

**AN INVESTIGATION OF
BORRELIA BURGDORFERI IN SOUTH AFRICA**

THESIS

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by

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DEDICATION

This is dedicated to my Mum and Dad,
for always believing in me and
giving me the chance to try.

ABSTRACT

Four commonly occurring genera of ticks in South Africa were tested for their capacity to acquire and transmit *Borrelia burgdorferi*, the causative agent of Lyme disease. Attempts were made to infect rabbits with a culture of *B. burgdorferi*, and tick transmission experiments were carried out using the rabbits as the host of infection. Only one rabbit was successfully infected. Histological changes associated with a *B. burgdorferi* infection were noted in this rabbit, but no spirochaetes were isolated. All other host animals failed to become infected with the *B. burgdorferi*. As a consequence transmission experiments with the local ticks failed.

Serological surveys conducted on rodents, horses and cattle using immunofluorescent and haemagglutination tests indicated that there is evidence that *Borrelia* species occur widely and that there is a possibility of *B. burgdorferi* occurring in South Africa.

Studies conducted on ticks from suspected endemic areas revealed the presence, in *Ixodes bakeri* only, of a spirochaete-like organism that reacted with monoclonal antibody H5332.

Shortcomings of the study are highlighted and proposals are presented to address the problem of identifying specific *B. burgdorferi* infections.

CHAPTER ONE

INTRODUCTION

1.1 HISTORY.

Lyme borreliosis and Erythema Chronicum Migrans (ECM) are terms referring to a bacterial disease, Lyme disease, that appears to have been first recognized in Sweden in 1909 (Garfield 1989) and subsequently discovered through most of the world. In 1909 Dr Arvid Afzelius described an annular skin lesion that developed at the site of an *Ixodes ricinus* tick bite, which he called erythema migrans (EM). In 1948 Dr Carl Lennhoff reported organisms morphologically similar to spirochaetes in sections of skin of patients suffering from ECM (cited in Garfield 1989).

In 1975, Dr Allen Steere at Yale University Medical School was contacted by a concerned mother from Old Lyme, Connecticut, USA where 12 children in a small community of 5000 people had been diagnosed as having juvenile rheumatoid arthritis (Steere *et al.* 1977). Further investigations revealed that 51 residents had a similar type of arthritis, which was subsequently called Lyme arthritis or Lyme disease. No common virus, bacteria or serological evidence could establish the exact cause of the disease. Although most patients had similar symptoms, only 13 described the occurrence of a red annular rash and only one had been bitten by a tick at the site of the rash. Assembling the epidemiological evidence concerning the disease, namely that all patients lived near the heavily wooded areas around the town rather than in the centre of it, that the disease peaked in summer, and that it was not transmitted throughout the town, Steere concluded that the disease was transmitted by an arthropod vector. Although not all the patients suffering from Lyme arthritis had ECM, Steere concluded that the disease was in some way related to the erythema migrans reported by Afzelius in 1909.

The aetiological agent of Lyme disease was not found until 1981, when it was accidentally discovered by Dr Willy Burgdorfer (Burgdorfer *et al.* 1982). Burgdorfer was carrying out a survey of rickettsia in ticks on Long Island, New York, when he noticed long coiled spirochaetes in the mid-gut smears of two *Ixodes dammini* ticks. He remembered the discovery of Lyme disease, the connection of the ECM to spirochaetes and the fact that neither the causative agent nor vector of the disease had been found. He then conducted indirect immunofluorescent antibody tests of the spirochaete with sera of several Lyme disease patients and it was agreed that this spirochaete was the causative agent of Lyme disease or Lyme arthritis. The spirochaete was subsequently named *Borrelia burgdorferi*, after its discoverer.

1.2 BACTERIUM.

The morphology of *B. burgdorferi* is typical of other borrelia species (Saint Girons and Barbour 1991). It is 3-30 μ m long, 0.2-0.3 μ m wide and has an average of seven irregular coils (Burgdorfer *et al.* 1982; Baranton 1990; Saint Girons and Barbour 1991; Wilske *et al.* 1991). It is gram negative, and stains with Giemsa and silver stains. Different strains of *B. burgdorferi* are associated with the various geographical areas where Lyme disease occurs and these different strains have species specific but strain variable outer cell membrane proteins (Stanek *et al.* 1990; Wilske *et al.* 1991).

Members of the genus *Borrelia* are distinguished by the presence, in the outer membrane of a protein having a molecular weight of 41kDa (Barbour 1984; Reik 1991). All *B. burgdorferi* spirochaetes have a membrane-associated protein of molecular weight 31kDa. This is commonly known as Outer Surface Protein A (Osp A) (Howe *et al.* 1985; Reik 1991; Wilske *et al.* 1991). The differences between the various European and North American strains of *B. burgdorferi* are related to the presence or absence of another protein which has a molecular weight of 34kDa (Barbour 1984; Stanek *et al.* 1990; Wilske *et al.* 1991). This protein has been named Osp B (Howe *et al.* 1985). Most European strains lack the Osp B component, but there is evidence that these strains contain another protein with a molecular weight of 21-22kDa, known as Pc, which the North American strains lack (Wilske *et al.* 1986; Stanek *et al.* 1990; Wilske *et al.* 1991).

The monoclonal antibodies that have been raised for serological testing for Lyme disease are closely associated with the Osp A and Osp B components. Monoclonal antibody H5332 binds to the Osp A protein and thus recognises *B. burgdorferi* isolates found in Europe (Barbour *et al.* 1983b). Recently a protein was discovered that could be used as a basic requisite when testing new possible isolates of *B. burgdorferi* (Simpson *et al.* 1991). This is a unique periplasmic protein called P22-A that is found in all North American and European isolates and it ranges between 20 and 23kDa.

1.3 CLINICAL SYMPTOMS.

Lyme disease is a multisystem disease that can be divided into three stages (Steere *et al.* 1977; Barbour 1988; Nicholson and Greene 1988; Magnarelli 1989; Huppertz 1990; Palmer and Small 1991; Rahn and Malawista 1991; Reik 1991). The disease can be misdiagnosed if any of the symptoms of the three stages are looked at without a full medical history of the patient.

The first stage in some patients is characterised by an erythema chronicum migrans (ECM). This term is now used to describe the typical rash at the site of the tick bite 3-14 days after being bitten, and not the disease itself, as first described in Sweden. This ECM is a large red circular rash spreading outwards from the bite, and later clearing in the middle. It may be accompanied by influenza-like symptoms: fever, chills, headaches and fatigue. These symptoms may be intermittent or change in nature, and may last for a couple of weeks. Often the ECM does not occur, and the influenza-like symptoms at the onset of the disease may then be misdiagnosed. Fifty percent of those patients with an untreated rash at the site of the bite develop similar annular lesions at other sites on the body (Barbour 1988).

The second stage of Lyme disease, which occurs a few weeks to a few months after the onset of the first stage, is characterised by nervous disorders, cardiac problems and arthritis. The neurological disorders include headache, stiff neck, photophobia and signs of encephalitis such as depression, memory difficulties and altered mental state (Palmer and Small 1991). These patients may develop peripheral neuritis in limbs, Bell's palsy (Steere *et al.* 1977), facial paralysis (Nicholson and Greene 1988) and symptoms resembling those of multiple

sclerosis (Burgdorfer 1991). These neurologic abnormalities may last for months or they may resolve completely without antibiotic therapy. They may however recur. Cardiac problems vary in severity with the main disorder being atrioventricular heart block. In 8% of patients there are disturbances in conduction and rhythm that lead to permanent damage (Burgdorfer 1991). The arthritis associated with the second stage is migratory from musculoskeletal pain to inflammatory arthritis (Rahn and Malawista 1991). Attacks are recurring and may endure for several weeks. If untreated bone and cartilage erosion can occur (Barbour 1988).

The third stage of Lyme disease is characterised by chronic arthritic attacks. These primarily involve the larger joints such as knees, shoulders and hips. The attacks are recurrent and may commence several years after contracting the disease. Some patients in this later stage of the disease may develop Acrodermatitis Chronica Atrophicans (ACA) of the skin at acral sites (Aberer and Klade 1991). ACA is presently rare in the USA (Rahn and Malawista 1991) but occurs more commonly in Europe, where Lyme disease has a longer history.

Lyme disease symptoms in animals are apparently not clearly divided into the 3 stages ordinarily observed in humans. Many of the natural reservoirs of the spirochaete, such as the White-footed Mouse and White-tailed Deer, do not show clinical signs of the disease. Some experimentally inoculated animal species have also failed to develop symptoms, although they can be shown to carry antibodies to *B. burgdorferi* (Johnson *et al.* 1984; Burgess 1986; Duray and Johnson 1986; Anderson *et al.* 1987; Wright and Nielson 1990). Other animals do however appear to develop symptoms similar to Lyme disease. Horses show the typical symptoms of arthritis and limb stiffness (Burgess and Mattison 1987; Cohn *et al.* 1988; Fivaz *et al.* 1990) and can develop a cough (Cohn *et al.* 1988; Marcelis *et al.* 1987) and conjunctivitis (Cohen *et al.* 1988). Cattle exhibit lameness along with loss of condition (Rothwell *et al.* 1989) and swollen joints (Burgess *et al.* 1987; Post *et al.* 1988; Rothwell *et al.* 1989). Dogs may also show signs of lameness, lethargy and stiffness (Grauer *et al.* 1988; May *et al.* 1990). The only ECMs seen in animals were found in experimental rabbits at the sites of infected-tick feeding or following infected-tick inoculation (Burgdorfer *et al.* 1982; Burgdorfer *et al.* 1983; Benach *et al.* 1984; Kornblatt *et al.* 1984b). Inbred

mice have also been shown to develop arthritis (Schaible *et al.* 1991) and swollen joints, and to experience locomotion difficulties (Schaible *et al.* 1989).

1.4 LABORATORY DIAGNOSIS.

Several laboratory methods are routinely used in testing for Lyme disease. None of these are however 100% conclusive. There are inconsistencies in both the results obtained using different methods of testing and between laboratories (Lane *et al.* 1990; Christenson and White 1991; Corpuz *et al.* 1991; Olsson *et al.* 1991).

1.4.1 Direct detection of *Borrelia burgdorferi*.

In testing for actual *B. burgdorferi* spirochaetes, several methods are commonly employed: cultivation and staining, which visualises the spirochaetes, and the polymerase chain reaction (PCR), a test which indicates the presence of a specific strand of DNA from the spirochaete in the sample.

1.4.1a Cultivation.

Cultivation is routinely employed when trying to isolate the bacteria from tissue or body fluids (Stanek 1991). This method is however seldom successful (Stanek *et al.* 1988), as the growth medium for *B. burgdorferi* is easily contaminated and the probability of culturing organisms decreases with the progression of the disease (Stiernstedt *et al.* 1991). Skin biopsies of ECM have the highest yield of spirochaetes and there is a 60-70% success rate of cultivation from this source. There is a 30-40% success rate from biopsies of ACA, 3% from blood and 7-10% from cerebrospinal fluids from patients with neurological symptoms (Stiernstedt *et al.* 1991).

1.4.1b Darkfield microscopy.

Darkfield microscopy is used to detect the movement of the spirochaetes in the blood or other body fluids. The disadvantages with this method are that it is not specific to *B. burgdorferi* and the spirochaetes are often missed (Nelson *et al.* 1991).

1.4.1c Histology and staining.

Spirochaetes of *B. burgdorferi* have been detected in several organs including the spleen, kidney, liver, heart and eyes (Duray and Johnson 1986). This is due to their staining with a number of stains such Giemsa (Burgdorfer *et al.* 1982; Lane and Burgdorfer 1986), silver stains (Reik 1991) and acridine orange stains (Lauer *et al.* 1981; Sciotto *et al.* 1983). The organisms do not occur in large numbers, however, and the stains used are not species specific (Kronvall and Myhre 1977; Sciotto *et al.* 1983). As a consequence no definite diagnosis can be made.

1.4.1d Polymerase Chain Reaction (PCR).

PCR is the most recently described method for detecting *B. burgdorferi* in suspected cases of Lyme disease. The method involves testing for a short DNA sequence specific to *B. burgdorferi*, using small DNA probes with base sequences complementary to the host DNA sequence. Investigations thus far have been very promising. Some researchers have developed a DNA probe that reacts with 17 of 18 *B. burgdorferi* strains tested, and which does not cross react with other *Borrelia* species (Rosa and Schwan 1989). This method can be used to test biological fluids directly (Debue *et al.* 1991) as well as organ tissue (Lebech *et al.* 1991). Further investigations need to be carried out on this method since it appears that earlier probes based on the Osp B gene are more specific than those based on the Osp A gene (Debue *et al.* 1991).

1.4.2 Indirect detection of *Borrelia burgdorferi*.

Indirect detection is based on the screening of serum and cerebrospinal fluids for antibodies to *B. burgdorferi* antigens. The main disadvantage of these methods is that the immune system takes a long time to react to the spirochaete, and if tested too early false negative results are obtained. The IgM response peaks approximately six weeks after exposure to *B. burgdorferi*, while the IgG response peaks months after the exposure (Berg *et al.* 1991; Murphy 1991; Ostrov and Athreya 1991). Serological responses rise and fall, which probably explains the intermittent nature of the symptoms of Lyme disease, and may yield false negative results. A further disadvantage of these indirect methods is the lack of specificity for *B. burgdorferi*, as the antibodies tested are known to cross react with other *Borrelia*

antigens or those of closely related species (Barbour *et al.* 1983b; Russell *et al.* 1984; Lane *et al.* 1990). As these methods are based on screening for antibodies rather than for the organism itself, patients may have antibodies to *B. burgdorferi* as a result of a past infection and the disease may no longer be active, leading to false positives.

1.4.2a Indirect Fluorescent Antibody (IFA) test.

IFA tests are commonly used for routine screening of sera. This method is also not species specific, and cross reactions occur between similar antigens such as those of *Treponema pallidum* and other *Borrelia* species (Russell *et al.* 1984; Lane *et al.* 1990; Murphy 1991; Stiernstedt *et al.* 1991). There is no standardized IFA test and there have been discrepancies in reports from different laboratories engaged in this work (Hedberg *et al.* 1987; Lane *et al.* 1990; Corpuz *et al.* 1991; Stiernstedt *et al.* 1991).

IFA tests using monoclonal or polyclonal antibodies are used for detecting *B. burgdorferi* in ticks and organs of experimentally infected animals (Anderson *et al.* 1983; Burgess *et al.* 1987; Godsey *et al.* 1987). There are two main monoclonal antibodies used: H5332 and H9724, which react specifically with antigenic determinants in different strains of the spirochaete. H5332 is the more specific, as it binds with most *B. burgdorferi* isolates, and shows no cross reactivity with other representative species of *Borrelia*, treponemes or leptospire (Burgdorfer *et al.* 1983; Barbour *et al.* 1985). H9724 reacts with antigenic determinants in eight species of *Borrelia* (Barbour *et al.* 1986).

1.4.2b Enzyme Linked Immunosorbent Assay (ELISA).

The advantages of employing the ELISA technique include greater sensitivity, electronically quantified colour changes that are not prone to subjectivity and the fact that more tests can be performed at a time (Craft *et al.* 1984; Russell *et al.* 1984; Corpuz *et al.* 1991). The specificity of the test depends on the antigen used. ELISAs conducted with flagellum antigen are more sensitive than those using whole-cell antigens (Hansen *et al.* 1988; Christenson and White 1991; Olsson *et al.* 1991). ELISAs, like IFAs, are not species specific and cross reactions occur with other *Borrelia* and treponemes (Magnarelli and Anderson 1991; Murphy

1991). A modified ELISA test based on a peroxidase enzyme labelled antigen shows no cross reactions to sera with *T. pallidum* (Eiffert *et al.* 1991). It can be performed with sera from any species of animal, no dilutions are needed and the distinction between the positive and negative sera shows a smaller number of borderline cases.

1.4.2c Western blots.

In the western blot technique, antigens are separated according to their molecular weights by polyacrylamide gel electrophoresis, transferred to nitrocellulose paper and allowed to react with test sera. The antibody-antigen reaction is then visualized by either enzyme- or radioimmuno-labelled anti-immunoglobulins. Western blots show the highest sensitivity of the indirect methods in detecting *B. burgdorferi* (Grodzicki and Steere 1988; Ostrov and Athreya 1991; Stiernstedt *et al.* 1991). The technique is however difficult to standardise. Results are difficult to interpret as several molecular weight bands must be matched to obtain a positive result (Ostrov and Athreya 1991). Ideally, this test is used in conjunction with another type of serological test system.

1.4.2d Haemagglutination and other tests.

Haemagglutination tests for *B. Burgdorferi* have been devised, but neither the specificity nor the sensitivity of these tests are known (Melby *et al.* 1990; Fujita *et al.* 1991). An enzyme immunoassay test for IgG levels based on a detergent extract of *B. burgdorferi* shows high specificity for patients in the late stage of Lyme disease but only a 43% specificity for those in the early stage when IgG levels are low (Bergstrom *et al.* 1991). An immunoperoxidase test that is quick and simple to perform and comparable to IFA and ELISAs could be used as a screening assay (Schmidli *et al.* 1989).

Thus there is a need to devise a standardized, highly specific test for Lyme disease. Until such a test is available, two or more independent tests should be run for each serum sample under investigation.

1.5 DISTRIBUTION, VECTORS, RESERVOIRS AND HOSTS.

Since Burgdorfer discovered the causative agent of Lyme disease, the disease syndrome and spirochaete have been found in many parts of the world (Figure 1 and Table 1). In each region, the spirochaete is transmitted and maintained by a variety of vectors and reservoirs (Table 2). The vectors are all blood sucking arthropods, with the main carriers being ticks of the Ixodidae family.

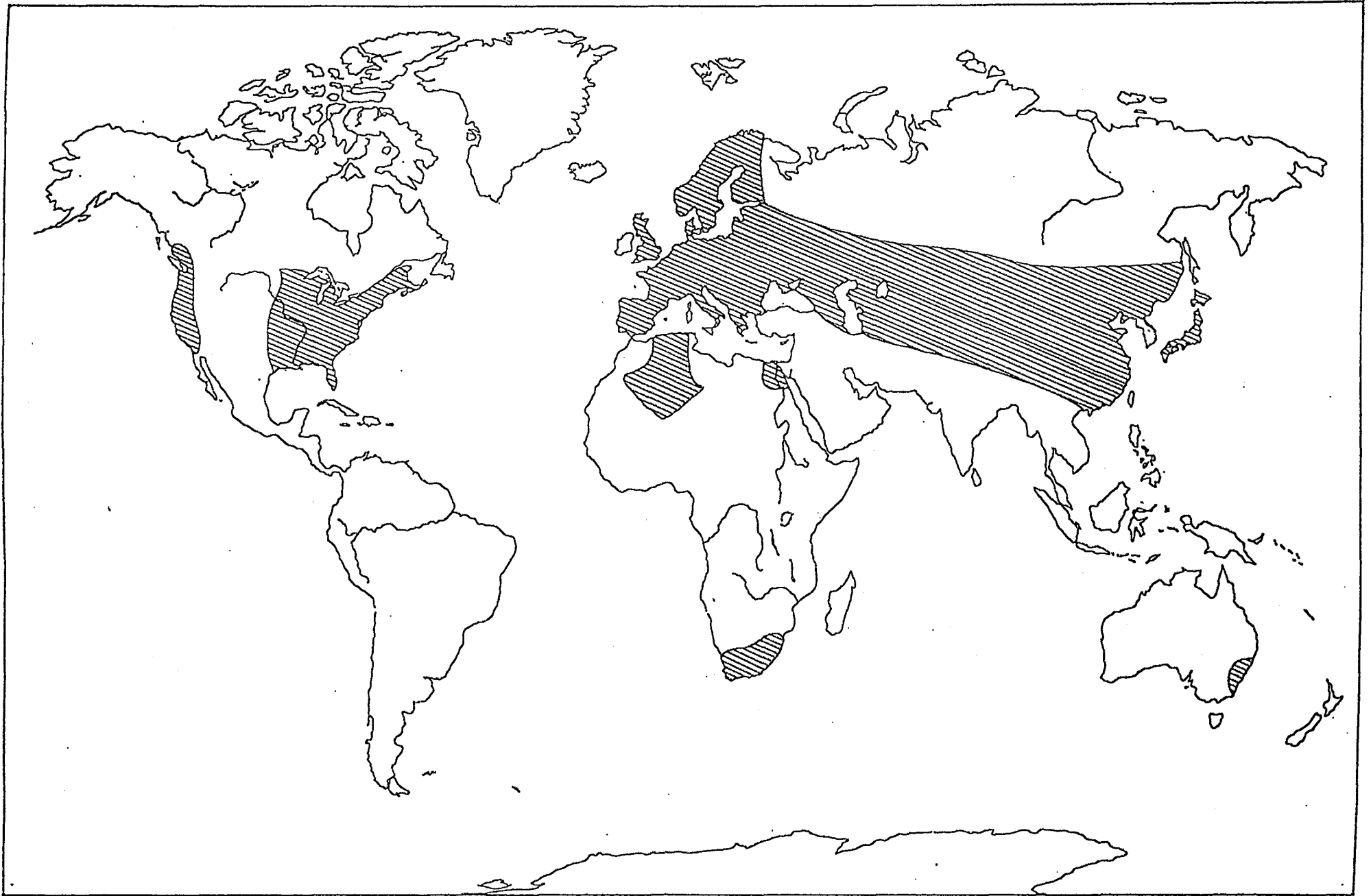


Figure 1. Distribution of *B. burgdorferi* spirochaetes and antibody titres recorded.

Table 1. Reported possible vectors of *B. burgdorferi*.

AMERICA		
Ticks	<i>Ixodes dammini</i>	Burgdorfer <i>et al.</i> 1982; Magnarelli <i>et al.</i> 1987a; Lane <i>et al.</i> 1991; Nelson <i>et al.</i> 1991; Magnarelli <i>et al.</i> 1991.
	<i>Ixodes pacificus</i>	Burgdorfer <i>et al.</i> 1985; Lane and Loye 1991.
	<i>Ixodes scapularis</i>	Piesman and Sinsky 1988.
	<i>Ixodes neotomae</i>	Lane and Burgdorfer 1988; Burgdorfer 1991.
	<i>Ixodes dentatus</i>	Anderson <i>et al.</i> 1989; Burgdorfer 1991.
	<i>Haemaphysalis leporispalustris</i>	Anderson and Magnarelli 1984; Lane and Burgdorfer 1988.
	<i>Dermacentor albipicicus</i>	Lane and Burgdorfer 1986; Rawlings 1986.
	<i>Dermacentor parumperlus</i>	Rawlings 1986.
	<i>Dermacentor variabilis</i>	Anderson and Magnarelli 1984.
	<i>Rhipicephalus sanguineus</i>	Rawlings 1986.
	<i>Amblyomma americanum</i>	Schulze <i>et al.</i> 1984; Teltow <i>et al.</i> 1991.
Deer Flies	4 species	Magnarelli <i>et al.</i> 1986b; Magnarelli and Anderson 1988.
Horse Flies	4 species	Magnarelli <i>et al.</i> 1986b; Magnarelli and Anderson 1988.
Bot Flies	1 species	Anderson and Magnarelli 1984.
Mosquitoes	5 species	Magnarelli <i>et al.</i> 1986b; Magnarelli <i>et al.</i> 1987b; Magnarelli and Anderson 1988.
Fleas	2 species	Anderson and Magnarelli 1984; Teltow <i>et al.</i> 1991.
Biting Flies	species unknown	Luger 1990.
EUROPE		
Ticks	<i>Ixodes ricinus</i>	Burgdorfer <i>et al.</i> 1983; Stanek <i>et al.</i> 1988; Hubálek <i>et al.</i> 1990; Hubálek <i>et al.</i> 1991.
Mosquitoes	species unknown	Hård 1966.
ASIA		
Ticks	<i>Ixodes persulcatus</i>	Ai and Xiaoming 1989; Ai <i>et al.</i> 1990; Uchikawa <i>et al.</i> 1991.
	<i>Ixodes ovatus</i>	Uchikawa <i>et al.</i> 1991.
	<i>Haemaphysalis concinna</i>	Ai and Xiaoming 1989.
AUSTRALIA Unknown	unknown	Stewart <i>et al.</i> 1982.

Table 2. Reported mammalian and avian hosts for *B. burgdorferi*.

AMERICA	rodents	Bosler and Schulze 1986; Spielman 1988; Magnarelli <i>et al.</i> 1988; Lane and Brown 1991; Lane and Loye 1991; Mukolwe <i>et al.</i> 1992.
	insectivores	Telford <i>et al.</i> 1990.
	lagomorphs	Lane and Burgdorfer 1988; Anderson <i>et al.</i> 1989; Burgess and Windberg 1989.
	raccoons	Anderson <i>et al.</i> 1983; Magnarelli <i>et al.</i> 1984.
	red foxes	Kazmierczak and Burgess 1989.
	coyotes	Kazmierczak and Burgess 1989; Burgess and Windberg 1989.
	gray wolves	Thieking <i>et al.</i> 1992.
	deer	Lane and Burgdorfer 1988; Magnarelli <i>et al.</i> 1986a.
	horses	Burgess and Mattison 1987; Marcelis <i>et al.</i> 1987; Cohan <i>et al.</i> 1988.
	cattle	Burgess <i>et al.</i> 1987; Post <i>et al.</i> 1988.
	dogs	Grauer <i>et al.</i> 1988; Greene <i>et al.</i> 1988; Greene <i>et al.</i> 1991.
	birds	Anderson and Magnarelli 1984; Anderson <i>et al.</i> 1986.
	humans	Magnarelli <i>et al.</i> 1988.
EUROPE	rodents	Vittoz <i>et al.</i> 1990; Anderson 1991.
	deer	Cutler 1990.
	cattle	Hovmark <i>et al.</i> 1986; Cutler 1990.
	sheep	Hovmark <i>et al.</i> 1986; Cutler 1990.
	dogs	May <i>et al.</i> 1990; May <i>et al.</i> 1991.
	humans	Burioni <i>et al.</i> 1988; Dekonenko <i>et al.</i> 1988; Abraham <i>et al.</i> 1991; Fahrer <i>et al.</i> 1991; De Marval <i>et al.</i> 1990.
ASIA	rodents	Miyamoto <i>et al.</i> 1991.
	human	Ai and Xiamong 1989; Ai <i>et al.</i> 1990.
AUSTRALIA	cow	Rothwell <i>et al.</i> 1989.
	human	Stewart <i>et al.</i> 1982.

1.5.1 Africa.

There have been numerous reported cases of Lyme disease in Africa. Haberberger and co-workers (1989) reported possible Lyme disease patients in residents at an oasis in Egypt. *B. burgdorferi* was not isolated and nothing is known about possible vectors, hosts or reservoirs. Possible transmission has also been reported in Algeria (Anonymous 1986).

There have also been possible Lyme disease reports in South Africa. In 1984 a tourist visiting this country was bitten by ticks whilst camping in Natal. When she fell ill, all serological tests performed, except for *B. burgdorferi* IFA and ELISA tests, were negative (Stanek *et al.* 1986). In 1982, a stable in Natal reported horses appearing stiff and having problems walking (Fivaz *et al.* 1990). The causative agent was not found and after treatment no further cases were reported. Following the appearance of literature on *B. burgdorferi*, sera from the horses and dogs at this stable were tested for *B. burgdorferi*, some five years after the outbreak of lameness in the horses. 61% of the horses and 55% of the dogs from the stable tested had antibody titres of 1:256 and higher (Fivaz *et al.* 1990). During 1988, the owner of the stable fell ill, showing Lyme disease symptoms (Botha *et al.* 1989; Fivaz *et al.* 1990). Serum tested by IFA, ELISA and Immunoblots confirmed Lyme disease. The vector at the stable is not known (Botha *et al.* 1989; Fivaz *et al.* 1990).

Although serological surveys have been carried out in South Africa, results are not conclusive due to probable false positive titres (Fiean 1989; Botha 1992). It is therefore unclear to what extent *B. burgdorferi* occurs in South Africa. All attempts to isolate the organism from ticks occurring in areas of suspected outbreaks have failed (Botha 1992). Further field investigation aimed at identifying possible vectors in South Africa is needed in view of these reported suspected outbreaks. Moreover, this country has a wide variety of tick species which include five tick genera in which spirochaetes have been found elsewhere in the world.

1.6 STUDY PROPOSAL.

Although there have been reported cases of suspected Lyme disease in South Africa based on serological evidence (Stanek *et al.* 1986; Fivaz *et al.* 1990; Botha 1992), it is not known which, if any, of the South African species of tick are capable of transmitting the organism and what the actual distribution of the disease is in this country - if it does in fact occur here. The identity of the possible reservoirs of infection is also a matter for conjectures and further investigation.

This research was undertaken with two aims. Firstly to investigate the capacity of some of our locally represented tick genera to transmit *B. burgdorferi*. The genera used have been clearly implicated as vectors elsewhere in the world. Secondly, to find serological evidence implicating certain local vertebrates as reservoirs of the pathogen. For this purpose horse, cattle and selected rodent species were investigated as possible hosts. Additionally, ticks were collected from the rodents and some of the horses included in the survey and examined for the presence of *B. burgdorferi*.

CHAPTER TWO

MATERIALS AND METHODS

2.1 CULTURE OF *BORRELIA BURGENDORFERI*.

A commercially available strain of *B. burgdorferi* (code no. 4861) was obtained from Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH, Braunschweig, Germany. This isolate was propagated in Barbour-Stoenner-Kelly culture medium (BSK II) (Barbour *et al.* 1983a; Preac-Mursic *et al.* 1991) supplied by Highveld Biological. The medium contained 860ml deionized water, 100ml GIBCO CMRL 1066 without glutamine, 5g neopeptone (Difco), 50g bovine serum albumin fraction V, 2g yeastolate (TC) (Difco), 6g HEPES buffer (Sigma), 0.7g sodium citrate (Mallinckrodt), 5g dextrose (Mallinckrodt), 0.8g sodium pyruvate (Sigma), 0.4g N-acetylglucosamine (Sigma), and 2.2g sodium bicarbonate (Mallinckrodt). To this was added 200ml of warm 7% (w/vol) solution of gelatin that had been sterilized for 15 minutes at 15 psi and 121°C, and 77ml slightly hemolysed rabbit serum. Media was dispensed into 13ml teflon lined screw cap test tubes. Each tube was inoculated with 0.1ml of media containing actively growing *B. burgdorferi* organisms and incubated at 28°C for 10 days.

2.2 RABBITS.

Tick-naive New Zealand Giant White cross Californian White rabbits were obtained from the Ciskei Rabbit Breeding Centre, Ciskei and the Animal Resource Centre, University of Durban Westville, Natal, South Africa. All rabbits were kept in individual cages at the Rhodes University Animal House, Grahamstown, South Africa until ready for use. They were fed on commercially available rabbit pellets containing 14% protein and water *ad libitum*.

2.3 TICKS.

Pathogen-free colonies of *Amblyomma hebraeum* and *Rhipicephalus simus* larvae were obtained from the South African Bureau of Standards, East London and were bred at the Tick Research Unit, Rhodes University, Grahamstown. A colony of *Haemaphysalis leachi* was reared from five engorged female ticks collected from a dog in Grahamstown and were maintained at the Tick Research Unit for the past two years. All three tick species were reared on rabbits. Ticks were fed by placing them in a cotton ear bag, which was put over one of the rabbit's ears and secured at the base with stretch plaster. Grooming was prevented by placing a rubber collar around the rabbit's neck. After repletion the engorged ticks were collected from the ear bag, kept in cotton wool-stoppered vials and allowed to moult/lay eggs. Adult *A. hebraeum* ticks were reared on cattle in the same manner. All repleted and questing ticks were kept in a tank in a constant environment room at 28°C with a 12 hour photoperiod, at a humidity of 85%. Humidity was maintained by a saturated solution of potassium chloride in the bottom of the tank.

Ixodes rubicundus larvae were kindly supplied by Dr. Leon Fourie, Department of Entomology, University of the Orange Free State, Bloemfontein. These larvae were kept at 15°C in a light-restrictive box with a humidity of approximately 97%. The humidity was maintained by a saturated solution of potassium dihydrogen phosphate (L. Fourie pers. comm.)

2.4 CONTROL RABBIT SERUM.

B. burgdorferi control serum was prepared by the method recommended by Prof. A. Kocan (Oklahoma State University, USA, pers. comm.). 0.5ml of 10% formalin in 0.1M phosphate buffered saline (PBS) pH 7.2 was added to 2ml of BSK II medium containing live *B. burgdorferi* spirochaetes. After 10 minutes, the suspension was centrifuged in a Sorvall RC-5 centrifuge using a SS-34 rotor at 20 000g at 25°C for 30 minutes. The pellet was then washed 3 times in 0.1M PBS (pH 7.2). The final pellet was resuspended in 1ml of 0.1M PBS (pH 7.2) and mixed with 1ml of Freund's complete adjuvant and injected intramuscularly into a naive rabbit. A booster inoculation of the same dose was administered four weeks later. Four weeks after the booster this rabbit was bled from the marginal ear

vein. Serum was collected and frozen until confirmed to show a positive titre by IFA and used as a control positive in the IFA tests.

As a negative control for the IFA tests, serum was collected from the blood of a parasite-free rabbit.

2.5 TRANSMISSION OF *BORRELIA BURGENDORFERI* BY TICKS.

Transstadial and transovarial transmission of *B. burgdorferi* were attempted using four species of ticks, viz. *A. hebraeum*, *R. simus*, *H. leachi* and *I. rubicundus*.

2.5.1 Artificial infection of rabbits with *Borrelia burgdorferi* and infestation with ticks.

After checking the viability of *B. burgdorferi* spirochaetes in culture after 10 days of growth using dark-field microscopy, eight adult rabbits were inoculated intravenously with 1ml of medium containing approximately 6×10^4 spirochaetes (Burgdorfer 1984). The number of spirochaetes was determined by plotting a standard curve using the number of Giemsa stained spirochaetes manually counted in serial dilutions of inoculum.

The eight *B. burgdorferi*-inoculated rabbits were then randomly divided into four groups (two per group): one group for each of the four tick species under evaluation. On days seven and 21 post-inoculation one rabbit from each of the three groups was infested with approximately 500 *B. burgdorferi*-free larvae of *A. hebraeum*, *H. leachi* and *R. simus* respectively. The second rabbit in each group was infested with approximately 100 *B. burgdorferi*-free nymphs of the same species. Rabbits were infested as described in section 2.3.

Ten percent of the engorged ticks recovered from the infected rabbits were examined for evidence of spirochaetes using the IFA test. Ticks were sterilized in 70% alcohol and washed in sterile 0.1M PBS (pH 7.2) before being smeared individually on microscope slides. Larvae were smeared whole on the slides, while nymphs and adults were dissected and portions of mid-gut were smeared. Slides were air-dried and fixed in acetone for 10

minutes. After air-drying a second time, the slides were wrapped in foil before being stored at -20°C (Lindsay *et al.* 1991; Magnarelli *et al.* 1987a; Nelson *et al.* 1991). The remaining engorged ticks were stored in cotton wool-stoppered vials in a tank at 25°C with a humidity of 85% until moulting. Moulded ticks were later used in transmission trials using susceptible rabbits.

Rabbits in the fourth group were each infested with approximately 30 *B. burgdorferi*-free *I. rubicundus* larvae on days seven and 44 after inoculation with *B. burgdorferi*. The rabbits were infested by placing the ticks in a plastic container glued to a shaved area in the lumbar region using Contact glue. Grooming by the rabbit was prevented by placing a rubber collar around its neck. Rabbits were kept in a constant environment room at 15°C with a 12 hour photoperiod. Engorged larvae were collected and processed for evidence of *B. burgdorferi* infection, as discussed above.

Engorged larvae were kept in cotton wool-stoppered vials at 15°C in a light restricted box with a humidity of approximately 97% (L. Fourie pers. comm.). Due to the low number of engorged larvae recovered, larvae from both infestations and both rabbits were pooled in the transmission trial.

2.5.2 Transstadial transmission.

The nymphs and adults from the larval and nymphal infestations were tested separately for the transstadial transmission of *B. burgdorferi* according to the following protocol.

Ten percent of the newly emerged nymphs and adult ticks from each infestation were prepared for evidence of infection with *B. burgdorferi* using indirect IFA, and stored as described in section 2.5.1.

The remaining ticks from each infestation on each of the *B. burgdorferi*-inoculated rabbits were then placed on naive rabbit hosts. All ticks except *A. hebraeum* adults and *I. rubicundus* nymphs were placed in ear bags and allowed to feed. The *A. hebraeum* adults and *I. rubicundus* nymphs were placed in chambers attached to shaved areas on the lumbar

region. Engorged nymphs and adults were collected after repletion. Ticks collected off each rabbit were stored separately as described in section 2.3.

2.5.3 Transovarial transmission.

Transovarial transmission of *B. burgdorferi* was evaluated by collecting the eggs laid by female *A. hebraeum*, *H. leachi* and *R. simus* ticks which had previously fed on *B. burgdorferi*-infected rabbits. Approximately 10% of these eggs were surface sterilized in 70% alcohol, crushed on microscope slides and prepared for indirect IFA testing, for evidence of *B. burgdorferi*. The larvae that emerged from the remaining eggs were placed on naive rabbits and allowed to engorge. Engorged larvae were collected and stored separately as described in section 2.3.

2.6 EXAMINATION OF TICK SMEARS FOR *BORRELIA BURGDORFERI* USING IFA.

Standard indirect IFA tests were carried out on the stored tick smears (Lindsay *et al.* 1991; Magnarelli *et al.* 1987a; Nelson *et al.* 1991). Slides were thawed at room temperature and then overlaid with 1:80 working dilution of murine monoclonal antibody, H5332, raised against strain B31 of *B. burgdorferi* isolated from an *I. dammini* (kindly donated by Dr A.G. Barbour, Department of Medicine, University of Texas, San Antonio, USA) (Barbour *et al.* 1983b; Barbour *et al.* 1985). This working dilution was assessed by performing a series of IFA tests on slides coated with different dilutions of *B. burgdorferi*. The dilution which showed the greatest fluorescence of spirochaetes was determined to be the working dilution. Slides were incubated at 37°C for one hour before being gently rinsed in distilled water (P. Botha pers. comm.) and soaked in 0.01M PBS (pH 7.2) for five minutes. This was repeated three times. After a final rinse in distilled water, the slides were overlaid with FITC-labelled anti-mouse immunoglobulin (whole molecule) (Sigma) diluted to 1:64 in a 0.05% solution of Evans Blue in distilled water. The slides were incubated, washed and rinsed as above, covered with a drop of mounting fluid and a cover slip, and examined for any fluorescing spirochaetes under a Zeiss fluorescence microscope with neofluar 40x and 100x objective lenses.

2.7 SEROLOGY.

Artificially infected rabbits were bled from the marginal ear vein prior to, and every seven days after, inoculation with *B. burgdorferi*. Blood was allowed to clot at 4°C overnight and after subsequent centrifuging in a Serle B400 bench-top centrifuge at 6000rpm for 10 minutes, serum was collected and stored in 1ml aliquots at -20°C. Naive rabbits used in the transmission experiments were bled prior to infestation, and again every seven days for seven weeks after infestation. The serum was frozen until tested.

Two serological tests were used to evaluate the presence of *B. burgdorferi* antibodies in rabbit sera, namely IFA (Anderson *et al.* 1983; Lane *et al.* 1990; Luther and Moskophidis 1990; Magnarelli *et al.* 1984; Russell *et al.* 1984) and haemmagglutination (Melby *et al.* 1990).

2.7.1 Indirect IFA.

Antigen coated slides were kindly donated by Prof. P. Howell (Department of Infectious Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa). Slides had been prepared according to the following method (Anderson *et al.* 1983; Lane *et al.* 1990; Russell *et al.* 1984): *B. burgdorferi*, strain 4681, was grown in BSK II for seven days. After satisfactory growth, spirochaetes were harvested by a 30 minute centrifugation of the culture at 21 000g at 15°C and three washings in 0.01M PBS (pH 7.2). Spirochaetes were diluted in 0.01M PBS (pH 7.2) to yield approximately 100 organisms per 40x microscope field. 20µl of this antigen was placed in each well of acetone-resistant multiwell fluorescent antibody slides and allowed to air-dry. The slides were then fixed in acetone for 10 minutes and air-dried. They were then washed in distilled water for 10 minutes, dried and stored at -70°C.

Each rabbit serum sample was tested using two-fold dilutions from 1:32 to 1:1204 (Burgess 1986). Diluent comprised 0.01M PBS (pH 7.2) with 0.1% Tween 20. 20µl of each dilution was placed in each well on an antigen-coated slide and incubated in a moist chamber for 30 minutes at 37°C. Slides were then rinsed in distilled water before being washed for five minutes in 0.01M PBS (pH 7.2) with 0.1% Tween 20. Rinsing and washing were repeated

three times. FITC-labelled goat anti-rabbit immunoglobulin (whole molecule) (Sigma) was diluted with 0.05% Evans Blue in distilled water to a dilution of 1:64. 10 μ l of conjugate was placed in each well. The slide was incubated, rinsed and washed as described above. The slide was then covered with mounting fluid and a cover slip and examined under a fluorescence microscope with 100x objective lens. End point titres were the highest dilution titres for which there was a definite fluorescence of spirochaetes. End point titres were considered positive if the dilution was > 1:64 (Magnarelli *et al.* 1986a; Greene *et al.* 1991). Sera from the positive control rabbit and a naive rabbit served as controls.

2.7.2 Haemagglutination.

Lymag (Diagast Laboratoires, Lille, France) test kits were used to test the rabbit sera for antibodies to *B. burgdorferi*. This kit tests sera against sheep erythrocytes sensitized with *B. burgdorferi*. The sera were also screened against non-sensitized sheep erythrocytes which served as controls for detecting non-specific agglutination. Test sera were screened at a dilution of 1:200 with supplied diluents in a 'U' bottomed microtitre plate to which sensitized and non-sensitized cells were added and left for three hours. Resulting agglutination was graded from 0 to +4. 0 was a tight ring or button sedimentation, indicating the serum had no *B. burgdorferi* antibodies. +4 was a strong positive uniform pellicule at the base of the well. Intermediates were graded according to the size of the pellicule in the well. Control positive and negative sera were provided in the assay kit and were run with each test.

Specificity of the Lymag haemagglutination for *B. burgdorferi* was evaluated by screening with sera from human patients positive for antibodies to *B. duttoni* (Donated by Prof. R. Swanepoel, National Institute for Virology, Johannesburg, South Africa) and *Treponema pallidum* (Donated by Prof. P. Botha, Medical Microbiology, University of the Orange Free State, Bloemfontein, South Africa).

2.8 IDENTIFICATION AND ISOLATION OF *BORRELIA BURGDORFERI* FROM RABBIT TISSUES.

Six weeks after the inoculation with *B. burgdorferi*, rabbits which had served as hosts for initial tick infestations were euthanased with 1.5ml of Eutha-naze (Centaur). The two rabbits that served as hosts to *I. rubicundus* were euthanased after ten weeks. Rabbits that were infested with the *B. burgdorferi* infected ticks in the transmission experiments were euthanased after eight weeks. After euthanasia, three sections of the liver, the kidney and the heart were aseptically removed from each rabbit. One tissue sample from each organ was immersed in 10% formalin in PBS (pH 7.2) for histological analysis, while another sample of organ measuring approximately 5mm x 5mm was quick frozen in liquid nitrogen, where it was stored until sectioning for identification of spirochaetes using IFA. The remaining tissue samples were used for culturing in an attempt to isolate spirochaetes.

2.8.1 Histology.

The organ samples from each rabbit immersed in 10% formalin in PBS (pH 7.2) were submitted to a veterinary consultant pathologist (Dr. L. Lange, Welgemoed, Cape) where they were sectioned and stained with Haematoxylin and Eosin and Warthin Starry silver stain. Sections were examined under a Nikon light microscope at 40x and 100x magnifications for any evidence of *B. burgdorferi* and any associated pathological changes.

The control positive and negative rabbits were euthanased after eight weeks and a section of kidney, liver and heart from each was submitted to the same consultant pathologist for histological analysis.

2.8.2 Identification of *Borrelia burgdorferi* using IFA tests.

The tissue samples frozen for sectioning were embedded in Cryomatrix (Shandon) and sections 8µm thick were cut at -27°C using a Mini Cryostat (Shandon). 10 serial sections were taken from each organ of each rabbit. Individual sections were laid on clean glass slides that had been coated with poly-L-lysine (Sigma) and air-dried. IFA staining was carried out as described for the staining of the tick smears in section 2.6.

2.8.3 Isolation of *Borrelia burgdorferi* from rabbit tissue.

The third sections of each organ removed for culturing were aseptically minced and placed individually in tubes containing 7ml of BSK II medium (Burgess *et al.* 1986; Burgdorfer and Gage 1987; Burgess *et al.* 1987; Barthold *et al.* 1988; Wright and Nielsen 1990; Lane *et al.* 1991; Schmitz *et al.* 1991). After six weeks of incubation at 28°C, all tubes of media were individually centrifuged in a Sorvall RC-5 centrifuge with a SS-34 rotor at 21 000g at 15°C for 30 minutes. Each pellet was washed 3 times in 0.1M PBS (pH 7.2). The final pellets were individually resuspended in 2ml of 0.1M PBS (pH 7.2). 0.1ml of each suspension was put on a slide and heat fixed. The slide was dehydrated in methanol for 10 minutes, air-dried and stained in 20% Giemsa for 30 minutes (Matton and Van Melckebeke 1990). After washing in distilled water, the slides were examined under a light microscope for spirochaetes at using a 100x objective lens.

2.9 SURVEYS.

2.9.1 Murine Survey.

A variety of wild rodents were trapped in Sherman traps baited with peanut butter, at three sites situated around Grahamstown (33°18'S 26°32'E). These sites were selected on the basis of the well established rodent populations known to exist at each site. After capture, the rodents were identified and kept in individual cages, each suspended over a tray of water to collect detaching ticks (Burgess *et al.* 1987; Matuschka *et al.* 1990; Telford *et al.* 1990; Matuschka *et al.* 1991). Recovered ticks were sterilized in 70% alcohol and then rinsed in 0.1M PBS (pH 7.2). They were identified to species level where possible and prepared for IFA testing by the method described to identify *B. burgdorferi*.

After eight days, the rodents were anaesthetized with ether and blood was collected by cardiac puncture. Sera were frozen at -20°C, ready for screening for *B. burgdorferi* antibodies using IFAT and haemagglutination.

2.9.2 Equine and bovine sera survey.

Twenty-seven horses at Stoneyhurst Stables, Hammersdale, Natal, South Africa (30°50'E 30°40'E) were bled by venous puncture in April 1992. Sera from these horses were

collected and stored at -20°C until they were screened for *B. burgdorferi* antibodies by IFA and haemagglutination tests. Attempts were made to correlate *B. burgdorferi* seroconversion and possible evidence of disease over a three year period, by comparing these test results with those performed by Prof. Botha (Fivaz *et al.* 1990) on sera collected from the same horses in June 1989.

Samples of ticks were collected approximately once every two weeks from stabled horses at Stoneyhurst over a two year period. The ticks were identified, prepared and screened for *B. burgdorferi* by IFA tests as described earlier.

Twenty-six horses at Trapalanda Riding School, Grahamstown were bled in March 1992. Sera from the horses were collected and stored at -20°C until they were screened for *B. burgdorferi* antibodies by IFA and haemagglutination tests.

Batches of bovine sera were collected randomly from both dairy and beef cattle in 1987 in an unrelated survey at six different farms around Grahamstown. These sera samples, which had been stored at -20°C , were screened for antibodies to *B. burgdorferi* using the Lymag haemagglutination test. IFA tests were not performed on the bovine sera due to the lack of funding for this study.

CHAPTER THREE

RESULTS

3.1 ARTIFICIALLY INFECTED RABBITS.

3.1.1 Serology.

Only one rabbit inoculated with *B. burgdorferi* in culture showed raised antibody titres on the IFA and haemagglutination tests (Figure 2 and Table 3). There was an increase in antibodies within seven days of inoculation and the highest titre (1:512) was reached after two weeks. Haemagglutination titres in another rabbit showed a slight rise at day 14 but this disappeared by day 21 (Table 3). This rabbit however showed no increase in titre on the IFA test. Sera collected prior to inoculation are not included in Table 3 due to insufficient amounts of sera collected.

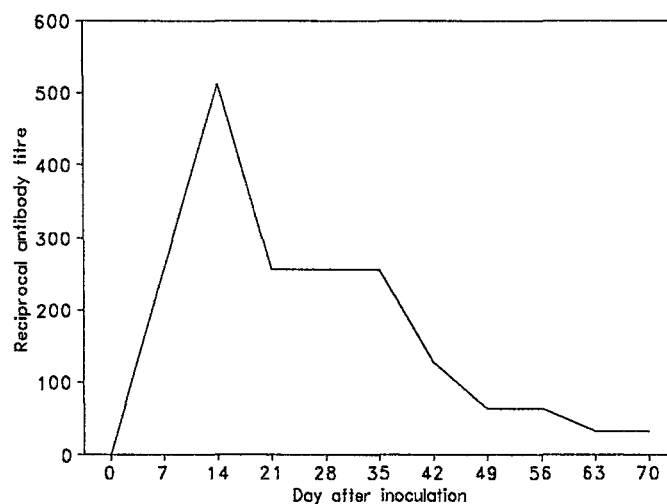


Figure 2. IFA titres to *B. burgdorferi* in Rabbit 1 following inoculation.

Table 3. Lymag haemagglutination test of sera collected at seven day intervals from two artificially infected rabbits following inoculation with *B. burgdorferi*.

Days after inoculation	Rabbit 1 (sera screened at 1:200)				Rabbit 2 (sera screened at 1:200)			
	a	b	c	Agglutination grade	a	b	c	Agglutination grade
7				+3				-
14				+2				+1
21				+4				-
28				+4				-
35				+4				-
42				+2				-
49				+2				-
56				+1				-
63				+1				-
70				+1				-
Control positive								
Control negative								

- a = diluting-absorbent plus test serum
 b = non-sensitized erythrocytes plus test serum
 c = *B. burgdorferi*-sensitized erythrocytes plus test serum
 - = no reaction
 + = grade of agglutination reaction.

3.1.2 Histology.

When comparing the histological sections of the kidney, liver and heart of the artificially infected rabbits and control rabbits, mild cellular changes were seen in the one artificially infected rabbit that had raised antibody titres to *B. burgdorferi* compared to the negative control rabbit.

Kidney: The artificially infected rabbit showed nephrosis with hyaline and haemoglobin cast formation (Figure 3a). There were no cellular abnormalities in the negative control rabbit (Figure 3b).

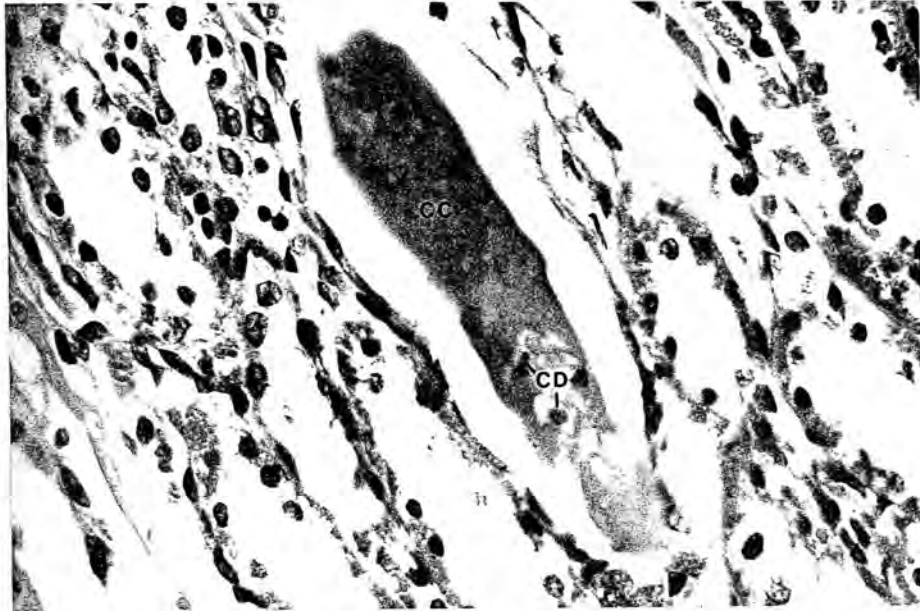


Figure 3a. Cellular cast (CC) with cell debris (CD) in the kidney of the seropositive rabbit artificially infected with *B. burgdorferi*. Mag. x 370.

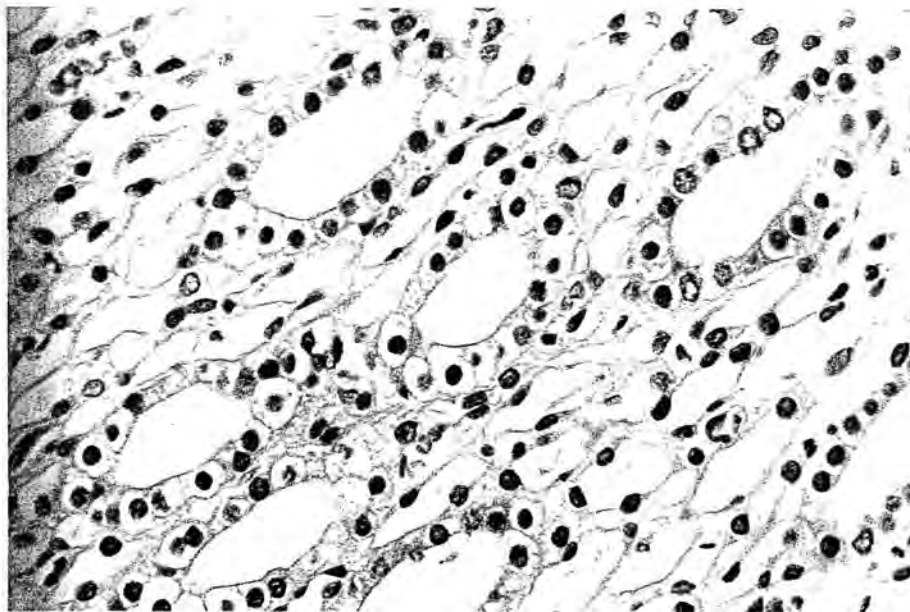


Figure 3b. The kidney cells of the negative control rabbit. Mag. x 370.

Liver: The artificially infected rabbit showed mild hepatosis and the portal tracts showed infiltration of lymphocytes and monocytes (Figure 4). There were no abnormalities in the negative control rabbit.



Figure 4. Mild hepatosis and lymphocytic infiltration of the portal tract in the liver of the rabbit artificially infected with *B. burgdorferi*. Lymphocytes (L) are shown. Mag. x 370.

Heart: The infected rabbit showed granular degeneration of the myocardial fibres together with infiltration of lymphocytes (Figure 5). There was no change in the heart of the negative control rabbit.



Figure 5. Granular degeneration of the myocardial fibres and round cell infiltration in the rabbit artificially infected with *B. burgdorferi*. Lymphocytes (L) and monocytes (M) are shown. Mag. x 370.

There were no significant histological changes in the other artificially infected rabbits. No spirochaetes were seen in any of these rabbit tissue samples, stained with Haematoxylin and Eosin, and Warthin Starry stains.

3.1.3 Identification of *Borrelia burgdorferi* using IFA tests.

IFA tests failed to demonstrate spirochaetes in any of the infected rabbits tissue samples.

3.1.4 Isolation of *Borrelia burgdorferi* from infected rabbits.

No organisms were isolated from the organs of the infected rabbits following incubation in culture medium.

3.2 TRANSMISSION OF *BORRELIA BURGDORFERI* BY TICKS.

3.2.1 Transstadial transmission.

None of the 10% of detaching *A. hebraeum*, *R. simus*, *H. leachi* and *I. rubicundus* larvae or nymphs tested for *B. burgdorferi* spirochaetes by IFA, appears to have acquired spirochaetes from artificially infected hosts. Similarly, none of the nymphs or adults that survived moulting contained spirochaetes, as tested by IFA. The naive rabbits serving as

hosts to the remainder of the nymphs and adults did not show any specific seroconversion to *B. burgdorferi* spirochaetes on IFA and haemagglutination tests. No *I. rubicundus* nymphs survived the moulting process to adults, so further possible transstadial transmission by this species of tick could not be evaluated.

























3.2.2 Transovarial transmission.

No *A. hebraeum*, *R. simus* or *H. leachi* female adult ticks passed spirochaetes transovarially to their progeny, and the naive rabbits serving as hosts to these progeny did not seroconvert. *I. rubicundus* adults were not available and transovarial transmission could not be evaluated.

3.3 LYMAG HAEMAGGLUTINATION TEST ON HUMAN SERA.

When the Lymag haemagglutination tests were performed on sera from human patients with *T. pallidum* and *B. duttoni* infections, cross reactions occurred between the antibodies to these organisms and *B. burgdorferi* antigens (Table 4).

Table 4. Lymag haemagglutination test results of sera from human patients with *T. pallidum* and *B. duttoni* infections.

Sera screened at 1:200	Patient	a	b	c
Sera with <i>T. pallidum</i> infections	A			
	B			
	C			
	D			
Sera with <i>B. duttoni</i> infections.	E			
	F			
Control positive				
Control negative				

a = diluting-absorbent plus test serum

b = non-sensitized erythrocytes plus test serum

c = *B. burgdorferi*-sensitized erythrocytes plus test serum.

3.4 SURVEYS.

3.4.1 Murine survey.

Three species of rodent were caught, viz. 22 *Rhabdomys pumilio* (Four-striped Field Mouse), 14 *Aethomys namaquensis* (Namaqua Rock Mouse) and 51 *Otomys irroratus* (Vlei Rat). Nineteen of these rodents had antibodies to *B. burgdorferi* when tested using the IFA test (Table 5a), while only four showed raised antibody titres when tested with the haemagglutination test (Tables 5a and 5b). The *R. pumilio* with raised antibodies to *B. burgdorferi* according to the haemagglutination test were seronegative using the IFA test. Sera from only two of the *O. irroratus* that reacted in the IFA test, reacted positively in the

haemagglutination test. The IFA titres were 1:128 and 1:256 respectively, while +4 reactions were recorded in the haemagglutination test.

Table 5a. Total number of rodents caught in the Grahamstown area and positive reactors with IFA and haemagglutination tests. Suspicious cases with dilutions of 1:128 are included.

Species of rodent caught	No. of rodents captured	No. of reactors (IFA tests)		No. of reactors (haemagglutination)
		1:128	1:256	
<i>Rhabdomys pumilio</i>	22	2	1	1
<i>Aethomys namaquensis</i>	14	0	1	0
<i>Otomys irroratus</i>	51	7	8	3

Table 5b. The haemagglutination test results of the sera from some of the rodents surveyed.

Species of rodent (sera screened at 1:200)												
<i>Rhodomys pumilio</i>				<i>Aethomys namaquensis</i>				<i>Otomys irroratus</i>				
a	b	c	Agglutination grade	a	b	c	Agglutination grade	a	b	c	Agglutination grade	
			+1				-				+1	
			-				-				+2	
			+1				-				+2	
			-				-				-	
			+1								-	
			+1								-	
			-								+4	
			-								-	
			-								-	
			+2								+2	
			-								-	
			-								-	
											-	
											-	
Control negative												
Control positive												

- a = diluting-absorbent plus test serum
- b = non-sensitized erythrocytes plus test serum
- c = *B. burgdorferi*-sensitized erythrocytes plus test serum
- = no reaction
- + = grade of agglutination reaction.

Ticks collected from these rodents included species of *Haemaphysalis*, *Rhipicephalus* and *Ixodes* (Table 6). Seven of the 221 ticks collected contained spirochaetes that reacted strongly with *B. burgdorferi* monoclonal antibody H5332 (Figures 6a, 6b and 6c). All seven of these ticks belonged to the genus *Ixodes*. Some of them were sent for identification to the Veterinary Research Institute, Onderstepoort, South Africa. All nymphs were identified as *Ixodes bakeri* while larvae were not identifiable.

All ticks containing spirochaetes were removed from hosts showing raised antibody titres to *B. burgdorferi* by IFA testing. Of the four infected *Ixodes* larvae collected from *R. pumilio*, three had fed on a host with an antibody titre of 1:256, while the fourth had fed on a rodent with a titre of 1:128. The one spirochaete-infected larva that fed on *O. irroratus*, together with one of the infected nymphs, were from a host with a *B. burgdorferi* antibody titre of 1:128. The second infected nymph's *O. irroratus* host also had a titre of 1:128. None of the hosts fed on by infected larvae, had antibodies to *B. burgdorferi*, when tested with the haemagglutination test.

Table 6. Number of ticks containing spirochaetes, collected during 1991 and 1992.

Species of rodent caught.	No. of ticks collected	No. of ticks with spirochaetes / No. of ticks tested					
		<i>Haemaphysalis</i>		<i>Rhipicephalus</i>		<i>Ixodes</i>	
		larvae	nymphs	larvae	nymphs	larvae	nymphs
<i>Rhabdomys pumilio</i>	98	0/14	0/2	0/33	0/5	4/39	0/5
<i>Aethomys namaquensis</i>	10	0/0	0/6	0/0	0/4	0/0	0/0
<i>Otomys irroratus</i>	113	0/21	0/9	0/19	0/41	1/9	2/14

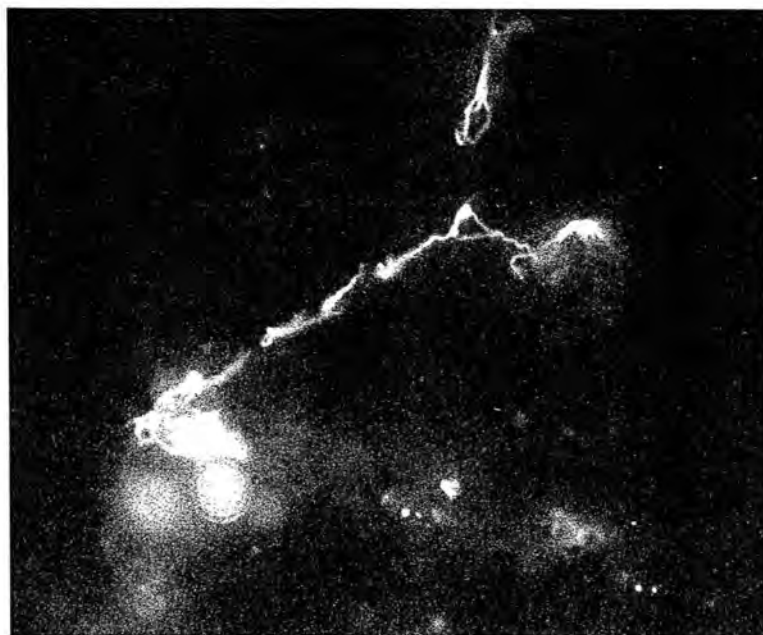


Figure 6a. Spirochaetes found in an *Ixodes* larva collected from *R. pumilio*.
Mag. x 3500.



Figure 6b. Spirochaetes found in an *Ixodes* larva collected from an *O. irroratus*.
Mag. x 3500.

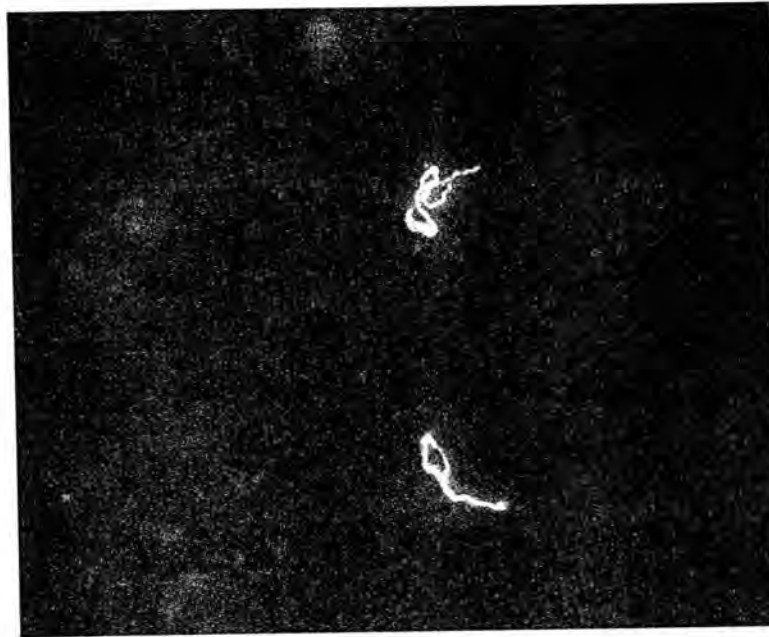


Figure 6c. Spirochaetes found in an *Ixodes* nymph collected from *O. irroratus*. Mag. x 3500.

3.4.2 Equine and bovine sera survey.

IFA test results from sera collected in June 1989 have been used with kind permission of Prof. P. Botha (Fivaz *et al.* 1990). When comparing the IFA test results from sera collected in June 1989 with those of April 1992, three horses showed a rise in *B. burgdorferi* antibody titres (Table 7). 'Dogs of War' rose from 1:128 to 1:512, 'Curwin' from negative to 1:128 and 'Wally' from negative to 1:256. 'Dogs of War' and 'Curwin' were unwell at the time of the bleed in April 1992. Both were lethargic, showing signs of stiffness and had lost condition. 'Dogs of War's' symptoms were more acute than 'Curwin's'.

The results obtained from the haemagglutination tests do not correlate with the IFA test results obtained from sera collected in April 1992 (Table 7a and 7b). Eleven horses were positive for *B. burgdorferi* antibodies using the IFA test, while only three tested definitely positive, and five were suspicious on the haemagglutination test. 'Dogs of War' had the highest titre by the IFA test while he was negative by the haemagglutination test.

Table 7a. Comparison of IFA titres and haemagglutination results for detection of *B. burgdorferi* antibodies in horses at Stoneyhurst Stables, Natal.

Horse tested	June 1989	April 1992		
	IFA* titres	IFA titres	Haemagglutination (screened at 1:200)	
			Agglutination grade	Well no. in Table 7b
Abo	1:128	-	+2	A1
Gay Splash	1:256	-	-	B1
Flash Gordon	1:128	1:64	+2	C1
Winslow Boy	1:128	1:64	-	D1
Lollipop	-	-	-	E1
Star Rose	1:256	1:128	+1	F1
Curwin	-	1:128	-	G1
Dreadnought	-	-	+1	H1
Cape Velvet	1:256	1:128	-	A2
Mickey	-	-	-	B2
Jasper	1:256	-	+4	C2
Wally	-	1:256	-	D2
Ecstatic	1:256	1:256	+3	E2
Business Trip	1:256	1:64	-	F2
Jackaroo	1:128	1:64	-	G2
Lift High	1:256	1:128	-	H2
Jupiter	-	-	-	A3
Coriander	-	-	-	B3
Dogs of War	1:128	1:512	-	C3
County Fair	-	-	-	D3
Spin the Dial	-	-	-	E3
Swag Man	-	1:64	-	F3
Sunny	1:256	1:128	-	G3
Misty	1:128	-	-	H3
Ultimate Choice	1:256	1:128	+1	A4
Fred	1:128	1:128	-	B4
Diamond King	1:256	1:256	+4	C4

* IFA tests performed by Prof. P. Botha (Fivaz *et al.* 1990).

- = no reaction.

Table 7b. Lymag Haemagglutination test results for *B. burgdorferi* antibodies in sera collected in April 1992 from horses at Stoneyhurst Stable, Natal. See Table 7a for horse names.

	1			2			3			4		
	a	b	c	a	b	c	a	b	c	a	b	c
A												
B												
C												
D												
E												
F												
G												
H												

- a = diluting-absorbent plus test serum
 b = non-sensitized erythrocytes plus test serum
 c = *B. burgdorferi*-sensitized erythrocytes plus test serum
 G4 = positive control
 H4 = negative control

Four hundred and thirteen *O. megnini* and 57 *R. evertsi evertsi* ticks were collected over a two year period from the horses at Stoneyhurst and tested for spirochaetes. Only three *O. megnini* contained organisms that may have been spirochaetes, but these had very low immunofluorescence when tested by IFA with *B. burgdorferi* monoclonal antibody. The remaining ticks tested contained no such organisms.

Seven of the 26 horses bled at Trapalanda Riding School, Grahamstown, showed slight reactions with the IFA test but did not have high titres, while sera from five of the horses reacted positively with the haemagglutination test and nine were suspicious cases (Table 8a and 8b). 'Soloman' was the only horse with high titres on both tests. 'Aubie' was negative by the IFA test, yet had a value of +4 for the haemagglutination test.

Table 8a. Comparison of IFA titres and haemagglutination results for detection of *B. burgdorferi* antibodies in horses at Trapalanda Riding School, Grahamstown.

Horse tested	IFA titres	Haemagglutination		Horse tested	IFA titres	Haemagglutination	
		Agglutination grade	Well no. in Table 8b			Agglutination grade	Well no. in Table 8b
Amber	-	+2	A1	Jupiter	-	+3	F2
Map Africa	-	-	B1	Prince	-	+1	G2
Windsurfer	1:128	+1	C1	Short note	-	+1	H2
Godslick	-	+2	D1	Bupa	-	-	A3
Barkley	-	-	E1	Aubie	-	+4	B3
Soloman	1:256	+4	F1	Lady Data	1:128	-	C3
Queen High	-	+3	G1	Peaches	1:64	-	D3
Santi-Ann	-	-	H1	Swiss Gate	1:256	+1	E3
Tupisa	-	+3	A2	Rampage	-	+1	F3
Rudolf	1:128	+1	B2	Aura	1:64	-	G3
Masked Ranger	-	+1	C2	Strong Haul	-	-	H3
Defin	1:128	+1	D2	Charlie Brown	-	-	A4
Topaz	1:128	+1	E2	Keen Edge	-	-	B4

- = no reaction.

Table 8b. Lymag Haemagglutination test results for *B. burgdorferi* antibodies in sera collected from horses at Trapalanda Riding School, Grahamstown. See Table 8a for horse names.

	1			2			3			4		
	a	b	c	a	b	c	a	b	c	a	b	c
A												
B												
C												
D												
E												
F												
G												
H												

- a = diluting-absorbent plus test serum
 b = non-sensitized erythrocytes plus test serum
 c = *B. burgdorferi*-sensitized erythrocytes plus test serum
 G4 = positive control
 H4 = negative control

In the bovine sera survey, all animals reacted positively with the haemagglutination test (Table 9).

Table 9. Haemagglutination test results of bovine sera collected in some Eastern Cape districts.

Humansdorp			Alexandria			Bathurst			Albany			Grahamstown			Bedford		
a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Control positive																	
Control negative																	

- a = diluting-absorbent plus test serum
- b = non-sensitized erythrocytes plus test serum
- c = *B. burgdorferi*-sensitized erythrocytes plus test serum.

CHAPTER FOUR

DISCUSSION

The demonstration of the transmission of a *B. burgdorferi* infection by means of a tick vector would need to be based on criteria which include confirmation of the viability of the pre-inoculated *B. burgdorferi*, establishment of a reliable route of infection, demonstration of seroconversion and pathological changes associated with the infection and evidence of the organism in the tick vector.

Several different methods have been used in attempts to infect experimental animals with *B. burgdorferi* spirochaetes. Four routes employing cultures of *B. burgdorferi* have been used: intraperitoneal (Duray and Johnson 1986); intravenous (Burgdorfer 1984); intradermal (Benach *et al.* 1984; Barthold 1991) and subcutaneous (Burgess 1986; Schaible *et al.* 1989; Schaible *et al.* 1991; Schmitz *et al.* 1991). Two further means of infection have been used with success. The first involves injecting the host with homogenised organs of ticks collected in Lyme endemic areas (Burgdorfer 1984; Kornblatt *et al.* 1984a; Kornblatt *et al.* 1984b). The second method, allowing *B. burgdorferi* infected ticks to feed on the experimental hosts, has proved to be the most successful way of infecting a host with infectious *B. burgdorferi* spirochaetes (Burgdorfer *et al.* 1982; Benach *et al.* 1984). A disadvantage with this method, however, is that several ticks may be required depending on the rate of infection within the source population. Furthermore, hosts would need to be tested by a reliable species specific method to ensure that it is *B. burgdorferi* with which the host animal has been infected, and not another related spirochaete.

As no *B. burgdorferi*-infected ticks were available, the experimental animals in the present study were infected intravenously with *B. burgdorferi* spirochaetes cultured *in vitro*.

Approximately 6×10^4 spirochaetes were injected into each host rabbit. This is more than the minimum number required to induce the disease in mice (Barthold 1991). Some researchers have shown that not all animals seroconvert after an initial inoculation of *B. burgdorferi* and in such cases a second inoculation is required (Greene *et al.* 1988; Stuen and Fridriksdóttir 1991). In the present study, a second inoculation was not given due to the small volume of uncontaminated culture that had been grown from stock culture. Availability of further stock culture was hindered due to the new export ruling in Germany. Decontamination of contaminated tubes of culture was attempted by forcing the inoculated media through sterile $0.22\mu\text{m}$ filters (P. Botha pers. comm.) but was not successful.

The IFA and haemagglutination test results showed that only one experimentally inoculated rabbit appeared to have acquired a *B. burgdorferi* infection. This rabbit showed a rise in antibodies to *B. burgdorferi* by day seven, peaking at day 14. It would appear that this was a rise in IgG titre, and as such it resembles the findings of Kornblatt and co-workers in their experimental infecting of rabbits (1984b). The increase in IgG titre in mice is of a similar pattern (Barthold *et al.* 1991), whereas in hamsters, the increase occurs two weeks later (Schmitz *et al.* 1991). The IgM titre activity was not monitored in this study, but it has been shown that the IgM titre increases almost immediately following infection and peaks before the IgG titre (Barthold *et al.* 1991; Schmitz *et al.* 1991). The IgM titre is much lower than that of IgG, and is therefore more difficult to detect. In the artificially infected rabbit, antibodies to *B. burgdorferi* decreased steadily after peaking, and at euthanasia the titre was very low.

The rabbit used to prepare positive control serum also showed a rise in antibody titre when bled eight weeks after inoculation. The titre, however, was not as high as that of the artificially infected rabbit. The method used to produce this positive control serum was performed according to the instructions of Prof. A. Kocan (Oklahoma State University, USA, pers. comm.), who advised that the antibody peak occurred eight weeks after inoculation. The titre at this point was 1:256: only marginally lower than the highest titre reached in the infected rabbit's serum.

In the period between inoculation with *B. burgdorferi* and euthanasia, none of the experimental animals in the present study developed the typical ECM rash which is sometimes a symptom of *B. burgdorferi* infection. This rash does not occur in all cases of *B. burgdorferi* infection and it appears to be difficult to reproduce a typical lesion in experimental animals. Lesions have been reproduced, but only in cases where the animal has been infested with infected ticks (Burgdorfer *et al.* 1982; Burgdorfer *et al.* 1983; Benach *et al.* 1984; Kornblatt *et al.* 1984a). No lesions have been detected at injection sites or any other part of the body after inoculation of animals with *B. burgdorferi* in media or PBS. An exceptional case was observed by Kornblatt and co-workers (1984b) when a lesion on a rabbit appeared at the injection site following intradermal inoculation with infected tick organ homogenate.

Due to the fact that not all animal and human Lyme disease sufferers develop the typical ECM rash following infection, it is unclear whether the rash is actually caused by the *B. burgdorferi* spirochaete, or is simply a reaction to the tick feeding. When ticks attach and feed on a host animal, an immune reaction in the animal is activated by the intrusion of the tick mouth parts and saliva. This immune reaction results in the formation of a lesion at the site of attachment (Kemp *et al.* 1982). The lesion recorded by Kornblatt and co-workers (1984b) following injection with infected tick organ homogenate may have resulted from some organ component, since the salivary glands were included in the homogenate injected. Histological changes in the lesion on this particular rabbit were similar to those that have appeared at non-*B. burgdorferi* infected tick feeding sites (Brossard and Fivaz 1982; Kornblatt *et al.* 1984a). It has however been shown by Binder and co-workers (1955, cited in Burgdorfer 1986) that an ECM developed in patients infected with skin samples taken from the peripheral zone of an ECM rash of a sufferer. These workers concluded that something in the rash was infectious but could not determine to what extent the lesion may have been due to an immune response to a substance or organism in the donor ECM rash. Although spirochaetes have been found in skin biopsies taken from lesions (Kornblatt *et al.* 1984b), they might not necessarily cause the rash and their presence could be an incidental result of the inoculation.

There is still no conclusive evidence to prove that the rash is caused by the spirochaete alone, and it is not clear why some Lyme disease sufferers develop the rash and others do not. Further investigation into the aetiology of the lesion is required before an understanding of the patchy distribution of lesions in populations can be obtained. There are no reports of skin biopsies being taken from the sites of inoculation where no lesions have formed to check for the presence of spirochaetes, and for any histological changes taking place. Further investigation might determine whether only certain strains of *B. burgdorferi* are capable of causing the skin lesion.

No rabbits in the present study showed signs of arthritis and it appears to be difficult to reproduce the typical *B. burgdorferi*-associated arthritis in rabbits (Benach *et al.* 1984; Kornblatt *et al.* 1984b), despite its demonstration in mice (Barthold *et al.* 1988; Barthold 1991) and hamsters (Duray and Johnson 1986). It is probable that higher doses of spirochaetes need to be administered to cause the arthritis. Moody and Barthold (1991) and Barthold and co-workers (1988) induced arthritis in mice with doses of 10^6 spirochaetes. Barthold and co-workers (1988) found, however, that the severity of the arthritis was not correlated with the levels of specific antibodies.

It is notable that there were basically no gross abnormalities in the thoracic or abdominal viscera except in the artificially infected rabbit which had raised antibodies to *B. burgdorferi*. This rabbit had a small amount of free, cloudy fluid in the abdominal cavity and the kidneys were mottled and granular in appearance.

The histological changes that took place in the rabbit showing raised antibodies to *B. burgdorferi* were similar to those found by other researchers in other animal species. Some tubules in the kidney of the seropositive infected rabbit were filled with hyaline casts and necrotic cellular debris and were similar to those of a naturally infected dog and cow (Burgess *et al.* 1987; Grauer *et al.* 1988). Hepatic and cardiac changes encountered in the seropositive experimental rabbit were similar to lesions reported elsewhere (Duray and Johnson 1986; Burgess *et al.* 1987; Schaible *et al.* 1989; Barthold *et al.* 1991). Although several workers have found that there is often no pathological change in the tissues of various

species following infection with *B. burgdorferi* (Kornblatt *et al.* 1984b; Burgess *et al.* 1986; Duray and Johnson 1986; Burgess and Patrican 1987), Schaible and co-workers (1989) have shown that the severity of the histological changes occurring in mice are often greater in immunodeficient specimens. The degree of histological change could be dependent on the individual infected animal's immune response. Nothing is known about the histology of organs in wild reservoir populations and about hosts of *B. burgdorferi* in the field. It is debatable whether histological changes in the kidney can be attributed to *B. burgdorferi* infection alone, since a kidney removed from a wild rodent containing spirochaetes was histologically normal (Bosler and Schulze 1986). It is possible therefore that the histological changes in the seropositive rabbit may have been due to a secondary contamination of the culture used in the inoculation, especially since no such histological abnormality was observed in the negative control rabbit, which received an inoculation of PBS only.

Various methods have been used for identifying spirochaetes in the tissues of animals infected with *B. burgdorferi*. Often however, spirochaetes are overlooked and the methods used are not sufficiently species specific to be reliable. Notwithstanding this, the IFA testing method used to test the tissues for the presence of *B. burgdorferi* in the present study is commonly used for the detection of *B. burgdorferi*, as the fluorescing spirochaetes are easily detectable (Burgess *et al.* 1987). No spirochaetes were seen in the organs of the rabbits that were artificially infected, or used in the transmission experiments. No spirochaetes were seen in the organs of the artificially infected rabbit that had raised antibodies to *B. burgdorferi*. In contrast to this finding, some workers have identified spirochaetes in the kidneys and livers of other species of animals experimentally or naturally infected (Bosler and Schulze 1986; Burgess *et al.* 1987; Grauer *et al.* 1988; Burgess and Windberg 1989; Wright and Nielsen 1990). Initially it was the author's opinion that the failure to detect spirochaetes in the tissues of the artificially infected rabbits in this study, was due to the possibility that the monoclonal antibody, H5332, used for the IFA testing in this case does not react with all *B. burgdorferi* isolates, particularly four European ones (Wilske *et al.* 1986; Stanek *et al.* 1990) and the isolate used for inoculation in this experiment is from Europe (Barbour *et al.* 1983a). However, the H5332 monoclonal antibody was screened against strain 4861 of *B. burgdorferi* used in the inoculation. Strong fluorescent reactions were seen, proving that

monoclonal antibody H5332 does react with the *B. burgdorferi* strain 4861. It therefore seems that no spirochaetes were present in the tissue.

The absence of *B. burgdorferi* infection in the experimental animals was confirmed following attempts to culture the spirochaetes from organs. Kornblatt and co-workers (1984b) were also unable to isolate organisms from the kidneys and livers of seropositive rabbits that had been inoculated with the agent. Culturing of spirochaetes from organs of wild hosts of other species (Anderson *et al.* 1986; Burgess and Windberg 1989; Mukolwe *et al.* 1992), as well as from organs of experimentally infected hosts (Johnson *et al.* 1984; Burgdorfer and Gage 1987) has generally proved to be successful.

Culturing of spirochaetes from the blood of the artificially infected rabbits was attempted but no results were obtained due to contamination of the culture media which persisted following filtration of the media through sterile 0.22 μ m filter. This method has however been successfully employed by a number of researchers (Kornblatt *et al.* 1984b; Burgdorfer and Gage 1987; Grauer *et al.* 1988).

Finally, although organisms have been seen in various tissues using Warthin Starry stain (Burgess and Patrican 1987), none were seen in the sections made from the organs of any of the rabbit hosts used in this study.

It is clear from these results that infection may have been established in only one rabbit, which seroconverted. The viability of the spirochaetes injected into the rabbits in the present study was confirmed prior to inoculation, using darkfield microscopy and staining with Giemsa. The failure of the remainder of the rabbits to seroconvert could be due to the fact that too few organisms were injected into the hosts to elicit an antibody response, although Barthold (1991), using smaller inoculation doses, found that only a few organisms are needed to infect a mouse. Furthermore, the lack of immune response could have been due to the fact that the spirochaetes were injected intravenously, where they may have been subsequently eliminated by phagocytes of the spleen, liver and lymph nodes. It has also been suggested that other antigens present in the media - such as the rabbit serum - may have

masked the immunogenic response to *B. burgdorferi* (Prof. D. Hendry, Department of Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa, pers. comm.).

It is notable that other researchers have been unable to infect horses with the same strain of *B. burgdorferi* used in this study (Prof. P. Howell, Department of Infectious Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa, pers. comm.). These horses were inoculated once with 2ml of medium containing viable spirochaetes and did not seroconvert. This phenomenon has been noted by other workers using different strains of the organism and it has been suggested that some strains of *B. burgdorferi* have lost their infectivity (Stuen and Fridriksóttir 1991). It is known that other species of *Borrelia*, such as *Borrelia hermsii* and *Borrelia recurrentis*, exhibit antigenic changes that are dependent upon their mode of culture (Barbour *et al.* 1982; Plasterk *et al.* 1986).

The possibility that *B. burgdorferi* changed antigenically during *in vitro* cultivation was first suggested by Schwan and Burgdorfer in 1987 when they noticed that a protein with a molecular weight of 34kDa in *B. burgdorferi* was prominent in unpassaged cultures. Following 15 passages in medium, this protein became undetectable. The loss of this protein however does not mean a loss of infectivity as some isolates of *B. burgdorferi* found in Europe do not have a protein of this molecular weight (Wilske *et al.* 1986; Stanek *et al.* 1990). The OspA and OspB which are species specific to *B. burgdorferi* are found on the 49 kilobase (kb) plasmid of the spirochaete's chromosomal DNA (Wilske *et al.* 1991). Investigations of the other plasmids of the DNA have shown that some become undetectable following several *in vitro* passages (Barbour 1988; Schwan *et al.* 1988). It is only the loss of some of these plasmids that results in the loss of infectivity. For example, Schwan and co-workers (1991) found that the Sh-2-82 strain lost a plasmid after 6 to 10 passages but still remained infectious. After 10 to 15 passages however the 7,6kb and 22kb plasmids were undetectable and the strain was no longer infectious. These specific plasmids do not appear to control the infectivity however, as it has been shown that strain B31 became non-infectious following the loss of the 38kb and 24kb plasmids (Barbour 1988).

It would therefore appear that the strain used to infect the rabbits in the present study was no longer infectious. When enquiries were made to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Germany where the stock culture originated, the company could not provide information on the number of times that the culture had been passaged, nor could it guarantee infectivity. Further investigations of the chromosomal DNA of the isolate used in this study would have to be done to determine if there has been a change in the infectivity of this isolate. It is also recommended that inoculation trials be performed on other possible hosts, such as mice or hamsters that appear to be readily susceptible to this isolate, to establish whether it was the choice of hosts that influenced the outcome of the experiment.

No conclusions can be drawn about the abilities of the four South African species of ticks tested to acquire and maintain a *B. burgdorferi* infection. This is due to the failure of attempts to artificially infect the rabbit hosts in this study.

Spirochaetes are found in the midgut diverticula of questing *Ixodes dammini* and *I. ricinus* ticks (Benach *et al.* 1987; Piesman *et al.* 1987; Ribeiro *et al.* 1987). After a tick has been feeding on a suitable host for approximately three days, the spirochaetes in the tick's midgut begin to divide (Benach *et al.* 1987; Gern *et al.* 1990). They then move into the haemocoel and salivary glands just prior to the rapid engorgement phase of the feeding (Ribeiro *et al.* 1987; Gern *et al.* 1990). This is when the host animal is thought to become infected as the spirochaetes are injected into the host with the tick saliva.

It has been shown that ticks only infect a host three days after feeding has begun (Piesman *et al.* 1991). This is the time taken for the spirochaetes to reach the salivary gland. This three day time lapse may be one of the reasons for the *B. burgdorferi* spirochaetes not being closely associated with members of the Argasidae family. Ticks belonging to this family feed repeatedly for short periods of time (a few minutes to one hour) (Howell *et al.* 1978; Oliver 1989; Aeschlimann 1991) and thus the three day developmental time of the spirochaete in the tick prevents successful infection of the host. This may explain why the argasid ticks *Otobuis megnini* collected from Stoneyhurst Stable would be unlikely vectors. It should also

be noted that ixodid larvae need to feed for eight hours before becoming infected with spirochaetes (Piesman 1991). This reduces the chances of argasid ticks being *B. burgdorferi* vectors.

Since the rabbits failed to become infected, it was clear that the ticks feeding on them would not be infected in turn, and that the transstadial and transovarial transmission experiments would fail.

Transstadial transmission of *B. burgdorferi* (for example from one life stage to the next through ecdysis), has been shown to occur in many of the important Lyme disease carriers in the world today, such as *I. dammini* (Benach *et al.* 1987; Piesman 1991), *I. pacificus* (Lane and Burgdorfer 1987), *I. scapularis* (Piesman and Sinsky 1988) and *I. ricinus* (Burgdorfer 1984). It should be noted that these ticks have a three-host life cycle, that is, each life stage feeds on a separate host animal, thus exposing many hosts to infection with *B. burgdorferi*. Some species of tick, for example *I. cookei* (Ryder *et al.* 1992) and *I. holocyclus* (Piesman and Stone 1991) are able to ingest the spirochaetes as larvae. The spirochaetes do not survive ecdysis, however, and the resulting nymphs are not infected.

Transovarial transmission is the process whereby the female tick passes spirochaetes on to her progeny via the egg. It appears to be an important transmission route in ticks such as *I. ricinus* (Burgdorfer *et al.* 1983; Stanek *et al.* 1988) and *I. pacificus* (Lane and Burgdorfer 1987) where there are records of up to 100% of the progeny of an infected female being infected with spirochaetes (Lane and Burgdorfer 1987).

Clearly, further investigation into the possible transstadial and transovarial transmission of *B. burgdorferi* by South African species of ticks using an infective strain of the spirochaete is required before any conclusions can be reached regarding Lyme disease transmission in this country.

Murine, equine and bovine surveys were carried out as part of this research to establish the possible presence of a natural reservoir of *B. burgdorferi* occurring in South Africa. Rodents

were surveyed as they are one of the main reservoirs of Lyme disease elsewhere in the world (Bosler and Schulze 1986; Magnarelli *et al.* 1988; Spielman 1988; Anderson 1991; Lane and Brown 1991; Lane and Loye 1991; Vittoz *et al.* 1990). To serve as an effective reservoir for Lyme disease, the host population must be naturally infected with *B. burgdorferi* and be host to a large number of parasites that are capable of transmitting the organism (Lane *et al.* 1991). In North America, the White-footed Mouse (*Peromyscus leucopus*) appears to be the main reservoir of Lyme disease since it is an important host to the immature stages of the tick vectors of *B. burgdorferi* (Anderson *et al.* 1983; Bosler *et al.* 1983; Magnarelli *et al.* 1984; Godsey *et al.* 1987; Magnarelli *et al.* 1988). Furthermore, up to 25% of the natural population was found to be infected with *B. burgdorferi* (Magnarelli *et al.* 1988) and laboratory experiments have shown that these rodents can remain infectious throughout their lives (Burgdorfer 1991). Other reservoirs found in America are several species of deer (Magnarelli *et al.* 1984; Lane and Burgdorfer 1986; Matuschka *et al.* 1991; Sigal and Curran 1991).

The area chosen for the rodent serological and tick survey is on the outskirts of Grahamstown, South Africa, where there was a reported case of a resident of the town contracting Lyme disease after being bitten by a tick whilst out jogging in this area (B. Fivaz pers. comm.). All the rodents trapped in the survey are distributed through most of South Africa (Smithers 1983), and are potential hosts to a wide variety of tick species. They therefore could all serve as competent reservoirs for *B. burgdorferi*.

The results of both the IFA and haemagglutination tests in the serological survey of these rodents showed that several of them appeared to have antibodies to *B. burgdorferi*. However, these two tests are not species specific (Russell *et al.* 1984; Lane *et al.* 1990; Luther and Moskophidis 1990; Melby *et al.* 1990; Fujita *et al.* 1991; Murphy 1991; Steirnstedt *et al.* 1991) and since other species of *Borrelia* occur in South Africa - such as *Borrelia theileri*, *Borrelia duttoni* and *Borrelia anserina* (Neitz 1956) - the antibodies detected may have been due to the presence of these species.

B. anserina causes a disease in domestic poultry and is carried by an argasid tick *Argas pericus* (Howell *et al.* 1978). None of these ticks was found on the rodents trapped in this survey, since they parasitize only poultry (Howell *et al.* 1978). Although this disease has been reported to occur in the Eastern Cape region of South Africa, it is unlikely that these rodents would have been exposed to *B. anserina* (cited in Neitz 1965, Table 2a).

B. duttoni causes relapsing fever in humans and is transmitted by another argasid tick *Ornithodoros moubata* (Howell *et al.* 1978). Although no ticks of this species were collected from the rodents in this survey, members of this tick species collected in the Eastern Cape are known to have been infected with this spirochaete (cited in Neitz 1965, Table 2a). It is therefore possible for the rodents to have been exposed to this organism, and the question of a rodent actually being a reservoir host for this disease was proposed by Zumpt in 1959. It is notable that serum from patients with relapsing fever cross-reacted with the *B. burgdorferi* antigen in the haemagglutination test.

B. theileri is transmitted by the ixodid ticks *Rhipicephalus evertsi evertsi*, *Boophilus decoloratus* and *Boophilus microplus* (Howell *et al.* 1978; Smith *et al.* 1978, Smith *et al.* 1985). These ticks parasitize mainly larger mammals such as cattle, sheep and goats, but the immature stages are sometimes found on wild hares and could be found on rodents (Howell *et al.* 1978; MacIvor 1986). As *B. theileri* is known to occur in the Eastern Cape region of South Africa, it is possible that the rodents have antibodies to *B. theileri* (cited in Neitz 1965, Table 2a). Whether these antibodies would cross react with tests for *B. burgdorferi* is not known as no isolates of *B. theileri* were available for investigation.

Ideally rodent sera should be screened against *B. burgdorferi*, *B. duttoni* and *B. theileri* using either western blot or PCR techniques, which provide a high degree of specificity. Additionally, cross reactivity between *B. burgdorferi* and other spirochaetal infections is known to occur in the IFA tests (Craft *et al.* 1984; Grodzicki *et al.* 1988). The possibility of the antibodies in the rodents actually being antibodies to a *Treponema* or *Leptospira* species cannot be ruled out. *Leptospira* species are known to occur in rodents and cattle in

the USA (Fuerst 1983; Duguid *et al.* 1978) and in dogs and mice in the Transvaal Province of South Africa (cited in Neitz 1965, Table 2a).

The key to the interpretation of the rodent serological results is the identification of the *Borrelia*-like organism that was found in the *Ixodes* species of tick in this study, as it is the most likely cause of the serological positives in the rodents. *Ixodes* is the most important genus of tick involved in *B. burgdorferi* transmission (Burgdorfer *et al.* 1985; Lane and Burgdorfer 1988; Piesman and Sinsky 1988; Anderson *et al.* 1989; Ai *et al.* 1990; Hubálek *et al.* 1990; Hubálek *et al.* 1991; Lane and Loye 1991; Lane *et al.* 1991; Magnarelli *et al.* 1991; Uchikawa *et al.* 1991). The identification at species level of each of the *Ixodes* ticks collected in the present study was not established, but a small sample was sent to the Veterinary Research Institute, Onderstepoort, South Africa where they were identified as *Ixodes bakeri*. There have been previous reports of this tick infesting the rodents *Otomys irroratus* and *Rhabdomys pumilio* (Clifford *et al.* 1976). Some of the other *Ixodes* collected could have been *Ixodes corwini*, which are also found in the Eastern Cape (Howell *et al.* 1978; Horak *et al.* 1987). The fact that all the ticks containing these fluorescing organisms were taken from hosts with raised antibody titres to *B. burgdorferi* by the IFA tests suggests an association between the organism and antibody titre.

The organism seen in the tick smears using the IFA test need not have been *B. burgdorferi* on the basis of its reaction with the monoclonal antibody H5332. This monoclonal antibody reacts with most *B. burgdorferi* isolates (Stanek *et al.* 1990), but there is a chance that it may not react with a South African isolate, if indeed one does occur. The organisms in the tick smear could have been another *Borrelia* species, such as *B. theileri*. Structurally *B. theileri* is similar to *B. burgdorferi* in that they both fall within the same size range (Theiler 1904; Burgdorfer *et al.* 1982) and they may both occur in different morphological forms, not the typical spirochaete coils but they may be overlapped or looped (Theiler 1904; Aberer and Duray 1991).

The organisms found in the *Ixodes* ticks in the present study need to be isolated and cultured for identification at species level. If they prove to be *B. theileri*, this would be the first

recorded presence of this organism in the *Ixodes* genus. If by chance the organisms are leptospiras, investigations should be made into possible tick transmission of this organism due to the fact that these are thought to be transmitted by contact alone (Fuerst 1983; Duguid *et al.* 1978). Finally, it is possible that the *Ixodes*-associated spirochaetes are in fact a new species which would require characterisation.

The serological survey at Stoneyhurst Stables in Natal has highlighted the complexity of the problem of interpretation of serological data. Of the 26 horses tested for Lyme disease in June 1989, 14 have shown a decrease in antibody titre three years later. Most of these have maintained low titres; Cohen and co-workers (1988) have shown that horses can remain sensitive to *B. burgdorferi* over a period of time, which provides a possible explanation for these low antibody titres.

The haemagglutination test does not appear to be as specific or sensitive as the IFA test. Cross reactions between the haemagglutination test agglutinogens and other antibodies in the horses sera seem to be occurring due to the higher number of reactive horse sera by this test compared to the IFA tests. 'Jasper', for example, recorded the greatest reaction in the haemagglutination test: a titre of greater than 1:200, yet in the IFA test, he was negative. 'Diamond King' also recorded a +4 reaction on the haemagglutination test, yet had an IFA titre of 1:256. It is therefore evident that the haemagglutination test is an inadequate screening method for *B. burgdorferi* infections.

As ticks are the main vectors of Lyme disease, and apparently transmitted the spirochaete to the owner and possibly one worker at the Stoneyhurst Stable (Botha *et al.* 1989; Fivaz *et al.* 1990), it was thought that spirochaetes would be found in ticks collected from the horses. This was not the case however. Of the 470 ticks tested over the two year period, only three of them may have contained organisms. It was not possible to confirm these as spirochaetes since the fluorescence was very weak compared to the fluorescence of the organisms found in the *Ixodes* ticks from the rodents. It is however known that members of the Argasidae family, to which *O. megnini* belongs, are associated with 16 of the 19 described species of *Borrelia* (Hoogstraal 1985). It should be noted that *O. megnini* and *R. evertsi evertsi* are the

only tick species found on these horses and, as other vectors such as flies and other biting insects are known to transmit *B. burgdorferi* (Anderson and Magnarelli 1984; Magnarelli *et al.* 1984; Magnarelli and Anderson 1988), investigations into isolating possible borrelias in these vectors should be conducted. It is also important not to overlook the fact that Callow, in 1967, found a spirochaete in the blood of a South African horse. This spirochaete was never described but he did note that it was smaller than the *B. theileri* found in cattle.

Seven of the horses tested at Trapalanda Riding School in Grahamstown could have been positive for a *Borrelia* species. However, there was no history of typical Lyme disease symptoms having been observed in these horses. Only 10 of the horses sera did not react with the haemagglutination test. This test has already been shown to be non-specific, and these results could be due to antibodies to another organism such as *B. theileri*. It should be noted that these horses were infested with *Boophilus decoloratus* and *R. evertsi evertsi* which are known to transmit *B. theileri* to horses in the Eastern Cape (cited in Neitz 1965, Table 2a).

The possibility of an endemic population of horses maintaining *B. burgdorferi* spirochaetes and thus being reservoirs should be investigated further. This endemic area could be important to public health due to the close association between humans and horses. It seems possible for transmission of *B. burgdorferi* to occur without a vector (Bosler and Schulze 1986; Marcelis *et al.* 1987; Rockstroh *et al.* 1989; Dorward *et al.* 1991) and patients exhibiting any typical Lyme disease symptoms that handle horses should be tested for the disease.

B. theileri commonly occurs in cattle throughout South Africa and could account for the positive reactions in the cattle tested by the haemagglutination test (Theiler 1904, 1906). Additionally, although a number of *Leptospira* occur in South Africa, no in-depth survey has been conducted on the disease in the Eastern Cape. The disease is sporadic in the Transvaal and Western Cape and there could well be seropositive cases of it in the Eastern Cape. It is clear that the possible presence of *B. theileri* and leptospires in the Eastern Cape could explain the high number of seropositive horses from Grahamstown.

The results of the murine, equine and bovine surveys have suggested the possibility of *B. burgdorferi* occurring in South Africa. If further investigations are conducted into the presence of *B. burgdorferi* in South Africa, care would have to be taken not to assume that any positive results are conclusive without precise screening for other related spirochaetes that also occur in this country.

CONCLUSIONS

The discovery in *Ixodes* ticks of organisms reacting with the monoclonal antibody H5332 was the most significant finding of this study and warrants further investigation. This organism needs to be isolated and positively identified. Even if it were not *B. burgdorferi* it could well be the first evidence of another borrelia or related spirochaete being carried in *Ixodes*.

Whether the more common South African ticks are capable of carrying and transmitting the Lyme disease agent *B. burgdorferi* has yet to be established. This should be investigated further using a more infective strain of *B. burgdorferi* in an appropriate host animal. Care must be taken to ensure that the animals have been successfully inoculated prior to experimentation by re-isolating the organism. Various problems were encountered in the course of this study, suggestions on how to overcome these are proposed throughout the discussion.

The murine and equine surveys have also shown that *B. burgdorferi* may occur in South Africa. Further investigations are required to confirm this, and the spirochaete would need to be isolated and properly identified. Although the rodent - horse relationship is typical of a *B. burgdorferi* reservoir, results from the bovine survey show that other organisms could complicate the testing for *B. burgdorferi* antibodies. It has been shown that antibody titre to the agent is maintained following an infection, and diagnosis of the infection should be made on symptoms and serological evidence.

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