually not transovarially transmitted (Uilenberg, 1983) the larvae obtained from the engorged ticks are in most cases clean of infections. The hemolymph test and larva squash were therefore done to complement the existing knowledge of the transmissibility of *C. ruminantium* by *A. variegatum* ticks. The organism has been observed in the haemocytes of infected *A. hebraeum* (Hart *et al;* 1991).

It has been reported that only larvae that engorged during pyrexia of the infected animal usually acquire infection (Beziudenhout, 1987). This may be the period of high rickettsiaemia. However, it has also been noticed that infection can be acquired when ticks feed on subclinically infected animals or animals that have recovered from clinical attack (Barre *et al;* 1987) or immune animals after reinfection (Hemobade and Leeflang, 1978). Even though infections can be acquired from these category of animals, the infection rate may not be as high as those ticks that acquire infection from reacting animals.

CHAPTER FOUR

4.0 STUDIES ON THE PREPARATION OF GROUND UP TICK SUPERNATANT (GUTS) VACCINE AND DETERMINATION OF MINIMUM INFECTIVE DOSE (MID)

4.1 Summary

The infected nymphae obtained from moulted larvae in chapter 3 were fed on a susceptible goat to initiate multiplication of the parasites and increase infectivity of the ticks. Crude *Cowdria* vaccine was prepared by grinding up the nymphae as described by Anon (1989). Few modications were made such as 10 minutes three times washing in cold sterile distilled water before being finally rinsed once in cold buffered saline (pH 7.4). The potency of the vaccine was confirmed by inoculating 2 ml into two parasite-free savanna brown goats which reacted with pyrexia of $\geq 40^{0}$C, eleven days post inoculation. The crude vaccine was serially diluted and varying concentrations of 7, 4, 0.25, 0.10, 0.125, 0.05 and 0.0125 infected nymphae per dose (N/dose) were obtained and inoculated intravenously (iv) into savanna brown goats.

Side reactions such as dyspnoea, staggering, salivation and death decreased propressively with decreasing concentration and were eliminated completely at 0.025 and 0.0125 (N/dose). The former was however more

effective than the latter. A dose of the crude vaccine containing 0.025 N/dose constitute therefore, the minimum infective dose (MID). The animal were not susceptible to homologous challenge given three weeks after primary infection

4.2 **Introduction**

When the South African initiated vaccination of susceptible domestic ruminants against heartwater 50 years ago, blood, vaccine prepared from Ball 3 strain of *Cowdria ruminantium* was used. The blood vaccine was bulky especially where large numbers of animals are involved, there is also the risk of transmitting other haemoparasites and it produces hemolytic anaemia in some vaccinated animals. Consequently Bezuidenhout (1981) produced the tick-derived vaccine which eliminated the problems associated with the blood vaccine. However, the tick derived vaccine which is essentially the ground up *Amblyomma variegativm* tick supernatant abbreviated as (GUTS) usually elicites side reactions which may include death of the vaccinated animals as it has to be administered intravenously. The side effects usually result from numerous tick materials in the vaccine. Consequently this creates limitations to its use. Meanwhile inactivated, attenuated and molecular vaccines (Bezuidenhout, 1993) have been tried but found to confer weak

protection against field challenge of the parasite and therefore less immunogenic than the blood and GUTS vaccine. The aim of this study is therefore to eliminate the side reactions associated with the GUTS vaccine and to determine the minimum infective dose (MID) that can be safely used for vaccinating savanna goats against heartwater.

4.3 Materials and Methods

4.3.1 Experimental animals

Thirty savanna brown goats were sourced locally, screened and found negative for blood and intestinal parasites. They were preconditions for three weeks and were fed with groundnut cake and hay. Water and salt leaks were provided ad libitum.

4.3.2 Preparation of crude vaccine and determination of minimun infective dose (mid)

The infected nymphae obtained in previous studies (Chapter 3) were used. They were pre-fed for four days on a susceptible goat using earbags. Infected nymphae usually retain infection during and after moulting (Walker and Olwage, 1987). Prior to use, the nymphae had been maintained for 7 months in incubator of 18°C and 80% relative humidity (RH) and had to be transfered to an incubator of 28°C and 80%

RH to make them active and facilitate attachment a night before the infestation of the goats. Pre-feeding of the ticks enhances parasite multiplication within the tick gut and enhances infectivity (Van der Merwe, 1979).

The procedures used in the preparation of the crude vaccine were similar to those of Burridge *et al.* (1984) and Anon (1989) with some modifications. These included 10 minutes of three times washing of the harvested nymphae in cold 70% ethanol and thrice in cold sterile distilled water before being finally rinsed once in cold phosphate buffered saline (pH 7.4).

The potency test of the vaccine was done by inoculating two millilitres of the vaccine each into two adult savanna brown goats purchased locally, screened for parasites, fed and pre-conditioned for three weeks as described earlier. The rectal temperatures of the goats were monitored daily for two weeks.

In determining the minimum infective dose, one vial (2cc) of the cryopreserved crude vaccine containing calculated number of 4 nymph/dose was rapidly thawed in lukewarm water and 0.4 ml was added

to 15.5 ml of cold phosphate buffered saline (P.B.S pH 7.4). This was mixed thoroughly to give 1:40 dilutions. Four other test tubes containing 16 ml of cold PBS were arranged on ice block and 16 ml of 1:40 dilutions was transfered to the first tube, mixed thoroughly to give 1:80 dilutions. Similar procedures were followed to prepare 1:160, 1:320 and 1:640 dilutions by serially - transfering 16 ml of the diluted vaccine from the preceeding dilutions to the next tube of the required dilutions containing 16 ml of buffer. The concentration of the crude vaccine (number of infected nymphae per dilution) was calculated (Appendix i). Twenty five of the experimental animals were inoculated with the crude vaccine as shown (Tables 4 to 8) and five served as controls. Each administration was carefully and slowly done with adrenalin used when serious side reactions were observed. Side reactions and signs following each inoculation were recorded. Reactions to primary infections were followed by homologous challenge three weeks later using the same or different concentrations. Absence of reaction to a primary inoculation was followed by another inoculation using higher concentrations.

Reactions to primary infection is treated by using long acting Terramycine at 20mg/kg intramuscularly at the second day of temperature reactions.

4.4 **Results**

By the fourth day of infestation, fully engorged nymphae had dropped into the earbags and were harvested. The goat was sprayed with acaricide (Pfizona, Pfizer plc) when the earbags were removed. The two animals that were inoculated to confirm the potency of the vaccine showed elevation of rectal temperature (T^o = 41.5°C) on the 11th day of inoculation. One of the goats died and Giemsa stained brain squash smears showed colonies of *C ruminantium*.

Observations on the tolerant levels of the animals to the different infective dosage levels are shown in Tables 4 to 8. Various side reactions were exhibited, the intensity of which varied. Inoculation of the concentrated forms (4 and 7.2 N/dose) resulted in reactions classified as violent and these included dyspnoea, staggering, urination, constant bleating, restlessness. In addition there were foamy salivation, laboured abdominal respiration, erect hair coat and thready pulse. While the animals that were inoculated with 4 N/dose recovered within two hours, a higher concentration of 7.2 N/dose caused the death of the animal (Table 4). Autopsy examination of the animal revealed an extensive perfusion of the trachea and lungs with foamy exudates. The heart chambers were completely devoid of blood.

Inoculation of 0.125 to 0.25 N/dose was accompanied by side reactions classified as moderate and they included weakness, restlessness and blinking of the eyes. The signs disappeared within 45 minutes. The use of adrenalin was not a necessity at these dose levels (Table 5). Only mild and transient reactions such as excitement, twitching of muscle were noticed when 0.0625 N/dose were administered (Table 6). Inoculation can be given at a faster rate at this concentration. Side reactions were completely eliminated when crude vaccines of 0.03125, 0.025 and 0.0125 N/dose were inoculated (Tables 7 and 8). The incubation period ranged from 9 to 14 days apart from animal No. 17B. The maximum febrile temperature recorded was 41.5°C. Incubation period (IP) was affected by the concentration of the inoculum, being shorter with the concentrated than the diluted vaccines. The animal (17B) which failed to react to primary challenge of 0.0125 N/dose reacted at a higher concentration of 0.10 N/dose. There were no clinical reactions within the three weeks observation period following the homologous challenge using 0.10 N/dose.

Table 4: Immunization of savanna brown goats against heartwater usisng concentrated crude vaccine (4 - 7.2 infected nymphae/dose)*

Animal No.	Reciprocal dilution	Concentration No. of infected nymph/dose	Days before onset of fever	Duration of fever	Maximum Temp. °C	Side reactions following inoculation
NN	Not diluted (ND)	7.2	-	-	-	Fatal
01A	ND	4	9	6	41.0	Violent
08A	ND	4	9	4	41.0	"
15B	ND	4	9	6	40.9	"
18B	ND	4	10	4	41.0	"

*Two mls of crude vaccine was given in all cases

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Table 5: Immunization of Savanna brown goats against heartwater using 0.10 and 0.25 N/dose (1/80)

Animal No.	Dose (mls)	Concentration (N/dose)	Days before onset of fever	Duration of fever (days)	Maximum Temp. (°C)	Side reactions
02B	5	0.25	14	5	40.5	Moderate
09A	5	0.25	14	4	41.0	"
11B	2	0.10	12	3	40.7	"
14B	2	0.10	11	4	41.2	"
17B	2	0.10	13	3	41.5	"
21A	2	0.10	10	3	41.3	"

Table 6: Immunization of Savanna brown goats against heartwater using 0.05 and 0.125 N/dose (1/160 dilution)

Animal No.	Dose (Mls)	Concentration (N/dose)	Days before onset of fever	Duration of fever (days)	Maximum Temp. (°C)	Side reactions
03A	5	0.125	14	3	40.8	Moderate
10B	5	0.125	14	4	41.5	"
12B	2	0.05	13	3	41.0	Mild
16A	2	0.05	13	4	41.0	"

Table 7: Immunization of Savanna brown goats against heartwater using 0.0625 and 0.025 N/dose (1/320 dilution)

Animal No.	Dose (Mls)	Concentration (N/dose)	Days before onset of fever	Duration of fever (Days)	Maximum Temp. (°C)	Side reactions
04A	5	0.0625	14	3	41.1	Mild
06B	5	0.0625	13	2	40.6	"
11A	2	0.025	11	4	41.4	None
12A	2	0.025	12	3	40.9	"
13B	2	0.025	14	3	41.3	"
20B	2	0.025	11	3	41.0	"

Table 8: Immunization of Savanna brown goats against heartwater using 0.0312 and 0.0125 N/dose 1/640 dilution

Animal No	Dose (Mls)	Concentration (N/dose)	Days before onset of fever	Duration of fever (Days)	Maximum Temp. (°C)	Side reactions
05B	5	0.03125	14	5	41.1	None
07A	5	0.03125	14	4	41.2	"
19A	2	0.0125	13	3	41.0	"
17B	2	0.0125	No reaction	None	None	"

4.5 **Discussion**

Bezuidenhout (1981) first produced Cowdria vaccine from infected ticks. Several advantages of this vaccine over the convectionally used blood form in Southern Africa were identified. Consequently hopes were rife that it would just be a matter of time before the vaccine gained general acceptability and probably replaced the blood form (Van der Merwe, 1979). The fatal shock reactions elicited by its intravenous (iv) inoculation generated calls by scientists for its withdrawal from use (Pipano, 1995). In Zaria, six sheep were lost after inoculation of GUTS vaccine (El-imairi, 1991). The various advances recorded in the development of *Cowdria* vaccines vis-avis the development of attenuated vaccine (Gueye *et al;* 1993), inactivated vaccine (Martinez *et al;* 1994) and the use of subunit molecular vaccine (Mahan *et al;* 1994) have further discouraged the use of GUTS vaccine. However, the emerging problems of reversal of attenuated vaccine to virulence, lack of complete protection by inactivated vaccine and the problem of high technology needed to produce molecular vaccine have brought forth the reality that GUTS vaccines can not be discarded now especially in the developing countires because of the simple technology needed for its production. Moreover, none of the modern vaccines has been able to provide the panacea for field problems associated with *Cowdria* vaccie. Inavariably therefore they also

have their problems just like the tick derive vaccines. The molecular vaccine is also thought to have narrow protection spectrum to be effective against wide variety of isolates usually encoutered on the field.

The shock reactions which constitute the greatest impediment to the use of tick-derived vaccine was eliminated in this study. Apart from the infectivity of the shock free dose (0.025 N/dose), it was also found to have effectively conferred immunity against homologous challenge. However; the quantification of the parasite or antigens within the vector was not possible. This could have assisted in evaluating the vaccine.

In spite of its shortcomings, tick-derived vaccine has more potential than any other forms of *Cowdria* vaccine especially in the developing countries because of the non sophiscated technology needed for its production. Moreover, the tick component of the vaccine may confer some immunity against the tropical bont tick. Du Plessis (1998) believed that immunogenicity parallels pathogenicity that is overt clinical signs are necessary for the development of solid immunity. Viewed under this background, inactivated/attenuated vaccines may not be able to confer strong immunity especially when vaccinated animals are exposed to barage of attacks by field strains of *C. ruminantium*.

CHAPTER FIVE

5.0 ISOLATION OF *Cowdria ruminantium* FROM SEDENTARY HERDS IN FOREST AND SAVANNA ZONES OF NIGERIA

5.1 Summary

Twelve *Amblyomma variegatum* ticks were collected from sedentary cattle and sheep in Akure (Forest zone) and twenty five from similar farm in Shika (Savanna zone). The ticks from Akure were ground up as described previously (page 111) with addition of phosphate buffered saline (PBS pH 7.4) at 0.8 ml per tick. Two ml of the supernatant was intravenously (iv) inoculated into a goat identified as 5256 and the rectal temperature monitored daily and the remaining supernant cryopreserved in liquid nitrogen. The animal had pyrexia (Temperature 40.5°C) 13 days inoculation (pi) and stabilate (CR7) was prepared and snap forezen in liquid nitrogen. The goat was sacrified the fourth day post reaction and the Giemsa stained brain squash smears had extensive *Cowdria* colonies. The ticks from Shika was similarly processed and 2 ml of the supernatant was also inoculated (iv) into goat 5253 while the remaing supernatant was cryopreserved in liquid nitrogen. The goat developed pyrexia 3 days (pi) (Temperature) 40°C) died and Giemsa stained smears of the brain had colonies of *C. ruminantium* which were scanty. Stabilate (CR 6) was however prepared prior to death.

5.2 Introduction

Isolates of *Cowdria ruminantium* are required for studies on heartwater disease. In Nigeria, the most widely studied isolate of *C. ruminantium* is D225 (Ilemobade, 1976) which has since been lost in the department due to technical problems to preserve it. Isolates of *Cowdria ruminantium* have been reported to vary in many respects (Bezuidenhout, 1987). This study aims at isolating *C. ruminantium* from some parts of the country.

5.3 Materials and Methods

Twenty nine partially engorged *A. variegatum* ticks (16 females and 13 males) were collected from the bodies of N'Dama cattle at the College of Agriculture Akure and Yankasa sheep at the Federal University of Technology, Akure farm. The ticks were put in Universal bottles with holes bored on the lids for ventilation. On arrival at Zaria, three days after collection, the surviving ticks (10 females and 2 males) were ground up as described previously (page 111) using pestle and morta with addition of phosphate buffered saline (pH 7.4) at 1.8 ml per tick. The crushed ticks were allowed to settle on ice and 16 ml of supernatant was recovered. A buck identified as 5256 was inoculated with 2 ml (0.8 tick/ml) of the supernatant intravenously. The remaining supernatant was

cryo-preserved in liquid nitrogen with dimethlsulphoxide (DMSO) added at 10% as cryo-protectant and labelled stabilate (CR 4). Rectal temperatures of the goat were taken early in the morning and late in the evening. Blood samples were also taken for white blood count. Confirmation of infection and infectivity was done by sacrificing the animal and examining the Giemsa stained brain squash smears for *Cowdria* colonies.

Twenty five *A. variegatum* ticks comprising of 9 partially, 2 fully engorged females and 14 males were collected from the body a sedentary cow in Shika. They were ground up as described previously; 30 ml of supernatant was recovered and 2 ml was inoculated into a buck 5253 intravenously. The remaining supernatant was cryo-preserved as stabilate (CR 6). The animal was monitored as stated above.

5.4 **Results**

The animal No. 5256 showed febrile reaction, an indication of infection, 13 days post inoculation (pi). Fever lasted for 3 days and 40.5°C was the maximum temperature attained. The animal was weak and feed consumption reduced. The main postmortem finding were accumulation of 1.5 mls straw-coloured fluid in the pericardial sac. The

lungs were also slightly congested. Giemsa stained brain squash smears showed very extensive *Cowdria* colonies with varying sizes (Plate III).

The goat identified as 5253 developed fever, 3 days pi attaining a maximum temperature of 40°C by the second day of fever. Three days pi the temperature dropped to pre-infection value of 38.5°C without chemotherapy. The appetite was retained but the animal appeared slightly sluggish and later died overnight.

Giemsa stained brain squash smears of the brain revealed *Cowdria* granules that were scanty and small colonies (Plate IV). Gross pathology of the animal included bilateral congestion of the lungs and accumulation of 2 ml straw-coloured fluid in the pericardial sac.



Plate III Giemsa stained brain squash smears of goat No. 5256 showing long and extensive colony of Akure isolate of *Cowdria ruminantium* (A and B). The colonies cut across the entire microscopic fields. The end of the colonies are indicated by two arrows. The granules are of uniform size



Plate IV Colonies of *Cowdria ruminantium* (Shika isolate) in Giemsa stained brain squash smear of goat No. 5253. A small colony of the parasite (arrowed) attaching to endothelial cell nucleus on the left is shown in "A". A rather unusually shaped colony (arrowed) which is made of four smaller units is shown in "B".

5.5 *Discussion*

***Cowdria ruminantium* can be isolated using several techniques.** These include pooling of blood collected randomly from ruminants and intravenously injecting it into susceptible animals (Camus and Barre, 1987). Feeding of moulted pooled immature wild ticks on susceptible animal (Jongejan, 1992), inoculation of blood from a clinically sick ruminants into a susceptible goat or sheep (Anon 1989; Hemobade, 1976) and pooling, homogenizing and injection of the supernatant of ticks collected on the field into another susceptible animal (Camus and Barre, 1987) are also other methods. The last method was used in this study because of its convenience. Infected ticks can retain their infectivity for a relatively long time under tropical condition as observed earlier.

Isolation of *Cowdria* organism from the vector was extensively used in the epidemiological studies of heartwater in the Caribbean. It is the most reliable method of confirming the presence of heartwater in a given area because of false positives that are usually recorded using serological techniques due to cross reactions with *Ehrlichia sp* (Du Plessis *et al;* 1993). Isolation of *C. ruminantium* from Akure in this study provides another information on heartwater in the South West zone in addition to previous reports of Isoun *et al.* (1974). The widespread

nature of *A. variegatum* in Nigeria is suggestive that a more detailed epidemiological study of heartwater is imperative. The disease might even be more widespread in the southern parts than is observed in the northern parts because of the abundance of wild life that can easily serve as alternative hosts to the vectors as reservoirs. Many authors have reported the occurrence of heartwater in wild and zoo animals in Nigeria (Okoh *et al;* 1986; Oyejide and Olaleye, 1984).

The level or intensity of colonization of brain capillaries by the forest isolate seems not to contribute much to the exhibition of signs and pathology that are usually observed in the course of the disease. The extensive invasion of the brain capillaries by the forest isolate in the infected goat is therefore in contrast to the mild signs and pathology observed in the animal. If toxins contribute to the pathology and signs of heartwater its production and the potency may vary from one isolate to another (Uilenberg, 1983).

CHAPTER SIX

6.0 IMMUNITY/CROSS IMMUNITY STUDIES BETWEEN SAVANNA AND FOREST ISOLATES OF *Cowdria ruminantium*

6.1 Summary

The savanna and forest isolates of *C. ruminantium* obtained previously (Chapter 5) were used to immunize some savanna brown goats by infection and treatment method. Lack of reaction to homologous challenge three weeks after the primary infection confirmed that the animals were immune. The immunized animals were cross challenged. It was observed that the Akure isolate cross protected animals against the Shika isolate and vice versa. However, an animal immunized to Akure isolate reacted and died when cross challenged with the Shika isolate. Shika isolate proved to be more pathogenic of the two isolates.

6.2 Introduction

According to Alexander (1931) and Uilenberg (1983) there is no antigenic difference among *C. ruminantium* isolates from different geographical areas. The belief changed when the mouse infective isolate, the Kumm strain, was isolated (Du Plessis and Kumm, 1971).

Kumm strain is highly pathogenic to sheep but not to cattle. Jongejan *et al.* (1988) also observed that the South African vaccine strain Ball 3 did not fully protect against an isolate from Senegal. Ten different stocks of *C. ruminantium* were tested, eight from South Africa, one from Mali and one from Comoro islands (Du Plessis *et al;* 1989). It was observed that only two (Ball 3 and Gemishuy) showed antigenic similarities of the eight South African stocks tested. In the Caribbean, isolates from Antigua and Guadeloupe differed when their MAPI gene sequence were compared (Reddy *et al;* 1996). Antigenic similarity has been reported with Nigerian isolates of *C. ruminantium* although, limited to northern parts (Audu *et al;* 1995; EL-Imairi, 1991). Isolates from the Southern and Northern parts have not been studied. Also cross protection studies between widely separated vegetational zones such as the Savanna and forest zones have not been reported. This is necessary because of likelihood of future control strategies involving the use of vaccine. The studies reported here are therefore conducted to assess the immunity/cross immunity abilities of *C. ruminantium* isolates from the forest and savanna zones of Nigeria.

6.3 Materials and Methods

Twelve savanna brown goats aged between 10 months and 1 year were purchased from a nearby market. Pre-infection screening and

feeding were as described in chapter 4. The animals were pre-conditioned for three weeks before use. They were divided at random into three groups of 4 animals per group. Eight animals were infected while four identified as (118, 5258, NNH, 5259) were left as uninfected controls. Four of the infected group identified as (5252, 5253, 5254, 5257) were infected intravenously with 2 ml of cryo-preserved stabilate of the savanna (Shika) isolate while four (5256, 5255, 0271, NNM) were similarly infected with the forest (Akure) isolate of C. *ruminantium*. Rectal temperature of the animals were monitored early in the morning on a daily basis. Three weeks after primary infection, the animals were challenged using homologous stabilates. Blood samples were taken twice a week and during reaction time for while blood cell count.

Animals 5252 and 5257 were heterologously challenged with 2 ml of cryo-preserved ground up tick stabilate from Akure and 5255 and 0271 were inoculated with Shika isolate three weeks after the secondary challenge. Physical inspection were done and the rectal temperatures monitored. Blood samples were taken twice a week for white blood cell count.

6.3 Results

The results of the experiments are presented in table 9.

Table 9: Immunity and cross immunity experiments between savanna and forest isolates of *Cowdria ruminantium* in savanna brown goats

		Primary infection Febrile reaction				Homologous challenge Febrile reaction				Heterologous Febrile reaction				
Animal No.	Isolate	Day of onset	Duration (days)	Maximum °C	Treatment	Day of onset	Duration (days)	Maximum °C	Treatment	Day of onset	Duration (days)	Maximum °C	Treatment	Remarks
5252	Shika	4	3	40.8	None	None	None	38.6	None	None	None	None	None	Survived
5253	Shika	3	2	40.0	None	-	-	-	-	-	-	-	-	Died 21 days P.
5254	Shika	2	2	40.0	None	None	None	38.9	None	-	-	-	-	Died 27 days P.
5257	Shika	3	2	40.5	None	None	None	38.7	None	None	None	None	None	Survived
5255	Akure	18	4	40.2	None	None	None	39.1	None	None	None	38.8	None	Survived
5256	Akure	13	3	40.5	-	-	-	-	-	-	-	-	-	Sacrificed
0271	Akure	19	1	39.6	None	None	None	38.7	None	None	None	39.0	None	Died overnight brain +ve
NNII	Akure	None	None	38.5	None	None	None	38.8	None	None	None	-	-	Survived
118	None	None	None	38.6	None	None	-	-	-	-	-	-	-	Control
NNM	None	None	None	39.0	-	-	-	-	-	-	-	-	-	Control
5258	None	-	-	39.0	-	-	-	-	-	-	-	-	-	Control
5259	None	-	-	39.0	-	-	-	-	-	-	-	-	-	Control

The animals inoculated with the savanna isolate reacted although at different times (Table 9; Fig. 7). Febrile reactions started in 5254, 2 days post inoculation (pi) while animals 5257 and 5252 reacted at 3 and 4 days pi respectively. The maximum rectal temperatures attained ranged from 40°C in 5254 to 40.8°C in 5257 and 5254 respectively. Two animals 5252 and 5257 were treated using terramycin long acting at 10mg/kg i/m while 5254 was not treated. Treatments were instituted on the second day of fever. None of the untreated animals survived beyond the fourth week of primary infection. Animal No. 5253 died 21 days pi.

Apart from the short incubation period, other clinical parameters observed in animal 5254 were either transient or mild. A maximum rectal temperature attained within 24 hours or reaction fell to 39.8°C in the next 24 hours and by the third day of thermal reaction, the temperature had fallen to pre-infection range of 38°C to 39.0°C. The main sign of reaction observed apart from pyrexia was weakness. There was no thermal reaction following the homologous challenge. However, a week later, a progressive loss of weight and stamina was noticed. The animal would fall when driven and rose with difficulty and in some cases had to be assisted to stand. Surprisingly however, the appetite was good and would continue to eat even on recumbency. The animal became emaciated

inspite of the good appetite. The animal died four weeks after the homologous challenge.

The gross pathology following the post mortem examination included the hyperaemia of the intestinal mucosa. The spleen was slightly enlarged and there was bilateral congestion of the lungs. The pericardial sac contained straw-coloured fluid measuring 3 ml. Giemsa stained brain squash examination revealed granules of *C. ruminantium* in the capillaries. The colonies were scanty and most of them showed abnormal features of poor delineation of individual granules. Some colonies were seen outside the capillaries and appeared encampsulated.

The treated animal survived the experiments. The temperature of animals Nos. 5252 and 5257 returned to pre-infection range the second day of treatment. There was no thermal response following homologous challenges (Table 9).

Of the animals infected with the forest (Akure) isolate (Table 9; Fig. 6), No. 5255 reacted 18 days pi. The duration of febrile reaction was 4 days and the maximum temperature was 40.2°C at the second day of febrile reaction. Weakness and slight diarrhoea were the accompanying

symptoms. The temperature returned to pre-infection values after five days of febrile response without chemotherapy.

Animal (0271) which was also infected with the Akure isolate showed thermala response at 19 days pi, with temperature of 39.6°C. There was no reaction following secondary (homologous) infection and the animal was apparently normal. The temperature of NNH, remained within the range of pre-infection values and those of the control animals (Fig. 6).

There was no reaction following the heterologous challenges in all the animals inoculated (Table 9; Fig. 8). However, more than month after the heterologous challenge, animal No. 0271 collapsed and died within an hour. The post mortem examination of the carcass showed no abnormal gross pathology in all the organs. However, scanty small sized colonies resembling colonies of Shika isolate were seen in the capillary endothelial cells when the Giemsa stained squash smears were examined.

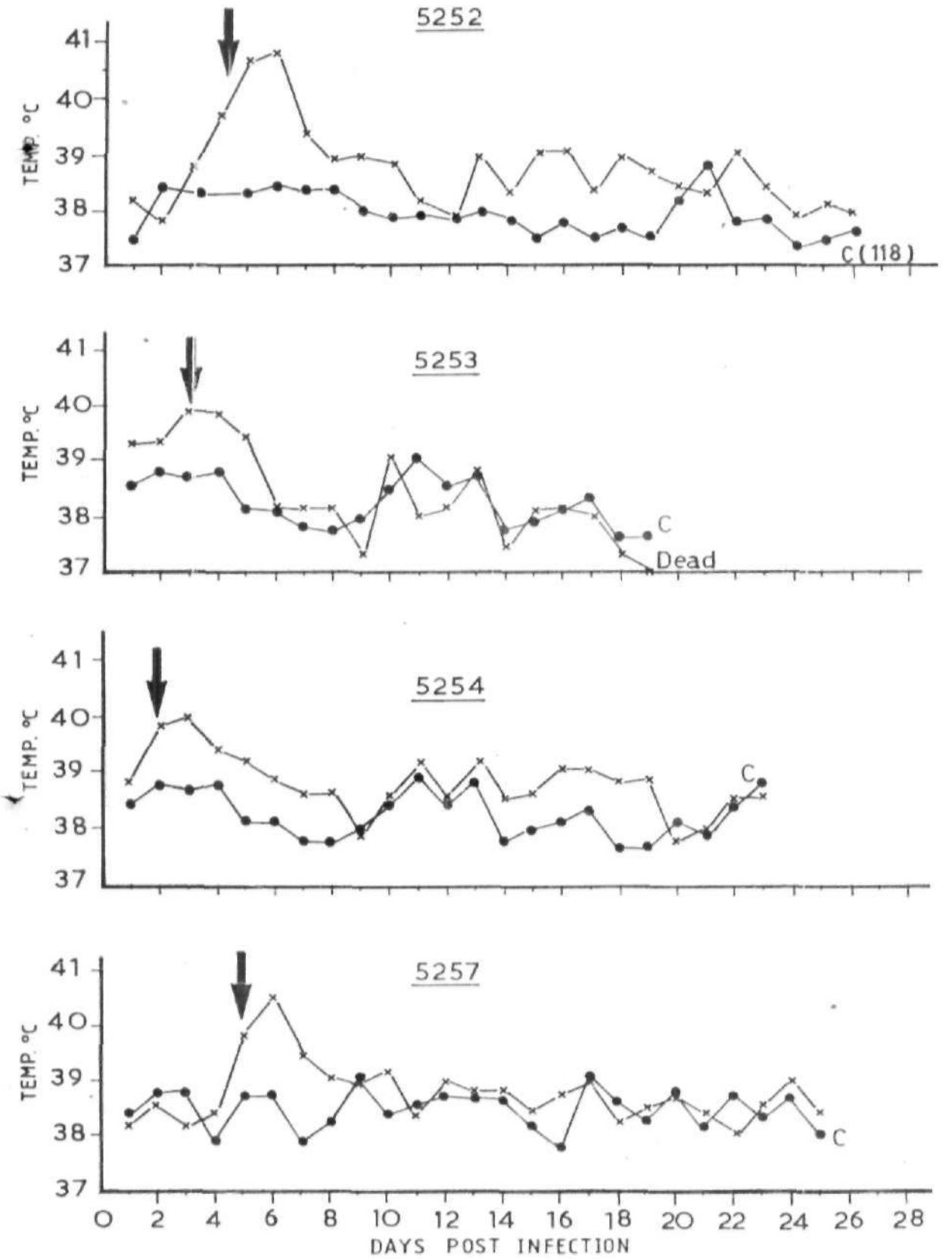


Fig. 6 Temperature profile of Savanna brown goats infected with Savanna isolate of Cowdria ruminantium (C = Control) (Arrow shows point of reaction).

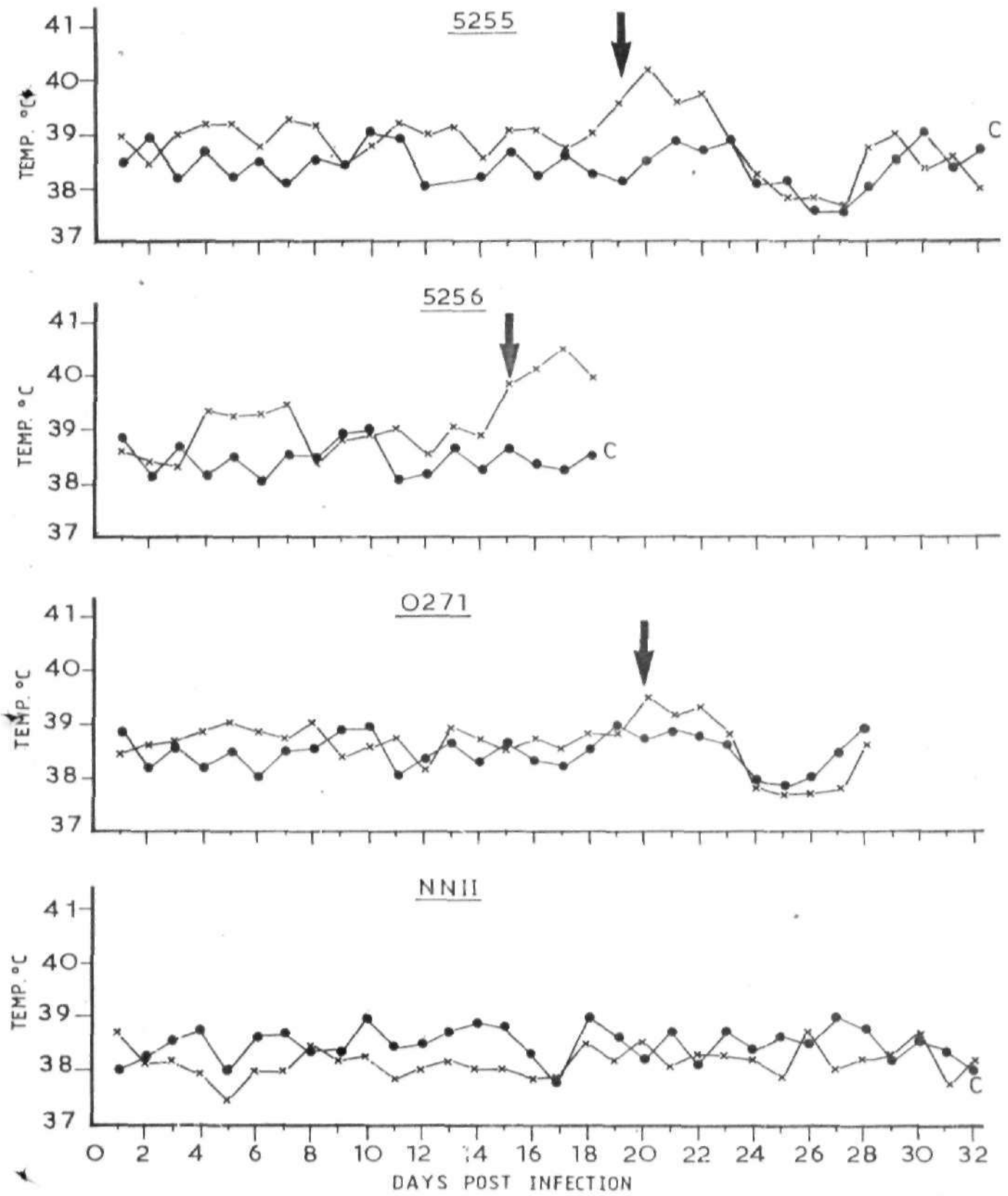


Fig. 7. Temperature profile of Savanna brown goats infected with Forest isolate of Cowdria ruminantium (C = Control),(Arrow shows point of reaction).

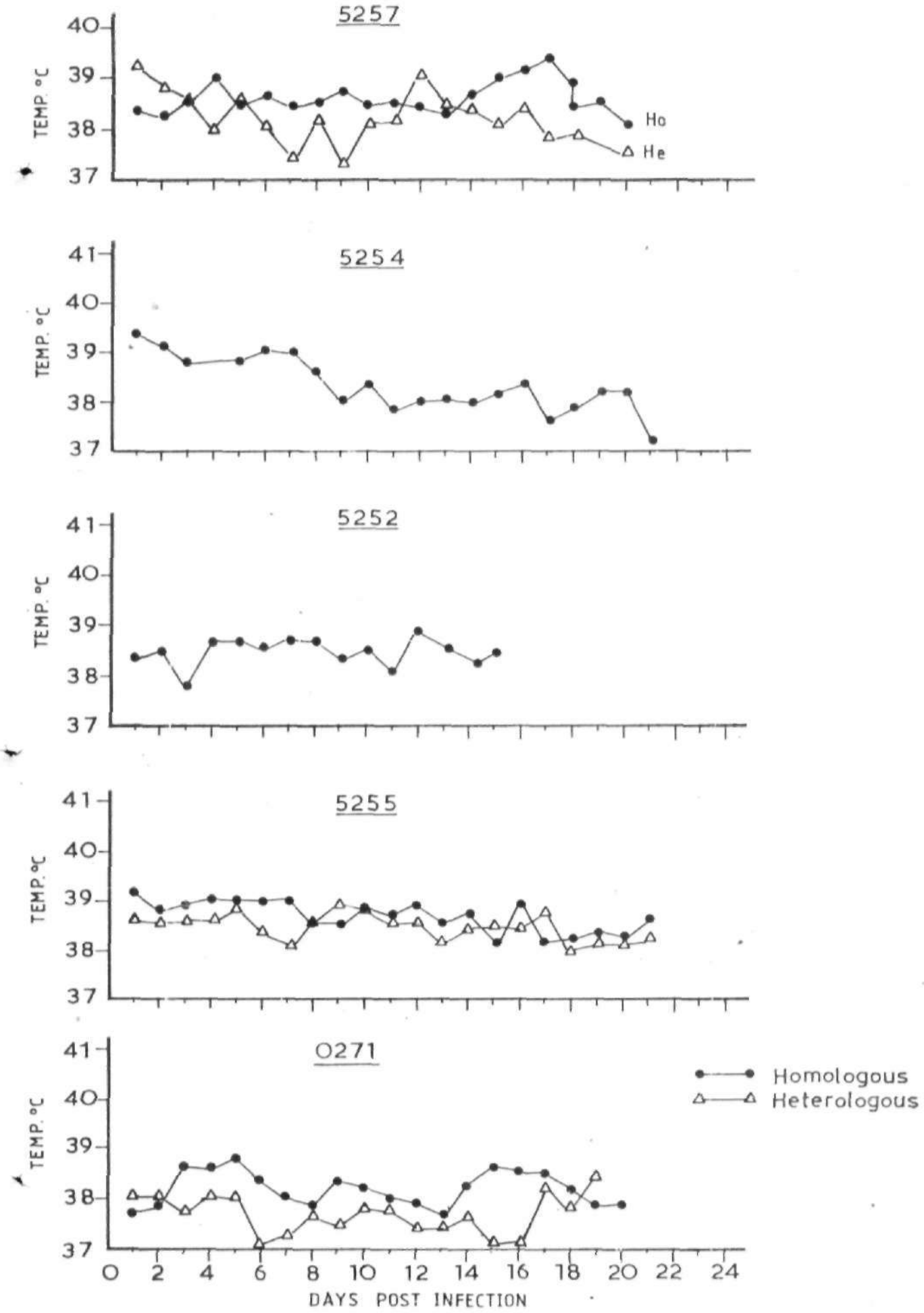


Fig. 8. Temperature pattern following Homologous and Heterogous challenges of Savanna isolate infected goats (5257, 5254, 5252) and Forest isolate infected goats (5255, 271).

6.5 **Discussion**

The savanna isolate from Shika appears to be more virulent than the forest isolate as observed in this study. Although, the febrile reactions elicited by the savanna isolate were curtailed in the untreated animals, the animals eventually died of the infection. An outstanding observation was the very short incubation period (ip) recorded with infection of the savanna isolate. This is one of the shortest (2 days) incubation period ever recorded during experimental infection. Using a Senegalese isolate, Gueye and Vassilides (1985), also recorded a short incubation period of 3 days during experimental infection. The much studied D225 (Hemobade, 1976) isolated from a research farm adjacent to the sedentary farm where the isolate used in the present study originated, gave an incubation period of a 4 to 12 days when infection was done with whole blood.

However, the D225 isolate gave ip of 8 to 17 days and 10 to 16 days when infections were done by lung macrophages and tick bite respectively (Hemobade, 1976). Audu *et al.* (1995) recorded incubation period of 8 to 22 days in savanna goats intravenously infected with blood stabilate (R64) also isolated from the same research farm in Shika.

Although the variations in the ages of the experimental animals and the concentration of the infective materials used in the different studies might contribute to the discrepancy in the incubation periods observed, the presence of different immunologic strains of different virulence cannot be ruled out. This could be an important epidemiological factor as animals have to be challenged by several infections to be able to develop solid immunity (Hilenberg, 1983).

Available records showed that the Shika isolate (D225, Hemobade, 1976, CR 64, Audu et al., 1995) and CR 6 of the present study produced acute infections in experimentally infected goats irrespective of their ages. Such animals if not treated usually die between 11 and 27 days of infection if they are less than 6 months (Audu *et al;* 1995) and between 3 to 6 days if they are older (Hemobade, 1976). Lack of terminal nervous signs in untreated animals reported in these studies agrees with the findings of Audu *et al.* (1995). This may be due to the relatively young age of the goat kids involved rathern than a non association of nervous signs with Shika isolates.

The findings of retained appetite and emaciation in terminally sick animals in this study can complicate ante-mortem diagnosis making hearwater to be confused with trypanosomosis in tsetse endemic areas.

These features have thus added to the diverse signs associated with heartwater in goats. The forest isolate produced mild reaction in savanna brown goats. Lack of febrile response in infected goats following a homologous challenge indicated that the isolate was immunogenic.

It is evident from these studies that goats which are immune to Shika isolate may not succumb to a challenge by the forest isolate, that is *Shika isolate cross protects animals against the Akure isolate*. Although, Akure protected most of the animals against Shika, one of the vaccinated animals (0271) was susceptible to Shika isolate. From the foregoing therefore, there are variations in clinical signs and characteristics of the colonies between the savanna and the forest isolates. The forest isolate *was only able to give animal 0271 partial protection against the* savanna isolate by the absence of febrile reaction. However, the organism succeeded in establishing colonies in the brain of the goat which eventually died. It is difficult to speculate that the two isolates (Akure and Shika) differ antigenically because only one animal was involved.

However, the various clinical pictures reported by various workers using different isolates from Shika (Hemobade, 1976; Audu *et al;* 1995; Ulayi *et al;* 1992) may suggest existence of various antigenic types in

Shika farms. Hemobade (1976) noticed that isolates D225 and D734 from Shika produced different clinical signs in savanna goats. While the former produced acute infection, the latter is associated with the subacute form of the disease. It is yet to be confirmed whether these were caused by variations in susceptibility or antigenicity.

Already natural diversity of *Cowdria* has been shown to exist by the identification of antigenically related but different strains such as the Kwanyanga, Kumm, Gardel and Mali strains (Logan *et al;* 1986). According to Williams and Vodkins (1987) *Cowdria* and *Ehrlichia* are the least understood of all the Rickettsia.

CHAPTER SEVEN

7.0 STUDIES ON THE CHARACTERISTICS OF *Cowdria ruminantium* ISOLATES FROM THE SAVANNA AND FOREST ZONES OF NIGERIA

7.1 Summary

The characteristics of the three isolates of *Cowdria ruminantium* Shika, Jaji isolates (Savanna zone) and Akure isolate (Forest zone) were compared. These were isolates used in the previous studies to prepare crude vaccine and immunize some experimental animals (Chapter 4 to 6). Their characteristics as regards morphology in Giemsa stained brain squash smears, clinical signs of goats to experimental infections and murinotropism (that is infectivity/pathogenicity in mice, rats and Guinea pigs).

Incubation periods of infection in savanna goats varied between 2-4 days, 9-14 days and 13-19 days for Shika, Jaji and Akure isolates respectively. The shapes of *Cowdria* colonies in the brain capillaries also varied. Jaji isolate produces coarse, coccoid and at times sand-like granules with the capillaries moderately colonized. Shika isolate produced small colonies which were scanty. While Akure isolate was

associated with large colonies of uniform sized granules. None of the isolates was infective for laboratory animals.

7.2 *Introduction*

Isolates of *C. ruminantium* vary in many respects. The level of colonization of brain capillaries also vary with isolates (Anon, 1989). In South Africa, differences in the characteristics of isolates from even the same locality had been reported (Du Plessis and Kumm, 1971; Mackenzie and McHardy, 1984). The Kumm isolate for instance has more affinity for peritoneal macrophages than the endothelial cells (Du Plessis and Kumm, 1971). The Kwanyanga stock produces mild infection and is not associated with nervous signs (Wassink *et al;* 1986) but causes high mortality in sheep (MacKenzie and Van Rooyen, 1981). The characteristics of the three isolates used in this study were therefore examined and compared especially as regards their infectivity for goats, laboratory animals and their morphology in Giemsa stained smears. It is assumed that the information from these studies will contribute to the understanding of our local isolates of which not much is known.

7.3 Materials and Methods

The origin of the *C. ruminantium* isolates.

Jaji isolate (Anon, 1989) was isolated from a clinically sick 6-month old

calf with nervous signs in a sedentary herd in Jaji, Kaduna state. Five millilitre of blood from the calf initiated heartwater in a goat kid which later died and the brain was positive for *Cowdria* granules. The origin and isolation of Shika and Akure isolates had been described previously in Chapter 5. Detailed examinations of the Giemsa stained brain squash smears of animals that died of experimental infections (Chapters 4, 5, 6) were done. Laboratory animals (Guinea pigs, rats and mice) were intravenously infected with 0.2 ml each of Savanna and forest isolates (0.1 ml was given intraperitoneally and 0.1 ml intravenously).

Three Guinea pigs were also infected with 0.2 ml of the Jaji stock. Rectal temperatures and visual inspections were done daily. The animals were sacrificed 2 weeks post infection. Homogenates of the liver and lungs of the sacrificed animals were prepared by crushing the organs in mortal and pestle using cold PBS (pH 7.4) as diluent. The homogenized materials were allowed to settle at room temperature and the supernatant drawn out and mixed with the blood. Two mls was injected into a parasite - free goat and monitored for two weeks. The goat was challenged with virulent stabilate two weeks after inoculation of the homogenized material if there was no reaction to confirm susceptibility of the animal.

7.3 **Results**

The three isolates showed differing characteristics. Incubation periods varied between 2-4 days, 9-14 days and 13-19 days for Shika, Jaji and Akure isolates respectively. Fever lasted longer (3-6 days) in animals infected with Jaji isolate reaching a maximum of 41°C within three days of reaction (Table 10). Maximum attainable temperatures were usually less than 41°C in Shika and Akure isolates infected animals and pyrexia lasted between 1 to 3 days.

The shapes and sizes of the colonies and individual granules varied among the isolates. The Jaji isolate has coarse, coccoid and a times sand-like granules. Brain capillaries are usually moderately colonized (Plate V). Colonies associated with the Shika isolates are usually very scanty in the brain capillaries of the infected animals. They are compact and individual granules may not be discernible (Plate VI). Some of the colonies appeared encampsulated. Few extracapillary colonies were observed. The forest (Akure) isolate on the other hand showed very extensive colonies which seemed to run across the entire microscopic field (Plate VII). The granules of such colonies are uniform in size and exhibit homogenous staining characteristics.



Plate V Colonies of *C. ruminantium* (Jaji isolate) in Giemsa stained brain smear. The big colony in "A" has large and conspicous granules. Three colonies (arrowed) can be found within the same locality. In "B" the colony (arrowed) has granules of small size

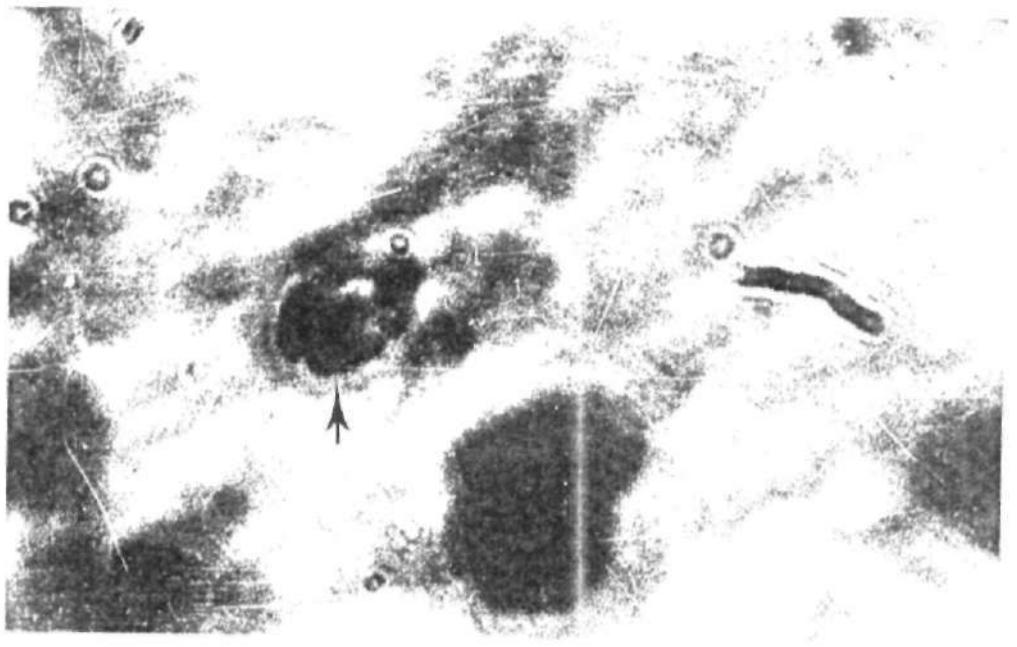


Plate VI Shika isolate of *C. ruminantium* (arrowed) in Giemsa stained brain smear. The colony is roundish, appears encapsulated and located slightly outside the capillary.



Plate VII *Cowdria ruminantium* colonies (Akure isolate) in Giemsa stained brain squash smears. In "A" the colony is located outside the brain capillary (C). In "B" the colonies are large and runs across the entire microscopic field as shown by the two arrows. The size of the granules and colonies vary.

Table 10: Characteristics of the *Cowdria ruminantium* isolates used for the studies

	Savanna isolates Jaji	Savanna isolates Shika	Forest isolates Akure
Origin Isolates	Blood from reacting Fulani calf inoculated into a goat (Anon. 1989)	Wild ground up tick supernatant inoculated into a goat	Ground up wild ticks sedentary herd in Akure
Incubation periods (days)	9 - 14	2 - 4	13 - 19
Maximum temperature (°C)	41 - 42	40.8	40.5
Duration of fever (days)	3 - 6	2 - 3	1 - 3
Nature of disease	Acute	Acute	Subacute
Course of disease	Classical acute signs in mature dogs died with 6 days if not treated	Goat kids show resistance but death results if untreated within 2 to 3 weeks of infection. Recovery occurs if treated.	Mild infection main clinical the sign is pyrexia, the temperature returning to normal
Main gross pathology	Hyperaemia of intestinal mucosa, hydropericardium, hydrothorax	Mild hydropericardium	Mild hydropericardium
Characteristics of colonies	Various sizes of colonies and granules which are mainly sand like and some course. The colonies are relatively many.	Scanty colonies	Very extensive colonies of different sizes and shapes
Infectivity for laboratory animals	Not infective for rats, mice and guinea pigs	Not infective for rats	Not infective for rats
Immunity	Immunity is usually confered following primary infection	DO	DO
Cross immunity	Not done	Goat immuned to Shika isolates are usually cross protected to Akure isolates	Some goats immuned to Akure are also immuned to Shika. However, one goat immuned to Akure was susceptible to Shika

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None of the infected laboratory animals reacted. Homogenates of tissues and organs prepared from them did not initiate any reaction in the recepient goats which reacted when challenged by virulent organism.

7.5 Discussion

The differing morphological characteristics of *Cowdria ruminantium* isolates including those that are from the same environment may contribute to some of the differences in the behaviour of the parasite such as variations in the ability to grow in culture (Yunker *et al:* 1988), non amenable to attenuation by several isolates (Jongejan *et al:* 1993c) and also variations in the course of disease produced in susceptible animals (Hemobade, 1976).

Although, existence of different morphological types have been extensively studied in vertebrate hosts (Cowdry, 1925a; Pienaar, 1970; Hemobade, 1976); in ticks (Kocan *et al:* 1986), and in cutlure (Prozesky *et al:* 1993), no detailed investigation has been conducted to find out if this can be linked to the behaviour of the parasites. Possibly some of the field isolates may be a composition of different strains. However such assumption needs to be confirmed probably by detailed studies using cloned parasites.

Several isolates have been made at different periods by our Department from the savanna vegetational zone. Isolates D225 and D734 (Ilemobade, 1976); CR 64, (Anon, 1989); CR 6 of the present study are from the same environment but they all seem to possess differing characteristics. However, the Jaji isolate (Anon, 1989) and Basawa isolate (Okaiyeto, 1995) which were isolated from sedentary zebu cattle located 60km apart, exhibit some similarities in terms of length of incubation periods, type of colonies found in brain and course of the disease. Although none of these isolates have been cultivated for detailed studies, in vivo studies done so far including the present studies, are suggestive that solid immunity through vaccination may be difficult to attain using only one local isolate. This seems to collaborate the studies of Du Plessis *et al.* (1989).

The forest (Akure) isolate shares some features with the savanna (Jaji isolate) in terms of the average length of incubation period, characteristics of colonies in Giemsa stained smears, although a milder form of the disease is produced by the Akure isolate. The reason for the mildness of the isolate in susceptible animals even in mature goats used cannot be easily explained. In the same vein, the high pathogenicity of Shika isolate despite the poor colonization of the brain cannot be

elucidated. Probably, pathogenicity of these isolates in susceptible animals depends on other factors possibly toxin production (Uilenberg, 1983) rather than the level of brain colonization.

The search for murinoptropic isolates of *C. ruminantium* has led to the discoveries of the Kumm, Kwanyanga, Nonile and Welgevonden strains that produced lethal infections in laboratory animals (Meckenzie and McHardy, 1984). Presently, no *Cowdria* isolates outside South Africa are known to be pathogenic to laboratory animals as those listed above, although, some isolates Senegal and Zeerust can be passaged through mice and still retain their infectivity for ruminants (Uilenberg, 1983). The cost and inconveniences associated with the use of domestic ruminants for *Cowdria* research will be minimised in Nigeria when local murinotropic isolates are discovered.

CHAPTER EIGHT

8.0 DIFFERENTIAL LEUCOCYTE COUNTS OF SAVANNA BROWN GOATS INFECTED WITH THE SAVANNA AND FOREST ISOLATES OF *Cowdria ruminantium*

8.1 Summary

The differential leucocyte counts of savanna brown goats (Nos. 5252, 5253, 5254, 5257) infected with the savanna isolate of *Cowdria ruminantium* and forest isolate (Nos. 5255, 5256, 0271) were monitored. Blood samples were collected in bottles containing EDTA (1 mg/ml). Thin blood smears were prepared stained with Giemsa and different leucocytes counted using standard procedures. The forest isolate infected animals showed slight increase in the percentage neutrophil but the lymphocytes showed wide variations. The Shika isolate infected animals showed a decrease in the percentage neutrophils while the lymphocyte level increased. The monocytes remain within the normal range while there is absence of eosinophils especially during pyrexia.

8.2 *Introduction*

The response of circulating white blood cells (WBC) during the course of heartwater in susceptible animals has been extensively studied

(Ilemobade and Blotkamp, 1978; Camus and Barre, 1982; Van Amstel *et al;* 1985; Okaiyeto, 1995). There has been no general agreement as regards the values of the total and differential leucocyte counts in heartwater infected animals (Uilenberg, 1983). Ilemobade and Blotkamp (1987a) and Camus and Barre (1982) reported an increase in lymphocytes in heartwater infected goats. However, Van Amstel *et al.* (1985) reported a decrease. Some haematological changes have been considered in the ante-mortem diagnosis of heartwater. Since some of the clinical parameters that are usually observed in heartwater are influenced by the characters of the isolate, this study is therefore meant to monitor the different leucocyte profiles in savanna goats especially those infected with the forest isolate of which there has been no previous report.

8.3 Materials and Methods

The source, handling and infection of the experimental animals used in this study had been reported in chapters (5 and 6). Animals identified as 5252, 5253, 5254 and 5257 were infected with Shika isolate while 027, 5255, 5256 were infected with the forest isolate.

Five ml of blood samples were taken through the jugular vein twice a week and during reaction to infection. They were collected in

bottles containing EDTA (1 mg/ml). Thin blood smears were prepared, stained with Giemsa and different leucocytes counted under oil immersion objectives using standard procedure as described by Schalm *et al* (1975).

8.4 Results

There is no much variation in the percentage differential leucocyte counts before, during and after reaction in forest isolate infected animals. However, the percentage neutrophils increased slightly in almost all the animals except in 0271 where it decreased during the transient pyrexia. The percentage lymphocytes showed a wide variation being unaltered during reaction in 5255, decreased slightly in 5256 and increased slightly in 0271 (Fig. 9).

The Shika isolate infected animals showed a decrease in the percentage of circulating neutrophils during pyrexia while the lymphocytes level increased (Fig. 10). However, the mean pre- and post-infection values did not vary much because soon after the pyrexia, the neutrophils and lymphocytes values returned to pre-infection range. The eosinophils were not found during reaction in all the reacting animals irrespective of the type of isolates used. The monocytes also remained within the normal range.

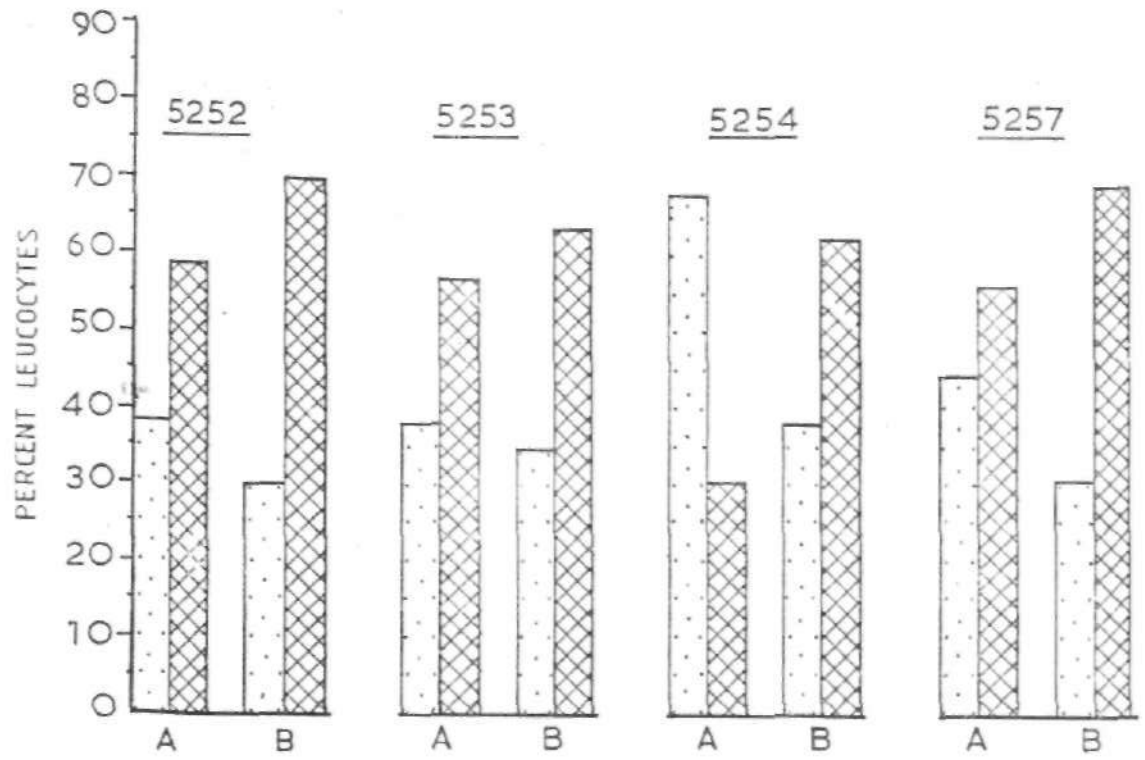


Fig. 9. Mean differential Neutrophil and lymphocyte counts of Savanna brown goats experimentally infected with Savanna isolate of Cowdria ruminantium.

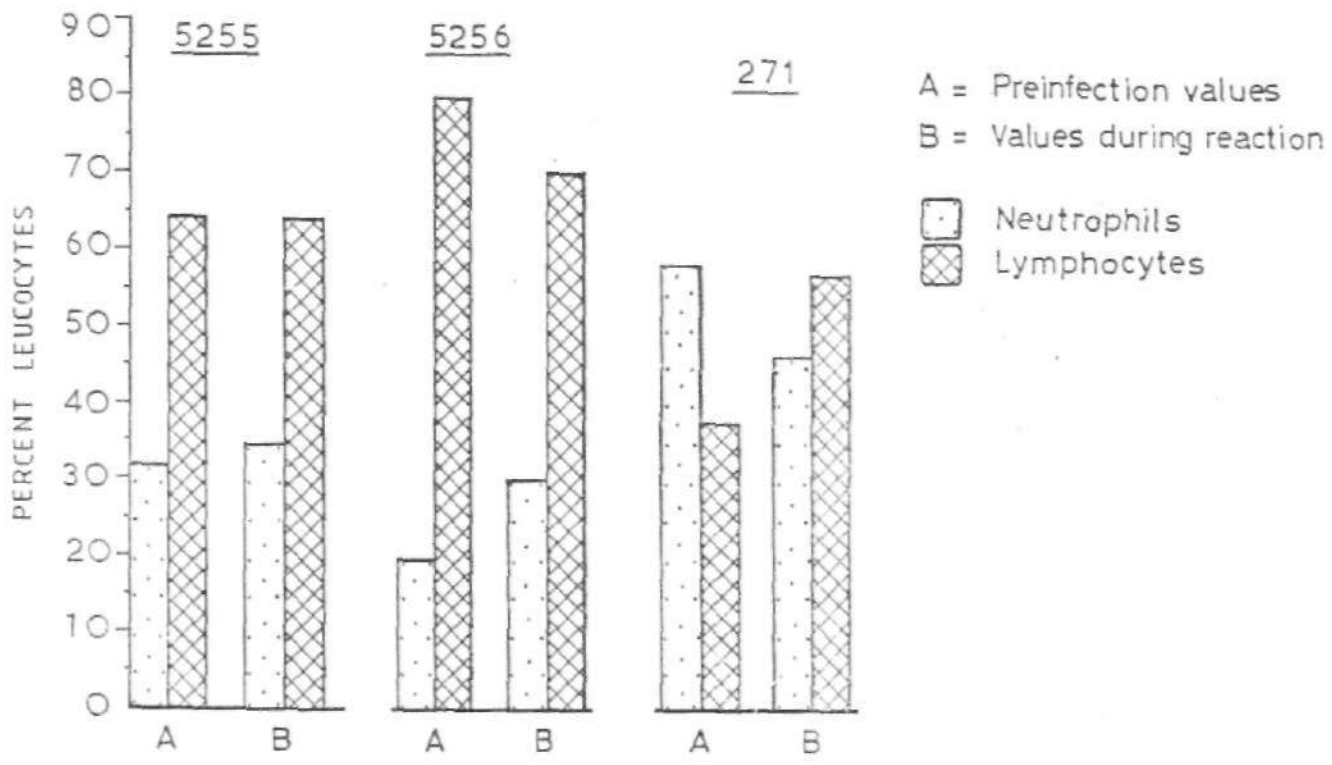


Fig.10. Mean differential Neutrophil and lymphocyte counts of Savanna brown goats experimentally infected with Forest isolate of Cowdria ruminantium.

8.3 Discussion

The increase in the circulating lymphocytes in the reacting goats infected with the savanna isolate is consistent with the findings of Ilemobade and Blotkamp (1978b) using the D225 savanna isolate. Although fewer animals were used in this study, a decrease in the neutrophil counts was observed, as against an increase by the above authors. While the observations of Ilemobade and Blotkamp (1978b) agreed with those of Camus and Barre (1982) using isolates from Guadeloupe, our own findings are consistent with those of Van Amstel *et al.* (1985) using a South African isolate.

Several factors may be responsible for the discrepancies observed such as type of isolate used, the age, breed, the immune status, susceptibility of the animals and length of confinement or other stress factors. These factors undoubtedly differed among all the authors and variations are therefore expected.

However, eosinopenia is a common factor in all the studies by the various authors. This has been attributed to stress factor (Ilemobade and Blotkamp, 1978b) which are usually imposed on the animals during the course of the experiments. Monocyte counts were found to remain

unaltered (Van Amstel *et al:* 1985b). Similar observation was made in the present study. Okaiyeto (1995) noticed a steady fluctuation in total and differential cell counts and reported that the neutrophil and monocyte count were not statistically significant in *Cowdria* infected West African Dwarf (WAD) goats. No alteration was also reported in total or differential counts in sheep infected with *C. ruminantium* (Clark, 1962).

Van Amstel *et al.* (1994) suggested that the depression of bone marrow activities could be responsible for some of the observed picture in the circulating white blood cells seen in heartwater infection. They also observed that severe stress could cause the release of endogenous corticosteroids into the circulation leading to the disappearance or reduction of some leucocytes.

CHAPTER NINE

GENERAL DISCUSSION

As part of the methodology for preparing tick-derived vaccine, laboratory colony of larvae of *Amblyomma variegatum* was established from engorged female ticks collected from resident cattle around Zaria. Absence of rickettsial bodies from the hemolymph, larval and egg squash smears was used to ascertain their clealiness from *C. ruminantium* infection. Lack of reaction from representative samples of the supernatant of the crushed larvae intravenously inoculated into a susceptible goat was used as an additional measure to confirm the clealiness of the tick. Absence of *C. ruminantium* from the larval progenies of the engorged female ticks supports the reports of several authors that *C. ruminantium* is usually not transovarialiy transmitted (Ilemobade, 1976; Uilenberg, 1983). The isolated report of Bezuidenhout and Jacobsz (1986) of transovarial transmission of *C. ruminantium* by *Amblyomma* species has therefore ever remains an exception than the rule. Studying the life cycle of *A. variegatum* in the laboratory, Ilemobade (1976) established specific moulting periods for the various immature stages of the ticks, although the observations made in the present studies conform with his findings, it was also noticed that oviposition and hatchibility of eggs were affected by the level of feeding of the female *A. variegatum* prior to incubation in

addition to temperature and relative humidity. This in turn affects the viability of the larval progeny. It was observed in this study that fully engorged female *A. variegatum* ticks are better for transmission experiments than the partially engorged one. Preparation of crude ground-up tick vaccine is not difficult. An infected nymphae can be kept in the preserving incubators of 18°C and 80% relative humidity for a long time and still retains its infectivity. This offers a good advantage in developing countries where facilities such as liquid nitrogen for keeping cryo-preserved vaccines is expensive to purchase and electricity supply not reliable enough to maintain the vaccine in frozen state. Most of the required recipes are common laboratory consumables. However, the materials have to be chilled to reduce mortality of the organism by heat generated during grinding. The side reactions precipitated by the intravenous inoculation of the vaccine is dependent on the nunmber of the ticks per the dose of the vaccine used. The higher the number of these ticks, the more intense the side reaction. It has been shown in this study that side reactions can be totally eliminated using a dose of the crude vaccine containing 0.025 to 0.03125 infected nymphae. However, the intensity of clinical reactions is not affected by the dose and reacting animals have to be covered by tetracycline to avoid mortality.

Isolation of *C. ruminantium* from Akure in this study added to the records of other workers (Zwart, 1984 unpublished data; Isoun *et al;* 1974) of the existence of the disease/parasite in the South-West zone of Nigeria. The impediment that could be created by this situation to animal production especially the use of exotic breeds to upgrade the local breeds has been documented (Isoun *et al;* 1974). Although there are no available reports as regards the pathogenicity of the southern isolates to indigenous ruminants, it has been shown that the Akure (forest) isolate used in this study produced mild reactions in savanna brown goats despite the extensive colonization of brain capillaries.

A savanna isolate (Shika isolate) obtained from Shika - Zaria produced very virulent reactions in savanna goats. An incubation period as short as 2 days was recorded - being probably the shortest incubation period recorded in in vivo inoculation in Nigeria. The isolate was pathogenic to young and adult goats, killing them in two weeks if they are not treated. It was noted that the parasites were scanty in the brain capillaries. The mildness of the Akure isolate despite its extensive brain invasion and the high virulence of the Shika isolate even though they are scanty in the brain, suggests that probably physiological mechanisms other than the brain damage (Clark 1962) may play a dorminant role in the

pathogenicity of *C. ruminantium*. Implication of toxins (Brown, 1990; Van Amstel *et al;* 1994) in the pathogenesis of heartwater which has been widely articulated of recent may be the predominant factor in the case of Shika isolate. If toxin is involved, its production may probably vary among isolates of *C. ruminantium*.

The *Cowdria* isolates obtained and used in this study were immunogenic and cross protective although the Akure isolate could not completely protect one of the animals against the highly virulent Shika isolate. Characterisation of isolates from different parts of the country may therefore be necessary to select a vaccine strain that has wider protection spectrum.

Comparative studies have revealed that Nigeria isolates of *Cowdria ruminantium* differ in respect of their origin. Such differences could create immunologic differences and thwart immunization efforts.

Attempts made in this study to obtain mouse-infective isolate of *C. ruminantium* failed. Further efforts should be made as the discovery of such strains will facilitate heartwater research in Nigeria.

Studies on the differential white blood counts of the infected animals could not produce a defined pattern as regards the level of the various circulating white blood cells. This agrees with the reports of Uilenberg (1983) that there is no clear pattern as regards the level of leucocytes in heartwater infected animals.

There is still a wide gap as regards our knowledge on heartwater and its vectors in Nigeria. Epidemiological studies and characterisation of *C. ruminantium* isolates from different parts of Nigeria are necessary. Findings from this study and reports from other workers show that isolates of *C. ruminantium* behave differently even from the same locality. Detailed studies on the local isolates will be more effective when the parasites are cultured.

RECOMMENDATIONS

The following recommendations are made for further investigations to enhance our understanding of heartwater and its causative agent in Nigeria.

1) Characterisation of isolates of *C. ruminantium* in Nigeria needs to be further pursued to enable us select a vaccine strain.

2) In vitro cultivation of our local isolates is needed especially the pathogenic Shika isolate and possible attempts at its attenuation, inactivation and characterisation.

3) Comparative studies of our local *Amblyomma species* especially their effectiveness in transmitting *C. ruminantium* from different ecological zones in Nigeria.

4) Detailed studies of *Amblyomma* species is also recommended. Probably some *Amblyomma species* other than *A. variegatum* may be encountered. This may contribute to the understanding of the epidemiology of the disease

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APPENDIX i

Calculation of the number of Nymphae per dose of the experimental vaccine.

No. of infected nymphae used = 220

Quantity of supernatant obtained = 54 mls

No. of nymphae per ml of supernatant = $\frac{220}{54}$ N

= 4 Nymphae/ml

Concentrated/Undiluted Vaccine:

Thus a ml of concentrated/undiluted vaccine

Contained 4 nymph = 4N/ml

2 mls contains = 8 nymph

Diluted Vaccine/Stabilate:

$\frac{1}{40} = \frac{4 \text{ n/ml}}{40} = \frac{1}{10} =$ 0.1 Nml

$\frac{1}{80} = \frac{4}{80} = \frac{1}{20} =$ 0.05N/ml

$\frac{1}{160} = \frac{4}{160} = \frac{1}{40} =$ 0.025N/ml

$\frac{1}{320} = \frac{4}{320} = \frac{1}{80} =$ 0.0125N/ml

$\frac{1}{640} = \frac{4}{640} = \frac{1}{160} =$ 0.00625N/ml

Appendix ii

Differential leucocyte counts of goat No. 5252 prior to infection

Days	Neutrophil (%)		Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
	Band	Mature			
1	0	37	60	3	0
2	0	40	58	2	0
3	0	38	59	2	1
4	1	40	57	2	0
5	0	37	62	1	0
6	1	42	56	0	1
Mean	0.33	39	58.70	1.70	0.33

Appendix iii

Differential leucocyte counts of goat No. 5252 experimentally infected with savanna (Shika) isolate

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	47	53	0	0
2	1	49	50	0	0
3*	0	30	70	0	0
4	0	40	59	1	0
5	0	42	58	0	0
6	1	42	56	0	1
7	0	45	55	0	0
8	0	39	59	1	1
9	1	40	56	2	1
10	0	45	54	1	0
11	1	40	57	2	0
12	0	44	55	1	0
Mean	0.33	41.92	56.83	0.67	0.25

* = Period of reaction (Pyrexia) to infection

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Appendix iv

Differential leucocyte counts of goat No. 5253 prior to infection

Days	Neutrophils (%)		Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
	Band	Mature			
1	0	35	58	0	7
2	0	45	53	1	1
3	0	39	56	1	4
4	0	40	56	2	2
5	0	40	52	2	6
6	0	35	60	1	4
7	0	43	57	0	0
Mean	0	40	56	0.86	3.43

Appendix v

Differential leucocyte count of
goat No. 5253 experimentally
infected with the Shika isolate *C. ruminantium*

Days	Neutrophils (%)		Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
	Band	Mature			
1	0	40	59	1	0
2*	0	38	60	2	0
3*	2	30	66	2	0
4	2	54	42	0	2
5	0	50	45	3	2
6	0	54	45	0	1
Mean	0.67	44.30	52.8	1.390	0.63

*, = Period of reaction (Pyrexia) to infection

Appendix vi

Differential leucocyte counts of goat No. 5254 prior to infection

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	46	49	1	4
2	0	48	50	2	0
3	0	67	33	0	0
4	0	50	48	2	0
5	0	60	40	0	0
6	0	58	41	1	0
7	0	60	38	2	0
Mean	0	55.57	49.83	1.13	0.57

Appendix vii

Differential leucocyte counts of goat No. 5254 experimentally infected with Shika isolate of *C. ruminantium*

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	64	35	1	0
2*	0	40	60	0	0
3*	0	36	63	1	0
4	0	45	55	0	0
5	0	46	54	0	0
6	0	40	60	0	0
7	0	45	55	0	0
8	0	55	45	0	0
9	0	46	54	0	0
10	0	48	50	2	0
Mean	0	46.5	53.10	0.4	0.0

* = Period of reaction (Pyrexia) to infection

Appendix viii

Differential leucocyte counts of goat No. 5255 prior to infection

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	31	68	1	0
2	0	42	58	0	0
3	0	35	65	0	0
4	0	40	58	2	0
5	0	38	62	0	0
6	0	40	60	0	0
	0	37.70	61.80	0.50	0

Appendix ix

Differential leucocyte count of
goat No. 5255 experimentally infected
with forest (Akure) isolate of *C. ruminantium*

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	40	55	1	4
2	0	39	61	0	0
3	0	30	68	2	0
4	0	35	65	0	0
5*	1	39	57	3	0
6	2	40	58	0	0
7	0	37	60	3	0
8	1	39	60	0	0
9	1	45	53	0	1
10	2	40	57	1	0
11	0	39	58	1	2
Mean	0.64	38.45	59.27	0.64	0.73

* = Period of reaction (Pyrexia) to infection

Appendix x

Differential leucocyte count of
goat No. 5256 prior to infection

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	36	60	3	1
2	0	40	58	2	0
3	0	37	60	1	2
4	1	39	59	0	1
5	0	40	60	0	0
6	1	38	58	2	1
Mean	0.33	37.7	59.20	1.30	0.83

Appendix xi

Differential leucocyte count of
goat No. 5256 experimentally infected
with forest (Akure) isolate of *C. ruminantium*

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	28	71	1	0
2*	0	30	70	0	0
Mean	0	29.0	70.5	0.5	0

* = Period of reaction (Pyrexia) to infection

Appendix xiii

Differential leucocyte count of
goat No. 0271 experimentally infected
with forest (Akure) isolate of *C. ruminantium*

Days	Neutrophils (%)		Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
	Band	Mature			
1	0	45	53	0	2
2	0	52	38	2	8
3	0	49	46	0	5
4	0	50	44	0	6
5*	0	41	59	0	0
6	1	60	34	0	5
7	0	48	46	0	4
8	0	50	43	1	6
9	0	48	50	0	2
10	0	42	56	2	0
Mean	1.3	48.5	46.8	0.5	3.9

* = Period of reaction (Pyrexia) to infection

Appendix xiv

Differential leucocyte counts of goat No. 118 (Control)

Days	Neutrophils (%)		Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
	Band	Mature			
1	1	38	60	1	0
2	0	39	58	3	0
3	0	46	51	3	0
4	0	40	55	1	4
5	0	48	50	1	1
6	2	38	58	2	0
7	2	36	60	0	0
8	1	46	53	0	0
9	0	43	54	0	3
10	0	30	69	0	1
11	1	43	50	0	6
12	0	43	50	0	4
13	0	42	57	0	1
Mean	0.54	40.90	55.80	1.23	1.53

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Appendix xvi

Differential leucocyte count of control animal 5259

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	40	59	1	0
2	1	38	58	2	1
3	2	41	57	0	0
4	0	37	60	2	1
5	1	38	59	0	2
6	2	41	57	0	0
7	1	38	59	1	1
8	0	39	59	2	0
9	0	42	56	0	2
10	1	43	53	1	2
11	2	40	57	1	0
12	1	37	62	0	0
13	0	36	64	0	0
Mean	0.85	39.23	58.50	0.76	0.70

Appendix xvii

Differential leucocyte count of control (NNM)

Days	Neutrophils (%) Band	Mature	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
1	0	39	60	1	0
2	1	36	62	1	0
3	0	40	60	0	0
4	0	38	61	0	1
5	1	42	55	0	2
6	2	37	60	1	0
7	0	41	57	2	0
8	0	36	64	0	0
9	1	38	60	0	1
10	0	40	59	1	0
11	2	42	56	0	0
12	1	48	54	1	1
13	0	40	57	2	1
Mean	0.62	39.40	58.85	0.70	0.46