THE ROLE OF RODENTS IN THE MAINTENANCE OF THE EAST AFRICAN TICK-BORNE RELAPSING FEVER AETIOLOGICAL AGENT, *BORRELIA DUTTONII*

BY

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ABSTRACT

A total of 250 rodents, 251 adult and nymph ticks and seven human blood samples were collected from Mvumi township in Dodoma region, Tanzania, between November, 2001 and February, 2002, and analysed for the presence of the tick-borne relapsing fever agent (TBRF), *Borrelia duttonii*. Cultures of the spirochetes were made in Barbour-Stoenner-Kellys (BSK-II) medium, and molecular characterization of the spirochetes was done by polymerase chain reaction (PCR). Spirochete cultures were successfully grown from two out of the seven human blood samples. No spirochetes grew in either of the cultures of the rodent blood or the tick haemolymph and crushed tick samples. These samples were negative for spirochetes by staining and direct microscopy. PCR detected Borrelia DNA from two of the seven human blood samples, and from one of either rodent (*Rattus rattus*), and tick samples. This is the first study on the possibility of rodents playing a role in the transmission of *Borrelia* spp in Tanzania. The detection of Borrelia DNA in rodents originating from this TBRF endemic area suggests the possibility of rodents playing a role in the maintenance of the TBRF agent, *B. duttonii*. Since it is generally accepted that only humans are the reservoirs of B. *duttonii,* this study calls for further investigation to determine the importance of commensal rodents and other mammals in the epidemiology of relapsing fever in Tanzania.

DECLARATION

I, Pax Jessey, do hereby declare to the Senate of Sokoine University of Agriculture that, this dissertation is my own original work and has not been submitted for a degree award in any other University.

Signature

Date 02-09-03

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LIST OF ABBREVIATIONS AND SYMBOLS

μ	micro-10^6
%	percentage
°C	degree celcius
bp	base pair
DF	dark-field
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleoside tri-phosphate
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immuno-sorbent assay
FAO	Food and Agricultural Organization
gm	gram
h	hour
IU	international units
LBRF	louse-borne relapsing fever
M	mole
Mg	milligram
$MgCl_2$	magnesium chloride
Min	minutes
mM	millimole
NaCl	sodium chloride

NaOH	sodium hydroxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	hydrogen ion concentration
pmoles	picomoles
rpm	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SUA	Sokoine University of Agriculture
TBE	tris-HCl, boric acid and EDTA
TBRF	tick-borne relapsing fever
TES	tris, EDTA and sodium dodecyl sulphate
TE	tris and EDTA
UV	ultra violet
V	volts
Vol	volume
v/v	volume by volume
X	times

CHAPTER ONE

1.0 INTRODUCTION

Tick-borne relapsing fever (TBRF) is a febrile disease characterized by headache, tachycardia and muscle pain. In East Africa this disease is caused by the spirochete *Borrelia duttonii*, which is transmitted through the saliva or coxal fluid of infected soft vector tick, *Ornithodorous moubata* (Walton, 1962) Untreated patients experience relapses of illness that are characteristic of the disease, and severe meningoencephalitic complications can ensue (Goubau, 1984). In West Africa, TBRF is caused by the spirochete *B. crocidurae* and is spread by the tick *Alectorobius sonrai*. A prospective study conducted in 1990 to 1992 by Jean-Francois Trape and others, in rural communities in Senegal, revealed a 10% prevalence in humans, and in Rwanda, a prevalence of 6% has been reported (Goubau, 1984).

Relapsing fever is one of the diseases which have been largely ignored in Tanzania. Most of the information about this disease available today, is based on research conducted in the 1960's (Walton, 1962).

TBRF is a public health concern due to the morbidity and mortality it causes. The mortality rates differ markedly between locations ranging from zero to 8% (Goubau, 1984). In Tanzania, a pioneer study was conducted in the mid eighties (Barclay and Coulter, 1990). This study revealed that between October 1985 and September 1986, 572 people were diagnosed with TBRF at Mvumi Hospital in central Tanzania. The risk groups were apparently children of less than five years, and pregnant mothers (Melkert, 1991). TBRF acquired during pregnancy poses a risk of loss of pregnancy of up to 50%. Among the complications of TBRF include: abortions, stillbirth and perinatal mortality (Barclay and Coulter, 1990). Jongen *et al.* (1997) reported a perinatal mortality of 436 per 1000 births to women with TBRF infection during pregnancy from Tabora region, while the risk of interruption of pregnancy has been reported to be 3% in some areas (Melkert and Stel, 1991).

An annual disease incidence of 5% has been recorded in Dodoma region, Tanzania (Talbert *et al.*, 1998), where cases of TBRF exceeded 60,000 per annum in 1994 and 1995. Barclay and Coulter (1990) estimated an annual TBRF incidence of 384 per 1000 children aged one year and 160 per 1000 in under five year olds in Mvumi village, Dodoma region. A prevalence study in nearby Makang'wa village in 1996 revealed that 3% of under fives and 7.5 % of pregnant women routinely attending MCH clinic presented blood smears with *Borrelia spp* (Talbert *et al.*, 1998). At Mvumi hospital, TBRF was reported as the sixth commonest cause of admission and

the seventh commonest cause of mortality among children (unpublished report, Mvumi hospital). The mainstay of diagnosis of relapsing fever borreliosis is the demonstration of the spirochetes in Giemsa-stained thick blood smear. However, thick smears from patients with spirochetemia are often negative due to low numbers of spirochetes in the blood stream (Lebech *et al.*, 1995).

Isolation and or visualization of the aetiological agent is often problematic because of the low numbers of spirochetes in infected tissues, their slow growth rates *in vitro*, and contamination by fast growing organisms (Barbour, 1984; Ewing *et al.*, 1994). For these reasons, culturing has to be supplemented with other diagnostic procedures of high specificity and sensitivity.

Spirochetes have been cultivated from blood and other different tissues of reservoir animals like feral rodents (Anderson *et al.*, 1985) and white-footed mice (Schwan *et al.*, 1988). Intraperitoneal inoculation of experimental mice with blood from human patients with TBRF is a more sensitive alternative approach for borrelial culture, however, this method is laborious and seldom performed routinely.

Diagnosis involving amplification of specific DNA segments by Polymerase Chain Reaction (PCR) is highly sensitive and specific, though expensive. PCR has been applied for the detection of Borrelial DNA in tissues of infected ticks, in animals and

in human specimens (Persing *et al.*, 1990; Armstrong *et al.*, 1992; Hofmeister *et al.*, 1992; McGuire *et al.*, 1992).

Control of TBRF has been carried out using several approaches, such as pesticide application (like house spraying with lambda-cyhalothrin), smooth plastering of floors and walls of rural houses, and improvement of housing and hygiene (Talbert *et al*, 1998). However, efficacy of these control strategies appears to have only a transient effect, as ticks re-appear within a relatively short time. All these control approaches, however, have targeted the O. *moubata* tick-vector but do not take into consideration the possibility of the existence of a non-human reservoir host for *B. duttonii*.

Currently, humans are the only established mammalian hosts for *B. duttonii* in East Africa (Goubau, 1984). Since different species of animals live in habitats where humans and ticks are present, there is the likelihood that, where ticks fail to access human blood, they may choose an alternative host to feed on. Rodents could be one such alternative host for the ticks, given the commensal behaviour of these small mammals. In this way, the TBRF agent would be transmitted between ticks and rodents, with ticks acting primarily as vectors while rodents act as reservoirs.

The possibility of rodents being reservoirs for *B. duttonii* is plausible since *B. duttonii* has been shown to produce a relapsing infection in experimental laboratory mice (Geigy and Mooser, 1955, Fukunaga Personal communication, 2002). *Borrelia spp* have been found in a blood smear from an elephant shrew (*Crocidura spp*) in Morogoro, Tanzania, however, it was not confirmed whether this spirochete indeed was B. *duttonii* (Machang'u and Kipanyula, Personal communication, 2001).
At present, data on human cases and prevalence of soft ticks in human houses provide the basis for risk assessment of TBRF in Tanzania. Although relatively few studies have been carried out to determine the prevalence *B. duttonii* in animals other than humans, studies suggest that birds and rodents may serve as reservoirs for Borrelia species (McLean *et al.*, 1993). A determination of a non-human reservoir host would provide valuable information on TBRF epidemiology and control.

Surveillance for Ornithodorous ticks in Tanzania has been done in the past, however, little is known on the prevalence of the Borrelia in the ticks themselves. The main objective of this study is therefore aimed to establish whether commensal rodents could be potential reservoir hosts for *B. duttonii* in Tanzania, thus broadening the existing knowledge on the epidemiology of TBRF and facilitating in the control of this disease. The findings of this study shall also contribute to an effective integrated control of the *B. duttonii* infections in affected rural and peri-urban communities.

The specific objectives of this study are:

1: To determine the presence of TBRF agents in ticks and rodents by microscopy and PCR.

2: To compare the molecular features of Borrelia isolated from ticks, rodents and humans in TBRF affected areas.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background

Relapsing fever is an acute infectious illness of humans caused by spirochetes of genus Borrelia, transmitted either by Ornithodorous ticks or lice (Warrel, 2000). There are two forms of relapsing fevers, namely, tick-borne relapsing fever (TBRF) and louse-borne relapsing fever (LBRF). LBRF is often epidemic, while TBRF fever is usually sporadic/endemic (Takahashi *et al.*, 2000).

LBRF can manifest as a severe disease characterized by a primary attack of fever, followed by up to four relapses (Cook, 1996). It may also be associated with dizziness, severe headache, and pain in the back, chest, abdomen, legs and joints. Nausea, vomiting, dysphagia, dyspnoea, and cough are also common. Hepatomegaly and jaundice are often associated with this disease, and epistaxis and petechial rashes occur in the skin and mucosa (Cook, 1996).

Humans are the reservoirs of LBRF, while body and head lice (*Pediculus humanus corporis* and *P. h. capitis*) are the vectors. The vectors ingest the infectious agent-*Borrelia recurrentis,* while feeding on blood of an infected human. Infected haemolymph may also be inoculated into the human body through the skin if the

louse is crashed onto an opening during scratching of an itching lesion caused by a louse bite.

War, famine and poverty favour the spread of LBRF (Warrell, 2000). Epidemics are known to occur particularly in times of war involving major population migrations, overcrowding, cold and poor hygiene. These conditions support heavy louse infestation. LBRF is also encountered in the tropics at high altitudes, where head lice play an important role in disease transmission (Cook, 1996). During the first half of the 20th century, there were at least 50 million cases of LBRF, with a 10% mortality in Europe, the Middle East, and Northern Africa.

Currently, the main endemic focus of LBRF is in the highlands of Ethiopia, where there is an epidemic of some 10,000 cases during the cool, rainy seasons. There have been outbreaks also in Sudan and Somalia (Petri, 1996). Between epidemics, *B. recurrentis* persists in mild or asymptomatic human infections. There is no known animal reservoir for LBRF (Warrell, 2000).

TBRF on the other hand, is a world-wide communicable disease spread by the bite or coxal fluid of soft ticks of the genus Ornithodorous, infected with spirochetal bacteria of the genus Borrelia, different from B. *recurrentis* (Walton, 1962). The disease manifests as an acute febrile illness in humans, with headache, malaise, myalgia,

arthalgia and fatigue. Another serious complication of TBRF include meningoencephalitis (Burgdorfer, 1999).

2.2 Aetiology of TBRF

At least seven species of the genus *Borrelia* are known to cause TBRF. These include: *B. duttonii*, which causes the East African TBRF, *B. crocidurae* of Senegal (Trape *et al.*, 1996), *B. turicatae*, *B. parkeri* and *B. hermsii* of North America, *B. persica* of Asia and *B. hispanica* of Spain and North Africa (Smith and Easman, 1990).

2.2.1 Morphological characteristics of the genus Borrelia

Borrelia are helical microorganisms measuring 0.2-0.5 X 3-20 μm of 3 to 10 loose loops. The cells are surrounded by a surface layer, an outer membrane and a cytoplasmic membrane. There are 7 to 30 periplasmic flagella (endoflagella), which originate at either end of the cell and wound about the protoplasmic cylinder to overlap in the middle of the cell. Periplasmic flagella have an essential role in motility (Goldstein *et al.*, 1996).

One stricking feature of the spirochetes is their capacity to efficiently swim in a viscous gel-like medium such as connective tissue where other bacteria are slowed down or immobilized. This invasive attribute may facilitate their passage through the extracellular matrix and cell junctions in infected tissues (Kimsey and Spielman, 1990).

The Borrelia are microaerophilic and chemoorganotrophic. They have a linear DNA molecule of about 1 million base pairs (bp), as well as circular plasmid-like DNAs and several small linear DNA molecules of 10-200kb, also called linear plasmids or minichromosomes.

The Borrelia are polyploid with each cell containing between 10-20 copies of the large chromosome and each of the linear plasmids. The extreme ends, or telomeres, of both the large and linear chromosomal DNA molecule, and the linear plasmids are covalently closed hairpins. The linear plasmids contain genes encoding the outer membrane lipoproteins, called the variable major protein (vmp) and outer surface protein (osp). Linear plasmids in the borreliae that cause relapsing fever also carry the genes responsible for antigenic variation (Barbour *et al.*, 1982).

The characterization of the Borrelia species is based on DNA-DNA hybridizations, rRNA gene restriction patterns, and or identification by monoclonal antibodies

(Wayne *et al.*, 1987). Other methods include polymerase chain reaction (PCR), multilocus enzyme electrophoresis (Boerlin *et al.*, 1992), 16S rRNA sequencing (Adam *et al.*, 1992), *fla* gene sequencing (Wallich *et al.*, 1992), serotyping (Wilske *et al.*, 1993), and plasmid profile analysis (Barbour, 1988).

2.2.2 Borrelial antigens

Borrelia expresses a number of outer surface proteins (Osps), which are encoded by genes located on linear and circular plasmids (Sadziene *et al.*, 1993b). The most common ones are OspA, OspB, OspC and OspD (Shoberg *et al.*, 1994, Marconi *et al.*, 1994). OspA, OspB and OspD genes are located on linear plasmid while the OspC gene is located on a circular plasmid. OspA has been tested as a vaccine candidate in mouse model systems and has been shown to confer both strain-specific passive and active protection from infection (Fikrig *et al.*, 1990). OspB has been shown to confer some degree of immunity to disease in a mouse model system, suggesting the potential importance of this lipoprotein in pathogenesis (Fikrig *et al.*, 1992). OspA and OspB may be involved in attachment of the Borrelia to human endothelial cells and in the penetration of these cells respectively (Comstock *et al.*, 1993, Sadziene *et al.*, 1993a).

Peac-Mursic and others (1992), found OspC to be a major candidate for a vaccine against Lyme borreliosis. OspC, which is found in both relapsing fever Borreliae and

Lyme disease Borreliae, is an immunodominant protein of the early humoral immune response in humans (Carter *et al.*, 1994).

2.3 Cultural requirements

Borrelia are difficult to grow in ordinary bacterial media. They require at least a temperature of 28^0C to 39^0C for optimal growth (Smith and Easman, 1990). Currently, the Bourbour-Stoenner-Kelly (BSK-II) medium is used to culture Borrelia (Cutler *et al.*, 1999). BSK-II medium contains antibiotic suppliments like rifampin (20-50µg/ml) and kanamycin (80µg/ml), to suppress contamination of Borrelia cultures with other fast growing bacteria. Growth in BSK-II medium is assumed to occur when there is a change in the colour and viscocity of the medium from clear red to yellowish turbid medium. Growth is confirmed under dark-field (DF) microscopy by observing the moving spirochetes.

2.4 Staining characteristics

Borrelia do stain well with Giemsa (Carter and Chengappa, 1991). At least 70% of patients show spirochetes in their blood during the febrile phase of the disease. Special techniques such as negative staining, DF microscopy, silver impregnation and fluorescent staining are used to visualize the spirochetes. The

immunofluorescence assays are used for the detection of specific IgG and IgM antibodies, in the diagnosis of *B. burgdorferi* infections (Carter and Chengappa, 1991).

2.5 Epidemiology of TBRF

The *Ornithodorous spp* feed on human blood during sleep at night. They usually cause a painless bite and feed for a relatively short time (less than 2 hours) before leaving the host. The affected individual is, therefore, frequently unaware of having received a bite (Jawetz, 1982). Infected ticks may, however, survive for up to two years without feeding, provided the humidity in the environment is low (Jawetz, 1982).

2.5.1 Geographical distribution

Tick-borne relapsing fever is an endemic disease commonly found in Africa, Asia and Central and South America. The disease assumes an endemic rather than epidemic status. In Central, East and South Africa, TBRF is variably present in human habitations wherever humans live collectively, including staging camps for migrant workers, in old camping sites and in certain types of houses (Cook, 1996).

2.5.2 Reservoir hosts

The vector ticks for TBRF belong to the *Ornithodorous* and *Ixodes spp*. Several species of the *Ornithodorous* ticks have been known to transmit borreliosis. These include: *O. parkeri*, *O hermsi*, *O. turicatae*, and *O. moubata* (Smith and Easman, 1990). The Ornithodorous ticks are common in arid areas and they live in cracks and crevices of walls and floors of houses. Ornithodorous ticks once infected, may maintain the infectivity status by transmitting the infectious agent to their offspring transovarially over a long period of time (Talbert *et al.*, 1998). The Borreliae are thus maintained in enzootic cycles that involve tick vectors and vertebrate reservoirs. In North Africa, the Eastern Mediterranean, Central Asia and North and South America rodents constitute the major reservoirs (Burgdorfer and Rawlings, 1999). Infection to humans is incidental (Cook, 1996).

2.5.3 Transmission of *Borrelia spp*

Infection to humans and other mammals is through contamination of the bite wound with coxal fluid or the saliva of the infected ticks (Talbert *et al.*, 1998). *Borrelia spp.* can also survive in lice and bed bugs (Cook, 1996). When the infected tick feeds on warm-blooded host, the spirochetes are transmitted from the infected salivary glands into the bite wound. The coxal organ secretes infected coxal fluid onto the skin from where spirochetes can penetrate (Weinman and Ristic, 1968). Accidental infection

via the conjunctiva is also possible, while transfusion, transplacental transmission and infection via intravenous drug administration have also been recorded (Cook, 1996).

The common hosts for Ornithodorous ticks, their geographical distribution and the respective Borrelial species they carry are shown in Table 1.

Table 1: Common hosts for *Borrelia* spp, their tick vectors and their geographical distribution

Tick	Host	Borrelia agent	Geographical area
Ornithodorous hermsi	Squirrels Chipmunks	*Borrelia hermsii*	Central and Western USA, Mexico
O. turicata	Goats Sheep Chipmunk	*B. turicatae*	Central and Western USA, Mexico
O. moubata	Human, various Domestic and Wild animals, Birds, tortoise	*B. duttonii*	East, Central and South Africa
O. parkeri	Squirrels, dogs	*B. parkeri*	Central and Western USA, Mexico
O. erraticus		*B. hispanica*	Mediterranean region, North and West Africa, Portugal and Spain
O. tholozani		*B. persica*	Meditterranean region, Tobruk, Cyprus, Israel, Iran, Kashmir and Sinkiang province-Western China
O.rudis (venezuelensis)		*B. venezuelansis*	Northern, South and Central America and Northern Argentina

Source: Smith and Easman (1990).

2.6 Pathogenesis of *Borrelia spp* in the mammalian host

The Borrelia enter the skin and subcutaneous tissues, from where they invade systemic and lymphatic circulations. They multiply in blood, after being phagocytosed by the reticuloendothelial system. There is no replication at extravascular sites, however, sequestration of platelets occurs in the bone marrow leading to thrombocytopaenia, which accounts for petecchial rashes in the skin commonly found in borrelial disease. Conjunctival vessels are congested and may bleed due to clumps of adherent Borrelia which become impacted in capillaries, where they enmesh red cells, causing capillary rupture (Cook, 1996).

Borrelia invade host tissues including the liver, spleen, heart, brain and kidneys (Cook, 1996). In the liver there is intrahepatic billiary obstruction leading to jaundice. Studies done by Sadziene *et al.*, (1996), have revealed that motility of the Borrelial organisms may be important for their pathogenesis.

Fever occurs following infection with large numbers of Borrelial parasites, and is attributed to heat stable outer envelope which stimulates mononuclear cells to produce pyrogens. A Jarisch-Hexheimer reaction may occur spontaneously, following treatment or consequent to accelerated phagocytosis of a large number of the Borrelia by neutrophils. Transient elevation in plasma concentrations of tumor necrosis factor, interleukin-6, and interleukin-8 may also occur (Petri, 1996). In

LBRF, the stimulus for cytokine release is the phagocytosis of the spirochetes, and subsequent release of a pyrogenic variable major protein (vmp). Phagocytosis can be enhanced following disease treatment with Benzyl Penicillin in which case the antibiotic attaches to penicillin-binding protein 1 as demonstrated in *B. hermsii* spirochetes and produces large surface blebs. The damaged spirochetes are then phagocytosed rapidly by neutrophils in the blood and by the spleen (Warrel, 2000). Disseminated intravascular coagulation may occur (Cook, 1996).

2.7 Pathogenesis of *Borrelia spp* in the vector tick

Ticks suck blood from Borrelia-infected individuals, and store it in their midguts. Shortly, after the onset of feeding, spirochetes migrate though the tick midgut epithelium and into the haemolymph where they multiply, and then invade tick tissues, including the salivary glands, the central ganglion, the two coxal organs, the two malpighian tubules, and the ovaries (Weinman and Ristic, 1968, Gern *et al.*, 1990). The spirochetes may, therefore, use the salivary route of transmission, although they may also be introduced into the host by regurgitation from the midgut (Burgdorfer *et al.*, 1989). However, spirochetes disseminating from the tick midgut are likely to encounter the adversity of a variety of biochemical, enzymatic and cellular barriers, which may impair the spirochetes (Burgdorfer *et al.*, 1989).

2.8 Clinical signs of TBRF

In humans, TBRF begins with sudden onset of fever. The initial episode lasts for about 4 days, during which large numbers of organisms may be demonstrated in the blood and urine. This may be followed by several episodes of relapses. Case fatality rate may reach 5% with higher susceptibility in pregnant women and young children (Talbert *et al*, 1998). The clinical symptoms and disease severity will depend on the immune status of the host, strain of the borrelia, and the phase of the attack (Carter and Chengappa, 1991).

Fever, headache, tachycardia, and muscle pain are characteristic of the disease. Other disease signs include malaise, arthalgia, fatigue, bronchopneumonia, ocular disease, jaundice, nausea and vomiting and macular rashes. These clinical signs disappear, but do recur after 7 to 14 days. TBRF can be included as an important differential diagnosis of malaria (Barclay and Coulter, 1990).

TBRF Borrelia are neurotrophic. Various neurological symptomes may result at the end of the first bout of fever, or during relapses. The most common neurological complication involves the cranial nerves, but other nerves may also be affected leading to deafness or ophthalmoplegia (Cook, 1996). Lymphocytic meningitis and occasionally subarachinoid haemorrhage may occur. Other cerebral symptomes include: hemiplegia, aphasia, encephalitis, optic atrophy, iritis and iridocyclitis.

Complications such as bronchitis, hepatic failure and arthritis may occur in association with the neural syndrome (Cook, 1996).

The Jarisch-Herxheimer reaction is not a recognized complication, but can occur (Bennet and Plum, 1996). This syndrome is characterized by a sudden rise of body temperature, restlessness and apprehensiveness, and intense chills which last for 10 to 30 minutes. The pulse, respiratory rates, and blood pressure rise sharply and may be associated with gastrointestinal symptoms, cough, limb pains, and delirium. Patients may die of hyperpyrexia. The flush phase which last for several hours, is characterized by profuse sweating, vasodilatation, a fall in blood pressure, and a slow decline in temperature (Warrel, 2000).

2.9 Immunity to TBRF

The antigenic specificity of the relapsing fever spirochetes is highly developed. This accounts not only for the fact that patients who have been infected with one type of relapsing fever borrelia strain l (e.g. European strain) can be infected with another strain (e.g. Indian or African type), but also that a patient who has recovered from invasion with a given strain may suffer from a relapse due to an antigenic variant of the same strain. The spirochetes can persist for extended periods in untreated individuals, even in the presence of an immune response. Chronic infection can occur

through a variety of mechanisms, including: limited exposure of antigenic targets, seclusion of the organisms into immune-previleged sites, local and or systemic suppresssion of immune responses that are harmful to the borrelial agent and antigenic variation (Stoenner *et al.*, 1982, Seiler and Weis, 1996).

The disappearance of the spirochetes from the blood stream occurs following the development of specific antibodies. In the course of natural disease, antibodies develop. These are mainly agglutinins, spirochetecidins and lysins, which are sufficiently powerful to overcome the blood infection and which lead to the disappearance of the spirochetes from circulation. The organisms may remain latent in the brain and other tissues, and when circulating antibodies decrease, spirochetes re-enter the blood circulation and give rise to a relapse.

Re-entry of spirochetes into circulation stimulates the production of fresh antibodies, which again lead to disappearance of the spirochetes from the blood. There is evidence that after the first attack, the spirochetes in the tissue undergo antigenic changes which render them unsusceptible to antibodies produced by the host against the original antigenic constitution (Warrel, 2000). This enables the new variants of the spirochetes to invade the blood a second time and give rise to a relapse. Up to nine distinguishable antigenic phases have been reported in a single tick- borne

borrelial strain (Smith and Easman, 1990). Tick-borne strains appear to have a greater potential for variation than louse-borne strains (Warrel, 2000).

The antigenic variation is a function of surface proteins that differ extensively in their primary structure. Plasterk *et al.* (1985), showed that in *B. hermsii* the expression of two variable major protein (VMP) types (serotypes 7 and 21) is associated with the rearrangement of the DNA in linear plasmids. It is suggested that both serotypes possess identical storage plasmids, which contain genes for both VMP 7 and 21, but differ in their expression plasmid. The expression plasmid contains a copy of the particular VMP gene adjacent to the expression site. The genetic switch may occur by the loss of one VMP gene and fusion of the other on the expression plasmid. In this way the Borrelia appear to posses, on the extrachromosomal DNA, a battery of silent and expressed surface protein genes which can be rearranged at high frequency without affecting the stability of the chromosome. It seems likely that the relapses are due to antibodies, which lead to a selection of organisms with modified DNA arrangement in linear plasmids that control VMP synthesis (Warrel, 2000).

2.10 Diagnosis of TBRF

Relapsing fever diagnosis rests primarily on epidemiological information, clinical findings, and laboratory findings such as microscopy, animal inoculation, and serology (Anderson *et al.*, 1998).

2.10.1 Microscopy

For microscopy, blood should be taken during the febrile stage and examined directly by DF illumination or after staining with Wright's or Giemsa stains. For staining, a thick film preparation is made from two drops (0.5ml) of blood on a slide. The spirochetes are seen in large numbers in blood taken during the febrile period, otherwise they are scarce, and are often coiled or clumped together.

2.10.2 Animal inoculation

The Borrelia can also be demonstrated via inoculation of blood from infected individuals into experimental animals, such as new born rats or white mice (Konishi *et al.*, 1993). The spirochetes will appear within 48 hours and may remain high for up to six days.

2.10.3 Growth in cell cultures

The Borrelia can be grown in cell cultures containing Sfl Ep cells (Konishi *et al.*, 1993), but can also be grown in the allantoic fluid of chicken embryo (Cook, 1996).

2.10.4 Serology

Serology is not a reliable method due to antigenic variation expressed by the Borrelia. Patient serum samples can be tested for species-specific immunoglobulin G (IgG) and IgM antibodies by indirect immunofluoresence, or ELISA using flagella antigens (Moter *et al.*, 1994).

2.10.5 Growth of *Borrelia spp* in culture media

Growth in culture media is difficult, however, if attempted, temperature of growth of 28^0C to 30^0C must be maintained. Blood taken during the febrile stage is inoculated into a BSK-II medium, and cultures are examined under DF microscopy periodically (usually bi-weekly, for 8 weeks) for the growth of spirochetes.

2.10.6 PCR amplification

Borreliosis can also be diagnosed by using PCR based methods. Blood, urine, cerebro-spinal fluid, synovial fluid or tissues from infected individuals serve as test samples (Lebech and Hansen, 1992). DNA from these samples are subjected to PCR amplification by using primers specific to *Borrelia spp.* The commonly employed primers are specific to the flagellin gene, the outer surface protein (Osp) and variable membrane protein genes (Takahashi *et al.*, 2000; Fukunaga and Koreki, 1995). The 16S rRNA sequence is also target in PCR amplification for Borrelia identification and classification (Fukunaga and Koreki, 1995).

2.10.7 Differential diagnosis

In a febrile patient with jaundice, petechial rash, bleeding, and hepatomegaly, the following disease conditions should be considered along with TBRF: LBRF, falciparum malaria, yellow fever, viral hepatitis, ricketssial infections and leptospirosis (Warrel, 2000). The major clinical differences between TBRF and LBRF are shown in Table 2.

Table 2: Major differences between tick-borne and louse-borne relapsing fevers

Sign/symptom	Tick-borne	Louse-borne
Parasite in peripheral blood	Scanty	Numerous
Paroxysms (days)	Relatively short, not more than 5-7. Often chronic, irregular fever	Relatively long-up to 10
Relapses	Two or more	Two or less, often none
Vomiting	Only with meningitis	Any stage
Other symptoms	Lethargy, loss of weight, debility	Diarrhoea, jaundice, coma, severe haemorrhage
Neurological complications	Common. Cranial nerve palsies	Infrequent
Ocular complications	Papilloedema with meningism	Infrequent
Illness	Less severe	More severe
Mortality (%)	Less than 10	May be high-up to 50

Source: Cook (1996).

2.11 Treatment and control

Relapsing fever can be treated with tetracyclines, penicillins and third generation cephalosporins (Anderson *et al.*, 1998). A single 500-mg dose of tetracycline may be as effective in clearing spirochetaemia of LBRF, although many physicians still treat with 500mg tetracycline every 6 hours for 5 to 10 days (Petri, 1996). Procaine penicillin (300mg) treatment has been reported to clear the spirochetaemia although it is slower than tetracycline (Petri, 1996). Barclay and Coulter (1990), found penicillin treatment to be satisfactory, with few apparent Jarisch-Herxheimer reactions and a low relapse rate in patients from Central Tanzania. Erythromycin is also effective and should be used in children rather than tetracyclines, which cause staining of the teeth (Petri, 1996).

Control of TBRF is achieved through the control of the vector ticks and reservoirs (rodents, squirrels, chipmunks and other wild animals and birds), and by treatment of infected individuals (Smith and Easman, 1990). Wearing of protective clothing and spraying of the tick habitats and living quarters with acaricides, such as lambda cyhalothrin, 3% Benzene hexachloride, 1% aldrin or 0.5% Diazinon® or Malathion®, will help to control the ticks. Improving housing by cementing and plastering may also reduce the incidence of TBRF significantly (Talbert *et al*, 1998).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This is a cross-sectional study in which tick samples and rodent blood and tissues were analyzed for the presence of *B. duttonii*. The findings were correlated with borrelia from humans within the study area.

3.2 Study area

The study was conducted in Central Tanzania (Dodoma region). This area has a dry climate and sandy soils that favour the survival of the vector tick, *O. moubata*. It is also one of the areas in Tanzania where TBRF is endemic (Talbert *et al*, 1998).

3.3 Sampling methods and sample size determination

Samples were taken from rodents, Ornithodorous ticks and humans. Both purposive sampling (patients complaining of fever), and convenience sampling (those willing to participate in the study) techniques were employed.

After getting ethical clearance from the Mvumi Hospital administration, blood from human patients with TBRF, who were attending hospital in the study area was

collected with the assistance of Mvumi Hospital technicians. The blood samples were inoculated in BSK-II medium, supplied kindly by Dr. Sally Cutler and Professor Fukunaga Masahito of the Veterinary Laboratory Agency, Woodham Lane, New Haw, Addlestone, Surrey, England, and the Laboratory of Molecular Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Japan respectively. Spirochete growth was detected by DF microscopy. Subculturing was done following growth of the primary culture. The Ornithodorous ticks were collected from houses and examined for the presence of spirochetes. For this purpose, the ticks were either examined in the hemolymph or crushed and slides prepared for microscopy. The tick tissue samples were also cultured in BSK-II medium. PCR was employed to detect Borrelial DNA in the ticks.

Blood smears of rodents found in human houses were Giemsa stained and examined by ordinary microscopy. Blood samples from these rodents were also cultured in BSK-II medium.

The optimal sample size (for rodents only) was determined by the formula adopted from Pfeiffer (1999);

$$n = Z^2 P (1-P) / d^2$$

where n = required sample size

P = expected prevalence (P = 50%)

d = desired absolute precision (d =95%)

Z = multipliers from the normal distribution (95% C.I.)

C.I = Confidence interval

3.3.1 Rodent specimen sampling

The rodents were trapped using peanut butter baited live traps (Sherman® traps; H.B. Sherman Traps, Inc. Tallahassee, USA), in and around human residences. A total of 250 commensal rodents were captured in two field trips conducted between November 2001 and February 2002 in five villages of Mvumi division in Dodoma region. Of these, 216 were *Rattus* spp, 32 were *Mastomys* spp, and one each of *Arvicanthis* sp and *Graphiurus* sp. Traps were laid in the late afternoon and examined the following morning, usually not later than 9.00 hours. A total of 5 traps were placed per each sampled household. Replacement of the traps was done if rodents were captured on the first day, so that the number of traps remained constant over the trapping session. Traps were left for two days before being transferred to another household. The area and the household where the rodents were captured, and the species of the captured rodent were recorded.

Blood samples were collected after anaesthetizing the rodents in a container using a cotton wool soaked with chloroform. The container was tightly capped, and after

being anaesthetized in the container for 2 min, the rodents were removed from the container and pinned on a dissecting rubber sheet. Then some blood was drawn from the heart by cardiac puncture using a 26 gauge needle and a 5ml syringe. The site of the puncture was cleansed with 70% ethanol before drawing the blood. The blood was dispensed directly into tubes containing 10ml of BSK-II culture medium, while observing sterility as much as possible. The cultures were examined by DF microscope twice a week for a period of eight weeks for growth of spirochetes.

Two drops (0.4ml) of blood from each rodent were, however, placed on a clean microscope slide, for DF examination for spirochetes. Duplicates of thick and thin blood smears were prepared from the blood of each rodent, air-dried, fixed in methanol for 3 min, stained with Giemsa for 30 min, and then examined under ordinary light microscope at X 1000 magnification. After drawing the blood, each rodent was dissected, and heart, liver or kidney were removed and placed in a tube containing 70% ethanol. These specimens were then stored at -20 $^{\circ}$ C, until used in PCR.

3.3.2 Tick and haemolymph sampling

A total of 251 live adult and nymph live ticks were collected in Mvumi township, in Dodoma district, where there is high prevalence of TBRF and high numbers of

Ornithodorous ticks. The ticks were collected from the floors and wall crevices of the houses and preserved live in collection tubes. The caps of the tubes were perforated to allow for air circulation. The ticks were left to engorge on rodents (*Cricetomys spp* and *Mastomys spp*) on the first month of collection. Out of 251 ticks collected, 188 ticks died while 63 ticks persisted for more than 10 months post collection, surviving on the blood meal that was given on the first month of collection.

Tick haemolymph samples were collected from each tick by clipping a front leg and letting a drop of the haemolyph onto a clean glass slide. The haemolymph was directly examined by DF microscopy for the presence of spirochetes. The slides were then air dried, fixed in methanol for five minutes, and after staining with Giemsa for 30 min, they were again examined for spirochetes under ordinary microscopy at X 1000 magnification.

3.3.3 Isolation of *Borrelia* spp from ticks

For the isolation of the Borrelia, ticks were externally disinfected by immersion in 70% ethanol for 5 min to reduce surface bacterial contamination, and then placed on a clean dissection rubber board pre-disinfected with 70% ethanol. The midgut, salivary gland, malpighian tube and coxal glands were removed and minced in PBS to prepare a homogenate. This homogenate was cultured in BSK-II medium. The

cultures were then incubated at RT and checked bi-weekly by DF microscopy for spirochete growth. The fresh homogenate was also directly examined for spirochetes under DF microscopy at X 1000 magnification.

3.3.4 Human blood sampling

Human blood samples were collected by laboratory technicians at Mvumi Mission Hospital from patients complaining of malaria-like symptoms and whose Giemsa stained blood smears were confirmed to be positive for Borrelia under light microscope. No history, however, was taken prior to blood collection, on whether the individuals had taken some medication prior to attending to hospital. Four drops of venous blood from patients with spirochetes in their blood (as evidenced by prior Giemsa staining of thick blood smears) were inoculated aseptically into tubes containing 10ml BSK- II medium, and mixed thoroughly. The cultures were left at RT and examined bi-weeky for 8 weeks for growth of spirochetes. Subculturing was further done for the positive cultures.

3.3.5 DNA extraction from rodent tissues

DNA extraction from rodent kidney, heart, and liver samples was performed as described by Sambrook *et al.*, (1989). Briefly, from each tissue, a 0.25gm sample

was taken and placed in a stomacher bag containing 100µl of extraction buffer, Tris-EDTA-SDS (TES pH 8.0 containing 0.12M Tris-hydroxymethyl aminomethane-TRIS, 0.1M ethylenediamine-tetraacetic acid-EDTA, and 1% sodium dodecyl sulfate-SDS; Appendix 1), and homogenized. The homogenate was transferred into 1.7 ml microcentrifuge tubes. Additional 400µl of TES buffer was added. Protenase K (25µl of 20mg/ml) was then added to the sample mixture, followed by incubation in a water bath for 3h at 60°C. A solution of 5M sodium acetate (0.4ml) was added and the mixture was then placed on ice for 40 min. This was followed by centrifugation at 12 000 rpm for 10 min. The supernatant was washed three times with equal vols of phenol-chloroform-isoamyl alcohol (24:23:1) and then once with an equal vol of chloroform-isoamyl alcohol (23:1). DNA was precipitated by addition of equal vol of isopropanol, followed by gentle shaking and centrifugation at 12 000 rpm for 15 min. The DNA pellet was cleaned by addition of 200µl of 70% ethanol (v/v). The ethanol was then decanted and the DNA left to dry at RT. The DNA was then re-dissolved in 50µl of Tris-EDTA (TE) buffer (10mM TRIS, 1mM EDTA, pH 8.0; Appendix 2) and preserved at -20°C until used in PCR. The same procedure was used to extract DNA from blood only that 0.25 ml of blood was used and the blood was not homogenized in the stomacher bag.

3.3.6 DNA extraction from ticks

Extraction of DNA from ticks for Borrelia detection was done as described by Fukunaga *et al.* (2001). Briefly, ticks were washed in 500µl of saline-EDTA (0.15 NaCl, 0.1M EDTA, pH 8.0), air dried and frozen in liquid nitrogen. The ticks were then thawed and crushed in a mortar and then suspended in 100µl of 0.02N NaOH, boiled for 2 min and then centrifuged at 14 000 rpm for 10 min. The aqueous phase was separated and stored at -20°C until used in PCR.

3.3.7 Extraction of DNA from borrelial culture

Extraction of DNA from the BSK-II culture was done by the boiling method as described by Corney *et al.* (1993), with some modifications. Briefly, DNA was obtained by taking 600µl of fresh borrelial culture in a 1.7 ml tube followed by centrifugation at 10 000 rpm for 15 min. The supernatant was discarded and the pellet re-suspended in 100µl TE buffer. This was followed by incubation in a water bath at 96°C for 10 min. The lysate was then stored at -20°C until used in PCR.

3.3.8 Quantitative and qualitative assessment of Borrelia DNA

Extracted DNA samples were run parallel with the lambda DNA in Tris-Boric acid-EDTA (TBE) buffered 0.8% agarose gel for 90 min at 70V, stained with ethidium bromide (0.5µg/ml) and visualized under uv light transilluminator UVP, (Ultra-

Violet Products-Scan Gabriel, CA, USA). The DNA sample was compared with standard DNA preparations to assess its purity and quantity (Budowle *et al.*, 2002).

3.4 Preparation of agarose gel

Agarose was prepared by mixing 0.8g and 2g of agarose powder (0.8% for DNA quantification and 2% for Borrelial DNA detection, respectively) with 1X TBE buffer (Appendix 3) to a total vol of 100ml. The agarose powder was dissolved by heating the mixture on a hot plate while stirring until a clear solution was formed. The solution was allowed to cool to 55°C, and then 0.5μg/ml of ethidium bromide (Sigma Chemicals CO St Luis, USA) was added, before pouring the molten agarose into the electrophoresis gel casting equipment fitted with combs. The gel was left for 30 min to set, and thereafter, the combs were carefully removed.

3.5 The PCR protocol

The PCR was performed as described by van Dam *et al.* (1999), in a 25μl total reaction vol containing 12.5μl PCR Master Mix, X2, Promega, (containing 50 units/ml of Taq DNA polymerase, pH 8.0, 400μM (each) dNTPs, 3mM $MgCl_2$), 2.5μl primer BBRNA8 (upstream primer), 2.5μl primer BBRNA14 (downstream primer), 5μl of DNA template and 2.5μl of nuclease-free water (Appendix 4). The primers were used at a concentration of 100pmole/μl each. To avoid evaporation, the

reaction mixture was overlaid with 25μl of mineral oil. After one cycle of 92°C for 3 min, 46°C for 1 min, and 72°C for 1 min 30s, the reaction mixture was subjected to 40 thermal cycles consisting of a denaturation step (94°C for 1 min), annealing step (46°C for 1 min), and extension step (72°C for 1 min 30s). In addition, the reaction was left at a holding temperature of 72°C for 10 min (Appendix 5). The sequences of the primer used are shown in Table 3 below.

Table 3: Oligonucleotide primers used in PCR, their sequence and position in rrs gene

Primers	Sequence	Position of the nucleotides in rrs gene
BBRNA8 (19bp)	5'- ACG CTG GCA GTG AGT CTT A-3'	33 to 51
BBRNA14(19bp)	5'- ATA TCA ACA GAT TCC ACC C-3'	702 to 684

3.6 Agarose gel electrophoresis of PCR products

For the determination of DNA quantity, quality, and analysis of PCR products, horizontal (flat bed) agarose gel elecrophoresis was performed. For the former, 5μl of the DNA sample mixed with 2μl of loading dye was introduced into the 0.8% agarose gel wells. The first well of the left lane was loaded with a DNA molecular

weight marker (size 100bp). The DNA marker was run parallel with the sample in a 1X TBE buffer at a constant voltage of 70V for 90 min. For the analysis of PCR products (i.e. detection of borrelial DNA), the same procedure was followed, except that, it was performed in a 2% agarose gel, and electrophoresis was done at 100V for 45 min. The gel was viewed under UV transillumination, and photographed with a polaroid camera system; VizDS –34 camera system (Sigma-Aldrich Techware-Dorset, UK).

3.7.Data scoring, interpretation and analysis

A *B. duttonii* culture sample was used to provide for DNA positive control in the PCR. A comparison was made between the fragment generated in the PCR of the positive control sample and the test samples. The data were analysed based on having or not having the expected DNA fragment (which was approximately 668bp DNA fragment).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Trapping of rodents

The number and distribution of the captured rodents was as shown in Table 4.

Table 4: Distribution by location of rodents captured in Mvumi locality

Rodent species	Village					
	Ikombolinga	Iringa-Mvumi	Makang'wa	Mvumi	Mvumi-Makulu	Total
Arvicanthis spp	0	0	0	1	0	1
Graphiurus spp	1	0	0	0	0	1
Mastomys spp	3	2	1	17	9	32
Rattus spp	11	15	24	123	43	216
Total	**15**	**17**	**25**	**141**	**52**	**250**

4.1.2 Screening for spirochetes in rodent blood smears

No borrelia was detected in any of the 250 blood smears examined, although other rodent blood parasites were detected (Appendix 6). These included trypanosomes (19.2%), which were identified as *Trypanosoma lewisi,* and bacterial organisms (7.2%).

4.1.3 Rodent blood cultures in BSK II medium

Following culture of blood in BSK II medium, no borrelial growth was observed from the 50 rodent blood samples after eight weeks of incubation (observation for spirochete growth was stopped after incubation period of eight weeks).

4.1.4 Culture in BSK II medium of homogenized tick tissues and haemolymph

There was no borrelial growth in any of the tick cultures after eight weeks of incubation. However, there were bacterial growth which presumably originated from the contaminants of the crushed tick salivary glands and the midguts contents. Cultures from the tick haemolymph were free from bacterial contamination.

4.1.5 Culture of human blood in BSK II medium

Two out of 7 human blood samples cultured in BSK II medium were positive for borrelial growth after examination by DF microscopy. The borrelial organisms were visible starting from day 10 post inoculation. However, in subsequent subculturing no borrelial growth was further achieved.

4.1.6 Determination of DNA quality and quantity

The comparison of the intensity and conformation of the electrophoretic bands of the extracted DNA against those of the molecular weight standards was as shown in Fig. 1. A sharp clear band of the DNA indicated a good quality DNA, while a smear indicated a poor quality DNA.

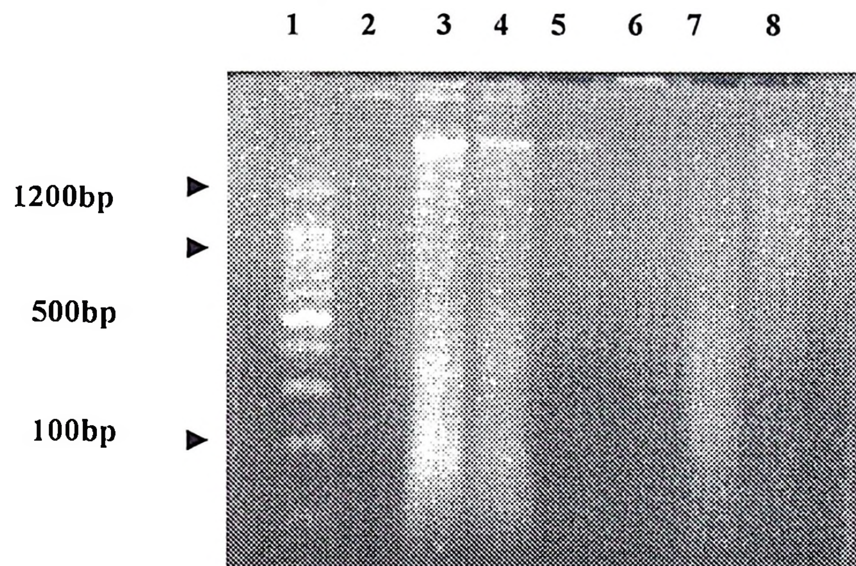

Figure 1: Agarose gel electrophoresis showing the intensity and conformation of extracted Borrelial DNA bands from TBRF affected human, tick and rodent samples, against DNA markers. Lane 1 contain the molecular weight DNA markers; lane 2 and 7 contain DNA samples extracted from blood of TBRF human patients; lane 3, 4, 5 contain rodent DNA samples; and lane 6 and 8 contain tick DNA samples

4.1.7 Rodent samples subjected to PCR

The rodent DNA samples that were extracted and subjected to PCR amplification are shown in Table 5.

Table 5: Rodent DNA samples subjected to PCR

Sampled rodent species	DNA source				
	Blood	Heart	Kidney	Liver	Total
Mastomys spp	1	2	1	0	4
Rattus spp	13	4	16	1	34
Total	**14**	**6**	**17**	**1**	**38**

4.1.8 Analysis of PCR products

The analysis of the PCR products was as shown in Fig. 2

Two out of the seven human samples (28.6%) produced a band of about 668bp. A band of the similar size was given by one out of the 38 rodent samples (2.6%) and by one out of the 40 tick (2.5%) DNA samples.

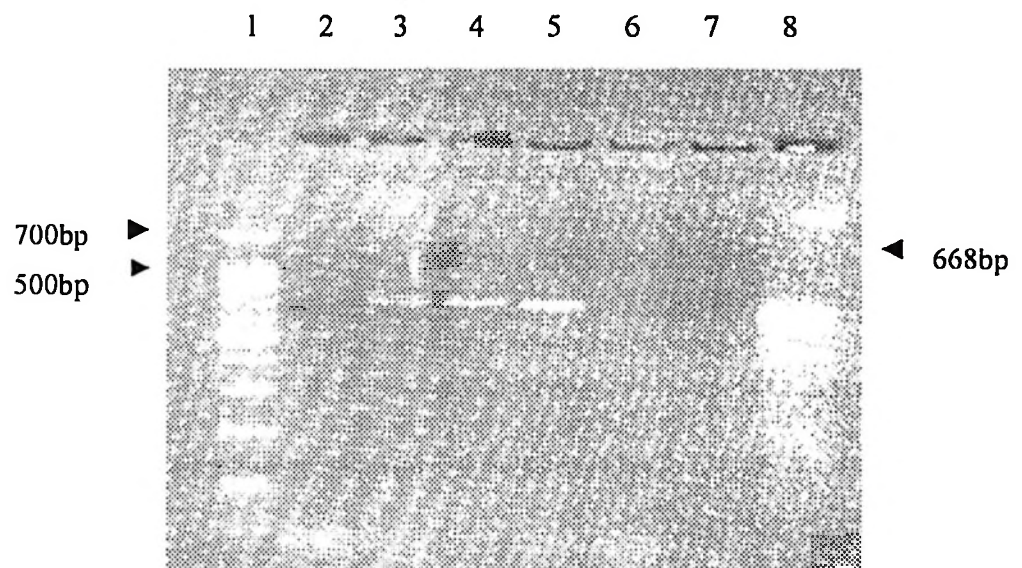

Figure 2: Agarose gel electrophoresis showing the PCR products using the 16S rRNA gene-based primers, BBRNA8 and BBRNA14. DNA molecular weight markers (lane 1); human negative samples (lane 2 and 6); human positive sample (lane 3); rodent blood sample (lane 4); tick sample (lane 5); negative control sample (lane 7); and positive *Borrelia duttonii* sample (lane 8)

4.2 Discussion

In this study a comparative study of Borrelial agents of TBRF was carried out in Mvumi, Dodoma district, which is a TBRF endemic area in Tanzania. Procedures used included dark field microscopy examination of tick haemolymph, Giemsa staining of rodent blood and tick haemolymph smears, culture of Borrelia from human blood in BSK II medium, and PCR for the detection of Borrelia from humans, rodent and tick samples. The major purpose of this study was, however, to investigate the possibility of rodents acting as reservoirs of the TBRF agent.

Direct microscopy and cultural studies did not reveal the presence of the Borrelial agent in rodent blood, however, PCR detected Borrelia DNA in the blood of one *Rattus rattus* suggesting that commensal rodents could be reservoirs for *B. duttonii* in the disease endemic areas. The PCR further detected Borrelia DNA in human samples from a clinically infected patient, and in tick samples.

The use of culture and PCR for Borrelia detection has been applied elsewhere (Holfmeister *et al.*, 1992) and have been recommended for longitudinal field studies to determine the status of infection of individual animals. These methods are also useful in prospective analyses of reservoir animals in the field.

Detection of relapsing fever Borrelia by PCR can be achieved by using primers targeting 16S rRNA gene since this gene is highly conserved among relapsing fever Borrelia (Banerjee *et al.*, 1987). Primers targeting the 16S rRNA gene were used because they produced reliable results, and moreover, if sequencing of the PCR product is done, different relapsing fever Borrelia can be distinguished (Banerjee *et al.*, 1987). Furthermore, 16S rRNA PCR products can further be characterized by restriction enzyme analysis.

4.2.1 Trapping of rodents

In the two rodent trapping sessions conducted between November, 2001 and February 2002, *R. rattus* (black rat) formed the biggest fraction of the capture (86.4%). Such a high capture of *R. rattus* was expected because this commensal rodent is almost entirely associated with human habitation and is replacing *Mastomys spp.* in human dwellings (FAO, 1994). *Mastomys spp.* also live in human residences or nearby-fields and feed on field and stored crops and, therefore, its prevalence in Mvumi villages was not unexpected. *Arvicanthis spp* and *Graphiurus spp* are essentially field rodents and only few captures would be expected from within human habitations (FAO, 1994). Among these rodent species captured, the *Rattus rattus* produced Borrelial DNA in the PCR procedure.

4.2.2 Tick collection

Household tick infestation rate in the studied area is known to be high (Talbert *et al.*, 1998). However, relatively few ticks were collected (n = 250). This is because certain families were reluctant to allow collection of ticks from their households, possibly because they felt tick infestation to be a "stigma". This attitude may have been attributed to previous TBRF awareness campaigns which were held in the area before this study. Also, during the second field trip conducted in February the rains had already started, therefore, people were able to plaster their houses with clay, and hence fewer ticks per household. It was noted that some of the collected ticks remained alive for more than 10 months in the laboratory without feeding, supporting the hypothesis that ticks can survive for a long time without a blood meal (Barclay and Coulter, 1990).

4.2.3 Human blood sample collection and inoculation

Out of seven blood samples from TBRF patients diagnosed to be TBRF infected (Giemsa staining of their blood smears), two gave positive BSK II cultures as established by DF microscopy. This relatively low growth success (28.6%) appears to support reports that Borrelia are difficult to grow even in a well balanced BSK II medium (Takahashi *et al.*, 2000.). However, since the media were supplied already constituted, the components of the media could have deteriorated somewhat with

time. This argument is supported by Pollack *et al.* (1993), who found that some batches of prepared media often fail to support the growth of the Borrelia. In addition, if Borrelia are present in only a few numbers, as could have been the case in this study, it is likely that the chances of growth would be reduced (Barbour, 1984). Moreover, some of the patients might have taken some antibiotics prior to blood collection which could have influenced negatively borrelial recovery in the blood samples in some of the cultures.

It was also observed that some of the cultures became highly contaminated and could not be further propagated. The bacterial contaminants may, therefore, have overwhelmed the slow growing Borrelia, hence causing their early death (Ewing *et al.*, 1994).

4.2.4 Detection of Borrelia by staining and by dark field microscopy

Following staining of the rodent blood smears no Borrelia was detected in any of the 250 rodent blood samples. This could be due to low numbers of borrelia in the samples, which however gave positive results in the more sensitive PCR analyses (Barbour, 1984; Lebech *et al.*, 1995). Giemsa staining is a useful method for examination of blood smears by conventional light microscopy, however, densities of 10^4 to 10^5 organisms per ml of blood need to be present for effective detection by this

method. The same number of organisms is required for detection by DF microscopy (Banerjee *et al.*, 1987). This same reason may account for the negative detection of Borrelia from tick smears and haemolymph. Piesman *et al.* (1986), observed a high frequency of false negatives in DF examination of spirochetes which may have contributed also to the negative result of Borrelia following DF microscopy of the of the tick haemolymphs.

4.2.5 Amplification of borrelial DNA

PCR has been shown to provide high sensitivity and specificity in detection of Borrelia DNA (Malloy *et al.*, 1990). A great advantage of PCR is that it can be performed directly with tissue samples, without requiring initial isolation and cultivation of bacteria in a growth medium. Detection of Borrelial DNA from blood cultures of human patients by PCR observed in this study compare positively to results published by Cutler *et al.* (1999).

In this study Borrelia DNA was detected in one out of 40 ticks tested by PCR method. Detection of Borrelia in Ornithodorous ticks in Tanzania agrees with the findings reported by Fukunaga *et al.*, (2001), who detected *B. duttonii* from ticks originating from Mvumi area. However, in contrast to their findings in which 11out of 13 ticks tested were positive for spirochetes, this study has shown fewer ticks

(2.5%) being infected with spirochetes. The explanation for this could be that the previously studied ticks originated from a population within one infected household, whereas in this study, the tested ticks originated from different villages and households. Another reason could be due to differences in the PCR protocols used. Nested-PCR, which is more sensitive (Hofmeister *et al.*, 1992), was employed by Fukunaga and others in the previous study.

Previous reports, have suggested that there are no non-human mammalian hosts for the East African TBRF (Goubau, 1984), yet others consider rodents as potential reservoirs (Burgdorfer and Rawlings, 1999). The findings of this study have shown a presence of Borrelia DNA in one *R. rattus* blood sample which strongly suggest the possibility of this rodent species playing a role in the maintenance of Borrelial agent of TBRF. To my knowledge this is the first report on the detection of Borrelial DNA in wild rodents from Tanzania. This finding also supports a previous report in which unspecified Borrelial organisms were found in the blood of a *Crocidura spp* captured in Morogoro Tanzania (Machang'u and Kipanyula, unpublished).

Borrelia duttonii have been grown experimentaly in mice (Cutler *et al.*, 1999). The detection of Borrelia DNA in a naturally infected rodent in Tanzania further supports the possibility of rodents acting as natural reservoir hosts for *B. duttonii*. Gerbils infected with *B. duttonii* from ticks collected from Mvumi, Dodoma, Tanzania, have

been shown to produce a blood population relapse similar to that observed in TBRF infected human patients (Fukunaga, Personal communication). This observation further supports the hypothesis that commensal rodents may act as reservoirs for *B. duttonii* in nature.

In this study Borrelia DNA was also detected in ticks using 16S rRNA gene-based primers. The 16S rRNA gene-based PCR has been used to identify and characterize *B. duttonii* isolates from Tanzania and elsewhere by other researchers (Ras *et al.*, 1996; Cutler *et al.*, 1999, van Dam *et al.*, 1999). The oligonucleotide primers used generated PCR products of 668bp, which is the expected band size for relapsing fever Borrelia (van Dam *et al*, 1999) with DNA samples obtained from humans, ticks and rodents. The results of this study, therefore, signify a broad applicability of these primer sets in the detection of Borrelia species in ticks, and other hosts, and therefore, are of great importance in the epidemiological studies involving TBRF in East Africa and elsewhere.

The Borrelial DNA detected in the rodent by PCR had the same size (of approximately 668bp) as those detected in humans and ticks in this study. It is likely, therefore, they are of the same strain, or possibly of a closely related species (Fukunaga *et al.*, 2001). Although the DNA sequences of the PCR products were not determined in this study, the amplification of Borrelial DNA from rodents originating

from TBRF endemic region in Tanzania suggests that the detected Borrelia species is *B. duttonii*. Further studies are needed to confirm the magnitude of rodent involvement in the epidemiology of TBRF in Tanzania, and also to study the prevalence of causal agent across the country.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

The present study has shown that rodents in Mvumi, which is a TBRF endemic area of Tanzania might harbour Borrelial agents of TBRF. The PCR amplified Borrelial DNA in the *R. rattus* captured in Mvumi, could be that of *B. duttonii* which is the causal agent of TBRF in the studied region. In this regard control of rodents could also contribute to the prevention of TBRF in disease endemic areas.

The infection of the Ornithodorous ticks with the Borrelia poses a risk to the residents in the studied area since a high number of the households are infested with these ticks. Efforts to combat TBRF should, therefore, continue to focus mainly on tick control.

As a follow-up of this investigation, it is important to conduct more studies on :

i) Isolation and characterisation of Borrelia from rodents and ticks in different parts of Tanzania where TBRF is known to be prevalent

ii) The extent to which rodents and other commensal animals may harbour and transmit *B. duttonii*

iii) Possible integrated pest management measures to control the East African TBRF in disease endemic areas.

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APPENDICES

Appendix 1: Preparation of Extraction buffer (0.2M Tris, 0.1M EDTA, 1% SDS)

Reagent	Quantity
Tris	24.22g
EDTA	37.22g
SDS	10.00g
Add distilled water to	1000ml

Appendix 2: Preparation of TE buffer (10mM Tris, 1mM EDTA, pH 8.0).

Reagent	Quantity
Tris	1.2g
EDTA	0.37g
Add distilled water to	1000ml

Appendix 3: Preparation of 10x TBE buffer pH 8.4

Reagent	Quantity
Tris base	108g
Boric acid	55g
EDTA	7.44g
Add distilled water to	1000ml

Appendix 4: Reaction Mixture for PCR.

Reagent	Quantity
PCR Mastermix	12.5µl
Primer BBRNA8	2.5µl
Primer BBRNA14	2.5µl
DNA template	5.0µl
Add nuclease free water to	25µl

Appendix 5: PCR Protocol

3 min 94°C; 1min 46°C; 1min 30 sec 72°C

1min 94°C; 1min 46°C; 1min 30 sec 72°C } 40 cycles

10min 72°C Hold

Appendix 6: Parasites detected during the screening of the rodent blood samples from Mvumi, Dodoma, Tanzania

Village	Rodent species	Trypanosomes	Bacilli bacteria	Cocci bacteria
Mvumi	Rattus	30	5	2
	Mastomys	2	1	1
Mvumi-Makulu	Rattus	10	2	2
	Mastomys	0	0	0
Iringa-Mvumi	Rattus	0	0	0
	Mastomys	0	1	0
Makang'wa	Rattus	5	2	2
	Mastomys	0	0	0
Ikombolinga	Rattus	1	0	0
	Mastomys	0	0	0
Total		48	11	7