1. INTRODUCTION

1.1 JUSTIFICATION OF THE PROJECT

Heartwater is a virulent, infectious, tick-borne and non-contagious disease affecting domestic and wild ruminants. It is characterized in domestic ruminants by a high fever, followed by severe nervous symptoms, hydro-pericardium and acute gastro-enteritis in its acute and peracute forms (Camus, Barré, Martinez & Uilenberg 1996). The causative agent is a *Rickettsia*, namely *Cowdria ruminantium*, which is transmitted by ticks of the genus *Amblyomma* (Camus *et al.* 1996). Heartwater appears to be of African origin, and occurs wherever particular *Amblyomma* vectors exist (Uilenberg 1996). The initial research on heartwater was carried out in South Africa, as there was a large and highly susceptible ruminant population in this country (Uilenberg 1996), and one of the main vectors, *Amblyomma hebraeum*, is indigenous to this region.

Provost & Bezuidenhout (1987) stated that heartwater is a major obstacle to the introduction of highly productive animals into Africa, and is also a significant problem when local animals are moved from heartwater-free to heartwater-infected areas. Heartwater is also a threat to the American mainland, following the introduction of the tick, *Amblyomma variegatum*, to the West Indies and other Caribbean islands (Barré, Uilenberg, Morel & Camus 1987).

Intensive tick control has been the method of choice for the control of heartwater in southern Africa since the end of the last century (Bezuidenhout & Bigalke 1987), but today decision makers are aware that these programmes are difficult to sustain, are costly and have adverse

effects on the immunity to the disease. Heartwater is not normally a problem in indigenous livestock which are born in endemic areas, and they soon acquire immunity through natural exposure when young (Norval 1981). In many instances the intensive dipping programmes have left the local cattle population susceptible to the various tick-borne diseases (TBD), resulting in massive stock losses due to TBD (Lawrence & Norval 1979). This has lead to a re-appraisal of various government strategies on dipping programmes in Africa.

There has also been a substantial increase in research efforts to give farmers alternative approaches to tick control. This has mainly been directed at increasing our knowledge of the epidemiology of heartwater, and the production of more effective vaccines. In order to use the vaccines effectively, one needs to characterize the *C. ruminantium* strains in the areas where the vaccine is being tested, and know the prevalence or infection rate of *C. ruminantium* in free-living, unfed ticks.

Peter (1995) stated that in an ideal epidemiological scenario, one would aim to collect free-living, unfed wild *A. hebraeum* ticks in order to get the most credible data. These ticks are truly representative of the field population, whilst attached feeding ticks may have acquired their infection during feeding on their present host. Peter (1995) was, however, not able to collect free-living, unfed *A. hebraeum* in Zimbabwe due to problems with the AAAP/CO_2 trap and eventually collected free-living, unfed *A. hebreum* attaching to sentinel hosts. One of the main objectives of this project was to collect free-living, unfed *A. hebraeum* with the AAAP/CO_2 trap, and have truly representative samples of the field population of ticks.

1.2 RESEARCH OBJECTIVES

1.2.1 OVERALL OBJECTIVES

To determine the *C. ruminantium* infection rate of free-living, unfed *A. hebraeum* ticks collected from heartwater endemic regions of South Africa.

1.2.2 SPECIFIC AIMS

- To develop a reliable Attraction-Aggregation-Attachment-Pheromone/Carbon Dioxide (AAAP/CO_2) trap to collect free-living, unfed *A. hebraeum* ticks in the field, and to test this trap at various field sites in South Africa.
- To determine the infection rate or prevalence of *C. ruminantium* in a statistically relevant sample of these free-living, unfed *A. hebraeum* by means of a *C. ruminantium*-specific Polymerase Chain Reaction (PCR) assay.

1.3 LITERATURE REVIEW

1.3.1 *C. RUMINANTIUM* IN THE HOST (RUMINANTS/WILDLIFE)

1.3.1.1 Characteristics of *C. ruminantium*

Cowdry (1925) first described the pathogenic agent as *Rickettsia ruminantium*. He considered the agent to be a *Rickettsia* because of its Gram-staining properties, its intracellular localization, its morphology and the fact that it was transmitted by an arthropod. The organism, which was later renamed *C. ruminantium*, may be found singly, in colonies or in clumps, and has a predilection for the cytoplasm of reticular cells, neutrophilic leukocytes and vascular endothelium (Mebus & Logan 1988). The organism is also very fragile and must be stored at -70°C or lower, using a cryopreservative.

C. ruminantium was successfully cultivated for the first time in tissue culture when Bezuidenhout, Paterson & Barnard (1985) grew it in a bovine umbilical endothelium cell line. Today it is routinely cultured at various research establishments throughout the world. Many different "stocks" (strains) of *C. ruminantium* have been described (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs 1988).

There appears to be a close phylogenetic relationship between *C. ruminantium* and *Ehrlichia* species (Dame, Mahan & Yowell 1992; Van Vliet, Jongejan & Van der Zeijst 1992), and this has limited the credibility of the serological tests for *C. ruminantium*. Sequencing of the gene coding for the 16S RNA gene has also revealed a close relationship between *C. ruminantium* and various *Anaplasma*, *Rickettsia* and *Ehrlichia* species (Dame *et al.* 1992; Van Vliet *et al.* 1992).

1.3.1.2 **Pathogenesis of the disease in the host**

The pathogenesis of heartwater is poorly understood (Bezuidenhout, Prozesky, Du Plessis & Van Amstel 1994). Vertebrate hosts are infected directly via the saliva of the attached ticks or by regurgitation of the gut material. Initial replication takes place in the reticulo-endothelial cells and in the macrophages in the regional lymph nodes (Du Plessis 1970). The organisms are disseminated by the blood stream, and have a predilection for endothelial cells of the brain (Bezuidenhout *et al.* 1994). Increased vascular permeability leads to effusion into the body cavities and consequent oedema, especially in the brain. Hydropericardium and nervous signs are a direct result of the oedema.

1.3.1.3 Main clinical signs of the disease

Under field conditions susceptible animals show signs of the disease 14 to 28 days after being introduced into a heartwater enzootic area. The incubation period in sheep and goats is normally 7 - 14 days, and in cattle 10 - 16 days (Mebus & Logan 1988). The course of the disease depends on the susceptibility of the host, and the virulence of the *C. ruminantium* isolate can be peracute, acute, subacute or subclinical (mild). Cows of the *Bos taurus* breeds are particularly prone to develop peracute heartwater, and these animals die within a few hours after developing a fever, often showing no other clinical signs apart from convulsions and marked respiratory distress (Bezuidenhout *et al.* 1994).

The acute disease is the most common form, and after the rapid onset of fever there is increased respiration, inappetence, hyperaesthesia, high stepping gait, twitching of the eyelids, chewing, abnormal tongue movement and individual muscle tremors. Nervous signs increase in severity, often with the limbs stretched out and showing paddling movements, opisthotonos, nystagmus and convulsions (Mebus & Logan 1988). The course of this form of the disease is usually less than seven days, and most of the animals die. The subacute disease is usually seen in animals which have some resistance, and is characterized by much milder signs, and only a small percentage of the affected animals die. In the subclinical form the signs are so mild, that there is only a transient fever and this is more often seen in animals with natural resistance to the disease.

Exotic breeds of goats, such as Angora and Saanen goats, are commonly affected by the peracute form of the disease. Clinical signs in goats are similar to those of cattle, but the nervous signs are less pronounced (Bezuidenhout *et al.* 1994). Black-headed Persian sheep

are thought to possess some natural resistance to heartwater and tend to develop a mild form of the disease (Alexander 1931). Clinical signs recorded in susceptible wild ungulates are generally similar to those recorded in domestic ruminants (Gradwell, Van Niekerk & Joubert 1976).

Mortality rates vary according to the stock (strain) of *C. ruminantium* involved, and the species, breed and age of the host. In untreated cases, mortality is usually in the region of 50% in exotic breeds of cattle, somewhat higher in exotic breeds of sheep and much higher in exotic goats (Uilenberg & Camus 1993).

1.3.1.4 **Immunity and immunization of the host**

1.3.1.4.1 Age resistance

Calves possess an age-linked resistance to bovine babesiosis and anaplasmosis, which is independent of the immune status of the dam, and may last nine months or longer. A state of endemic stability to these TBD is possible if the infection rate in the field ticks is sufficiently high to ensure that all calves develop active immunity before resistance wanes (Uilenberg & Camus 1993).

An age resistance also occurs in cowdriosis, but it is of shorter duration, lasting only the first four weeks of life in calves, and the first week in lambs and kids (Du Plessis & Malan 1987). The resistance is not absolute, as infection of some calves less than three weeks of age and some lambs and kids of less than one week may result in fatal disease (Neitz & Alexander 1945).

1.3.1.4.2 Breed resistance

Bos taurus cattle, especially those with no exposure to heartwater infection, are less resistant to infection than *Bos indicus* breeds (Uilenberg 1983). The resistance of local Zebu breeds is probably due to inherited resistance acquired through years of natural selection (Bezuidenhout *et al.* 1994). Conglutinin in the serum of cattle appears to be involved in non-specific protection against heartwater (Du Plessis & Bezuidenhout 1979).

Sheep and goats appear to be more susceptible to heartwater than cattle, and there is a great range in different breed susceptibilities which seldom develop good immunity. Merino sheep are particularly susceptible, whilst Persian sheep show a degree of natural resistance. This resistance to heartwater can also be inherited, such as in cross-bred goats (Donkin, Stewart, MacGregor, Els & Boyazoglu 1992).

Wild animals show varying degrees of immunity to *C. ruminantium*, depending on the species and their previous exposure to *C. ruminantium*. Some species such as eland (*Taurotragus oryx*), blesbuck (*Damaliscus pygargus phillipsi*), springbok (*Antidorcas marsupialis*) and black wildebeest (*Connochaetes gnu*) can develop clinical disease, whereas the majority of wild ruminants are refractory to the disease or become subclinical carriers (Peter, Anderson, Burridge & Mahan 1998).

1.3.1.4.3 Mechanisms of immunity to *C. ruminantium*

Although much research has been conducted into the serological responses of the various domestic animals to *C. ruminantium*, it is thought that the humoral response is not the most important immune mechanism. Alexander (1931) injected sheep with hyperimmune serum,

but was not able to protect the sheep from consequent challenge with *C. ruminantium*. Du Plessis (1970) also injected heartwater susceptible sheep with gamma globulins from immune sheep, but these sheep were not protected against heartwater challenge.

The immunity appears to be mainly cell-mediated, and the transfer of spleen cells from immune mice conferred protection against homologous challenge in recipient mice (Du Plessis 1981). Cellular immunity in heartwater is likely to be mediated directly via cell cytotoxic effects, and indirectly through the production of T cell cytokines which induce an anti-rickettsial state (Mahan, Smith & Byrom (1994). Mahan *et al.* (1994) also demonstrated that the addition of mitogen-stimulated bovine T cell supernatants to bovine endothelial cell culture inhibited intracellular development of *C. ruminantium*. The authors suggested that the active agent was gamma-interferon. Circulating levels of alpha-interferon have been shown to be higher in cattle resisting experimental *C. ruminantium* infection (Totté, Blankaert, Zilinwabagabo & Werenne 1993a; Totté, de Gee & Werenne 1993b; Totté, Jongejan, de Gee & Werenne 1994; Totté, McKeever, Jongejan, Barbet, Mahan, Mwangi & Bensaid 1998).

1.3.1.4.4 Different stocks (strains) of *C. ruminantium*

There is a great variation in the pathogenicity of the various stocks (strains) of *C. ruminantium*. Certain stocks of *Cowdria* differ considerably in their pathogenicity to mice, sheep, cattle and goats, and this pathogenicity is often independent of geographical differences (Du Plessis, Van Gas, Olivier & Bezuidenhout 1989). Some stocks are pathogenic to mice (Crystal Springs, Kümm, Kwanyanga, Nonile & Welgevonden), and some non-pathogenic to cattle (Kümm & Mara), (Du Plessis *et al.* 1989; Byrom *et al.* 1993).

Almost all stocks give partial to complete cross-immunity, however, total absence of cross-protection is known to exist between certain stocks (Van Winkelhoff & Uilenberg 1981; Uilenberg 1983; Du Plessis *et al.* 1989; Jongejan, Thielemans, Briere & Uilenberg 1991). The recognised antigenic diversity of different stocks may have important repercussions for vaccine production or development.

1.3.1.4.5 Vaccination/immunization of the host against *C. ruminantium* (Historical)

It has long been recognized that animals which recover from heartwater acquire an immunity to the disease (Alexander 1931). Many of the early attempts to produce a vaccine failed, and it was not until 1931 that Alexander described an intravenous blood vaccine which produced clinical heartwater in the host. The inoculated animal had to be treated with tetracycline once the temperature began to rise (Van Amstel & Oberem 1987). The infection and treatment of immunization/vaccination is still in use in South Africa today (Bezuidenhout *et al.* 1994). It is used especially when animals are brought into endemic areas from heartwater-free regions.

The main problem with this method of immunization is the timing of the treatment. If the treatment is given too early no immunity develops, and if too late, the animal dies (Bezuidenhout *et al.* 1994). Very young animals can be immunized without temperature monitoring because of their age-linked resistance. Cattle develop immunity to heartwater following one vaccination, provided they are then put onto heartwater veld. With sheep and goats this immunity may last for less than one year if not re-enforced by natural infection.

A small proportion of vaccinated animals, usually less than 5%, fail to develop immunity after vaccination (Lawrence, Malika, Whiteland & Kafuwa 1995). It may be that the vaccine is not protective because of field strain differences with the vaccine strain which do not exhibit cross-immunity. Lawrence *et al.* (1995) vaccinated 1 094 cross-bred Zebu cattle in Malawi and found that more than 50% of the vaccinated cattle did not react to the vaccine and six (0.6%) cattle died during vaccination. Van der Merwe (1979) vaccinated 2 743 cattle in South Africa and most of these reacted to the vaccine (±98%), and there were also some mortalities (0.83%).

Because it is necessary to inject animals intravenously (I/V) and to monitor the temperature daily, it is difficult to give mass immunization. The block method of immunization, first described by Fick & Schuss (1952) and Poole (1962), is widely practised in South Africa, especially when vaccinating large numbers of kids and lambs (Bezuidenhout *et al.* 1994). The procedure involves treating vaccinated animals on a specific day, irrespective of whether the animals have reacted or not. In South Africa it is generally advised that *Bos taurus* cattle be treated on day 14, *Bos indicus* cattle on day 16 and sheep and goats on day 11 and 12, respectively (Bezuidenhout *et al.* 1994). The main criticism of this method is that one is uncertain whether the vaccinated animals developed a temperature reaction and whether immunity develops or not.

A further disadvantage of the vaccine is that the organisms are very labile, and need to be stored at -70°C, using special facilities which are not available on farms and in other rural areas. Apart from the difficulties experienced in giving an intravenous vaccine, blood-

borne pathogens can also be transmitted (Uilenberg & Camus 1993). There are also reports of anaphylactic reactions to the vaccine (Lawrence *et al.* 1995), and when using the vaccine in does and kids a high mortality (close to 20%) might be experienced (Gruss 1983).

1.3.1.4.6 New developments with the heartwater vaccine

It is therefore clear that there are a multitude of problems when using the infection and treatment method of immunization for heartwater, but in spite of all the disadvantages, exotic stock cannot be maintained in endemic areas without immunization, unless excellent tick control can be sustained (Uilenberg & Camus 1993). If progress is to be made, then a user-friendly, inactivated vaccine, that can be given subcutaneously (s/c), has to be developed, and the successful growth of *C. ruminantium* (Bezuidenhout, Paterson & Barnard 1985) in cell culture has made this goal more achievable.

Jongejan (1991) showed that a live, attenuated Senegalese stock of *C. ruminantium* vaccine could be used to protect sheep against certain strains of heartwater. Martinez, Maillard, Shiekboudou & Coisne (1993) vaccinated nine goats s/c with an inactivated elementary body preparation of *C. ruminantium* (Gardel stock) mixed with Freund adjuvant. Six of the nine goats were protected on challenge as opposed to none of the controls.

Mahan, Andrew, Tebele, Burridge & Barbet (1995) immunized three groups of five Merino sheep from a heartwater-free area with five inoculations of a s/c inactivated vaccine. Group 1 were vaccinated with the vaccine only, Group 2 with vaccine and Freund's complete and incomplete adjuvants, and Group 3 were the control group. None of the sheep in Group 2

died, but there was a 50% and 60% mortality in Groups 1 and 3, respectively. Clearly the sheep vaccinated with the inactivated vaccine plus the adjuvant were protected.

Mahan, Kumbula, Burridge & Barbet (1998a) did extensive laboratory and field trials using 178 sheep to test the inactivated *C. ruminantium* vaccine against heterologous and field strains. They demonstrated that the vaccine with Freund's adjuvant protected sheep against lethal intravenous challenge and against laboratory-raised infected ticks. The vaccination was also able to protect against heterologous strains from geographically diverse areas and against natural tick challenge. The Freund's adjuvant has, however, been shown to cause numerous clinical problems and would not, therefore, be the adjuvant of choice. Montanide ISA 50 was shown to be the most effective adjuvant for the vaccine (Mahan *et al.* 1998a).

More recently Nyika, Mahan, Burridge, McGuire, Rurangirwa & Barbet (1998) demonstrated that the Major Antigenic Protein-1 (MAP-1) gene of the Crystal Springs stock, cloned into an eukaryotic expression vector VCL 1010 induced a degree of homologous protective immunity in mice.

Nearly all the developments and testing of the inactivated vaccines for heartwater have been tested in sheep. Future developments must be extrapolated to cattle before the vaccine can bring practical benefits to farmers.

1.3.1.5 Reservoir hosts

There appear to be three important reservoirs of heartwater (Norval, Andrew, Yunker & Burridge 1992):

- Ruminant carriers,

- Free-living, unfed *Amblyomma* ticks, and
- Attached male *Amblyomma* ticks.

C. ruminantium is widely spread throughout the host and vector populations, and this appears to be the reason why enzootic stability to heartwater is common in areas where *Amblyomma* ticks are prevalent throughout the year (Norval *et al.* 1992).

The early investigators (Alexander 1931; Neitz 1939; Ilemobade 1978) found that blood from infected cattle and sheep, when injected I/V into another ruminant host, was infective for a variable period of 35 to 60 days. Andrew & Norval (1989a) demonstrated that sheep, cattle and African buffalo (*Syncercus caffer*) could remain carriers for as long as 250 days. Peter , Mahan, Barbet, Norval & Burridge (1995) showed that this reservoir state in cattle can last for up to a year.

Many wildlife species are considered to be reservoirs of heartwater, and these have been reviewed by Oberem & Bezuidenhout (1987), and more recently by Peter, Anderson, Burridge and Mahan (1998a). As many as 28 different wildlife species have been implicated as hosts of *C. ruminantium*, but few have been conclusively proved to be susceptible to infection. Eight wildlife species have been experimentally proven to be susceptible to *C. ruminantium* infection, and seven of these have become carriers of the infection (Peter *et al.* 1998a).

Wildlife hosts play a very important role in the epidemiology of the disease. These asymptomatic carriers can infect free-living ticks (Norval *et al.* 1992), and although intensive tick control may lead to the localized eradication of *Amblyomma* ticks, it is

unlikely to do so if alternative hosts for the ticks are available (Norval *et al.* 1992). This also has important consequences for veterinary quarantine decision makers, and makes the translocation of potentially infected animals over long distances into heartwater-free areas such as the United States of America (USA) more important, and they should be carefully screened (Peter *et al.* 1998a).

With the improvement of diagnostic tests for *C. ruminantium*, more and more wild animals will probably be shown to be important carriers of diseases. Recently, Kock, Van Vliet, Charlton & Jongejan (1995) used the PCR assay to demonstrate that the blood and bone marrow of tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala (*Aepyceros melampus*) were positive for *C. ruminantium*.

1.3.1.6 Treatment of clinical cases

Historically, a variety of different drugs have been used to treat clinical cases of heartwater, and these have been reviewed by Van Amstel & Oberem (1987). The tetracyclines, especially oxytetracycline (10 to 20 mg/kg live mass), are the most effective drugs used for specific treatment of the disease. Long-acting tetracyclines have the advantage that only a single injection is usually sufficient, whilst with the short-acting preparations at least two injections, 24 to 48 hours apart, are required (Uilenberg & Camus 1993). Treatment of heartwater during the early febrile stages presents very few problems if a diagnosis can be made soon enough. If, however, as is commonly the case, treatment is delayed until nervous symptoms are present, then the prognosis becomes poor (Van Amstel & Oberem 1987). Various drugs, such as diuretics, can also be used to reduce the oedema, and corticosteroids can be used in the supportive treatment of clinical cases (Van Amstel & Oberem 1987).

1.3.1.7 Main post mortem signs

Lesions in cattle, sheep and goats are very similar and differ only in their severity and frequency (Prozesky 1987). Severe hydropericardium and hydrothorax, and in some cases ascites, are striking changes in the most fatal cases of the disease (Bezuidenhout *et al.* 1994). A moderate to severe lung and brain oedema is also present (Prozesky 1987). In spite of marked clinical signs in goats, post mortem lesions are not always present (Uilenberg & Camus 1993).

1.3.2 THE DIAGNOSIS OF HEARTWATER

1.3.2.1 Clinical signs

Unlike many other TBD, *C. ruminantium* is not detectable in a blood smear, making a specific confirmation of heartwater in the living host very difficult. The early investigators relied heavily on the clinical signs seen in domestic stock. These included a variety of nervous manifestations, many of which are not pathognomonic for the disease (Van der Pypekamp & Prozesky 1987). Fever is present throughout the course of the disease, and the nervous signs include inco-ordination, exaggerated high-stepping gait, chewing movements, as well as blinking and twitching of the eyes (Synge 1978). As the disease progresses certain signs, such as hyperaesthesia and scours, become more prominent and eventually affected animals collapse in galloping convulsions (Synge & Scott 1978).

There are, however, a wide range of other diseases of ruminants which have similar clinical signs to heartwater (botulism, babesiosis, theileriosis, anaplasmosis and coccidiosis) (Camus *et al.* 1996; Uilenberg 1971), as well as various neuro-toxicoses of plant origin (Coetzer, Kellerman & Naude 1985).

1.3.2.2 **Detection of the organism (antigen) in the live animal**

1.3.2.2.1 Biopsy

The biopsy method, described by Synge (1978) for the diagnosis of heartwater in goats, involves collecting brain sections for histopathology. This method has been used successfully by a number of field workers (Camus & Barré 1987a), but requires specialized techniques.

1.3.2.2.2 Xenodiagnosis

- Inoculation of blood

In the past sub-inoculation of blood into a susceptible ruminant was often necessary to confirm the presence of *C. ruminantium*. If it was present, the recipient animal reacted within 16 days (Synge & Scott 1978). This method was used as one of the confirmatory tests for the presence of *C. ruminantium* in the Caribbean (Burridge, Barré, Birnie, Camus & Uilenberg 1984).

- Inoculation of tick homogenates

The inoculation of a supernatant of homogenized *Amblyomma* ticks into a susceptible animal, can also demonstrate the presence of *Cowdria* (Camus *et al.* 1993).

- Tick feeding transmission

Cowdria-free laboratory-reared *Amblyomma* nymphs can also be fed on suspected infected hosts. After feeding, the ticks are allowed to moult and the resulting adults are fed on a heartwater susceptible host to see whether they have become infected (Camus *et al.* 1996).

Intrastadial transmission of *C. ruminantium* has been demonstrated by infecting heartwater-free *A. hebraeum* ticks after feeding on heartwater infected hosts (Andrew & Norval 1989). Intrastadial transmission has also been achieved with male *A. variegatum* (Kocan, Norval & Donovan 1993).

1.3.2.2.3. Post mortem signs and Histopathology

It is much easier to detect the *C. ruminantium* organism in a dead host, as access to the brain of the animal is then possible. In addition, the pathological changes associated with heartwater are well-documented (Bezuidenhout *et al.* 1994), and include a characteristic hydropericardium, hydrothorax and lung oedema. Examination of crush smears of the endothelial cells of the renal glomeruli or of the cerebral cortex will show colonies of *C. ruminantium* (Purchase 1945). The *Cowdria* organisms can be demonstrated in brain smears even in an advanced state of putrification, but detection of the organisms in treated animals can be difficult (Bezuidenhout *et al.* 1994).

C. ruminantium has also been demonstrated in histological sections of various organs in ruminants (Cowdry 1926), and in various endothelial cells (Jackson 1931). Detailed histopathological and electron-microscopic studies have been done (Du Plessis 1970; 1975), but demonstration of the organism remains difficult outside of the endothelial cells of the kidney and the brain. Various stains have been used to demonstrate *C. ruminantium*, but those incorporating toluidine blue or Giemsa are preferred (Prozesky 1987).

1.3.2.3 **Detection of antibodies**

1.3.2.3.1 Development of antibodies to *C. ruminantium*

Early research workers attempting to develop serological tests to determine the kinetics of antibody production to *C. ruminantium*, were hampered by the inability to produce adequate quantities of antigens. The successful cultivation of *C. ruminantium in vitro* in endothelial cell cultures (Bezuidenhout *et al.* 1985) provided a pure source of antigen for the various diagnostic tests, and the tests became more repeatable.

Semu, Mahan, Yunker & Burridge (1992) experimentally infected cattle with a Zimbabwean (Palm River) and a South African (Ball 3) isolate of *C. ruminantium*. A specific antibody response was detected by an indirect fluorescent antibody test (IFAT) using *C. ruminantium*-infected bovine aortic endothelial (BAE) cell cultures as antigen. The first detection of antibodies to *C. ruminantium* generally coincided with the peak of the febrile reaction, and the antibodies remained detectable for a period of 8 - 30 weeks. Peak reciprocal antibody titres in both groups ranged between three and six weeks post-infection. Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen (1986) used an Enzyme-linked immunosorbent assay (ELISA) to demonstrate that IgG-specific antibodies to *C. ruminantium* were detectable in cattle and sheep up to 22 weeks post-inoculation.

Mice develop a specific immune response following infection with mice pathogenic strains of heartwater, and the duration of immunity in the case of the Kümm strain can be as long as 18 months (Du Plessis 1985; Stewart 1987). Byrom, Mahan & Barbet (1993) found that mice antibody to *C. ruminantium* develops in the second week post-infection, and the antibody level is dependent upon the inoculation dose.

The exact role of antibody in the protection of domestic animals has been debated over a long period. However, neither passive transfer of immune sera in ruminants (Alexander 1931) or mice (Byrom *et al.* 1993) appears to reduce the severity of the disease. Cell-mediated immunity is presumed to be more important than humoral immunity in protecting against heartwater (Mahan *et al.* 1994).

1.3.2.3.2 Isolation and culture of *C. ruminantium*

For many years all efforts to propagate *C. ruminantium in vitro* failed (Uilenberg 1983). The first successful culture method was that of Bezuidenhout *et al.* (1985). This provided the basic information necessary for the refinement of techniques and to the supply of organisms required for molecular and serological studies (Byrom & Yunker 1990). Problems were, however, often experienced with the special irradiation and chemical retardation deemed necessary to delay cell growth. Byrom & Yunker (1990) reported that the addition of 0.4% glucose to the medium greatly improved the culture conditions, and *C. ruminantium* could be passaged routinely in untreated bovine vascular endothelial cells.

Byrom, Yunker, Donovan & Smith (1991) described a culture technique where no cell growth retardation by irradiation or chemicals was used. They used heparin-derived plasma samples obtained from heartwater-infected animals exhibiting a prolonged high temperature. They demonstrated that by using this technique the infection rate of cultures increased from 1% to 25%. More recently Smith, Anderson, Burridge, Peter & Mahan (1998) infected various wild animal culture cells with *C. ruminantium* by inoculation of plasma from infected animals or infected bovine cell cultures.

1.3.2.3.3 Immunogenic proteins of *C. ruminantium* (MAP-1)

With the advances in the culture of *C. ruminantium*, and the great improvement in *Cowdria*-specific immunological techniques, various antigenic proteins have been identified, namely the 21, 27, 32, 40, 46, 58, 85 and the 160 kilodalton (kDa) proteins. Any of these antigenic proteins may be used in the serological tests or recombinant vaccines for heartwater. Jongejan & Thielemans (1989) first described a 32-kDa immuno-dominant protein referred to as MAP-1 (Barbet, Semu, Chigagure, Kelly, Jongejan & Mahan 1994). This protein was also conserved within nine geographically different stocks of *Cowdria*, and was considered a good candidate for antigen in sero-diagnostic tests (Jongejan & Thielemans 1989).

Due to the various problems with the IFAT such as cross-reactions to various *Ehrlichia* spp., new serological diagnostic techniques have been developed. Most of these new tests use ELISA immuno technology and are based on the use of the immuno-dominant MAP-1 32-kDa surface protein of *C. ruminantium* (Barbet *et al.* 1994). Several other immunogenic proteins have also been identified (Mahan 1995), but the 32-kDa remains the most immuno-dominant. Most of these immunogenic proteins are surface exposed, and this facet would be important in the research and development of new vaccines and diagnostic tests for heartwater (Mahan 1995). Monoclonal antibodies are also available for the 21 and 32-kDa proteins (Jongejan, De Vries, Nieuwenhuijs, Van Vliet & Wassink 1993).

Two immunogenic regions of the MAP-1 protein, have recently been described (Van Vliet *et al.* 1995) and designated MAP-1A and MAP-1B. MAP-1A reacted to *C. ruminantium*

antisera and *E. ovina* antisera, whilst MAP-1B reacted only with *C. ruminantium* antisera. The gene encoding MAP-1 of the Senegal isolate of *C. ruminantium* has also been cloned and expressed in *Escherichia coli* (Van Vliet *et al.* 1994). This allowed identification of the immunogenic regions on MAP-1, and an evaluation of their possible use in the construction of a specific diagnostic test.

1.3.2.3.4 Antibody detecting diagnostic tests

- Indirect fluorescent antibody test

Du Plessis (1981) was the first researcher to use an IFAT to detect antibodies to *C. ruminantium* in ruminants and mice. He used infected mouse peritoneal cells as antigen and the test was known as the mouse IFAT, and the test was used extensively in South Africa and the Caribbean. Logan, Quintero, Whyard & Mebus (1985) used infected neutrophils and macrophages in an IFAT and a direct Fluorescent antibody test (FAT). Semu *et al.* (1992) used bovine endothelial cells infected with *C. ruminantium* in their IFAT to detect antibodies in experimentally infected cattle (refer to section 1.3.2.3.1).

With the advances in cell culture techniques the IFAT's became more sophisticated. Martinez, Swinkles, Camus & Jongejan (1990) used cultured bovine endothelial cells as antigen, and the test could detect antibodies as early as two weeks after infection and for 30 weeks after the initial infection.

b. 14442413
i. 14745562

- Immunoblots

Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet (1993) used the immunoblot diagnostic assay, which was first described by Jongejan & Thielemans (1989), to test for heartwater antibodies in cattle, sheep and goats in Zimbabwe. They used this test because of the known sensitivity limitations of the IFAT and the ELISA. Questions, however, arose as to the credibility of the results obtained with the immunoblot diagnostic assay, especially when many "positive" samples were found in heartwater-free areas of Zimbabwe (Mahan *et al.* 1993).

- Enzyme-linked immunosorbent assays
- Competitive ELISA

A competitive ELISA (c-ELISA)was first described by Jongejan, Thielemans, De Groot, Van Kooten & Van der Zeijst (1991), which detected *Cowdria* specific antibodies in goat, sheep and cattle sera. The c-ELISA used a monoclonal antibody directed against the immunodominant MAP-1 antigenic protein. The c-ELISA detected antibodies in 55 out of 70 (79%) goats experimentally infected with eight different *Cowdria* stocks. There was a 89% agreement between the results of the IFAT and the c-ELISA, and there were apparently, no cross-reactions with *Ehrlichia phagocytophila* antibodies in goat sera, and *Anaplasma marginale* in bovine sera.

- Indirect ELISA's

Two indirect ELISA's (i-ELISA) have also been described. Martinez, Coisne, Sheikboudou & Jongejan (1993) working in the Caribbean, stated that the overall sensitivity of their

i-ELISA varied between 97 and 98%. The specificity showed only 3% false positives, and was lower for sheep than for cattle and goats. Soldan, Norman, Masak, Paxton, Edelston & Sumption (1993) also used an indirect ELISA to monitor the seroconversion to *C. ruminantium* in Malawian Zebu calves. This ELISA test used a detergent soluble fraction of *C. ruminantium* organisms. This fraction was reported to have a reduced cross-reactivity to *Ehrlichia ondiri* and *E. phagocytophila*. There was no published information on the sensitivity and specificity of the test (Soldan *et al.* 1993).

Recent work (Van Vliet *et al.* 1995; Mahan *et al.* 1998c) with the MAP-1B ELISA test, seems to indicate that the problems with cross-reactivity with *Ehrlichia* have been partially solved by using the MAP-1B ELISA test (refer to section 1.3.2.3.7).

1.3.2.3.5 Advantages of the use of serological tests (Epidemiological surveys)

- IFAT (Surveys)

One of the main objectives of the various serological tests would be to determine the prevalence of *C. ruminantium* antibodies in domestic animals. Du Plessis (1981) first used the IFAT to determine the relationship between the dipping of cattle and immunity to heartwater. Du Plessis & Malan (1987) then used the IFAT to determine whether endemic stability to heartwater was present or not at certain selected farms in South Africa. Asselbergs, Jongejan, Langa, Nevis & Afonso (1993) also used the IFAT with infected endothelial cell culture antigen to test goat and cattle sera collected from various provinces in Mocambique. Overall 30% of the goat and 43% of the cattle sera were positive to *C. ruminantium*. There was also a noticeable difference between the sera from the north of

Mocambique where *A. variegatum* is prevalent (10% goats, 20% cattle), and the south of Mocambique where *A. hebraeum* predominates (63% goats, 59% cattle).

Camus & Barré (1987b) used the IFAT with the Kümm and Gardel stocks as antigen to determine which islands in the Caribbean were infected with *C. ruminantium*. Most of the areas where *A. variegatum* was present were positive, but of concern were certain islands which were shown to be positive, where no *A. variegatum* were found.

- c-ELISA (Surveys)

De Vries, Mahan, Ushewokunze-Obatolu, Norval & Jongejan (1993) used the c-ELISA and the IFAT to determine if there was any correlation between the antibodies to *C. ruminantium* in cattle and the distribution of *Amblyomma* ticks in Zimbabwe. They reported that in *Amblyomma*-infested areas 52% (c-ELISA) and 26% (IFAT) of the sera were positive for *C. ruminantium* antibodies. In the *Amblyomma*-free areas 11% (c-ELISA) and 10% (IFAT) were positive. The latter result was of some concern because no heartwater was present in these regions, therefore, some other organism, possibly also transmitted by the ticks, must have been responsible for the false positives.

- i-ELISA (Surveys)

The indirect ELISA was also used in two important serological surveys for heartwater in widely differing geographical areas in the Caribbean and Africa (Malawi). Camus *et al.* (1993) published their findings on a survey conducted in Guadeloupe and various islands in the Caribbean. The authors of this major work had to conclude that the serological results

were difficult to interpret due to various factors. At Antigua and Guadeloupe where *A. variegatum* was widespread, the domestic ruminants had a high percentage of positive results. On the other nine islands, however, 4% of the sera were positive and these "positive sera" must have been due to other organisms related to *C. ruminantium*.

Soldan *et al.* (1993) also used the indirect ELISA to test for antibodies to *C. ruminantium* in calves in Malawi, and they compared the antibody levels to heartwater in dipped and undipped cattle. They reported that by 12 months of age almost all the undipped calves had seroconverted to *C. ruminantium*, compared with 41% in the dipped group. They also concluded that a state of enzootic stability exists to *C. ruminantium* in undipped Zebu cattle in the study area in Malawi.

1.3.2.3.6 Disadvantages of the use of serological tests for *C. ruminantium*

As early as 1987, Du Plessis, Camus, Oberem & Malan warned of serious problems with the interpretation of the IFAT due to cross-reactions with related *Ehrlichia* organisms. The specificity was first questioned when positive IFAT results were obtained from sera in the Caribbean where no *A. variegatum* occurred. This was followed with sera from Namibia where again no heartwater was known to exist. Du Plessis *et al.* (1987) believed that *E. bovis*, which is common in Africa and transmitted by various *Hyalomma* and *Rhipicephalus* species, must have been the cause of the false positive reactions.

Holland, Logan, Mebus & Ristic (1987) and other workers demonstrated that there was a close serological relationship between *C. ruminantium* and various members of the genus

Ehrlichia. Du Plessis, Bezuidenhout, Brett, Camus, Jongejan, Mahan & Martinez (1993) proved that five different serological tests for heartwater, at five different laboratories, all showed cross-reactivity with *Ehrlichia* species. De Vries *et al*. (1993) in their serological survey of the antibodies to *C. ruminantium* in Zimbabwe, found high background in the field sera, and they believed that this was due to closely related organisms which recognize similar epitopes in the 32-kDa protein to *C. ruminantium*.

Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet (1993) did an extensive investigation into the sensitivity and specificity of the 32-kDa-based serological tests. They used immunoblotting techniques on sheep sera from Zimbabwe to investigate whether any of the sheep had in fact been previously exposed to heartwater. Eleven immunoblot-positive and six immunoblot-negative sheep from heartwater-free areas in Zimbabwe were compared regarding their susceptibilities to challenge with *C. ruminantium*. Prior to challenge, *C. ruminantium* could not be detected in any sheep by transmission to *A. hebraeum* ticks or by PCR conducted with plasma samples. All sheep were equally susceptible to the challenge, and infection was confirmed by brain biopsy, necropsy, PCR and transmission to ticks. Mahan *et al*. (1993) suggested that the immunoblot "positive" reactions of the sera were not due to previous *C. ruminantium* infection, but serological cross-reactions with other positive reactors such as *E. canis*, *E. equi*, *E. phagocytophila*, *E. bovis* and *E. ovina*.

They also reported that there was a higher frequency of immunoblot "positive" reactors in heartwater-free areas of Zimbabwe, which appeared to be associated with minimal tick control. Tick species which occur in these areas, such as *Rhipicephalus evertsi evertsi* and various *Hyalomma* species are known to transmit *E. bovis*, *E. canis* and *E. ovina*. Savadye, Kelly & Mahan (1998) later collected *R. evertsi evertsi* adults from these areas of Zimbabwe with "false positives" and fed these ticks on seronegative sheep. The sheep seroconverted to the MAP-1 antigen, but were again totally susceptible to challenge with *C. ruminantium*.

In conclusion the disadvantages of the serological tests for *C. ruminantium* are mainly due to poor sensitivity and specificity, which include serious cross-reactions with various closely related *Ehrlichia* species.

1.3.2.3.7 Recent developments of the serological tests (MAP-1B ELISA technology)

Recent developments (Van Vliet *et al*. 1995; Mahan, Semu, Peter & Jongejan 1998c) with the MAP-1B ELISA test, however, appear to have at least partially solved the cross-reactions with *Ehrlichia*. Van Vliet *et al*. (1995) showed that the MAP-1 contains at least two immunogenic regions MAP-1A and MAP-1B. The MAP-1A contains conserved epitopes which cross-react with *Ehrlichia* species. The MAP-1B region, however, contains one or more epitopes with a high degree of specificity to *C. ruminantium*, and only antibodies to *E. canis* and *E. chaffeensis* reacted with MAP-1B. Mahan *et al*. (1998b) stated that *E. canis* and *E. chaffeensis* are only found in dog and human sera respectively, and should not interfere with routine ruminant diagnostic serological tests.

Mahan, Semu, Peter & Jongejan (1998c) evaluated the MAP-1B ELISA and the immunoblot assay, and compared their specificity and sensitivity on goat, sheep and cattle sera, from heartwater-free and heartwater-endemic areas.

In the heartwater-free areas the MAP-1B ELISA detected only 1% (bovine), 4% (caprine) and 3% (ovine) positives in the various sera. The immunoblot assay detected 40% (bovine) and 83% (ovine) positive sera in the same heartwater-free areas. In heartwater-endemic areas, the MAP-1B ELISA detected 33% (bovine) and >90% (caprine) positive sera.

It was clear that the immunoblot test detected many "positive" samples in heartwater-free areas. The MAP-1B ELISA test did not detect many false positives (1 - 4%) in cattle, goat and sheep sera in the heartwater-free areas, and clearly the specificity was greatly improved. In heartwater-endemic areas, however, the sensitivity of the MAP-1B ELISA was much more difficult to interpret, and cattle and goat sera showed wide differences in sera-prevalence (33% - >90%).

In conclusion great strides have been made with the refinement of serological tests for *C. ruminantium* over the past decade. As a result, the serological tests have increased in sensitivity and specificity, and as a consequence they can now be used with more confidence in serological surveys.

1.3.2.4 Molecular assays to detect *C. ruminantium*

1.3.2.4.1 Molecular probes (DNA/RNA)

■ Principles of molecular probes

Nucleic acid hybridization can be used to identify specific micro-organisms by using Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA) probes. The principles involved in the use of the molecular probes are illustrated in Figure (Fig.) 1.

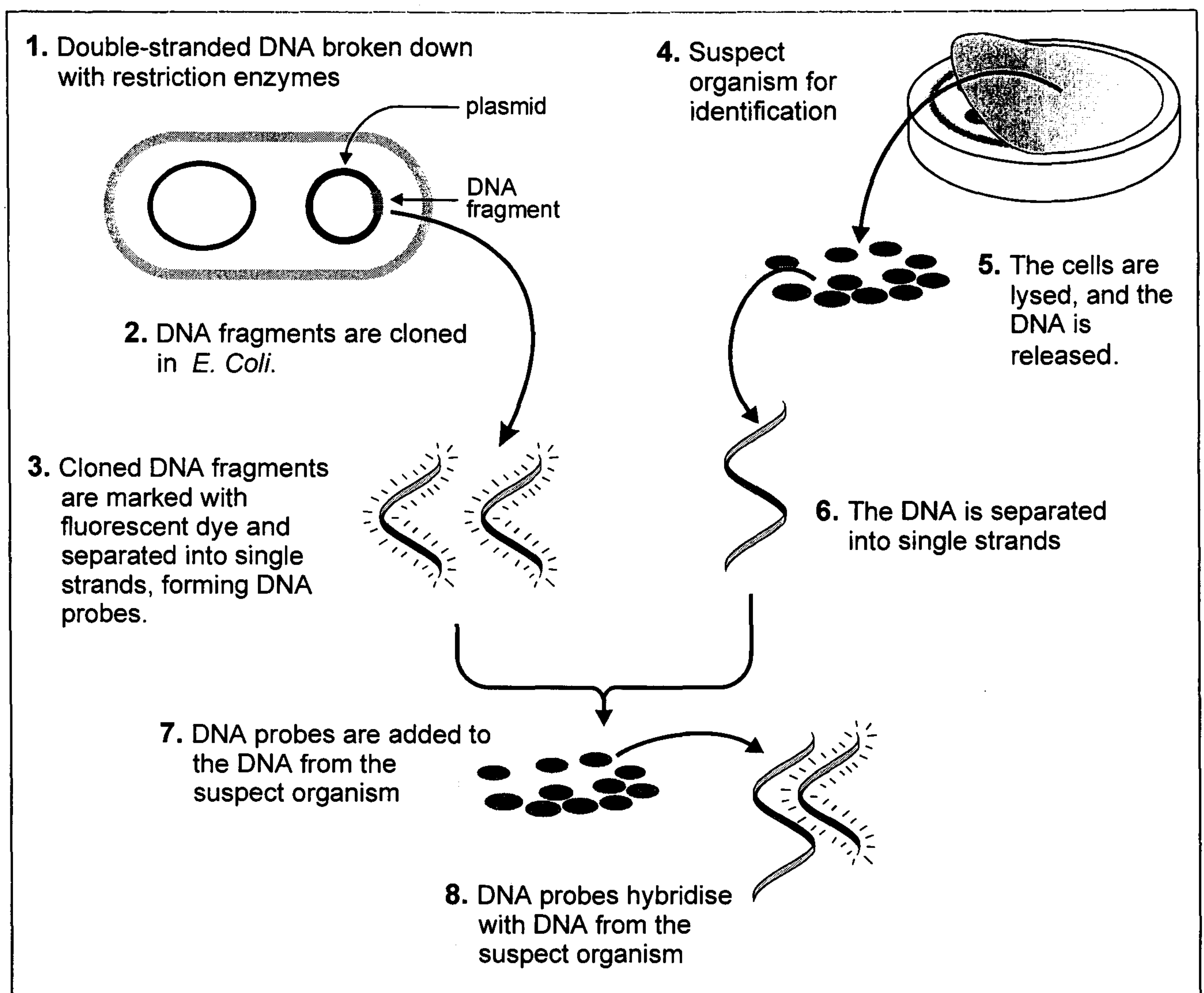

Fig. 1 Principles of a DNA probe used to identify an unknown organism

(Adapted from Tortora *et al.* 1995)

Double-stranded DNA for the probe is initially broken down with restriction enzymes (1), and the small species specific DNA-fragment cloned in *E. coli* (2). The cloned DNA fragments are then marked (3) with fluorescent dye or radioactivity and separated into single strands (DNA probes). These DNA probes can be reacted with target DNA, grown in culture from the suspected organism, which has been blotted onto paper (4 - 6). If the suspected organism is there, the DNA probe will hybridize with its DNA, and this will be detected by radioactivity or fluorescence of the probes.

- Advantages of molecular probes

Molecular probes have certain advantages over conventional diagnostic methods. They are very sensitive, for example, the DNA probe for the diagnosis of *Plasmodium falciparum* malaria can detect 10 picograms (pg) of purified DNA and this is equivalent to only 100 parasites (Barker, Laksami, Rooney, Alecrim, Dourade & Wirth 1986). The *Anaplasma marginale* probe (Eriks, Palmer, McGuire, Allred & Barbet 1989) is 4 000 times more sensitive than existing conventional types of detection. The probes are also very specific and a workable probe should only detect the specific organism and not related organisms. This is because the mechanism of the probe is based on the recognition of the specific DNA structure. The probe for *P. falciparum* for example, only recognizes *P. falciparum* and not two closely related species *P. vivax* and *P. cynomolgi* (Barker *et al.* 1986).

Another advantage of molecular probes is that they give a very quick diagnosis, and are of increased use in the diagnosis of organisms which take a long time to culture or cannot be

cultured. The time taken to process samples is also reduced considerably with molecular probes. Barker *et al.* (1986) stated that with the DNA probe for malaria, a 1 000 samples a day could be done as opposed to only 60 per day with conventional blood smears. Molecular probes can detect antigen directly, and one gets a good indication of present infections as opposed to serological tests which cannot distinguish past and present infections. Finally the sensitivity of the DNA probes can also be exponentially increased through the use of amplification technology such as Polymerase Chain Reaction (PCR).

- Disadvantages of molecular probes

The molecular probe technology is technically demanding, and requires expensive equipment and well-trained laboratory staff. This obviously adds to the cost per sample, so for economic reasons a large throughput of samples is required to bring down the running costs. Although the molecular probes are very sensitive, they cannot detect the very low numbers of organisms circulating in certain reservoir hosts. This was demonstrated by Peter (1995) who found that the Zimbabwean heartwater (pCS20) probe for *C. ruminantium* was not sensitive enough to detect the very low numbers of *C. ruminantium* circulating in reservoir hosts and field ticks.

- Use of molecular probes in medical epidemiological surveys

Much of our present veterinary epidemiological knowledge was gleaned from medical fields. Barker, Banchongaksorn, Courval, Suwongkerd, Rimwungtragoon, Srittong & Wirth (1994) reported on a major epidemiological survey for malaria carried out in Thailand. Their

studies showed that the major advantage of the DNA probe in large-scale epidemiological work was the time saved. Barker *et al.* (1994) were able to increase the time-saving factor by 20 to 40 times, when compared with conventional methods. DNA probes have also been used by research teams to detect *Leishmania* and *Trypanosoma cruzi* in various other epidemiological surveys (Barker 1990).

1.3.2.4.2 Use of molecular probes to detect TBD closely related to *C. ruminantium*

- Anaplasmosis

Much of the early innovations and development of molecular probes to detect anaplasmosis was done by American research workers, and the technology was then transferred to Africa. Goff, Barbet, Stiller, Palmer, Knowles, Kocan, Gorham & McGuire (1988) developed a 2-kilobase (Kb) DNA fragment from a cloned *A. marginale* gene to be evaluated as a DNA probe. They reported that the sensitivity of the probe exceeded that of blood smears, and believed it would be helpful in identifying chronic carrier animals. The probe was species-specific, and did not hybridize with *A. ovis* DNA. Eriks *et al.* (1989) later reported on the development and use of a RNA probe able to detect the very low parasitaemia present in chronic carriers of anaplasmosis. The probe was an RNA transcript made from a 2-Kb fragment of a gene encoding a portion of an *A. marginale* surface protein. The probe was able to detect *A. marginale* in red blood cells 12 days before microscopic diagnosis, and was 4 000 times more sensitive than the latter. Shompole, Waghela, Rurangirwa & McGuire (1989) described the use of a pA012A DNA probe which identified *A. ovis* in goats in Kenya. *A. marginale* and *A. centrale*, which both occur in Kenya, did not hybridize to the probe.

- Babesiosis

DNA probes for *Babesia bigemina*, *B. bovis* and *B. caballi* have been developed recently, and are sensitive enough to detect clinical infection but not carrier status (Reddy & Dame 1992). A RNA-based probe for the detection of carrier status in *B. bigemina* has been tested and can detect the very low numbers of *Babesia* organisms present in carrier animals (Reddy & Dame 1992).

- Theileriosis

Conrad, Iams, Brown, Sohanpal & Ole-MoiYoi (1987) used three different DNA probes from a genomic library of *Theileria parva* to distinguish the five major stocks of *T. parva* in Kenya. The probe was the most useful in distinguishing between all five stocks, and did not hybridize to *Theileria mutans*, *Theileria taurotragi* or *Theileria annulata* (Conrad *et al.* 1987).

1.3.2.4.3 Molecular probes for *C. ruminantium*

The development of molecular probes for *C. ruminantium* closely followed those for anaplasmosis, and a lot of the highly technological developments were in the USA, Kenya and Zimbabwe. Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet. Burridge & McGuire (1991) reported on two cloned DNA probes which identified *C. ruminantium* in *A. variegatum* ticks. These two probes were evaluated, and the pCS20 probe was shown to be more sensitive and specific than the *C. ruminantium* probe (pCR9) from Kenya. More

recently Allsopp, Hattingh, Vogel & Allsopp (1999) evaluated a panel of eight different molecular probes used for the detection of *Cowdria* and *Ehrlichia* species. The development and evaluation of the probes for *C. ruminantium* are discussed in detail in Chapter 3 (section 3.1).

1.3.2.4.4 Polymerase chain reaction

- Principles of PCR

Very small quantities of DNA can be amplified with a method called PCR, to produce usable quantities of DNA for analysis. The procedure was originally developed by Mullis & Co-workers and he received the Nobel Prize for this work in 1993, and today the technique is standard practice in laboratories throughout the world.

- Advantages of PCR

The PCR is a very fast, highly sensitive and specific diagnostic procedure which makes it very useful in epidemiological surveys which require extreme levels of sensitivity and specificity (Azad, Webb, Carl & Dasch 1990). It also has other advantages over conventional assays as it does not require frozen samples. DNA from dry dissected organisms, as well as archival specimens and alcohol-preserved samples can be used. The PCR has found wide application in various fields of molecular biology. In human diagnostics it is widely used for the pre-natal diagnosis of inherited diseases, and by evolutionary biologists and forensic pathologists to detect minute quantities of DNA.

- Disadvantages of PCR

The very sensitivity which makes the PCR a useful technique, is also its most serious

limitation. Because of exponential amplification, targets can be achieved from any air-borne cross-contaminants and can present a serious risk of "false positive" amplification. By far the most common source of "false positive" contamination are products of previous PCR reactions. The sequence of the target material used for the PCR must also be known in order to develop the primers.

- The use of PCR in epidemiological surveys

In the last decade there has been a vast list of reports on PCR techniques in epidemiological surveys world-wide. In the veterinary diagnostic field, the PCR has found most credibility with those organisms which have traditionally been difficult to culture, some of these are described below.

African swine fever virus (ASFV) is enzootic in certain areas of Europe and often difficult to identify. Steiger, Ackermann, Mettraux & Kihm (1992) developed a PCR to detect ASFV which is rapid and safe. Lopez, Osorio & Donis (1991) also developed a PCR for bovine viral diarrhoea which was historically difficult to distinguish from numerous, similar viruses of bovines. Gerritsen, Olyhoek, Smits & Bokhout (1991) have reported on a rapid, specific and sensitive PCR for *Leptospira interrogans* in bovine urine. As culturing of *Leptospira* organisms could take up to six months to confirm a negative sample, this rapid PCR test would have practical benefits to dairy farmers.

1.3.2.4.5 The use of PCR to detect TBD closely related to *C. ruminantium*

- Anaplasmosis

Stich, Bantle, Kocan & Fekete (1993) collected haemolymph from the severed legs of

Dermacentor andersoni ticks, and *A. marginale* DNA was detected by a PCR assay in all the pooled haemolymph samples of ticks which had fed on infected hosts. *A. marginale* was also detected in adult ticks which had fed one day earlier on an infected calf. Stich *et al.* (1993) suggested that this test would be used to detect *A. marginale* in field collected ticks. They also concluded that the PCR/haemolymph test is a simple, rapid and sensitive method to detect *A. marginale* in ticks. The use of haemolymph samples also circumvents any cross-reaction and Taq-polymerase inhibition which were experienced with midgut specimens (Stich *et al.* 1993)

- Babesiosis

The serological techniques used to diagnose bovine babesiosis do not consistently detect carrier animals, and do not specifically eliminate cross-reactions between *B. bigemina* and *B. bovis*. The use of specific DNA probes and nucleic acid hybridization had several advantages over conventional microscopic, serological and sub-inoculation techniques. According to Figueroa, Chieves, Johnson & Buening (1992) the DNA probe to detect *B. bigemina* in carrier cattle lacked sensitivity. They described the development of a PCR-based assay which detected latently infected cattle over an 11-month period. The PCR-based test was 10 000 times more sensitive than the non-radioactive probe. The authors believed that the high specificity and sensitivity of this PCR assay would provide a valuable tool for epidemiological studies.

- Theileriosis

There have been numerous reports on the use of the PCR assay to detect various *Theileria*

species in domestic animals. Bishop, Sohanpal, Kariuki, Young, Nene, Baylis, Allsopp, Spooner, Dolan & Morzaria (1992) demonstrated that the PCR using *T. parva*-specific primers was capable of detecting parasites present at low parasitaemias in carrier cattle. D'Olivera, Van der Weide, Habela, Jacquiet & Jongejan (1995) used the PCR technique to detect *T. annulata* in blood samples from carrier cattle, and De Kok, D'Olivera & Jongejan (1993) used the PCR assay on alcohol-preserved specimens to detect *T. annulata* in *Hyalomma* ticks. Both confirmed that the PCR specifically amplified the 372-base pair (-bp) theilerial RNA target.

1.3.2.4.6 Detection of *C. ruminantium* in *Amblyomma* ticks

- Conventional methods

The morphology and distribution of *C. ruminantium* in *A. hebraeum* was first described by Cowdry in 1925. He used conventional Giemsa staining techniques to demonstrate colonies of *C. ruminantium* in the gut epithelial cells, and sometimes in the lumen of the alimentary tract of the tick. These findings were later confirmed with fluorescent antibody staining techniques, and light and electron microscopy in *A. hebraeum* (Bezuidenhout 1984), and *A. variegatum* (Kocan, Morzaria, Voigt, Kiarie & Irwin 1987). Yunker, Kocan, Norval & Burridge (1987) reported that *C. ruminantium* colonies in the midgut of *A. hebraeum* could be distinctively stained with a Mallory's phloxine-methylene blue stain. They predicted that the stain could be applicable in detecting infected ticks collected in the field.

Kocan, Bezuidenhout & Hart (1987) described the presence of two types of *C. ruminantium*, electron dense and reticulate forms, in the gut epithelial cells of nymphal *A. hebraeum*. They

also observed the first *C. ruminantium* in the salivary glands of known infective *A. hebraeum*. An ELISA for detecting infection in *A. hebraeum* nymphs has been described, but has not been widely used (Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen 1986).

- The use of the pCS20 probe/PCR assay to detect *C. ruminantium* in *Amblyomma* ticks

A pCS20 probe for *C. ruminantium* detection in *Amblyomma* ticks has been developed and tested in the field (Waghela *et al.* 1991), but was found to lack the sensitivity to detect the very low numbers of the organisms found in field ticks. A PCR assay was developed by the University of Florida/United States Agency for International Development/Southern African Development Community (UF/USAID/SADC) Heartwater Project in Harare, Zimbabwe, to detect *C. ruminantium* in *Amblyomma* ticks. This will be discussed in detail in Chapter 3 (section 3.1).

Peter, Deem, Simbi, Barbet, Norval, Kelly & Mahan (1995a) evaluated the PCR assay as a method of detecting these very low rickettsaemias in carrier animals and *Amblyomma* ticks. The PCR assay detected *C. ruminantium* in both *A. hebraeum* and *A. variegatum* ticks from different geographical areas. The PCR assay was also specific for *C. ruminantium*, and did not detect other haemoparasitic TBD of domestic animals, or DNA from closely-related serologically cross-reactive organisms such as *E. canis*. Peter *et al.* (1995a) concluded that the PCR supercedes all older detection methods such as microscopy and xenodiagnosis, because of its high sensitivity, specificity and speed.

- The use of the PCR to detect *C. ruminantium* in ruminant and wildlife hosts

Mahan *et al.* (1993) were the first to use the PCR for detection of *C. ruminantium* in infected sheep plasma. They demonstrated that many immunoblot positive sheep from heartwater negative areas of Zimbabwe, were heartwater negative and were in fact, infected with tick transmitted *Ehrlichia* species and not *C. ruminantium* (refer to section 1.3.2.3.6). Mahan *et al.* (1993) demonstrated that the PCR assay can be used to distinguish pre- and post-*Cowdria* infection in the plasma of sheep. They showed that in the pre-infection sheep (uninfected) there was no PCR amplification of the *Cowdria* specific 279-bp DNA products. After challenge with *C. ruminantium*, the 279-bp DNA product was amplified by PCR.

Peter *et al.* (1995a) were able to use the PCR on ticks which had been feeding on recovered carrier cattle, and these ticks were then shown to be infected with *C. ruminantium* by using the DNA probe and the PCR assay. Peter (1995) also demonstrated that *C. ruminantium* can be detected in carrier cattle, by using the DNA probe on the cattle and the PCR assay on the ticks which had fed on the cattle.

Koch *et al.* (1995) developed a PCR assay to detect *C. ruminantium* in whole blood and bone marrow samples in clinically normal, free-ranging Zimbabwean wild ungulates. Prior to this work there was no reference to the use of whole blood or bone marrow for the PCR detection of *C. ruminantium*. PCR inhibition by elements in the haemoglobin was thought to be the reason for negative PCR results. Koch *et al.* (1995) also found positive PCR in tsessebe (*Damaliscus lunatus*), waterbuck (*Kobus ellipsiprymnus*) and impala *(Aepceros melampus*). They suggested that these wild animals could be important reservoirs for *C. ruminantium*.

1.3.3 THE *AMBLYOMMA* TICK VECTOR: ECOLOGICAL ASPECTS

1.3.3.1 Important tick vectors

Only ticks of the genus *Amblyomma* have been shown to be capable of transmitting *C. ruminantium*. *Hyalomma truncatum* (Du Plessis 1990), and *Rhipicephalus evertsi evertsi* (Savadye *et al.* 1998) have, however, been implicated of transmitting *Cowdria*-like organisms which may interfere with serological surveys.

Heartwater occurs only where its vectors are present, and ten *Amblyomma* tick species are capable of transmitting the organism in Africa. The major species are *A. variegatum* and *A. hebraeum*. Species of lesser importance include *A. pomposum* (Angola), *A. lepidum* (East Africa), *A. astrion* (mainly a buffalo tick), *A. cohaerens* and *A. gemma*, both of which occur in East Africa (Walker & Olwage 1987). *A. sparsum*, *A. tholloni* and *A. marmoreum* are regarded as accidental vectors on domestic hosts and normally feed on wild animals. Only *A. variegatum* has spread to other parts of the world, and is now well-established in the West Indies (Burridge 1985). Three American species of *Amblyomma* (*A. maculatum*, *A. cajennense* and *A. dissimile*) have been shown to be capable of transmitting *C. ruminantium* (Uilenberg 1982; Barré *et al.* 1987; Bezuidenhout 1987; Jongejan 1992).

1.3.3.2 Distribution of the vectors

The distributions of the *Amblyomma* vectors and potential vector species in Africa and the Americas have been well-documented (Walker & Olwage 1987). *A. hebraeum* is restricted to southern Africa (Mocambique, Swaziland, Botswana and South Africa), and in Zimbabwe it co-exists with *A. variegatum*. Walker & Olwage (1987) confirmed that three *A. hebraeum* males were collected in Tanzania, but believed these to have been transported there by birds.

A. variegatum is the most common *Amblyomma* species in Africa, and in southern Africa is widespread in Mocambique, Botswana and Zimbabwe. It has spread to Madagascar, presumably with mainland cattle, many years ago. *A. variegatum* has also spread to various islands in the Caribbean, presumably by the importation of tick-infested cattle from West Africa (Burridge 1985).

Amblyomma pomposum is almost entirely restricted to Angola, and its distribution overlaps that of *A. variegatum*. *Amblyomma lepidum*, *A. cohaerens* and *A. gemma* are common in East Africa and parts of Sudan, Ethiopia and Somalia. *A. lepidum* has been implicated in the transmission of heartwater to sheep and goats in Sudan (Karrar 1960). *A. cohaerens* and *A. astrion* are closely associated with buffalo (Walker & Olwage 1987). *A. gemma* is common in East Africa, and has recently been shown to be a vector of *C. ruminantium* (Wesonga, Mukolwe & Rurangirwa 1993).

A. marmoreum, in the adult stage, is almost exclusively a tick of reptiles (tortoises, snakes and monitor lizards) (Walker & Olwage 1987). The immature stages are found on domestic and a variety of wild animals (Horak, MacIvor, Petney & De Vos 1987). *A. marmoreum* has also been shown to transmit *C. ruminantium* (Bezuidenhout 1987), and has recently been discovered on tortoises in the USA (Allan, Simmons & Burridge 1998). *A. tholloni* is exclusively found on elephants, although MacKenzie & Norval (1980) reported that in Zimbabwe domestic animals were infested with immature ticks of this species.

1.3.3.3 Population dynamics of the *Amblyomma* species

1.3.3.3.1 South Africa

South Africa has a long history of ticks and TBD because of the historical importance to the stock industry. The only *Amblyomma* species of economic importance in South Africa is *A. hebraeum*. In 1900, Lounsbury confirmed the long-standing suspicion that *A. hebraeum* was the vector of *C. ruminantium* in South Africa. Much of the early work was conducted in those regions of South Africa where heartwater was a major problem for the farmers. It was soon realized that more information about the life cycle and seasonal occurrence of the tick was necessary in order to control it. This led to the establishment of Research Centres at Onderstepoort, Grahamstown and Pietermaritzburg.

Alexander (1931) stated that farmers in the Eastern Cape region of South Africa blamed their heavy stock losses (2 000 - 3 000 sheep and goats per annum), to the introduction of *A. hebraeum* from Zululand where the tick was endemic. Heartwater also had devastating effects on the highly susceptible Angora goat flocks, and this resulted in increased research into the life cycle and population dynamics of *A. hebraeum*. Norval (1974; 1977) was responsible for the early research on the ecology, life cycle and population dynamics of *A. hebraeum* in the Eastern Cape Province. He concluded that the life cycle of *A. hebraeum* can last for up to three years, the adults were more prevalent in mid- to late summer, while the immature stages were found throughout the year, and concluded that the absence of nymphal activity was due to a decrease in photoperiod (Norval 1977).

To investigate the dynamics of various tick populations in the Eastern Cape, a detailed five-year survey was then conducted at the Tick Research Unit in Grahamstown. *A. hebraeum* adults were found to be present on cattle throughout the year, but the highest numbers were encountered in summer. This was followed by larval peaks in late summer and early winter, and two nymphal peaks, one in early midsummer and the other in winter (Rechav 1982). Rechav concluded that the life cycle in the Eastern Cape probably only lasted one year and not three years as suggested by Norval (1977).

Horak & Williams (1986), also working at the Tick Research Unit in Grahamstown, reported that helmeted guinea fowls (*Numida meleagris*) were important hosts of *A. hebraeum* immatures, especially the larval stages. Howell, Petney & Horak (1989) also looked at the four-striped grass mouse (*Rhabdomys pumilio*) as a possible host of *A. hebraeum*, but concluded that it was not a significant carrier of these ticks. MacIvor (1982) noted that there was a clear relationship between foot abscesses and the seasonal abundance of *Rhipicephalus glabroscutatum* and *A. hebraeum*, the two most important tick species found on the feet of Angora goats in this region.

An early investigation in KwaZulu-Natal on the tick infestation of livestock by Baker & Du Casse (1967) confirmed that *A. hebraeum* was common on cattle on the farms studied. The adult *A. hebraeum*, in this study, were active from the beginning of September and their numbers only declined towards the end of January. They also noted that although Theiler (1962) had concluded that most immature *A. hebraeum* occur on birds, in their study, a significant proportion also occurred on cattle, and then especially on their limbs.

Much of the early work on tick taxonomy and population dynamics at the Onderstepoort Veterinary Institute was initiated by Gertrud Theiler (1948; 1962), and this culminated in a publication on the ixodid ticks found in this region (Theiler 1962). Londt, Horak & De Villiers (1979) working in the Naboomspruit district, in the Northern Province of South Africa, collected ticks off cattle and only 4% of these were *A. hebraeum*. Although they were unable to conclude exactly when the various peak abundances occurred, they hypothesized that the peak larval period was in late summer/autumn, the nymphal peak in winter/spring and the adult peak periods August or September to January, or February.

Horak has published extensively on the ectoparasites associated with wild animals in South Africa. Some of his more important findings associating *A. hebraeum* with these animals are summarized below:

- The size of the host animal does not appear to have a strong influence on the total tick loads, but with certain exceptions, the larger hosts tended to carry more adult ticks (Horak 1995). Two exceptions to this observations were, warthogs (*Phacochoerus aethiopicus*) which are relatively small animals yet carry fairly large burdens of *A. hebraeum*, and blue wildebeest (*Connochaetes taurinus*) which carried virtually no ticks (Horak 1995). The smaller hosts, such as helmeted guinea fowls (*Numida meleagris*), and scrub hares (*Lepus saxatilis*), tended to carry more immature ticks.
- Certain host species, such as blue wildebeest, seem to have a natural resistance to infestation, and as a consequence carry very low tick burdens (Horak, De Vos & Brown 1983).

- A complete absence of *A. hebraeum* on gerbils (family *Gerbillinae*) and veld rats (*Aethomys* spp.) and very low numbers on striped mice (*Rhabodomys pumilio*). Rodents probably play no role in the epidemiology of heartwater (Howell *et al.* 1989; Horak 1995).
- Burchell's zebra (*Equus burchelli*), shot in the Kruger National Park (KNP), were quite good hosts of all stages of *A. hebraeum*, and they were present on these zebras throughout the year (Horak, De Vos & De Klerk 1984).
- Warthogs must be considered a preferred host of *A. hebraeum*, especially the adult stages (Horak, Boomker, De Vos & Potgieter 1988).
- *A. hebraeum* was the most abundant larval tick collected by drag sampling in the KNP (Spickett, Horak, Braack & Van Ark 1991).

Rechav & De Jager (1991) found that *A. hebraeum* was one of the three most abundant ticks on goats in the Northern Province of South Africa. Adult *A. hebraeum* were present on the goats during summer but absent during winter. The nymphs were present during most of the year, but absent in July (Rechav & De Jager 1991).

1.3.3.3.2 Zimbabwe

The history of tick and TBD in Zimbabwe has been extensively reviewed (Lawrence & Norval 1979). The first recorded heartwater case was in 1927, and it was thought that the disease had been introduced from South Africa (Jack 1942). In 1930, the bont tick (*A. hebraeum*) had spread to large areas of the western lowveld region of Zimbabwe (Norval,

Perry, Meltzer, Kruska & Booth 1994). Between 1942 and 1975 the vector was subjected to a very intensive dipping campaign, and in 1975 only three isolated foci of the tick remained (Norval *et al.* 1994). The disruption of dipping during seven years of conflict in Zimbabwe lead to the breakdown of dipping services, and therefore to the spread of *A. hebraeum* in large areas of Zimbabwe (Norval *et al.* 1994), and this spread has continued up to the present (Bruce &Wilson 1995; Peter, Perry, O'Callaghan, Medley, Shumba, Madzima, Burridge & Mahan 1998).

A. hebraeum has always been absent from the highveld regions of Zimbabwe, and it was thought that the climatic conditions in these areas were not conducive to the survival of *A. hebraeum* (Theiler 1962), as well as the lack of alternative hosts in the region (Norval 1983). *A. hebraeum* immatures can feed on a wide range of domestic hosts, as well as wild animals and the translocation of the latter hosts to game reserves may have played an important role in the rapid spread of heartwater in Zimbabwe.

Norval, Andrew & Meltzer (1991) looked at the seasonal occurrence of *A. hebraeum* in Zimbabwe and concluded that adults and nymphs were present on cattle at all times of the year, and they showed less seasonal periodicity than the other three host ticks in the region. Mooring, Mazhowa & Scott (1994) investigated the effects of rainfall on tick challenge at the Kyle Game Reserve near Masvingo, Zimbabwe, and noted that in the case of *A. hebraeum*, only the larval stage could be correlated with the monthly rainfall pattern. The distribution of *A. variegatum* in Zimbabwe has remained static since the early part of the century, and is restricted to the north-western region (Norval *et al.* 1994).

1.3.3.3.3 *A. variegatum* in Zambia

The population dynamics of *A. variegatum* in Zambia are well-documented, with the adults active during the rainy season, and peak abundance on cattle from November to January (MacLeod 1970; Pegram, Perry & Schels 1984). The larvae are present from March to May, and the nymphal population during the cool, dry season May - June (Pegram *et al.* 1984). *A. variegatum* is one of the most important tick species in Zambia, and has been reported from a wide variety of habitat types (Mangani 1997). Very few *A. variegatum* larvae were, however, collected by Zieger, Horak & Cauldwell (1998), and only about 200 of the nearly 40 000 larvae collected by drag-sampling at Mtendere Game Ranch in Central Province, Zambia, were *A. variegatum*.

1.3.3.3.4 *Amblyomma* species in Botswana

Both *A. hebraeum* and *A. variegatum* are present in Botswana, but *A. hebraeum* causes the greatest economic problems (Chimbombi 1997). This is usually when heartwater-susceptible cattle are moved to the market area bordering South Africa, where *A. hebraeum* is found. *A. variegatum* is found in the extreme north of Botswana, which borders Zambia and Zimbabwe.

1.3.3.3.5 *Amblyomma* species in Mocambique and Angola

Heartwater occurs throughout both countries, but mainly during the rainy season. In southern Mocambique *A. hebraeum* is the main heartwater transmitter, and *A. variegatum*

is found in the north of the country (Mavale 1997). In Angola *A. variegatum*, *A. pomposum* and *A. astrion* are the most common *Amblyomma* species (Gomes 1993). *A. variegatum* is only found in the eastern region of Angola, whilst *A. pomposum* is far more widely spread throughout the southern and northern regions of Angola (Ricardo 1997). *A. pomposum* is found in those areas of Angola where most of the domestic animals are kept, and is the most important vector of heartwater. Adults occur throughout the year, but the tick loads are heavier on cattle during the rainy season (Gomes 1993).

1.3.3.3.6 *Amblyomma* ticks in Swaziland

Heartwater occurs in all four agro-ecological zones in Swaziland, and the incidence of the disease follows that of the vector *A. hebraeum* (Dlamini 1997). Heartwater is the second most important disease affecting cattle, and *A. hebraeum* is present at nearly 80% of the diptanks surveyed in the Lowveld and Lubombo region (Dlamini 1997). *A. hebraeum* is also common on wild animals in Swaziland, and Gallivan (1995) found that adult ticks were common on the five species of ungulates studied. Gallivan, Culverwell, Girdwood & Surgeoner (1995) also found that impala (*Aepyceros melampus*), at the Mlawula-Mbuluzi-Simunye Nature Reserve were infested with *A. hebraeum* adult ticks, and these ticks cause a loss of body condition.

1.3.3.3.7 *Amblyomma* species in East Africa (Kenya, Tanzania, Uganda and Burundi)

A. variegatum on Ruzinga Island in Western Kenya appears to have only one generation per annum (Punyua, Latif, Nokoe & Capstick 1991). The high adult infestations of cattle with *A. variegatum* reached a peak between January and February, and were followed by a

sudden drop with the onset of the rainy season (Punyua *et al.* 1991). In Uganda, Kaiser, Sutherst & Bourne (1982) found that *A. variegatum* was the second most common tick on Zebu cattle, although all species of ticks were present throughout the year, with no seasonal variation in abundance of the two- and three-host species (Kaiser *et al.* 1982).

Kaiser *et al.* (1982) working in Burundi, gave a detailed record of the population dynamics of the ticks on the local "Ankole" cattle. The life cycle of *A. variegatum* in this region is closely linked to the rainfall, and the adults peak after the heavy rain in September to January (Kaiser *et al.* 1982). The larvae follow after a four-month delay, and the nymphs are present in the dry season (Kaiser *et al.* 1982). Twelve *Amblyomma* species have been identified in Tanzania, but only *A. variegatum* and *A. gemma* are important vectors of heartwater (Otaru 1997).

1.3.3.3.8 *Amblyomma* species in West Africa (Mali, Nigeria and Senegal)

Teel, Bay & Ajidagba (1988) found *A. variegatum* to be a very common tick in southern Mali, and the adult ticks were found to increase after the rains, followed by the larvae and nymphs in the winter/dry season. Bayer & Maina (1984) found that *A. variegatum* was a dominant tick species in their survey in Nigeria. In Senegal, Gueye, Mbengue, Diouf & Sonko (1993) and Gueye, Mbengu & Diouf (1994) found that *A. variegatum* was a common tick on the cattle, sheep and goats which they sampled.

1.3.3.4 **Pathogenesis / Life Cycle in the tick**

In infected ticks the organisms have been seen in the epithelial cells of the midgut (Cowdry

1925). Cowdry (1925) believed that transmission occurred by regurgitation of the gut contents during the feeding of the tick. More recent information suggested that *C. ruminantium* is injected into the host with the tick's saliva (Kocan, Bezuidenhout & Hart 1987). Organ suspension of infected ticks demonstrated that all organs in the tick were infected with *C. ruminantium*, except the brain, haemolymph and ovaries (Bezuidenhout 1988).

1.3.3.5 Behaviour of the vectors in the field

Unfed nymphs and adults of *A. variegatum* and *A. hebraeum* do not ascend the vegetation to await passing hosts, as occurs in so many other ixodid tick species. For many years it was not known where the adult and nymphal stages of *A. hebraeum* survived during the long, unfavourable, non-parasitic period of the life cycle. It is now well-documented that these ticks seek shelter beneath the debris on the soil surface and only become active in response to CO_2 and AAAP (Norval, Yunker & Butler 1987).

The unfed ticks are able to locate suitable hosts from distances of up to 25 metres. The ticks also attach preferentially to suitable hosts, which carry attached fed males secreting AAAP. This largely eliminates their chances of attaching to unsuitable hosts (dipped hosts with no males, or hosts with good grooming behaviour), on which they would be unlikely to survive (Norval, Andrew, Yunker & Burridge 1992). Game ranches where cattle share grazing with large wild animals, which naturally have a high prevalence of pheromone secreting male ticks, make the control of *A. hebraeum* extremely difficult.

1.3.4 *COWDRIA RUMINANTIUM* IN THE TICK VECTOR

1.3.4.1 Infection rates of *C. ruminantium* in the tick vector

In order to advise government policy makers on the use of strategic control, more information on the population dynamics of the *Amblyomma* vector species and their infection rates is required (Norval 1988). Many surveys on the *Amblyomma* ticks of Africa have been published, and we can now, with a certain degree of confidence, predict the presence, or absence, of the *Amblyomma* vectors in specific areas, and at specific times of the year. However, this is not the case with the infection rates in free-living or non-parasitic ticks, probably because of the difficulty in detecting with confidence the presence of *C. ruminantium* in *Amblyomma* ticks. In the past it was generally assumed that the heartwater infection rate in tick populations was low, as domestic ruminants could escape infection with heartwater whilst living in endemic regions (Currasson & Delpy 1928; Alexander 1931; Bonsma 1944; Neitz 1939; Uilenberg 1971).

The infection rates in most vector-borne diseases of domestic animals and man have been published and some of these are summarised in Table 1. It is noticeable that most of these infection rates are less than 5%. The infection rates of *C. ruminantium* in *Amblyomma* ticks obtained by the different research teams in Africa and the Caribbean, are summarized in Table 2. It is clear from Table 2 that a wide variety of different methods have been used for determining the infection rates, but only one (Peter *et al.* 1999) uses free-living, unfed *A. hebraeum* collected with a AAAP/CO_2 trap and processed with the PCR assay.

TABLE 1: Infection rates of some important vectors of domestic animals and man which have been reported in the literature

AUTHORS/ YEAR	VECTOR	PARASITE	DISEASE	INFECTION RATE IN VECTORS
Jupp **1994	Mosquito	RVF virus	RVF	0.01%
Plowright *et al.* **1994	*Ornithodoros* spp. Tampans	ASF virus	ASF	0.3 - 1.7%
Mahoney & Mirre 1971	*Boophilus* spp. ticks	*Babesia bigemina*	Redwater Bovine babesiosis	0.23%
Mahoney & Mirre 1971	*B. microplus*	*Babesia bovis*	Redwater	0.04%
Leitch & Young 1981	*R. appendiculatus*	*Theileria parva*	ECF	1 - 2 %
Dallwitz *et al.* 1986	*Boophilus* spp. ticks	*Anaplasma marginale*	Anaplasmosis	0 - 100%
Burgdorfer *et al.* 1979	*Ixodes ricinus*	Swiss *Rickettsia*	Spotted fever group	8.4%
Burgdorfer *et al.* 1985	*Ixodes pacificus*	*Borrelia burgdorferi*	Lyme Disease	1 - 2%
Magnarelli *et al.* 1986	- *Ixodes damini*	"	"	10.5%
	- *Dermacentor albipictus*	"	"	0.6%
	- *Amblyomma americanum*	"	"	3.5%
Rijpkema *et al.* 1994	*Ixodes ricinus*	"	"	13 - 46%
Piesman *et al.* 1986	*Ixodes damini*	*Babesia microti*	Human babesiosis	11 - 47%

** Coetzer, Thompson & Tustin. 1994. *Infectious Diseases of Livestock*. Oxford University Press

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TABLE 2: Infection rates of *Cowdria ruminantium* in *Amblyomma* ticks reported in the literature

AUTHORS & YEAR	**STUDY AREA**	**TICK SPECIES**	**METHOD OF COLLECTION**	**METHOD TO DETECT *Cowdria***	**FORMULA TO DETECT INFECTION RATE**	**TOTAL SAMPLE SIZE**	**LIFE CYCLE STAGE**	**INFECTION RATES (% POSITIVES)**
Uilenberg 1971	MADAGASCAR	***A. variegatum***	Field exposure	Sheep blood injected into cattle	-	-	-	1% estimate
Du Plessis 1981	SOUTH AFRICA	***A. hebraeum*** Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = 240		13/240 5.4%
Du Plessis & Malan 1987	SOUTH AFRICA 23 farms in heartwater endemic regions	***A. hebraeum*** Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = average 6 ticks per cow	-	1.6% - 30% (mean 7%)
Du Plessis *et al.* 1992	SOUTH AFRICA Mara	***A. hebraeum*** Adults	Off cattle	Tick homogenate injected into mice - confirm IFA	-	n = 240	-	0% - 5% (mean 2.9%)
Peter *et al.* 1999	SOUTH AFRICA KNP	***A. hebraeum*** Adults	AAAP trap	PCR assay	-	n = 292	A	1.7%
Norval 1981	ZIMBABWE Various communal grazing areas	***A. hebraeum*** Engorged nymphs	Off cattle	Adults on sheep	Pooled ticks fed on 7 sheep	108		2/7 sheep died 28% positive
Norval *et al.* 1990	ZIMBABWE - Mbizi - Lemco	***A. hebraeum*** Unfed adults & nymphs Nymphs	AAAP trap Off cattle	Pooled samples fed on sheep Adults on sheep	$P = 1 - \left(\frac{n - x}{n}\right)^{1/m}$	32 40 80 49 36	M F N M F	0 → 45% 20 → 36% 0 → 13% 10 → 25% 20 → 34%
Peter 1995	ZIMBABWE Manifest Farm	***A. hebraeum*** Adults	Off sentinel cattle	PCR assay on ticks	-	n = 512		1.76%
Peter 1997	ZIMBABWE - Southern lowveld	***A. hebraeum*** Adults and nymphs	Off cattle	PCR assay on ticks	-	A = 441 N = 95	A M F N	11.48% 10.5% 12.45% 3.16%
Gueye *et al* 1993	SENEGAL - Zones: North Guinean, Niayes, South Sudan	***A. variegatum*** Adults and nymphs	Off cattle	Tick supernatant injected intra-venously into 30 sheep	3 pools of ticks 1 RO = $\frac{\text{No of ticks}}{\text{Total ticks}}$ **Inf.** x 100		A N	11% 7.8%
Camus & Barré 1987b	CARIBBEAN (Guadeloupe)	***A. variegatum*** Adults	Partially engorged ticks off cattle	Individual tick homogenate injected into mice - confirm IFA	- Pooled ticks	200 19 pools	-	3/200 Pos (1.5%) 12/19 Pools Overall (1 - 2%)

A = Adult **N** = Nymphs **M** = Male **F** = Female **Inf.** = Infected **KNP** = Kruger National Park

1.3.4.1.1 Infection rates in South African ticks

Du Plessis (1981; 1985) was the first researcher in South Africa to attempt to find a method to detect the percentage of ticks infected with *C. ruminantium* (refer to Table 2). He collected 240 adult *A. hebraeum* (50% males and females) off cattle throughout the heartwater-endemic regions of South Africa. These ticks were then homogenized and injected either intraperitoneally (I/P), or I/V into mice, and the mice either seroconverted to *C. ruminantium* or died of heartwater (Du Plessis 1985). Thirteen out of the 240 ticks tested were positive for *C. ruminantium* (mean of 5.4%), and of these only one mouse died of heartwater and 12 seroconverted. The results of the various experiments ranged from 2.5% to 20% prevalence (Du Plessis 1985).

Du Plessis & Malan (1987) did an extensive epidemiological study of 23 farms, all of which were in heartwater-endemic regions of South Africa. Six adult *A. hebraeum* ticks were collected off each cow in the survey, and the homogenates of these ticks were injected I/V into mice. A mean of 7% (range 1.6% to 30%) of these ticks were positive for *C. ruminantium*. Du Plessis & Malan (1987) also concluded that the *A. hebraeum* burdens on the cattle were the most important determinants of heartwater immunity. They also showed that many of the farms with a high infection rate in the ticks, also had higher seropositivity to *C. ruminantium*. They concluded, however, that the latter relationship was not absolute (Du Plessis & Malan 1987).

In 1992, Du Plessis, Loock & Ludemann carried out an important epidemiological study at the Mara Experimental Farm in the Northern Province of South Africa. Two hundred and

forty adult *A. hebraeum* (125 males and 115 females) were collected and injected into mice. Positive results ranged from 0% to 5%, and never exceeded 5%. They concluded that the seropositivity of calves at six months of age was related to their tick burdens and the infection rates of these ticks. In other words, the higher the *A. hebraeum* burdens and tick infection rates, the more seropositive and immune the calves were. In this particular study 97% of the calves were found to be serologically positive, which indicated exposure to *C. ruminantium* or a closely-related organism. These seropositive calves were not necessarily immune to heartwater.

Du Plessis *et al.* (1992) went a step further, and proposed that if an average of ten adult *A. hebraeum* ticks were found on the calves in a heartwater-endemic area, then 83% of the calves would have seroconverted to *C. ruminantium* at six months of age. If 15 ticks were found on the calves than 97% of them would be serologically positive (Du Plessis *et al.* 1992). They stated that the present tick burdens of ten ticks per animal throughout the year at Mara, coupled with an infection rate in the tick of 3%, was enough to maintain endemic stability to heartwater. They concluded that higher tick infection rates and lower tick burdens, would suffice to induce endemic stability.

More recently Peter, Bryson, Perry, O'Callaghan, Medley, Mlambo, Smith, Horak, Burridge & Mahan (1999) collected 292 adult *A. hebraeum* with the AAAP trap near the Skukuza rest camp in the KNP, and tested them with a PCR assay. Of these ticks 1.7% were positive for *C. ruminantium*.

1.3.4.1.2 *C. ruminantium* infection rates in Zimbabwean ticks

In 1981 Norval collected 108 engorged *A. hebraeum* nymphs from cattle kept on communal grazing in a heartwater-endemic region in the southern lowveld region of Zimbabwe. These nymphs were allowed to moult and the adult ticks were then fed on seven heartwater susceptible sheep, and two (28%) of the seven sheep died of heartwater. Norval (1981) was not able to give an exact infection rate in the ticks after these experiments, but he concluded that the prevalence was high.

Norval, Andrew & Yunker (1990) working on the Lemco Ranch and the Mbizi Quarantine Station in the southern lowveld of Zimbabwe, conducted further experiments in order to calculate the infection rate using a mathematical formula (Norval *et al.* 1990). At Mbizi they collected 152 *A. hebraeum* ticks; 32 were male, 40 female and the remaining 80 were nymphs. Thirty-nine pools of free-living, unfed ticks were then fed on 18 heartwater-susceptible sheep (Norval *et al.* 1990). The infection rates in the various pools of ticks were calculated, using a formula adapted from researchers in mosquito epidemiology (Chiang & Reeves 1962). The infection rate in the male ticks ranged from 0 - 45%, in female ticks from 20 - 36% and in nymphs from 0 - 13%.

At Lemco Ranch engorged nymphal ticks were collected off weaner calves, allowed to moult and 22 pools of adult ticks were fed on 14 sheep. The infection rate at Lemco ranged from 10 - 25 % in the males and 20 - 34% in the females. These results from Zimbabwe were

considerably higher than findings in South Africa and the Caribbean (Du Plessis *et al.* 1992; Camus & Barré 1987).

More recently Peter (1995) working at Manifest Farm in the south-western lowveld region of Zimbabwe, an area considered to be heartwater-endemic, collected *A. hebraeum* adult ticks (n = 512) which had attached onto sentinel cattle. He used the PCR assay to detect *C. ruminantium* and 1.7% of the ticks were positive. Peter (1997) also collected adult and nymphal ticks (adults = 441, nymphs = 95) off cattle, again in the southern lowveld of Zimbabwe and these were also tested with the PCR assay. The overall infection rate in the adult ticks was 11.48% (10.5% in the males and 12.45% in the females), and in the nymphal ticks 3.16%.

1.3.4.1.3 *C. ruminantium* infection rates in Senegalese ticks

Gueye, Mbengue, Dieye, Diouf, Seye & Seye (1993) are part of a research team that has, over a number of years, been studying the population dynamics and various epidemiological aspects of Senegalese ticks. *A. variegatum* has been found to be the most economically important vector of *C. ruminantium* in that country. Gueye *et al.* (1993) collected unfed *A. variegatum* adults and nymphs off cattle in the Niayes, South Sudan and North Guinean regions of Senegal where the ticks were most prevalent.

Some of these ticks were initially fed on rabbits and then divided into three groups, homogenated and injected I/V into 30 susceptible sheep. Using a formula they deduced that 11% of the adults and 7.8% of the nymphs were infected with *C. ruminantium*.

1.3.4.1.4 *C. ruminantium* infection rates in Madagascan ticks

One of the early investigations into the infection rates of *C. ruminantium* in *A. variegatum* was published by Uilenberg in 1971. He noted that most of the cattle in Madagascar were exposed to large populations of the heartwater vector *A. variegatum*, but did not acquire immunity by two years, and sometimes five years of age. He concluded that the infection rate in the free-living, unfed ticks was very low (less than 1%), and as a consequence, few of the cattle were exposed to the organism (Uilenberg 1971).

1.3.4.1.5 *C. ruminantium* infection rates in Caribbean ticks

Camus & Barré (1987b) did extensive epidemiological studies on various aspects of *A. variegatum* in the Caribbean. They collected adult ticks off local cattle and injected individual and pooled homogenated ticks into mice, using the method of Du Plessis (1985). They found that three out of the 200 ticks (1.5%) were positive using IFAT.

1.3.4.2 **Inoculation Rate / Principle of Endemic Stability to TBD**

1.3.4.2.1 *Boophilus*/Babesiosis Model

Much of the early research on the endemic stability to TBD was conducted in Australia, using *Bos taurus* cattle infested with *Boophilus microplus* which were infected with *Babesia bovis* and *Babesia bigemina* (Mahoney 1969; Mahoney & Mirre 1971; Mahoney & Ross 1972). The researchers noted that in places where ticks were abundant there was endemic stability to babesiosis, and clinical cases were rare (Mahoney & Ross 1972). They developed predictive models for the disease, based on the assumption that one infection

confers immunity for life. This relationship between cattle and *Babesia* was maintained chiefly by infection of calves (Mahoney & Ross 1972), and for complete herd immunity, transmission of infection must be adequate to infect all calves in order to avoid clinical illness. Disturbance of this epizootiological balance occurs in places where tick populations have been reduced by dipping or by climatic changes (Mahoney & Ross 1972).

The inoculation rate represents the average probability that an animal in a herd of cattle will be infected with *Babesia* or other TBD (Mahoney & Mirre (1971). The inoculation rate (**h**) is a very important parameter in the maintenance or loss of endemic stability to a TBD. The inoculation rate is dependent on the number of ticks biting per day (**m**), the proportion of these ticks which are infected with the organism (**a**), and the proportion of bites which successfully transmit infection (**b**). The resultant formula to calculate the inoculation rate is illustrated below:

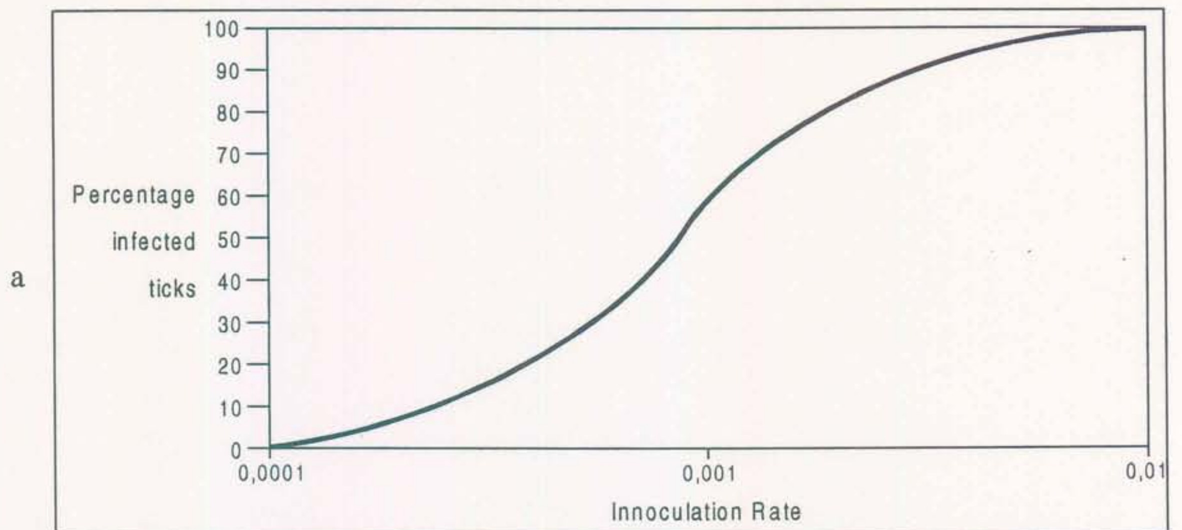
$$\mathbf{h} = \mathbf{mab.}$$

Mahoney & Ross (1972) concluded that the endemic stability to *Babesia* in cattle was dependent on the inoculation rate, and when the inoculation rate was high enough, then all calves became infected whilst being protected by colostral immunity, and clinical disease would be minimal and endemic stability was achieved (Mahoney 1974). If the inoculation rate was not sufficiently high, then not all the calves were infected, and endemic instability and clinical disease should follow (Mahoney 1974). Mahoney & Ross (1972) were then able to illustrate this process graphically (Fig. 2a).

The relationship between the level of vector challenge, disease incidence, case fatality and antibody prevalence to TBD is illustrated in Fig. 2b (Perry & Young 1995; O'Callaghan, Medley, Peter & Perry 1998). It is clear that with the increase of the inoculation rate, the number of animals which are immune to the disease also increases (endemic stability). The inoculation rate can also be substituted with percentage serologically positive animals, and a similar endemic stability graph can be produced (Fig. 2b).

Norval, Fivaz, Lawrence & Daillecourt (1983) produced a model for bovine babesiosis in Zimbabwe. They divided the country into five different epidemiological zones according to the percentage of serological positive sera (Norval *et al.* 1983). The disease-free regions (0% positive), the minimal disease areas (1 - 20% positive), the enzootically unstable regions (21% - 60% positive), the regions approaching enzootic stability (61 - 80% positive), and finally the enzootically stable regions (81 - 100% positive).

a



b

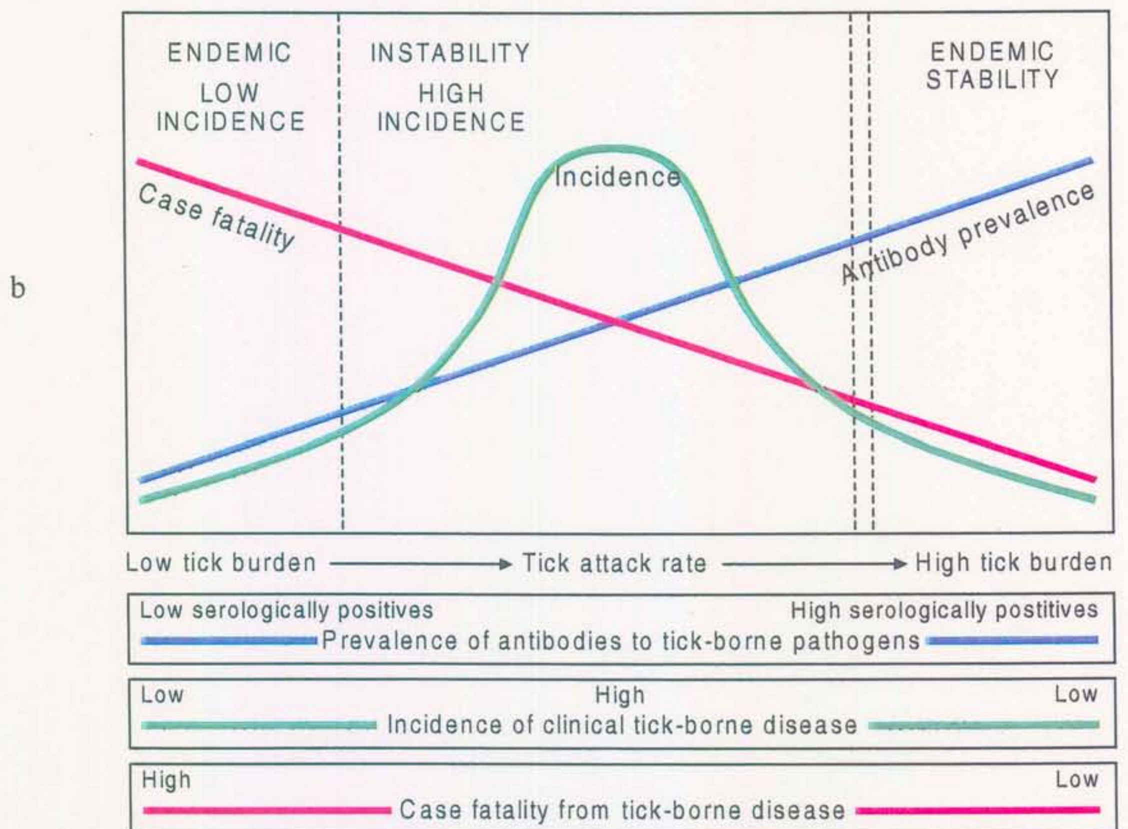


Fig. 2a Percentage infected ticks *versus* inoculation rate, the percentage of calves infected with *Babesia* by nine months of age is illustrated graphically (Adapted from Mahoney & Ross 1972)

Fig. 2b Endemic stability and endemic instability to tick-borne diseases (Adapted from Perry & Young 1995; O'Callaghan, Medley, Peter & Perry 1998)

Mahoney & Ross (1972) found that there were many different factors which could affect the endemic stability to TBD. Changing any of these factors would have serious repercussions for the endemic state of the TBD, some of these factors are listed below:

- Breed of cattle:

  Bos taurus cattle were shown to have three times as many *Boophilus* ticks as *Bos indicus*. In a study carried out by Mahoney, Wright, Goodger, Mirre, Sutherst & Utech (1981), increased tick burdens raised the inoculation rate in European cattle, which then had better endemic stability to *B. bigemina*.
- Climatic conditions for the ticks:

  Soil and temperature conditions had the potential to affect the tick infection rates and consequent stability to babesiosis (Mahoney *et al*. 1981)
- Tick control:

  Tick control reduced the inoculation rates below the minimum level for endemic stability in European cattle, and this resulted in clinical babesiosis.

In South Africa the creation of an endemically unstable situation for bovine babesiosis is mainly due to unfavourable climatic conditions, and the injudicious control of ticks (De Vos & Potgieter 1983). These authors also reported that when tick control was poor there was enzootic-instability to *B. bovis*, while endemic stability to *B. bigemina* was evident. Good tick control reduced *B. bovis* to low levels, but this increased the risk of *B. bigemina* outbreaks (De Vos & Potgieter 1983).

1.3.4.2.2 Endemic stability to heartwater

Historically heartwater was controlled like so many other TBD by dipping, but there was little recognition of the importance of the immunity of the host. In 1945 Neitz & Alexander introduced the first practical field vaccination for heartwater, and for many years this was used in conjunction with dipping. Although these early workers were aware of the importance of breed resistance and the practice of raising domestic stock on heartwater veld, they were not able to convince the commercial farmers to routinely vaccinate and use heartwater-resistant stock.

In Zimbabwe during the civil war (Norval 1981; 1983) cattle could no longer be dipped, and this resulted in high mortalities due to TBD. This led to a re-assessment of the role of dipping in the control of TBD in Zimbabwe, and to more reliance on enzootic stability developed through frequent disease exposure.

Animals which were not dipped, and were heavily tick infested, such as on communal grazing areas (CGA), acquired infection at an early age. They were protected by innate immunity and if repeatedly challenged, become immune. Clinical disease due to TBD on CGA in southern Africa was rare (Tice, Bryson, Stewart, Du Plessis & De Waal 1998), and was only noticed when susceptible or intensively dipped animals were moved into these areas.

1.3.4.3 **Tick transmission dynamics**

All proven vectors of heartwater are three-host ticks, and transmission has been shown to be transstadial only except for a single report on transovarial transmission (Bezuidenhout & Jacobsz 1986). Thirteen species of *Amblyomma* have been shown to transmit *C. ruminantium* experimentally (Jongejan 1992). Bezuidenhout (1987) reviewed the natural transmission of heartwater, and listed all the proven vectors. *A. variegatum* and *A. hebraeum* were the two most important vectors of *C. ruminantium*, but transmission of the disease appeared to be possible by all *Amblyomma* species normally associated with ungulates in the adult stage (Camus *et al.* 1996). In Mozambique *A. hebraeum* appeared to be a better vector of heartwater than *A. variegatum* (Asselbergs *et al.* 1993).

The hardiness and exceptional longevity of many *Amblyomma* species make them ideal reservoirs of the organism as it can persist in the tick for as long as 15 months (Ilemobade 1978). Male *A. hebraeum* play an important role in the transmission of heartwater as they stay on the host for long periods and they are thus more likely to become infected from an infected host than a female tick. Male *A. hebraeum* can also detach from a dead host and re-attach to another host, and transmit the infection (Norval *et al.* 1992).

Recent work at the UF/USAID/SADC Heartwater Project in Zimbabwe has introduced some new concepts to the accepted view on the transmission of heartwater. The "tick attack rate" (TAR) was used to describe the number of new ticks which attach daily onto animals, and is an important parameter in *C. ruminantium* infection dynamics (Peter 1997). Tick infectivity decay (TID), is the rate at which the infectivity of the clinically infected ruminant

host declines after recovery (Peter 1997). The TID is a new concept, and very little quantitative information was available, but this data will be important for developing epidemiological models of heartwater.

1.3.4.4 Vertical transmission of heartwater

In many heartwater-endemic regions of southern Africa, calves do not become infested with *Amblyomma* ticks until they are three months and older (Du Plessis *et al.* 1992; Norval, Donachie, Meltzer, Deem & Mahan 1995; Deem, Norval, Yonow, Peter, Mahan & Burridge 1996b). If calfhood immunity wanes at four weeks of age, and the initial tick transmission of *C. ruminantium* by *Amblyomma* ticks to calves does not occur, then endemic stability to heartwater would not seem to be possible (Deem *et al.* 1996a). There is, however, strong evidence that heartwater does occur in an endemically stable situation in large areas of Africa, especially where dipping is not carried out (Bezuidenhout *et al.* 1994; Norval *et al.* 1992).

- Deem, Norval, Donachie & Mahan (1996a) demonstrated that vertical transmission of *C. ruminantium* from cows to their calves does occur under natural heartwater-endemic conditions, and that these calves become reservoirs of infection for *Amblyomma* ticks in the area. They believed the epidemiology of heartwater was driven by both vector and vertical transmission. The establishment and maintenance of endemic stability may in fact only be possible because of the high rate of vertical

transmission, which occurred during the perinatal period when there was a high tolerance to clinical disease (Deem *et al.* 1996a). This recent finding of Deem *et al.* (1996a) that vertical transmission of *C. ruminantium* can occur, would obviously have dramatic implications for epidemiological models for heartwater.

1.3.4.5 Epidemiological models for heartwater

Accurate models for the various climatic factors are available for many of the African tick species. The CLIMEX model of Norval, Perry, Kruska & Kundert (1991) was applied to both *A. variegatum* and *A. hebraeum*. Perry, Kruska, Mahan & Pfeiffer (1995) were able to predict the changes in the distribution of *A. hebraeum* in Zimbabwe, which had occurred in the last few decades using their Climex model. Randolph & Rogers (1995) were also able to use satellite imagery to produce population and disease transmission models for both *R. appendiculatus* and *Amblyomma* spp., using currently available population dynamics data.

Various heartwater models have been produced at different stages, which explain the accepted epidemiological principles at that time, and these models have had to be updated as new information was discovered. Deem *et al.* (1996b) produced three different epidemiological models (Fig. 3, 4 and 5), which attempt to explain the principles held during these periods (Fig. 3 - 1985, Fig. 4 - 1993 and Fig. 5 - 1996).

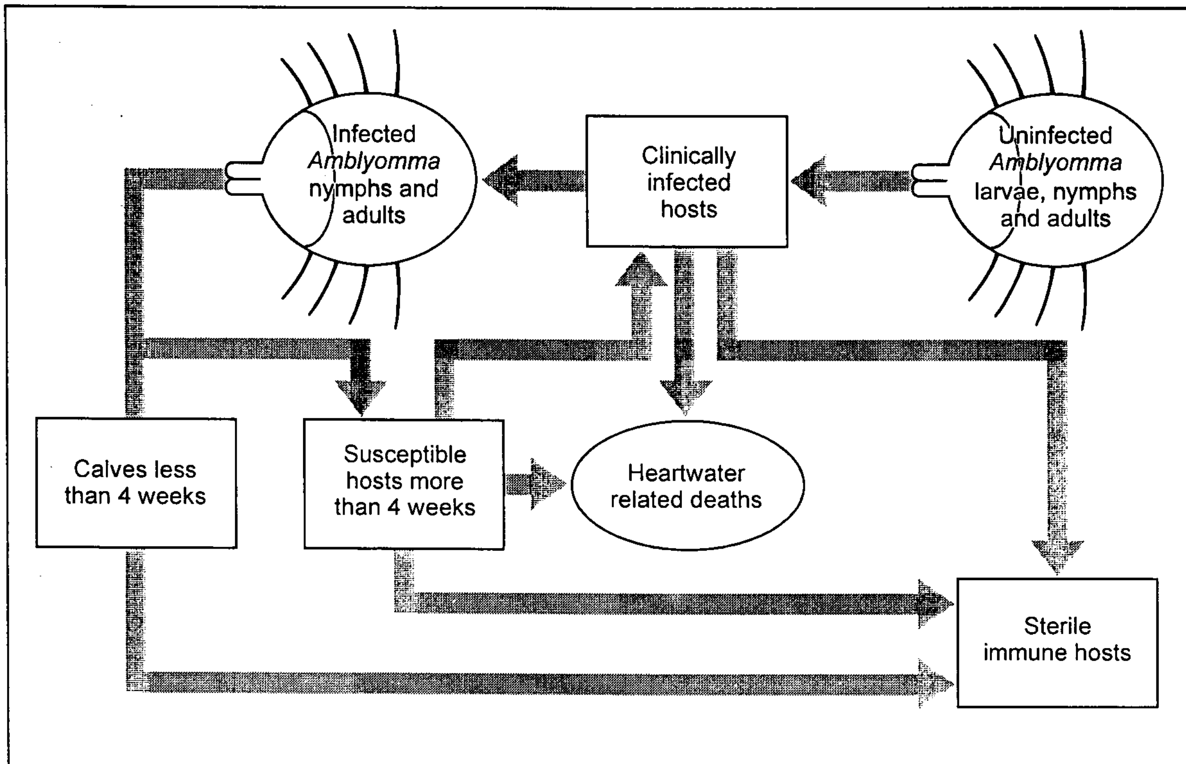


Fig. 3: The epidemiology of heartwater prior to 1985 (Deem *et al.* 1996b).

The first model was the accepted epidemiological model prior to 1985, and was built upon the view held at the time, namely that the dynamics of heartwater did not allow for the development of endemic stability to the disease (Deem *et al.* 1996b). The early model was based on the belief that hosts were only infective for a short period during the clinical reaction, and this led to the very low infection rate in free-living ticks (Alexander 1931; Neitz 1939).

In the second model (Fig. 4), cognisance is taken of the important findings that vertebrate hosts and free-living *Amblyomma* ticks (Norval, Andrew & Yunker 1990), can be long term carriers of *C. ruminantium* and these are included in the model (Andrew & Norval 1989a).

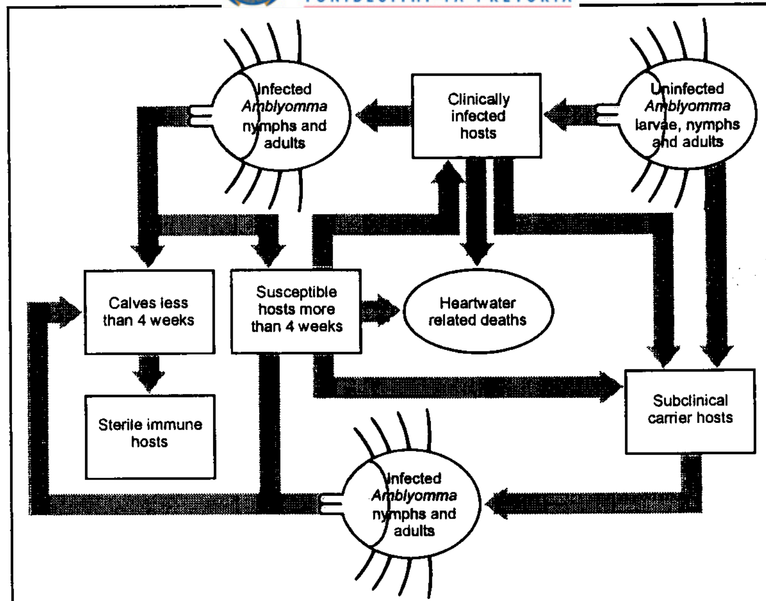


Fig. 4: The epidemiology of heartwater prior to 1993 (Deem *et al.* 1996b)

In Fig. 5 the importance of subclinical carriers and both vertical and tick transmission in establishing and maintaining endemic stability is emphasized (Deem *et al.* 1996b). Carrier calves can also infect clean *Amblyomma* ticks and remain asymptomatic when first infested with *C. ruminantium* infected ticks.

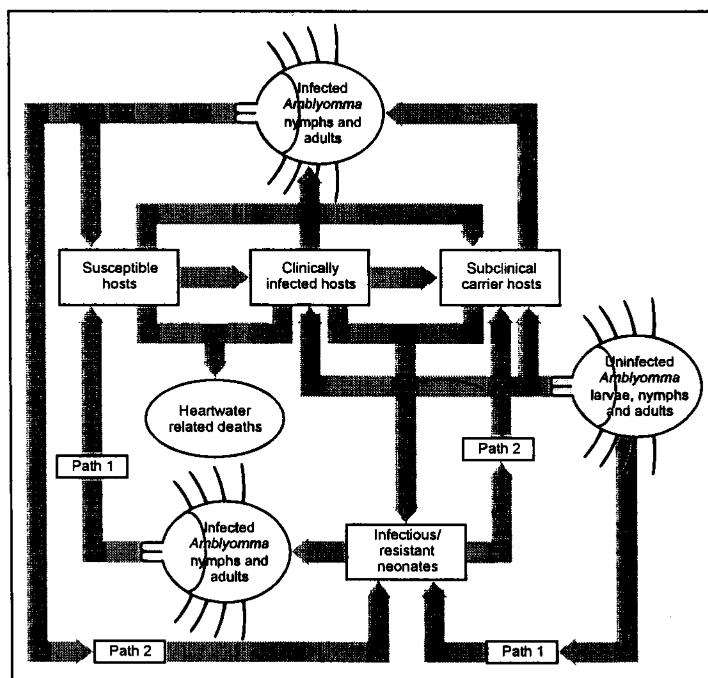


Fig. 5: The epidemiology of heartwater prior to 1996 (Deem *et al.* 1996b)

O'Callaghan *et al.* (1998) have produced a mathematical model which incorporates six mutually exclusive categories of infection which should include all possible host components involved in heartwater. This mathematical model has three components: a deterministic compartmental model of *C. ruminantium* transmission, a mammalian host demographic model and a tick vector population dynamics model. The model will be used to achieve a better understanding of the transmission dynamics of *C. ruminantium*, particularly with reference to the testing of new vaccines in the field (O'Callaghan *et al.* 1998).

Quite recently Yonow, Brewster, Allen & Meltzer (1998) published their 1998 models of heartwater epidemiology. They adapted a malaria epidemiology model and two detailed biological models for heartwater, and predicted various scenarios on their three models. Their results indicated that endemic stability, with a high level of immune carrier infection, in a herd of cattle was likely to be the norm for heartwater. Analyses with the models showed that endemic stability can only be prevented by keeping the cattle *Amblyomma*-free, and even with one tick per host per day, 30% of the cattle could be infected with heartwater.

Yonow *et al.* (1998) concluded that heartwater would readily establish and maintain itself in a cattle population. This would lead to a high percentage of the cattle becoming infected, and the disease should be allowed to spread throughout the cattle population. This would produce immunity in the herd and the risks of the disease minimised. Livestock mortality associated with converting herds from heartwater-susceptible to heartwater-carrier could be kept at a minimum by using heartwater vaccines. Tice *et al.* (1998) have also shown that the process to endemic stability does not always lead to outbreaks of clinical disease, and that direct damage by the ticks themselves is often the biggest problem (Kiwanuka, Stewart, Bryson, De Waal, Tice, Schoeman & Pettey 1995).

1.3.5 THE USE OF TRAPS TO COLLECT FREE-LIVING, UNFED TICKS

There are many different methods of collecting free-living, unfed adult and nymphal ticks and some of these are summarized in Table 3. Most of the traps for adult and nymphal ticks use some form of CO_2 as an attractant. This may be a direct CO_2 source such as dry ice, or indirect such as animals breathing CO_2. Other forms of trapping involve vacuuming the animal's nest for ticks or taking soil samples of the burrow. Some adult and nymphal ticks can also be collected by dragging the vegetation with a flannel apparatus, which the ticks attach to.

1.3.5.1 **Different methods of collecting free-living, unfed adult ticks**

1.3.5.1.1 CO_2 trapping devices

The early workers in the USA demonstrated that CO_2 emitted by dry ice was attractive to certain species of ticks in the field (Garcia 1962). They were later able to collect ticks and fleas from mammal burrows with these CO_2 traps (Miles 1968). Wilson, Kinzer, Sauer & Hair (1972) described a CO_2 platform trap, which was effective in collecting all stages of *A. americanum*, and demonstrated the advantages of this type of trap over flagging with flannel cloths to collect free-living, unfed ticks.

TABLE 3: Methods used by various authors to collect adult and nymphal ticks in the field

AUTHORS	YEAR	AREA	TICKS	METHOD OF COLLECTION	SAMPLE SIZE	OBJECTIVE
Garcia	1962	USA	-	CO_2 trap		To collect ticks with a CO_2 trap
Miles	1968	USA	-	CO_2 trap		To collect ticks from mammal burrows
Wilson *et al.*	1972	USA	*A. americanum*	CO_2 platform trap		To collect *A. americanum* in the field
Eads *et al.*	1982	USA	*D. andersoni*	CO_2 platform trap		To collect released laboratory-reared *D. andersoni* in the field
Bulter *et al.*	1984	USA/ Caribbean	*Ornithodoros* spp.	Various vacuum-type traps		To collect burrow dwelling ticks to test for ASF
Gray	1985	Ireland	*Ixodes ricinus*	CO_2 trap		To collect released *I. ricinus* (A/N) ticks in the field
Hess & De Castro	1986	Kenya	*A. variegatum*	AAAP trap (No CO_2)		To collect laboratory-reared *A. variegatum* with a AAAP trap
Norval *et al.*	1987	Zimbabwe	*A. hebraeum* (A)	Various	n = 2 000	To test the attractiveness of various stimuli to unfed laboratory-reared ticks (A)
Norval *et al.*	1988	Zimbabwe	*A. hebraeum* (N)	Various	n = 2 000	To test the attractiveness of various stimuli to unfed laboratory-reared ticks (N)
Ginsberg & Ewing	1989	USA	*Ixodes damini* *A. americanum*	Various		To compare the flagging, walking, trapping and host collection methods to collect ticks
Norval *et al.*	1989	Zimbabwe	*A. hebraeum*	Calves with AAAP trap		To see whether AAAP calves attract *A. hebraeum*
Norval *et al.*	1989	Zimbabwe	*A. hebraeum*	AAAP/CO_2 trap		To test whether laboratory-reared *A. hebraeum* (A) ticks are attracted to the AAAP/CO_2 trap
Kinzer	1990	USA	*A. americanum*	Flagging + CO_2 sticky trap		To compare flagging + CO_2 traps to collect *A. americanum* in the field
Norval *et al.*	1990	Zimbabwe	*A. hebraeum* (A/N)	AAAP/CO_2 trap	n = 152	To collect unfed field *A. hebraeum* (A/N) ticks
Norval *et al.*	1991	Zimbabwe	*A. hebraeum* *A. variegatum*	AAAP/CO_2 trap		To test if *A. hebraeum* and *A. variegatum* are attracted to similar components of the AAAP/CO_2 trap
Norval *et al.*	1992	Zimbabwe	*A. hebraeum* *A. variegatum*	AAAP/CO_2 trap		To test if laboratory-reared *A. hebraeum* and *A. variegatum* (A/N) are attracted to CO_2 and AAAP/CO_2 traps
Falco & Fish	1992	USA	*Ixodes damini*	Various		To compare mice, drag sampling and CO_2 traps to detect immature *I. damini*
Barré	1997	Caribbean (Quadeloupe)	*A. variegatum* (A/N)	AAAP/CO_2 trap	1 440 (A) 2 700 (N)	To test the AAAP trap in the Caribbean in order to collect released ticks (A/N), under variable conditions at different times of the year.

A = Adult **N** = Nymphs

Eads, Smith & Maupin (1982) working with released populations of *Dermacentor andersoni* in Colorado, USA, devised a platform-type CO_2 trap, which could be filled and re-charged with 650 g of chipped dry ice, and checked on a daily basis for ticks. They found that up to 76% of the marked released ticks could be recovered from a distance of six metres, but when the distance was increased to 12 metres, catches were poor.

Wilson *et al.* (1972) used one kilogram of dry ice over a seven-day period, but recovered only 5% of marked *A. americanum* released at six metres from the trap, and only 3.5% released at 12 metres. Clearly, there were substantial differences in the attractiveness of CO_2, for these two species of ticks with *A. americanum* showing much less response.

Butler, Holscher, Adeyeye & Gibbs (1984) integrated the technology used by previous workers, and designed a complex chemical CO_2 system, and various vacuum extraction systems, to suck out debris from warthog burrows, and this was then filtered and the live ticks separated. They showed that multiple sampling of the burrows at 3 - 4 day intervals, produced more than 2 000 ticks per burrow (Butler *et al.* 1984).

Gray (1985), working in Ireland with free-living, unfed and released adult, nymphal and larval *I. ricinus*, re-designed the trap of Wilson *et al.* (1972), to take much more CO_2 (12 kg), so that it could be dispersed for seven days. The CO_2 disperses in the area surrounding the trap and any field ticks that were attracted were caught on sticky tape as they go up the slopes. A total of 4 777 larvae, 1 092 nymphs and 772 adults were caught with this trap,

and this was substantially more than a blanket-dragging method used as a control (Gray 1985). The trap would attract ticks over a maximum distance of 3.5 metres. This was less than that recorded by Wilson *et al.* (1972) and Eads *et al.* (1982), for ticks of other species in the USA.

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1.3.5.1.2 Pheromone trapping devices

Pheromones are information-bearing chemicals used by animals to modify behaviour. These chemical signals are used by a wide variety of animals to influence many aspects of their behaviour, e.g. mating, food gathering, assembly and other vital activities (Sonenshine, 1991). Four different categories of tick pheromones have been recognized since the pioneering discovery of the first tick sex pheromone, 2.6-dichlorophenol [cited in Sonenshine (1991)]. These include assembly, AAAP, sex and primer pheromones (Sonenshine, 1991).

Feeding male *A. hebraeum* emit an AAAP and other host odours which are extremely attractive to unfed males and females of the same species (Norval *et al.* 1989). Ether extracts of fed males and O-nitrophenol were also attractive to these ticks. Male *A. hebraeum* stay on the host for considerable periods, and heavily infested animals would be more attractive to unfed adult ticks than dipped hosts with no pheromone producing males. Male ticks do not transfer to new hosts under normal circumstances, but would leave a dead host and attach to a living one (Andrew & Norval 1989b).

The AAAP induces attachment of ticks to the host's body at sites where feeding males are attached. This pheromone is volatile, soluble in organic solvents, produced solely by males,

and is only produced by members of the genus *Amblyomma* (Sonenshine 1991). Gladney, Grabbe, Ernst & Oehler (1974) and Rechav *et al.* (1977; 1978), were the first groups to carry out experiments with natural extracts of AAAP. Rechav *et al.* (1978), working in the Eastern Cape Province of South Africa, applied highly concentrated diethyl-ether extracts from feeding *A. hebraeum* males to specific sites on cattle. The cattle were then released into a camp where a large population of free-living, unfed *A. hebraeum* was known to occur. They were able to demonstrate that unfed adult ticks were attracted to the treated areas on the cattle, and this was the first recorded evidence of wild, unfed ticks being attracted to a pheromone extract. Rechav *et al.* (1978) thought, however, that the effect of the pheromone was only short-lived (four days), and that ticks responded only over a short distance.

Norval *et al.* (1987) released thousands of adult *A. hebraeum* in a heartwater-endemic region of Zimbabwe, in order to determine which factors stimulated them to emerge. Large volumes of CO_2 from blocks of dry ice, placed upwind from the release site, were the most attractive of the stimuli (Yunker & Norval 1991). The CO_2 did not, however, lure the adult or nymphal ticks to the source, but rather caused them to emerge from their shelters and to run erratically on the surface (Yunker & Norval 1991).

In subsequent field trials in Zimbabwe, various chemicals were tested for their attractiveness to *A. hebraeum*. It was found that adult and nymphal ticks responded positively to the volatile extract of the AAAP produced by feeding males, and this response was enhanced by the addition of CO_2 (Yunker & Norval 1991).

Norval *et al.* (1987) stated that wild *A. hebraeum* adult ticks were attracted over a distance of three metres, to cattle carrying pheromone secreting males. Herds or flocks infested with *Amblyomma* males will emit large quantities of CO_2 and AAAP, and are likely to attract wild, unfed nymphs, as well as free-living, unfed adult *A. hebraeum* (Norval *et al.* 1992). The practice of herding domestic stock during the day and holding them in enclosures overnight is thus likely to increase the exposure of these animals to unfed ticks (Norval *et al.* 1992).

Males of *A. hebraeum* remain on the host for several months (Norval 1974), and are capable of mating with several females. Only after the males have been feeding for a week or longer, do they secrete sufficient AAAP to attract females, other males and nymphal *A. hebraeum* which then attach. As a consequence, these ticks tend to accumulate on suitable hosts in clusters, formed at pre-dilection sites of male attachment (Norval, Andrew & Yunker 1989a).

The responses to both CO_2 and AAAP appear to be part of a selection process that ensures that ticks attach preferentially to hosts on which they are more likely to feed successfully (Norval *et al.* 1989a). Obviously, cattle which are treated with acaricides will have less pheromone secreting males and, presumably, less immunity to heartwater. In 1989 Norval, Butler & Yunker confirmed that host location in adult *A. hebraeum* involved two main responses. Firstly, a response to CO_2 which initiates searching actively, but is non-directional, and secondly, a directional response to AAAP.

They also designed two field traps to collect laboratory-reared *A. hebraeum*, both of which used CO_2 from a cylinder passing over fed AAAP producing males (Norval *et al.* 1989b). Norval, Peter, Yunker, Sonenshine & Burridge (1991) also tested a variety of potential chemicals as long-range attractants for *Amblyomma* ticks, and they concluded that AAAP contained compounds that served as long-range attractants, short-range attractants that induce aggregation, and also attachment stimulants. The major component of the AAAP of *A. hebraeum* appeared to be O-nitrophenol, and the AAAP attracted both *A. hebraeum* and *A. variegatum* (Norval *et al.* 1991).

Norval *et al.* (1992) found that with the AAAP/CO_2 trap large numbers of released adults (64%) were recovered at four metres, but this was not the case with CO_2 alone (7%). As the distance was increased to over ten metres, 30% of adult ticks were still collected with AAAP/CO_2, as opposed to 0% with CO_2 alone.

Barré, Garris & Lorvelec (1997) working with *A. variegatum* in the Caribbean, used an AAAP/CO_2 trap to collect ticks. The trap, consisted of a piece of white cloth and a plastic cup of dry ice (250 g), and various attractants, including tick pheromone and cattle hair. As only a small quantity of CO_2 was used, it had to be replaced every two hours. This study involved laboratory-reared ticks, and was not directed at wild tick capture, and the maximum attraction distance reported for this trap was eight metres.

In conclusion, it would appear from the literature that there are few reports of sustained collections of wild *Amblyomma* adult ticks in the field, and most of the collections to date have been based on the release and capture of laboratory-reared ticks.

1.3.5.2 Different methods of collecting free-living, unfed nymphal ticks

Nearly all of the known, proven experimental vectors of *C. ruminantium* can become infected in the larval stage and the infection passed on to the nymphal stage (Bezuidenhout 1987). *A. hebraeum* and *A. variegatum* in the nymphal stage would then transmit *C. ruminantium* in the adult stage, i.e. the nymphs are infective. In other species of *Amblyommas* (*A. sparsum* and *A. cajenense*) the nymphs lose the infection and the adults cannot transmit heartwater (Bezuidenhout 1987).

Unfed *A. hebraeum* nymphs could be expected to be most abundant in areas where cattle rest at night (Norval 1988), and one would hence look for *A. hebraeum* nymphs in shaded areas near kraals where cattle are kept overnight. Unfed nymphs and adult *A. hebraeum* have not been successfully collected by conventional sampling techniques such as blanket-dragging, flagging or standard CO_2 traps (Norval *et al.* 1992). Norval *et al.* (1987) found that the presence of high concentrations of CO_2, mimics the breath of large hosts, and causes nymphs and adult *A. hebraeum* to emerge from and engage in host seeking activity, but required the addition of pheromone to be more effective. Adult *A. hebraeum* are large and conspicuous, and can easily be collected as they run over the soil surface. Nymphs, however, cannot be readily collected in this manner as they are much smaller, are darkly coloured, and consequently not easily visible on the ground (Norval, Yunker, Gibson & Deem 1988).

Norval *et al.* (1988) released 2 000 nymphs into camps at the Mbizi Quarantine area in

Zimbabwe. They then tried various methods to recover the ticks. Flagging with a drag apparatus was not effective, but it was improved by prior stimulation with CO_2. Vacuuming, following CO_2 exposure, was the most successful way of collecting the nymphs. However, a strong source of CO_2, e.g. dry ice, was needed to attract the nymphs (Norval *et al.* 1988). With released nymphs, 83% were recovered at ten metres with CO_2, and 88% with AAAP/CO_2. The response of the nymphs to CO_2 alone, obviously differed to that of the adults, which showed poor response. The AAAP trap was able to attract nymphs from up to 25 metres from the source (Norval *et al.* 1992).

Rechav & Whitehead (1978) in their field trial with AAAP mixtures on cattle, found that wild, nymphal *A. hebraeum* were also attracted to demarcated treated areas on cattle. Many other reports (Gray 1985; Falco & Fish 1992; Kinzer 1990) have documented the use of traps for collecting nymphs of other species, most have little relevance to this project, as these nymphal ticks quest, and are easily caught with flagging and other means. Other methods besides trapping, used to collect nymphal ticks, are the use of tracer animals, trapping rodents, and the use of sentinel cattle (Peter 1995).

1.3.5.3 **Different methods of collecting free-living, unfed larval ticks**

Ticks acquire *C. ruminantium* whilst feeding on a reacting or carrier animal. Most of the larval stages of the proven vectors of heartwater can transmit *C. ruminantium* transstadially to the nymphal stage. The transmission from the nymph to adult is, however, variable and depends on the ability of the species involved (Bezuidenhout 1987).

Most larval ticks, including *A. hebraeum*, quest and are easily collected off the vegetation with the various forms of drag-sampling. *C. ruminantium* is not normally transmitted transovarially (Bezuidenhout & Jacobsz 1986), so very few larvae of *A. hebraeum* would be infected with *Cowdria*. This stage of development is less important in the epidemiology of the disease. The larvae feed on numerous hosts which could also carry heavy burdens of *A. hebraeum* larvae, and could be sub-clinically infected with *C. ruminantium* (Horak & Williams 1986; Horak *et al.* 1987).

Spickett *et al.* (1991) using drag-sampling in the KNP, collected 18 199 ticks of which 99.53% were larvae. This would imply that this form of sampling is not efficient for adult and nymphal ticks in southern Africa, and especially for those species which do not quest. This is certainly not the case in other parts of the world where other tick species occur, and L'Hostis, Dumon, Dorchies, Boisdron & Gorenflot (1995) reported on a survey in France where blanket-dragging collected 34 adult, 900 nymphal and 2 628 larval *I. ricinus*.. In Ireland Gray (1985) collected a large sample of all stages of *I. ricinus* with both a CO_2 trap and blanket-dragging. There have been few reports on the use of a CO_2 trap to collect larval ticks in South Africa. This is probably because of the ease with which these ticks are collected by dragging, as opposed to the difficulty of seeing these very small ticks when they are attracted to CO_2 traps. The larvae also quest on the grass stems and blades, and are unlikely to be found on the ground during the day.

Various workers (Ginsberg & Ewing 1989; Kinzer *et al.* 1990; Falco & Fish 1992; Shulze, Jordan & Hung 1997) have critically analysed live tick sampling methods. Ginsberg & Ewing (1989) concluded that those ticks which seek hosts in leaf litter, are poorly

sampled by attachment onto the investigators' clothing (walking samples), and these ticks can better be sampled by dragging at ground level. Dry ice-baited traps caught far more *A. americanum* than *I. damini* in areas where the latter ticks predominated on flagging samples (Ginsberg & Ewing 1989).

Falco & Fish (1992) compared mice trapping, drag-sampling and CO_2 traps to collect the various stages of *I. damini* in an endemic lyme disease area in the USA, and they concluded that drag-sampling is best for nymphs, and CO_2 traps best for the larvae. Kinzer (1990) on the other hand, compared the efficacy of flagging and CO_2 traps to collect *A. americanum*, and found that CO_2 traps captured more adults and nymphs than did flagging. The traps were more precise and produced less variability between samples. Schulze *et al.* (1997), however, have recently reported that various biases have to be taken into account when looking at the populations of *I. scapularis* and *A. americanum* in the USA. Walking surveys were more efficient for collecting *I. scapularis* adults, but drag-sampling more effective for collecting *A. americanum* adults. Clearly, this contradicted the work of Kinzer *et al.* (1990) and Ginsberg & Ewing (1989).

To conclude one can accept that there are many different methods of trapping larval, nymphal and adult free-living, unfed ticks. It is important, however, that one should carefully analyse the strengths and weaknesses of each method, before using it on a large scale to collect free-living, unfed adult and nymphal ticks. With *A. hebraeum* there have been no sustained collection surveys of adult and nymphal ticks, but it is clear that pheromone/CO_2 traps would be far more successful in collecting adult and nymphal ticks than dragging methods.