DIFFERENTIAL DIAGNOSIS OF TICK-BORNE DISEASES AND POPULATION GENETIC ANALYSIS OF *BABESIA BOVIS* AND *BABESIA BIGEMINA*

MARTIN CHITOLONGO SIMUUNZA, BVM, MSc

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Division of Veterinary Infection and Immunity

Faculty of Veterinary Medicine

University of Glasgow

Author's declaration

The work presented in this thesis is entirely my own and carried out with the help of those people mentioned in the acknowledgements. It has not been previously submitted to any university for the award of a degree.

Martin C. Simuunza

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Abstract

Tick-borne diseases are a constraint to livestock production in many developing countries. They are responsible for high morbidity and mortality resulting in decreased production of meat, milk and other livestock by-products. The most important tick-borne diseases of livestock in sub-Saharan Africa are East coast fever (caused by *Theileria parva*), babesiosis (caused by *Babesia bigemina* and *Ba. bovis*), anaplasmosis (caused by *Anaplasma marginale*) and heartwater (caused by *Ehrlichia ruminantium*). Despite their economic importance, information on the epidemiology of these diseases in many countries is often lacking or inadequate, resulting in inappropriate disease control strategies being implemented.

The availability of specific, sensitive and cost-effective diagnostic methods is important in the design and implementation of effective disease control strategies. In this study PCR assays based on the 18S and 16S rRNA gene sequences, that could identify *Theileria* / *Babesia* and *Anaplasma* / *Ehrlichia* pathogens of cattle respectively, were developed. In addition, PCR assays based on the β-tubulin gene that could detect *T. parva*, *Ba. bigemina*, *Ba. bovis* and *T. taurotragi*, and PCR assays based on the cytochrome b gene that could diagnose infection by *Ba. bigemina* and *Ba. bovis* were also developed. When the 18S and 16S rRNA gene PCR assays were combined into a multiplex PCR assay, *Ba. bigemina* and *E. ruminantium* DNA did not amplify and some non-specific bands were observed following agarose gel electrophoresis. The β-tubulin gene multiplex PCR assay for the diagnosis of *T. parva*, *Ba. bovis* and *Ba. bigemina* worked relatively well when used on laboratory-derived parasite DNA preparations. However, when it was used on field samples collected on FTA cards, multiple non-specific bands were observed after agarose gel electrophoresis of the PCR products.

The 18S and 16S rRNA gene PCR assays were used for an epidemiological study of tick-borne diseases of cattle in Central and Eastern Zambia in the wet and dry seasons. All the disease pathogens under study (*T. parva, T. mutans, T. taurotragi, Ba. bovis, Ba. bigemina, Anaplasma spp* and *E. ruminantium*) were prevalent in all the regions of the country in both seasons. However, variation was observed in the prevalence of these pathogens between the regions and the seasons. A number of risk factors, associated with the occurrence of tick-borne pathogens in cattle and the tick burdens observed on cattle in the wet season were determined. A negative association was observed between the number of co-infecting pathogens and the erythrocyte packed cell volume (PCV) of carrier cattle.

Using recently available genome sequences, mini- and microsatellite markers were developed for population genetic analysis of *Ba. bovis* and *Ba. bigemina* parasite populations. *Ba. bovis* isolates from Zambia and Turkey and *Ba. bigemina* isolates from Zambia were used in the population genetic analysis. High levels of genetic diversity were observed for both parasites. Population genetic analysis of the Zambian and Turkish *Ba. bovis* populations, using eight genetic markers showed that the two populations were sub-structured. The Zambian population comprised a single randomly mating population, while the Turkish population comprised two genetically distinct subpopulations. Population genetic analysis of the *Ba. bigemina* parasites from Zambia showed that this parasite population was in linkage disequilibrium. Further, analysis of the *Ba. bigemina* population using STRUCTURE showed that it was genetically sub-structured into five distinct subgroups. However, the resulting sample size of each subgroup was too small to definitely determine whether they were panmictic. These results provide an improved understanding of the epidemiology of bovine *Babesia* parasites in Turkey and Zambia.

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Abbreviations and Symbols

A	adenine
ANOVA	analysis of variance
BAC	Bacterial Artificial Chromosome
B-cells	B-lymphocytes
BLAST	basic local alignment search tool
BM86	*Boophilus microplus* 86 kDa antigen
BM91	*Boophilus microplus* 91 kDA antigen
bp	base pairs
Bv80	a 77 kDa spherical protein in the Australian *Ba. bovis* strains
BvVA1	*Ba. bovis* spherical body protein
C	cytosine
CAT	card agglutination test
$CD4^+$	cluster of differentiation 4 – positive
$CD8^+$	cluster of differentiation 8 – positive
C.I.	confidence interval
C-terminus	carboxyl terminus of a protein
CTL	cytotoxic T-lymphocyte
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleic triphosphates
DR Congo	Democratic Republic of Congo
e.g.	example given
ECF	East Coast fever
ELISA	enzyme linked immunosorbent assay
ERIC-PCR	entorobacterial repetitive intra-genic consensus sequence-PCR
FAM	blue florochrome used to label PCR primer
F_{ST}	standard measure of genetic differentiation among populations
FTA	Flinders Technology Associates
G	guanine
GDP	gross domestic product
gp45	*Ba. bigemina* 45 kDa merozoite surface antigen
gp55	*Ba. bigemina* 55 kDa merozoite surface antigen
GS500	Genescan^{TM} 500 size marker
H_e	estimated heterozygosity
HGE	human granulocytic ehrlichiosis
Hsp90	heat shock protein 90
IFAT	indirect antibody fluorescent test
IF-γ	Interferon-gamma
IL-12	inter-leukin 12
I^S_A	standard index of association
ITM	Infection and Treatment method
K	probable number of subpopulations or clusters
Kb	kilobase (one thousand bases)
kDa	kilodalton
km^2	kilometre squared
L	95 % confidence interval (linkage analysis)
L(K)	likelihood distribution; estimates number of clusters in STRUCTURE
LAMP	loop mediated isothermal amplification
LD	linkage disequilibrium
LE	linkage equilibrium
L_{MC}	95 % confidence interval (linkage analysis / Monte Carlo simulations)
Mb	megabase pairs

MCMC	Marcov chain Monte Carlo
MLG (s)	multi-locus genotype (s)
mm	millimetres
msa	*Ba. bovis* merozoite surface antigen gene
MSA-1	*Ba. bovis* merozoite surface antigen 1
MSA-2	*Ba. bovis* merozoite surface antigen 2
MSPs	*A. marginale* major surface proteins
^{o}C	Degrees Celsius
Omps	outer membrane proteins
OR	odds ratio
p	*p value*: type I error (probability of wrongly rejecting null hypothesis)
P104	*T. parva* 104 kDa microneme-rhoptry protein
p150	*T. parva* microsphere antigen
p67	*T. parva* 67 kDa sporozoite antigen
PBS	phosphate buffered saline
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PCR-RFLP	PCR - restriction fragment length polymorphism
PCV	packed cell volume
PIM	*T. parva* polymorphic immunodominant molecule
Pr(X/K)	posterior prob. of genotype (X) given the subpopulation (K) of origin
PRA	polymerase chain reaction - restriction analysis
qPCR	real time polymerase chain reaction
R^2	coefficient of determination
RAP-1	Rhoptry associated protein 1
RAPD	random amplification of polymorphic DNA
RAS	*Rhipicephalus appendiculatus* serpin proteins
RBC	red blood cells
REP-PCR	repeative extra-genic palindromic elements – PCR
RIM36	*Rhipicephalus* immuno-dominant protein 36
RLB	reverse line blot
RNA	ribonucleic acid
ROX	fluorescent tag (red) for size standards in capillary electrophoresis
rRNA	ribosomal ribonucleic acid
SD	standard deviation
spp	species
SPSS	statistical package for social sciences
T	thymine
Taq	*Thermus aquaticus* polymerase
TBDs	tick-borne diseases
T-cells	T-lymphocytes
T_m	Melting temperature
Tpr	*T. parva* repeat; multi-copy locus found in *T. parva*
TRP	*Rhipicephalus appendiculatus* saliva protein
U	unit
URL	uniform resource locater, website address
US$	United States Dollars
USA	United States of America
V_D	observed variance of mis-match values (linkage analysis)
V_e	expected variance of mis-match values (linkage analysis)
VMSA	*Ba. bovis* variable merozoite antigen
µg	microgrammes
µl	microlitres
µM	micromolars
64TRP	*R. appendiculatus* 64 kDa saliva cement protein

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CHAPTER ONE

Introduction

1.1 Introduction

Tick-borne diseases (TBDs) are a constraint to livestock production in many developing countries of the world. They are responsible for high morbidity and mortality resulting in decreased production of meat, milk and other livestock by-products and the loss of draught power. They are also a significant impediment to the improvement of indigenous breeds of cattle, sheep and goats, since they prevent the introduction of more productive exotic breeds. TBDs of ruminants are responsible for significant economic losses to the livestock industry in countries where they occur. In a review, de Castro (1997) estimated the annual global costs associated with ticks and TBDs in cattle to be between US$ 13.9 billion and US$ 18.7 billion. In Africa, tick-borne diseases are considered to be the most important animal disease problem (Minjauw & McLeod 2003; Young *et al.* 1988). In Zambia alone, between 1997 and 2000, approximately 89,000 cases of tick-borne disease were reported in cattle, of which 19,420 were fatal (Makala *et al.* 2003).

Many pathogenic organisms are transmitted by ticks, but the most important in the Southern, Central and East African region are protozoan and rickettsial pathogens. Diseases caused by *protozoan* parasites in cattle include malignant theileriosis (corridor disease / East Coast fever (ECF) and babesiosis while rickettsiae cause diseases known as anaplasmosis and cowdriosis (Makala *et al.* 2003). Despite the economic importance of these diseases, information on their epidemiology in many African countries, including Zambia, is either inadequate or completely absent. Much of sub-Saharan Africa provides suitable ecosystems for a number of tick-borne pathogens, where different parasite species share similar habitats and/or vectors and often occur within single vertebrate hosts concurrently. Understanding the interactions that may occur among these pathogens in both cattle and the vector may help in the design of cost-effective integrated control measures to combat these diseases. Another important aspect of these TBD parasites that requires deeper understanding is the extent of genetic exchange that exists within natural populations. This is of practical importance since, if present at a significant level, the resultant sexual recombination would generate a high degree of genetic diversity within populations. Understanding the levels of genetic diversity and the structure of parasite populations is critical for predicting responses to selective pressures such as vaccination

and drug treatment, both locally and in the wider context. This study therefore set out to investigate the diagnosis and epidemiology of TBDs of cattle in Central and Eastern Zambia, and to study the population genetic structures of *Babesia bovis* and *Ba. bigemina*.

1.2 Geographic and climatic characteristics in Zambia

Zambia lies between longitudes 22°E and 34°E and latitudes 8°S and 18°S. A map of Zambia, showing the Provinces and the neighbouring countries is shown in Figure 1.1. The country has a total area of 75.262 million hectares of which 0.920 million is covered by lakes, swamps and rivers. Zambia is landlocked and shares its borders with eight other countries: Angola, Botswana, DR Congo, Malawi, Mozambique, Namibia, Tanzania and Zimbabwe. Administratively, Zambia is divided into nine provinces: Southern, Western, Luapula, Northwestern, Northern, Central, Lusaka and Eastern Provinces (Figure 1.1). Due to their close proximity, Lusaka and Central Provinces are together referred to as Central Zambia in this study.

Zambia has a tropical climate that is influenced by altitude. Three distinct climatic seasons occur: hot and dry from September to November, warm and wet from December to April, and cool and dry from May to August. Rainfall is greatest in the Northern part of the country with an average annual precipitation of 1400 mm that decreases progressively towards the south with an average annual precipitation of 600 mm. Mean relative humidity increases from below 5 % in the hot dry season to above 75 % in the rainy season. Mean minimum temperature ranges from 5 to 10°C in June/July in Central, Southern and Western Zambia and 10 to 13°C in Eastern and Northern parts of the country. Mean maximum temperature ranges from 30 to 35°C in October in Central Zambia, whilst those in the Northern and Eastern plateaus are between 25 and 30°C.

1.3 Livestock production in Zambia

Livestock production is an important socio-economic activity in Zambia and contributes significantly to the national gross domestic product (GDP) (Anon 2003). Cattle are the most important livestock in Zambia and can be divided into three breeds or types: European (*Bos taurus*), indigenous (*Bos indicus*) and the crosses of indigenous and taurine breeds. There are three main types of indigenous traditional cattle: (i) Barotse, a long-horned Sanga type in the Western Province, (ii) Tonga, a medium-horned Sanga type in the Southern and Central Provinces and (iii) Angoni, a short-horned Zebu type in the Eastern Province (Pegram *et al.* 1986). The total cattle population in the country is currently estimated at 2.9 million (Anon 2007).

Figure 1.1 Map of Zambia

Source: http://www.fao.org/docrep/X5598E/Zambia.GIF

Figure legend

----- Provincial boundaries
___ Rivers
___ International boundaries
• Towns

In Zambia, livestock production is broadly classified into the traditional sector (85 %) and the commercial sector (15 %). Traditional livestock farming is the preserve of rural families which are often resource poor (Chilonda *et al.* 1999) and is conducted mostly in Western, Southern, Central and Eastern Provinces. This is mainly based around the indigenous Sanga and Zebu breed types and is characterised by extensive communal grazing, overstocking and overgrazing. Generally, the productivity of traditional cattle is low, with average calving rates estimated at 53 %, adult mortality rates of 8 % and calf mortality rates averaging 25 % annually. Disease control is limited and in most cases tick control is intermittent or absent. Because of the extensive production system, herd inter-mixing and poor disease control, a variety of cattle diseases, including TBDs, are prevalent in the traditional sector (Chilonda *et al.* 1999). Most of the diseases are enzootically stable in these animals, allowing year-round contact between the bovine host, parasite and vector (Jongejan *et al.* 1988). Adult animals are therefore invariably infected carriers but immune to clinical episodes of disease.

Commercial cattle production units are found on large-scale cattle ranches with free range and / or controlled grazing for intensive production. The main breeds of cattle in the commercial sector are Boran, Afrikander, Sussex, Gelbvieh, Brahman, Friesian and Simental. There are over 50,000 dairy cows, representing 10 % of the commercial herd (Chilonda *et al.* 1999). Cattle productivity in the commercial sector is high with low calf mortality rates (1 - 2 %) and relatively high reproductive rates of between 65 and 70 % annually (Chilonda *et al.* 1999). Tick control is practised intensively leaving the animals highly susceptible to TBDs if challenged. In contrast to the traditional sector, these diseases can be clinically important and can reach epidemic proportions when intensive control measures break down (Chilonda *et al.* 1999).

1.4 Important tick-borne diseases of livestock

1.4.1 Babesiosis

Babesiosis is caused by intra-erythrocytic protozoan parasites of the genus *Babesia* which infects a wide range of domestic and wild animals and occasionally man. The disease is distributed world-wide (Bock *et al.* 2004a). The genus *Babesia* belongs to the phylum Apicomplexa, and family Babesiidae (Allsopp *et al.* 1994; Levine 1971). There are over one hundred reported species of *Babesia* but those of greatest importance for cattle are *Babesia bigemina* and *Ba. bovis* (Bose *et al.* 1995). The disease is found in most tropical and sub-tropical countries between latitudes 40 ^{o}N and 32 ^{o}S (Bock *et al.* 2004a).

1.4.1.1 Vectors of *Ba. bigemina* and *Ba. bovis*

Babesiosis, caused by *Ba. bigemina* and *Ba. bovis*, is transmitted vertically by one-host ticks of the genus *Boophilus*. Although the five species of this genus have recently been placed within the genus *Rhipicephalus* (Horak *et al.* 2002), the name *Boophilus* has been retained to indicate a subgenus (Barker & Murrell 2002; Horak *et al.* 2002). To avoid confusion, the old nomenclature will be used in this thesis. The main vectors of bovine babesiosis are *Bo. microplus*, *Bo. annulatus* and *Bo. geigyi* which transmit both *Ba. bovis* and *Ba. bigemina,* and *Bo. decoloratus* which transmit only *Ba. bigemina*. Only *Bo. microplus* and *Bo. decoloratus* are found in Eastern, Central and Southern Africa. *Rhipicephalus evertsi* also transmits *Ba. bigemina* (Bock *et al.* 2004a). Climate, soil and cattle biotypes modulate the capacity of the land to support *Boophilus* tick populations and in turn, influence the babesial infection rate in cattle and ticks (Guglielmone 1995). The distribution and ecological preferences of the *Boophilus* ticks in Africa have been described by Estrada-Pena e*t al*. (2006). The minimum temperature and rainfall requirements are similar for both *Bo. microplus* and *B. decoloratus*. They both require low rainfall between May and October, and high rainfall between November and March. However, despite these similar requirements, the two species do not occur together due to interspecies competition (Estrada-Pena *et al.* 2006). The shorter life cycle and the absence of specific host resistance to Bo. microplus gives it a greater population growth potential compared to *Bo. decoloratus* (de Waal & Combrink 2006). In Zambia, *Bo. microplus* was only found in Northern and Eastern parts of the country, while Bo. decoloratus was found in all parts of the country, except in those areas where *Bo. microplus* occurs (Jongejan *et al.* 1988; Pegram *et al.* 1986). No recent studies have been done to determine the current distribution of these parasites and their vectors and it likely that their distribution has changed given the rampant movement of cattle.

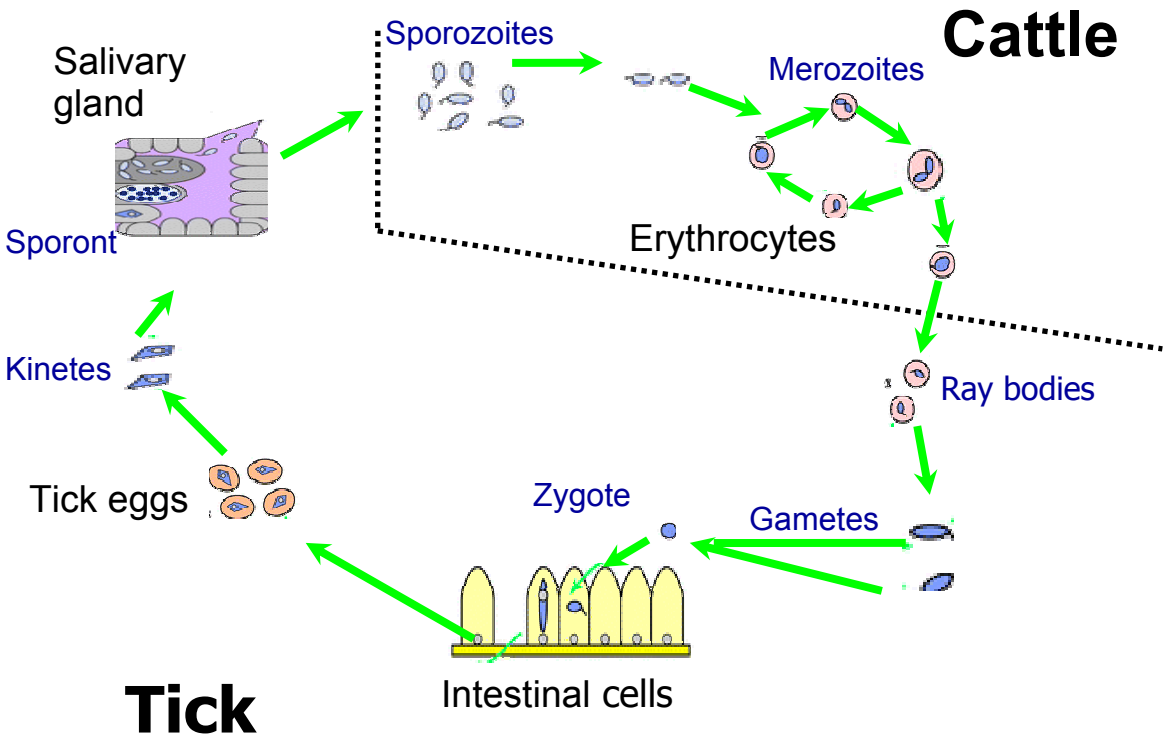
1.4.1.2 Life cycle *of Ba. bovis* and *Ba. bigemina*

Babesia parasites are not characterised by a life-cycle that is specific for all members of the genus (Bock *et al.* 2004a; Mackenstedt *et al.* 1995). The development of *Ba. bovis* and *Ba. bigemina* follow similar patterns in adult *Boophilus spp* (Mehlhorn & Shein 1984; Potgieter *et al.* 1976; Potgieter & Els 1977a). Features of the life-cycle of the two parasites are shown schematically in Figure 1.2. *Babesia* spp do not parasitize any other vertebrate cell other than the erythrocyte (Hodgson 1992). After entering the erythrocyte, the sporozoite transforms into a trophozoite from which two merozoites develop by a process of merogony (binary fission) (Potgieter & Els 1977b). Since there have been more detailed

Figure 1.2 Lifecycle of *Babesia* parasites

Cattle are infected by feeding ticks, which inoculates sporozoites that invade erythrocytes where they transform into trophozoites that divide by binary fission (merogony). The erythrocyte membrane breaks down and the released merozoites invade new cells resulting in an intra-erythrocytic cycle. Following a tick blood meal, gametocytes develop in the tick gut, which fuse to form diploid zygotes. Zygotes invade the digestive cells and probably basophilic cells where they under go successive round of multiplication before emerging as haploid kinetes. The kinetes migrate to many other organs including the ovaries where further division occurs. After egg hatching, the kinetes migrate to the salivary gland where they transform into multi-nucleated stages (sporogony) which later form sporozoites.

Figure 1.2 Lifecycle of *Babesia* parasites



studies of the development of *Ba. bigemina* than have been done for *Ba. bovis* (Gough *et al.* 1998; Mackenstedt *et al.* 1995), most of the details given in this section will refer to this parasite species. In *Ba. bigemina,* Mackenstedt *et al*. (1995) identified an ovoid type of merozoite they called gamont precursor, which unlike the other piroplasms studied, had diploid DNA levels. These gamont precursors do not undergo any further development until they are taken up by the tick. Changes experienced in the passage from host blood to the midgut of the tick vector stimulates the development of two populations of ray bodies from the gamonts (gametocytes) (Gough *et al.* 1998). The ray bodies undergo further multiplication within the erythrocytes that continues after they have emerged. Large aggregates of multinucleated ray bodies are generated, but once division is complete, single-nucleated ray bodies that are now haploid and assumed to be gametes (Mackenstedt *et al.* 1995), emerge from the aggregates and fuse together in pairs (syngamy) (Gough *et al.* 1998) to form a spherical cell (zygote). The zygotes, which are diploid, selectively infect the digestive cells of the tick where they probably multiply and then invade basophilic cells (vitellogenin synthesising cells). Further multiplication by schizogony occurs with the development of kinetes (vermicules) that escape from the basophilic cells into the haemolymph (Agbede *et al.* 1986). These motile club-shaped kinetes, which are now haploid, infect a variety of cell types, including oocytes where secondary cycles of schizogony take place. Thus transovarial transmission occurs with further development taking place in the larval stage. This is an important adaptation as *Boophilus* ticks are one host ticks. Kinetes enter the salivary glands and are transformed into multinucleated stages (sporogony) and these then break up to form sporozoites (Mackenstedt *et al.* 1995). In all species, sporozoite development usually only begins when infected larval ticks attach to the host. In *Ba. bigemina* some development takes place in the feeding larvae, but infective sporozoites take about 9 days to appear and therefore only appear in the nymphal and adult stages. Transmission can occur throughout the rest of the nymphal and adult stages of the ticks. In the case of *Ba. bovis*, the formation of infective sporozoites usually occurs within 2 to 3 days after larval attachment.

1.4.1.3 Pathogenesis and clinical signs of babesiosis

Despite being closely related and transmitted by the same *Boophilus* ticks*, Ba. bovis* and *Ba. bigemina* cause remarkably different diseases in cattle. In *Ba. bovis* infections*,* the disease pathology can be both due to over-production of pro-inflammatory cytokines and the direct effect of red blood cell destruction by the parasite. During an acute infection, macrophages activated by the parasite produce pro-inflammatory cytokines and parasitocidal molecules (Brown & Palmer 1999). The outcome of infection is related to the

timing and quantity of production of these substances. Over-production of inflammatory cytokines results in severe pathology leading to vasodilatation, hypotension, increased capillary permeability, oedema, vascular collapse, coagulation disorders, endothelial damage and circulatory stasis (Ahmed 2002; Wright *et al.* 1989). Although stasis is induced in the microcirculation by aggregation of infected erythrocytes in capillary beds, probably, the most deleterious pathophysiological lesions occur from the sequestration of parasitised erythrocytes in microcapillaries of the lungs and brain. This results in cerebral babesiosis and a respiratory distress syndrome associated with infiltration of neutrophils, vascular permeability and oedema (Bock *et al.* 2004a; Brown & Palmer 1999). Progressive haemolytic anaemia develops during the course of *Ba. bovis* infection. While this is not a major factor during the acute phase of disease, it contributes to the disease process in more protracted cases. The clinical signs associated with *Ba. bovis* infections are fever, inappetence, depression, increased respiratory rate, weakness and a reluctance to move. Haemoglobinuria is often present (red water). Anaemia and jaundice develop especially in more protracted cases. Cerebral babesiosis is manifested by a variety of signs of central nervous system involvement and the outcome is almost invariably fatal (Bock *et al.* 2004a)

In *Ba. bigemina* infections, pathogenesis is almost entirely related to rapid, and sometimes massive, intravascular haemolysis. Coagulation disorders, cytoadherence and the hypotensive state seen in *Ba. bovis* are not features of *Ba. bigemina* infections (Bock *et al.* 2004a). The pathology relates more directly to the destruction of RBC. Haemoglobinuria is present earlier and is more consistent that in *Ba. bovis* infection. There is no cerebral involvement and recovery in non-fatal cases is usually rapid and complete. However, in some cases the disease can develop very rapidly with sudden and severe anaemia, jaundice and death, which may occur with little warning (Bock *et al.* 2004a)

1.4.1.4 Epidemiology of babesiosis

The prepatent period for *Ba. bigemina* is usually 12 to 18 days after tick attachment (Bock *et al.* 2004a). As *Ba. bovis* does not persist in an infective form in ticks beyond the larval stage (Mahoney & Mirre 1979), the prepatent period is generally 6 to 12 days and peak parasitaemia is reached 3 to 5 days after that (Bock *et al.* 2004a). However, unlike *Ba. bigemina*, heat stimulation of the larval ticks prior to attachment (37 ^{o}C for 3 days and 30 ^{o}C for 8 days) enables transmission of *Ba. bovis* immediately upon attachment and this can lead to shortened prepatent periods particularly in hot climates (Dalgliesh & Stewart 1982).

The prevalence of infection and the occurrence of disease are determined by complex interactions between the bovine host, vector and parasite (Jonsson *et al.* 2008). Bock *et al*. (1997, 1999b) found that *Bos taurus* cattle are more susceptible to *Ba. bovis* than *Bos indicus* breeds, while there was no significant difference in susceptibility to an Australian *Ba. bigemina* isolate between the two types of cattle. Breeds of cattle that are indigenous to *Babesia*-endemic regions often have a certain degree of natural resistance to these diseases and the consequences of infection are not as serious as those for exotic *Bos taurus* breeds. In addition, in tropical areas with a high vector population, natural exposure usually occurs at an early age when these animals are naturally protected, allowing acquired immunity to develop and these cattle are therefore immune to subsequent challenge as adults (Bock *et al.* 2004a). In a recent study, Magona *et al*., (2008) observed a significant negative association between age and the risk of seroconversion to *Ba. bigemina* in Zebu cattle kept under natural tick challenge in Uganda. This phenomenon was explained by a large number of older cattle remaining seropositive due to continued exposure to infected ticks, thus reducing the proportion of susceptible individuals in the population (Magona *et al.* 2008). This scenario represents a state of endemic stability, defined as the situation where all calves below the age of 6 months have been in contact with the parasite and where clinical diseases is rare (Yeoman 1966). In situations where cattle do not come in contact with the disease earlier in life, an inverse age resistance to babesiosis exists with young animals possessing innate resistance while older animals are fully susceptible (Jongejan *et al.* 1988; Mahoney *et al.* 1973). Passively acquired immunity from colostrum lasts two months but this is followed by innate immunity from 3 to 9 months of age. Therefore, calves exposed to babesiosis early in life rarely show clinical signs but develop long lasting immunity (Mahoney *et al.* 1973).

1.4.2 Theileriosis

Theileriosis is a disease of *Bovidae* caused by infection with protozoan parasites of the genus *Theileria.* The genus *Theileria* encompasses a number of protozoan species that affect domestic livestock and other mammals. They are classified in the phylum apicomplexa along with *Babesia*, *Eimeria*, *Plasmodium* and *Toxoplasma*. Of the five species known to infect cattle, the two most economically important are *T. annulata*, the cause of tropical theileriosis, which is widespread throughout the Mediterranean basin, the Middle East and Asia, and *T. parva* which causes East coast fever (ECF), a highly fatal disease of cattle in Eastern, Central and Southern Africa (Norval *et al.* 1992). The other species of bovine *Theileria*, *T. mutans*, *T. taurotragi* and *T. velifera* are usually benign, although *T. mutans* and *T. taurotragi* can occasionally cause losses (Binta *et al.* 1998).

With over one million animals dying each year from ECF in sub-Saharan Africa, theileriosis presents one of the most important threats to livestock production in the tropics and is a major constraint on the livelihoods of millions of rural farmers (Minjauw & McLeod 2003).

1.4.2.1 Vectors of *T. parva*

Theileria parva is transtadially transmitted by the nymphs and adults of the brown ear tick, *Rhipicephalus appendiculatus,* after picking up the disease as larvae and nymphs respectively (Konnai *et al.* 2006; Mulumba *et al.* 2001). *R. appendiculatus* is also the vector of *T. taurotragi* (Jongejan & Uilenberg 2004). The tick has been recorded in large areas of Eastern, Central and Southern Africa, with a range stretching from Southern Sudan in the north, to South Africa in the south (Perry *et al.* 1990). Short and Norval (1981) showed that the seasonal occurrence of *R. appendiculatus* is largely dependent on the timing period of the adult stage (due to behavioural diapause) and this is regulated by the combined influences of temperature, humidity and day length. In parts of Africa where there is a well defined rainy season (e.g. Nyabubinza, Tanzania; Chaya, Zambia; Lilongwe, Malawi) there is an obvious association between the onset of the rains and adult tick activity, and in these regions only one generation of ticks per annum is observed. In other parts of Africa where rain falls throughout the year (e.g. Mwanza, Tanzania) adults may be present all year round (McCulloch *et al.* 1968; Newson 1978). In Zambia there is only one generation of ticks per year. Adults ticks occur from December to April, larvae between March and May and nymphs between May and September (Pegram *et al.* 1986).

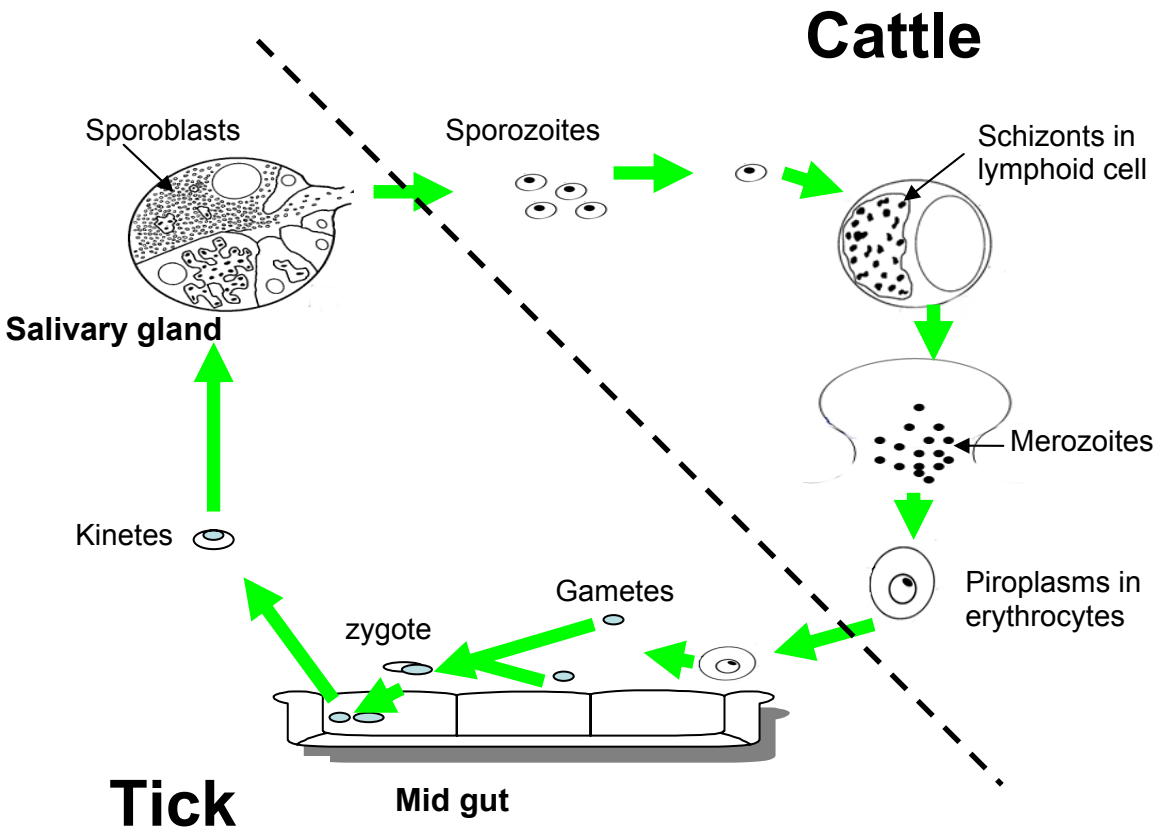
1.4.2.2 Life cycle of *Theileria*

Theileria spp have a complex life cycle that involves several morphologically distinct developmental stages in the tick and mammalian host (Fawcett *et al.* 1982; Shaw & Tilney 1992). The current view of the life cycle of *Theileria* is presented schematically in Figure 1.3. The infectious stage of the parasite, the sporozoite, is introduced into the bovine host in the saliva of the ticks feeding as nymphs or adults. They enter lymphoid cells where they develop into schizonts, which induce the host cell to proliferate and are then disseminated throughout the body by the normal circulation of the host's lymphoid cells. After a period of growth and division in the host lymphoid cells, the schizont gives rise to numerous uninucleate merozoites which leave the lymphoid cells to invade erythrocytes. In non-lymphoid transforming species of *Theileria* (e.g. *T. mutans*), there is no intralymphocytic multiplication, and the parasites multiply almost exclusively in the erythrocytes. The intraerythrocytic stages of the parasite, the piroplasms, are ingested in

Figure 1.3 Life cycle of *T. parva*

Cattle are infected by feeding ticks, which inoculate sporozoites that invade lymphoid cells to form multinucleated schizonts. Infected cells proliferate with a proportion of schizonts differentiating into merozoites. Following destruction of host cell membranes, merozoites are released into the blood stream where they invade erythrocytes to become piroplasms, the infective stage for feeding ticks. Following a blood meal, gametes develop in the tick gut, which fuse to form zygotes. The zygotes transform into motile kinetes. Zygotes and kinetes are the only stages where the parasite is considered diploid. Kinetes migrate into salivary glands, where after several rounds of asexual multiplication (sporogony) sporozoites are formed.

Figure 1.3 Life cycle of *T. parva*



the blood meal by the feeding ticks and released in the gut lumen. There, they divide into macro- and microgametes (Schein *et al.* 1977) which fuse to form a zygote. The resulting zygotes enter the lining of the gut epithelium where they develop into motile kinetes. Following the moult of the tick stage, the kinetes migrate through the gut wall into the haemocoele and make their way to the salivary gland where they become intracellular and transform into sporoblasts (Fawcett *et al.* 1982).

1.4.2.3 Pathogenesis and clinical signs of ECF

After sporozoites are injected into susceptible cattle by the feeding tick, they invade host lymphocytes where they rapidly differentiate into schizonts (Fawcett *et al.* 1982). This process is associated with transformation of the infected cells to a state of uncontrolled proliferation. By associating with the mitotic spindle, the parasite divides in synchrony with the host cell, resulting in each daughter cell inheriting the infection (Rocchi *et al.* 2006). This phase of parasitic and host cell division is associated with the severity of pathology and clinical signs seen in *T. parva* infections. Some pro-inflammatory cytokines have been found to be up-regulated during the acute phase *T. parva* infection when the parasite is proliferating and are thought to contribute to the severity of the disease (Yamada *et al.* 2009). The disease is characterised in initial stages by swelling of the lymph nodes draining the ear surface which is the predilection feeding site for feeding ticks. The incubation period is usually between 7 to 10 days after natural infection, with fever (39.5 ^{o}C or higher) from about day 10. Fever is a consistent feature. Schizont infected cells disseminate to lymph nodes and other organs including the interstitial tissues of the lungs, the gastro-intestinal tract and the kidneys. The main symptoms of ECF are fever, swelling of lymph nodes, anorexia, and dyspnoea. Finally the lung tissues are damaged by inflammatory infiltration, leading to severe pulmonary oedema, thereby causing death of the host (Gwamaka *et al.* 2004).

1.4.2.4 Epidemiology of ECF

The occurrence and distribution of *T. parva* is closely related to that of its vector, *R. appendiculatus*. Differential climatic conditions influence tick population dynamics and the transmission possibilities of *T. parva*, creating a range of epidemiological situations in different areas (Lessard *et al.* 1990; Norval *et al.* 1991). Three different transmission scenarios of *R. appendiculatus* have been described in relation to rainfall and temperature and correspond to the number of tick generations per year. The East African situation is characterised by year-round transmission due to the presence of the adult vector ticks throughout the year. In the transitional zone, where climatic conditions are not favourable

for the year-round presence of adult ticks, rainy season and dry season adult tick transmission peaks may prevail (Norval *et al.* 1991). The occurrence of only one tick generation per year in the Southern part of the *R. appendiculatus* range reduces the adult transmission period to four months which coincides with the rainy season (Norval *et al.* 1991), making the epidemiological situation more unstable. However, in these areas, nymphal transmission between May and September still plays an important part in the epidemiology of the disease (Mulumba *et al.* 2001). In years of low rainfall, high ECF sero-prevalences in September, attributed to nymphal transmission have been reported in Southern Zambia (Fandamu *et al.* 2005). In an experimental challenge, Marcotty *et al.,* (2002) found that nymphs were able to induce a lethal infection, providing a large number of them attached to the host.

Two additional factors which are important in the epidemiology of theileriosis are the host type and density. The African cattle population consists of different breeds which show varying levels of tick resistance and parasite susceptibility (Norval *et al.* 1988). *Bos taurus* breeds are the most susceptible to tick infestation and *T. parva* infection, whereas indigenous Sanga and Zebu types of cattle in endemic areas show a degree of resistance to the parasite (Ndungu *et al.* 2005). Animals that recover from the disease, either naturally or following treatment, remain carriers, and provide a reservoir of parasite infection for feeding ticks (Kariuki *et al.* 1995).

1.4.3 Anaplasmosis

Bovine anaplasmosis is an arthropod-borne, haemolytic disease of ruminants caused by the rickettisial haemoparasite, *Anaplasma marginale*. Another less pathogenic species of *Anaplasma* called *A. centrale* which does not cause severe clinical signs in cattle was described by Theiler (Kocan *et al.* 2000). *Anaplasma* is globally the most prevalent tick-borne pathogen of cattle, with regions of endemicity on six continents (Futse *et al.* 2003), and is to be found in tropical and sub-tropical areas including North and South America, Africa, the Caribbean, Russia, European countries bordering the Mediterranean and the Middle East and Far East (Kocan *et al.* 2000). The pathogen is classified within the Order Rickettsiales and the family *Anaplasmataecoe* which was recently reorganised to include the genera *Anaplasma*, *Ehrlichia*, *Wolbachia* and *Neorickettsia,* based on the genetic analysis of the 16S ribosomal RNA genes, groELS and surface protein genes (Dumler *et al.* 2001). The genus *Anaplasma* now includes the pathogens *A. marginale*, *A. centrale*, A. *ovis*, *A. bovis* (formerly *Ehrlichia bovis*), *A. phagocytophilum* (formerly *E. phagocytopylum*, *E. equi* and the agent of human granulocytic ehrlichiosis (HGE)) and

A. platys (formerly *E. platys*) (Dumler *et al.* 2001; Kocan *et al.* 2004). Erythrocytes are the only known cell type to be infected by *A. marginale* in the animal host. Infected red cells are phagocytosed by the reticulo-endothelial system resulting in a mild to severe anaemia together with icterus, but without haemoglobinaemia and haemoglobinuria (Kocan *et al.* 2000).

1.4.3.1 Vectors of *A. marginale*

Transmission of anaplasmosis can occur mechanically by biting flies and blood contaminated fomites or biologically by ticks (De Wall 2000). Biological transmission of anaplasmosis is effected by ticks and approximately 20 species of ticks have been implicated throughout the world, including *Boophilus* spp, *Rhipicephalus* spp, *Hyaloma* spp, *Demacentor* spp and *Ixodes* spp (De Wall 2000; Jongejan & Uilenberg 2004; Kocan *et al.* 2004; Uilenberg 1995). Biological transmission can occur from one tick life cycle stage to the next (trans-stadial) or when a tick feeds on more than one host in the same life cycle stage (intra-stadial) (Kocan *et al.* 2003). Intra-stadial transmission of *A. marginale* is mainly effected by male ticks (Kocan *et al.* 2004). Trans-ovarial transmission of *A. marginale* has only been reported for *D. andersoni* (Howell *et al.* 1941). Mechanical transmission frequently occurs via blood-contaminated fomites, including contaminated needles, dehorning saws, nose tongs, tattooing instruments, ear tags devices and castration instruments. In addition to mechanical and biological transmission, *A. marginale* can also be transmitted from cow to calf transplacentally (Kocan *et al.* 2003).

1.4.3.2 Life cycle of *Anaplasma*

Infected erythrocytes taken into the tick with the blood meal provide the source of *A. marginale* infection in the gut cells. After development of *A. marginale* in the gut cells, many other tissues become infected, including the salivary glands from where the rickettsiae are transmitted to vertebrates during tick feeding (Ge *et al.* 1996; Kocan *et al.* 1992b; Kocan *et al.* 1992a). At each site of infection in ticks, *A. marginale* develops within membrane bound vacuoles or colonies. The first form of *A. marginale* seen within the colony is the reticulated (vegetative) form that divides by binary fission forming large colonies that contain hundreds of organisms. The reticulated form then changes into the dense form, which is the infective form and can survive extracellulary. Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands (Kocan *et al.* 2004). In the vertebrate host, the only known site of replication is the bovine erythrocytes. Within the erythrocytes, membrane bound inclusion

bodies contain from 4 to 8 rickettsiae, and as many as 70 % or more erythrocytes may become infected during acute infection or disease (Kocan *et al.* 2004).

1.4.3.3 Pathogenesis and clinical signs of anaplasmosis

Once infected, the number of infected erythrocytes increases logarithmically and clinical disease associated with anaemia is consistently observed when 40 to 50 percent of the erythrocytes have been removed. Destruction of erythrocytes by the reticuloendothelial system results in development of icterus without haemoglobinaemia and haemoglobinuria (Kocan *et al.* 2000). Acute anaplasmosis, caused by *A. marginale*, is characterised by a progressive haemolytic anaemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle.

1.4.3.4 Epidemiology of anaplasmosis

Several studies have been carried out to determine the susceptibility of different breeds of cattle to infection with *A. marginale* (Bock *et al.* 1997; Bock *et al.* 1999a; Wilson *et al.* 1980). Results from these studies show that both *B. taurus* and *B. indicus* cattle are equally susceptible to the disease. Calves are less susceptible to infection by *A. marginale* and when infected rarely develop clinical disease. In a cross-sectional study in Tanzania, Swai *et al*. (2005), found that the seroprevalence of anaplasmosis increased with the age of the animal. Calves that recover from the disease develop life-long immunity (Guglielmone 1995). Cattle that survive acute disease develop persistent infection characterised by cyclic low-level rickettsaemia but life-long immunity. These animals serve as a reservoir of *A. marginale,* providing a source of infective blood for both mechanical and biological transmission (Guglielmone 1995; Kocan *et al.* 2003). Anaplasmosis is the most widespread TBD in Zambia, found in all nine provinces (Jongejan *et al.* 1988).

1.4.4 Heartwater

The tick-borne rickettsia, *Ehrlichia ruminantium* (formerly called *Cowdria ruminantium*) is an obligate intracellular bacterium that parasitises vascular endothelial cells, neutrophils and macrophages of the mammalian host and causes a disease in ruminants called heartwater (or cowdriosis) (Peter *et al.* 1995). The genus *Ehrlichia* belongs to the Order Rickettsiales, and was recently placed within the family *Anaplasmataecoe,* together with the genera *Anaplasma*, *Neorickettsia* and *Wolbachia* (Dumler *et al.* 2001). The disease is found throughout most of sub-Saharan Africa and neighbouring islands, and at least three islands in the Caribbean (O'Callaghan *et al.* 1998; Uilenberg 1996).

1.4.4.1 Vectors of *E. ruminantium*

Heartwater is transmitted transtadially by ticks of the genus *Amblyomma* of which twelve species are known to be able transmit the disease, although the most important vectors are *A. variegatum* and *A. hebraeum* (Uilenberg 1996; Walker & Olwage 1987). Only nymphs and adults are able to transmit the disease after being infected as larvae and nymphs, respectively (O'Callaghan *et al.* 1998). Transtadial transmission can occur via adults infected as larvae without reinfection as nymphs (Norval *et al.* 1990). Vertical transmission of heartwater from dam to calf has also been demonstrated (Deem *et al.* 1996b).

1.4.4.2 Life cycle of *E. ruminantium*

Information on the development of *E. ruminantium* in both mammalian and tick hosts is limited. *E. ruminantium* occurs within membrane-bound colonies in both vertebrate and tick host cells. The major site of development of *E. ruminantium* in ticks appears to be midgut epithelial cells (Kocan *et al.* 1987; Kocan 1995). Subsequent stages invade and develop in the salivary gland acini cells of the vector. The development of the stages of the organism that are transmitted seems to be coordinated with the feeding cycle of the ticks (Kocan *et al.* 1987). In the vertebrate host, the spread of the parasite from the infection site is poorly understood. It has been proposed that the initial development of the organism occurs mainly, but not exclusively, in reticulo-endothelial cells. Then the parasitised reticulo-endothelial cells rupture and the organism is released into the general circulation where it invades endothelial cells (Du Plessis 1970; Prozesky & Du Plessis 1987). Depending on the host, the organism seems to have a predilection for endothelial cells in certain organs. In ruminants, the highest concentration of bacterial organisms are found in the brain followed by kidneys (Prozesky & Du Plessis 1987).

1.4.4.3 Pathogenesis and clinical signs of heartwater

The pathophysiology of heartwater is poorly understood. Postmortem findings associated with heartwater include hydrothorax, pulmonary oedema, ascites, hydropericardium, cerebral oedema, oedema of the lymph nodes and splenomegaly (van Amstel *et al.* 1987). The transudate and the oedema are presumed to be caused by increased capillary permeability, although the mechanisms are not known (Deem 1998). Damage to the endothelial cells and alveolar capillaries is limited and the often mild cytopathic changes seen in parasitised endothelial cells suggest that the organism itself may not be the cause of the increased vascular permeability (van Amstel *et al.* 1988). Frequently observed clinical pathological changes in heartwater include progressive anaemia, marked decline in thrombocytes, fluctuations in total and differential white blood cell counts, increased total

bilirubin, and an increase in total serum proteins (van Amstel *et al.* 1987). Clinical signs of heartwater range from mild to transient fever in subclinical cases, to death without premonitory signs in peracute cases. The acute form of the disease is characterised by sudden onset of fever, tachycardia, inappetance and neurological signs (hyperaesthesia, high-stepping gait, twitching eyelids, chewing, abnormal tongue movement and individual muscle tremors). Haemorrhagic diarrhoea is commonly reported (Van de Pypekamp & Prozesky 1987).

1.4.4.4 Epidemiology of heartwater

Endemic stability is a common epidemiological state of heartwater in sub-Saharan Africa. This is due to the occurrence of the long-term carrier state in ruminants (Andrew & Norval 1989) and a high *E. ruminantium* infection rate in *Amblyomma* ticks in the field (Norval *et al.* 1990). It had been known for a long time that new-born lambs and calves possessed an age-related resistance to heartwater which was independent of the dam's immune status and that this resistance was of short duration lasting about three to four weeks (Du Plessis 1984; Du Plessis & Malan 1988; O'Callaghan *et al.* 1998). However, it has been demonstrated that colostrum plays a significant role in calf-hood immunity to heartwater and that in endemic areas, this immunity lasts beyond the previously reported age of four weeks (Deem *et al.* 1996a; Norvl *et al.* 1995). In addition to the transplacental route, there is also evidence that infected dams are able to pass on heartwater infections to their offspring early in life through infected colostrum, and that vector ticks feeding on these calves are able to pick up the infection and transmit it to susceptible hosts (Deem *et al.* 1996b).

1.5 Control of tick-borne diseases

Available methods of controlling TBDs include tick control, immunisation and chemotherapy. The choice of which control method to use is influenced by geographical location, prevailing socio-economic factors, cost and the ease of application of the method (Kocan *et al.* 2000; Mugisha *et al.* 2008).

1.5.1 Tick control

Methods available for controlling ticks include the use of chemicals, tick resistant breeds and more recently immunisation. Control of ticks on animals is not only directed at preventing transmission of disease but also aims at reducing direct losses associated with tick infestation such as damage to hides, reduction in weight gain and drop in milk production (Minjauw *et al.* 1997).

1.5.1.1 Chemical control

Chemical control measures against ticks date back to an era before ticks were proven to be vectors of disease, and to this day it remains the mainstay of control for TBDs (George *et al.* 2004). Prolonged use of chemicals to control ticks has resulted in various problems such as tick resistance, residues in animal products, environmental pollution and high costs (Ghosh *et al.* 2007). Intensive use of acaricides interferes with enzootic stability, creating a susceptible population of animals which are liable to disease epidemics whenever there are disruptions to control programmes (Kocan *et al.* 2000; Lawrence *et al.* 1980). These concerns have reinforced the need for alternative approaches to control tick infestations (Ghosh *et al.* 2007).

1.5.1.2 Tick resistant breeds

Genetically resistant animals, i.e. animals which show a heritable ability to become immunologically resistant to tick infestation, are a vital component of many tick control strategies (Willadsen 2004). They are particularly important in the control of *Boophilus* ticks on cattle in Australia. However, this approach is not without its difficulties. For the hosts of many tick species, resistance may simply not develop and it may be difficult to breed for tick resistance while preserving other desirable production characteristics, such as high milk yield in dairy cattle.

1.5.1.3 Anti-tick vaccines

The feasibility of controlling tick infestations through immunisation of hosts with selected tick antigens was first demonstrated by Allen and Humphreys (1979). They based their trials on the crude concept that ticks feeding on an appropriately immunised host might ingest antibodies specific for antigens within the digestive tract and reproductive organs of the tick, producing deleterious effects on the feeding and reproductive behaviour. By vaccinating cattle using crude antigens extracted from the midgut and reproductive organs of partially fed female *D. andersoni* ticks, they found that ticks fed on immunised cattle weighed significantly less, laid fewer eggs and very few eggs hatched compared to the control group (Allen & Humphreys 1979). Since then, a number of tick antigens have been discovered in several tick species (de la Fuente *et al.* 2007; Imamura *et al.* 2008; Labuda *et al.* 2006; Mulenga *et al.* 1999; Willadsen 2004). Two distinct types of antigen have been explored for vaccine development (Nuttall *et al.* 2006). The first are conventional antigens that are secreted in saliva during tick attachment and feeding on the host, the so called exposed antigens. These are taken up at the tick feeding site by host dendritic cells, which process and present them to T lymphocytes, priming a cell- or antibody-mediated immune

response (Larregina & Falo, Jr. 2005; Nithiuthai & Allen 1985; Willadsen 2001). The second type are the concealed antigens which are normally hidden from the immune response (Willadsen & Kemp 1988). Typical concealed antigens are those found on the tick gut wall which interacts with specific immunoglobulins taken up with the blood meal. Concealed antigens are considered potential vaccine candidates if they encounter immunoglobins entering the haemolymph (or gut) and are associated with cells or tissues that perform some vital function for the tick (Nuttall *et al.* 2006).

So far, only two *Boophilus microplus* concealed antigens, BM86 (Rand *et al.* 1989) and BM91 (Riding *et al.* 1994), have been developed commercially. The vaccine containing BM86 antigen is marketed as TickGuard^{TM} in Australia and GAVAC^{TM} in Cuba (de la Fuente *et al.* 1998; Willadsen *et al.* 1995). A number of exposed antigens have been suggested as anti-tick vaccine candidates. For example, Mulenga *et al.* (1999) characterised a 29 kDa salivary gland-associated protein from *Haemophysalis longicornis* and vaccination with recombinant protein led to a significant reduction in weight, 40 % and 50 % in larvae and nymphs respectively, post–engorgement. A 15 kDa protein (64 TRP) from *R. appendiculatus* was identified as a putative cement protein involved in attachment and feeding, and vaccination of cattle with the recombinant protein resulted in reduction of nymphal and adult infestation rates by 48 and 70 % respectively (Labuda *et al.* 2006). In a recent experiment, Imamura *et al.* (2008) vaccinated cattle with a cocktail of two recombinant *R. appendiculatus* serpin proteins (RAS-3 and RAS-4) and a 36 kDa immuno-dominant protein of *R. appendiculatus* (RIM36) reporting 39.5 % and 12.8 % adult female tick mortality rates in vaccinated and control groups, respectively. Although the vaccine could not prevent ticks from transmitting the disease, the appearance of *T. parva* in peripheral blood was delayed by 1 to 2 days in vaccinated animals.

Control of ticks by vaccination has the advantages of being cost effective, reducing environmental contamination and prevents the selection of drug-resistant ticks that results from repeated acaricide application. In addition, development of vaccines against ticks using multiple antigens that could target a broad range of tick species may prevent or reduce transmission of other pathogens (de la Fuente *et al.* 2006; de la Fuente & Kocan 2006).

1.5.2 Vaccination against TBDs

Two main approaches have been pursued in attempts to develop vaccines against TBDs, namely administration of live or inactivated parasites and the use of defined antigens (sub-unit vaccines) predicted to represent targets of protective immune responses.

1.5.2.1 Live and inactivated vaccines

The rationale for immunisation of cattle against TBDs using live vaccines has been based on the observation that animals which recover from disease are immune to subsequent challenge (de Waal & Combrink 2006; Morrison & McKeever 2006). Current methods used to vaccinate cattle against TBDs include the infection and treatment method against ECF (Radley *et al.* 1975a) and heartwater (Uilenberg 1983), and the use of attenuated vaccines against babesiosis and tropical theileriosis (de Waal & Combrink 2006; Pipano & Shkap 2000). The non-virulent *A. centrale* has been used to immunise cattle against anaplasmosis (Dalgliesh *et al.* 1990).

In the 19th century, it was noticed that animals that recovered from natural *Babesia* infection usually developed a durable, long-lasting immunity and that infection with blood from recovered animals did not precipitate such a severe form of the diseases in recipient animals (Connoway & Francines 1899). This feature has been exploited in many countries to immunise cattle against babesiosis. Blood vaccines incorporating live attenuated strains have been used for many years in Australia, South Africa, Argentina, Brazil, Uruguay and Israel (de Waal & Combrink 2006; Shkap *et al.* 2007). Splenectomised calves are the most popular source of immunogens. For *Ba. bovis*, attenuation is achieved by rapid passage of the parasite strains through susceptible splenectomised calves. Attenuation is not guaranteed, but usually develops after 8 to 20 calf passages (Bock *et al.* 2004a). The virulence of *Ba. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This phenomenon has been used to obtain avirulent strains by infecting calves, splenectomising them after three months and then using blood taken from the ensuing relapse to infect another calf and repeat the process (Bock *et al.* 2004a). Parasites attenuated *in vitro* have been used successfully in Argentina (Bock *et al.* 2004b). The vaccines are marketed as a chilled or frozen blood preparation containing *Ba. bovis, Ba. bigemina* and *A. centrale* (Dalgliesh *et al.* 1990). The use of frozen or chilled attenuated blood vaccines has overcome some of the earlier problems that were associated with blood collected from recovered animals (i.e. the carrier blood donor system), as it has allowed production of standardised vaccines (Dalgliesh *et al.* 1990; de Waal & Combrink 2006; Pipano 1995). In South Africa, the frozen *Babesia* vaccines have recently gained in

popularity over the chilled because of the lower production costs, prolonged shelf-life and on-demand availability (de Waal & Combrink 2006). Despite their ability to confer protection to vaccinated animals, these vaccines have a number of problems which have limited their use (de Waal & Combrink 2006). Severe post-vaccination reactions may occur in some highly susceptible animals, especially adult European cattle breeds and high yielding dairy cows. It is therefore recommended to only vaccinate animals less than nine months old which still have some innate resistance to the diseases (Bock *et al.* 2004a). Additionally, there was an increase in the number of breakthroughs reported in Australia between 1989 and 1993. These breakthroughs were attributed to changes in the field populations of *Ba. bovis,* although the host's immune non-responsiveness may also have been involved (Bock *et al.* 1992). Other problems associated with these vaccines include loss of viability, potential spread of *Babesia* following vaccination, and spread of other latent infectious in blood (Bock *et al.* 2004a; de Waal & Combrink 2006; Pipano 1995). Lastly, parasitic infections are common in many parts of the World, however, little is known about how the immune response to one species is affected by concurrent infection with other pathogens. A range of antagonistic and synergistic interactions has been shown in hosts co-infected with helminth and protozoan parasites, which might have implications for the effectiveness of live vaccination (Christensen *et al.* 1987; Graham *et al.* 2005; Graham *et al.* 2007).

Over 100 years ago, Theiler demonstrated that cattle could be infected with *T. parva* by transfer of infected cells from tissues of clinically affected cases, and that recovered animals were immune to subsequent challenge (Theiler 1911). Based on the results of Theiler and other observations, Spreull undertook a field trial in which he vaccinated 283,000 cattle with spleen and lymph node cell suspensions from *T. parva* infected cattle (Spreull 1914). Unfortunately, about 25 % of the animals died as a result of infection established by the immunisation procedure. However, 70 % of the cattle that survived were immune to subsequent challenge. Radley *et al.* (1975) described the infection and treatment method (ITM) in which simultaneous administration of long-acting tetracycline and defined doses of cryo-preserved sporozoites was found to induce immunity against *T. parva*. While this protocol resulted in solid immunity to challenge with homologous parasite isolates, it did not offer complete protection against heterologous challenge. Subsequent experiments demonstrated that immunisation with a mixture of three selected parasite isolates (Muguga, Kiambu 5 and Serengeti), known as the Muguga cocktail, resulted in immunity against a range of heterologous isolates (Radley *et al.* 1975b). This vaccine has been deployed extensively in Tanzania and Uganda and to a lesser extent in

Malawi and Zambia (McKeever 2007). In addition, experimental immunisation with a single parasite isolate (Marikebuni) provided protection against challenge from a number of heterologous isolates (Morzaria *et al.* 1987). This parasite stock has been used to successfully vaccinate cattle in the field in Kenya (Morrison & McKeever 2006). In the Eastern province of Zambia, thousands of cattle have been immunized using the local *T. parva* Katete stock while in Zimbabwe, the local *T. parva* Bolani stock has been used successfully both with and without oxytetracycline (Di Giulio *et al.* 2009; Uilenberg 1999). There is strong evidence that protection following vaccination is mediated by major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (McKeever *et al.* 1994; Morrison *et al.* 1987).

Vaccination has been used extensively to control anaplasmosis in most parts of the world and represents the most effective control measure for the disease (Kocan *et al.* 2000). Vaccination is achieved using killed or live vaccines and is directed towards preventing morbidity and mortality but does not prevent cattle from becoming infected upon challenge (Kocan *et al.* 2000; Shkap *et al.* 2008). Both live and killed vaccines are dependent on blood as a source of antigen. Infection of cattle with attenuated or less pathogenic *A. centrale* live vaccines has been most widely used for vaccination against anaplasmosis in South Africa, Israel and Australia (Dalgliesh *et al.* 1990; de Waal & Combrink 2006; De Wall 2000; Kocan *et al.* 2000). Immunised cattle develop sub-clinical infections and become persistent carriers, which results in protection against clinical anaplasmosis upon challenge with *A. marginale*. However, *A. centrale* does not provide effective cross-protection against *A. marginale* isolates from widely separated geographical areas (Brizuela *et al.* 1998). Additionally, attenuated live *A. marginale* vaccines have been used in South America and California (Henry *et al.* 1983; Kocan *et al.* 2000). These vaccines induce sub-clinical infection in calves which go on to develop immunity to clinical anaplasmosis (Henry *et al.* 1983). Tick cell culture-derived *A. marginale* antigens have been used to stimulate partial protection in a manner similar to erythrocyte-derived antigens (de la Fuente *et al.* 2002; Kocan *et al.* 2001). As with *A. centrale*, immunising with isolates of *A. marginale* may not be cross-protective in widely separated geographical areas. Attenuated vaccines also bear the risk of becoming virulent after successive passage through cattle by ticks (Kocan *et al.* 2000). A killed *A. marginale* vaccine was developed and marketed in the USA in the 1960s (Brock *et al.* 1965), although this has since been withdrawn and is currently unavailable. This vaccine was effective when used against local isolates but not against those from other areas. However, its utility was hampered by the

expensive purification process, the requirement of booster immunisation and difficulty with standardisation and quality control (Kocan *et al.* 2000).

Vaccination against heartwater involves infecting animals with cryo-preserved sheep blood containing virulent *E. ruminantium* organisms of the Ball 3 isolate, followed by antibiotic treatment when fever develops (Bezuidenhout 1989). This strain of the parasite does not provide broad protection against virulent field isolates and there has been a long-standing search for better and safer vaccines (Shkap *et al.* 2007). An attenuated vaccine was developed by passage of the South African virulent *E. ruminantium* Welgevonden isolate in a continuous canine macrophage-monocyte cell line (Collins *et al.* 2003). After more than 50 passages the cultures failed to induce disease when inoculated in mice or sheep and the immunised animals were solidly immune to subsequent lethal homologous needle challenge. In the Gambia, a trial was conducted to evaluate protection conferred to sheep against heartwater following immunisation with inactivated (Gardel stock) and attenuated (Senegal stock) *E. ruminantium* vaccines (Faburay *et al.* 2007). The local *E. ruminantium* (Kerr seringe) was used as the challenge material. The inactivated and live attenuated vaccines provided 43 % and 100 % protection, respectively against virulent challenge. In a subsequent field trial, the attenuated vaccine protected 75 % of sheep against virulent tick challenge that was fatal for all the control sheep (Faburay *et al.* 2007).

1.5.2.2 Subunit vaccines

Practical limitations imposed by using live parasites for immunisation have led to efforts to develop subunit vaccines based on defined parasite antigens. It is expected that the availability of complete genome sequences of *T. parva* (Gardner *et al.* 2005), *Ba. bovis* (Brayton *et al.* 2007), *A. marginale* (Brayton *et al.* 2005) and *E. ruminantium* (Collins *et al.* 2005) will accelerate the process of identifying candidate antigens.

The search for vaccine candidate antigens of *Babesia* parasites has focused mainly on merozoite surface antigens that are functionally relevant, immunodominant in natural immune cattle and are present among different parasite strains (Bock *et al.* 2004a; Brown *et al.* 2006). To date a number of candidate antigens have been identified (Brown *et al.* 2006). Such antigens include *Ba. bovis* merozoite surface antigen 1 (MSA-1) (Hines *et al.* 1995b), *Ba. bovis* merozoite surface antigen 2 (MSA-2) (Mosqueda *et al.* 2002; Wilkowsky *et al.* 2003), *Ba. bigemina* merozoite surface proteins gp45 and gp55 (Mcelwain *et al.* 1991), *Ba. bovis* and *Ba. bigemina* RAP-1 (Norimine *et al.* 2002; Wright *et al.* 1992), and the *Ba. bovis* and *Ba. bigemina* small heat shock protein (Hsp20) (Mosqueda *et al.* 2004; Norimine *et al.* 2004).

Immunisation of cattle with recombinant MSA-1 induced antibodies that were capable of neutralising invasion of erythrocytes, however the immunised cattle were not protected against virulent *Ba. bovis* challenge (Hines *et al.* 1995b). MSA-2 proteins have been shown to be expressed on the surface of both merozoites and sporozoites, and specific antibodies to MSA-2 block initial binding and subsequent erythrocyte invasion by the parasite (Mosqueda *et al.* 2002). Immunisation of cattle with native *Ba. bigemina* gp45 proteins also induced partial protection against challenge (Mcelwain *et al.* 1991). However, transcription of this gene has only been identified in a Mexican strain. The gene is absent in the Puerto Rico and St. Croix strains and is not transcribed in the Texcoco *Ba. bigemina* strain (Carcy *et al.* 2006; Fisher *et al.* 2001). Due to its abscence in some of the strains, this antigen is not an ideal candidate for vaccine development. RAP-1 is a 60 kDa antigen of *Babesia* that is recognised by antibodies and T cells from naturally immune cattle (Norimine *et al.* 2002; Rodriguez *et al.* 1996). Native RAP-1 protein isolated from parasite preparations and recombinant *Ba. bovis* RAP-1 have been shown to induce partial protection against homologous challenge (Wright *et al.* 1992). Native *Ba. bigemina* RAP-1a protein conferred partial protection, defined by reduction in parasitaemia following challenge with a homologous *Ba. bigemina* strain (Brown *et al.* 1998; Mcelwain *et al.* 1991). Hsp20 is expressed in sporozoites and merozoites of both *Ba. bovis* and *Ba. bigemina* (Mosqueda *et al.* 2004; Norimine *et al.* 2004; Vichido *et al.* 2008). Examination of *Ba. bovis* Hsp20 revealed that *Ba. bovis* and *Ba. bigemina* share at least one epitope that was conserved among all *Ba. bovis* strains tested, identified by a mono-specific mouse serum and monoclonal antibody (Brown *et al.* 2001). However, only one of three post-infection sera recognised the 20 kDa native *Ba. bovis* or recombinant Hsp20 protein on immunoblot analysis, indicating that Hsp20 is not serologically immunodominant during infection. T cell epitopes were also identified in Hsp20 following recovery from infection or immunisation with recombinant Hsp20 (Norimine *et al.* 2004). $CD4^+$ T cells from cattle that recovered from infection or immunisation with recombinant Hsp20 plus IL-12 produced high amounts of IFN-γ *ex vivo* when stimulated with *Ba. bovis* antigens (Brown *et al.* 2001; Norimine *et al.* 2004).

The host responses to *T. parva* infection have been subject to detailed investigations that have yielded information on the nature and specificity of protective responses, and provided immunological tools that have been recently used to identify candidate antigens for vaccination (Morrison & McKeever 2006). Current efforts to develop a subunit vaccine against *T. parva* have targeted both sporozoite and schizont antigens. One sporozoite surface antigen (p67) (Musoke *et al.* 2005), 6 schizont antigens (Graham *et al.* 2006) and

the polymorphic immunodominant molecule (PIM), expressed by both the sporozoite and schizont stages of *T. parva* (Bishop *et al.* 2005), have been identified as possible vaccine antigens. In a two site trial in Kenya, Musoke *et al*. (2005) reported a 47 % and a 52 % reduction in the severity of ECF in the two locations (coastal region and Central Kenya respectively) following vaccination of cattle with a recombinant sporozoite surface antigen p67 or an 80 amino acid terminal portion of the molecule (p67C). The ability of 5 of the 6 *T. parva* schizont antigens to induce protective immune responses was tested in experiments involving the use of the prime-boost protocols, priming with plasmid DNA or recombinant canarypox virus followed by a single boost with recombinant vaccinia virus (Graham *et al.* 2006). A number of naïve cattle immunised with these antigens showed cytotoxic T lymphocyte (CTL) responses that significantly correlated with survival from a lethal parasite challenge. Additionally, marginal T helper responses were detected in 39 % of the vaccinated animals although these did not correlate with the presence of CTL (Graham *et al.* 2006). Recently Ververken *et al*. (2008) demonstrated that immunisation of cattle with recombinant PIM alone induced antibody and $CD4^+$ T cell responses in addition to inducing $CD8^+$ cytotoxic T cells specific for *T. parva* in the absence of other CTL dominant *T. parva* antigens. Of the two calves that were immunised with this recombinant antigen, the calf that showed a CTL response survived a lethal challenge while the one that did not and the control calf died.

Recent studies have provided details about the nature of the immune response of cattle to *A. marginale*, as well as definition of key antigens that appear to play a role in the immune response (Kocan *et al.* 2003; Palmer *et al.* 1989). Efforts to develop a subunit vaccine for anaplasmosis has mainly focused on the proteins found on the outer parasite membrane (Kocan *et al.* 2003; Palmer *et al.* 1999). In the outer membrane of *A. marginale*, six major surface proteins (MSPs) were initially characterised: MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 (Barbet *et al.* 1987; Kocan *et al.* 2003; Oberle *et al.* 1988; Palmer & McGuire 1984). Cattle immunised with initial bodies or their outer membranes showed a significantly lower reduction in packed cell volume (PCV) and lower rickettsaemia after challenge with virulent *A. marginale*, as compared to non-immunised animals (Montenegro-James *et al.* 1991; Rodriguez *et al.* 2000; Tebele *et al.* 1991). However immunisation of cattle with recombinant MSPs, individually or collectively, has not been able to recreate the immunoprotective capacity of the whole outer membranes (Brayton *et al.* 2006). Recently a number of novel outer membrane proteins (omps) have been described (Brayton *et al.* 2005; Lopez *et al.* 2005; Noh *et al.* 2008) and immunisation of cattle with *A. marginale* surface protein complexes was able to equal the protective

immunity induced by whole outer membrane proteins in terms of reduction in acute bacteremia and anaemia (Noh *et al.* 2008).

Efforts to develop a recombinant vaccine against heartwater have been focussed on the outer membrane proteins (Mahan *et al.* 1999). The *E. ruminantium* immunodominant major antigen protein 1 (MAP1) has been found to be immunoprotective in a mouse model system when expressed using a DNA vaccine strategy, against challenge with a lethal dose of *E. ruminantium* (Nyika *et al.* 1998). In a subsequent study, Nyika *et al*. (2002) found that boosting DNA vaccine-primed mice with recombinant MAP1 protein significantly augmented protection on homologous challenge in various trials from 13 – 27 % to 53 – 67 %. Pretorius *et al*. (2002) immunised mice against heartwater using a DNA vaccine based upon groES and groEL antigens. Western blot analysis using sera from infected mice indicated that both GroEL and GroES proteins were expressed, however none of the mice vaccinated with the GroEL DNA vaccine survived challenge and only 10 % of those vaccinated with the GroES DNA vaccine survived a lethal challenge (Pretorius *et al.* 2002). A cocktail of four *E. ruminantium* genes cloned into a vaccine vector and used to immunise sheep engendered 100 % protection against subsequent needle challenge with a homologous isolate and another 5 heterologous isolates. However, when sheep immunised with this cocktail were exposed to field challenge in a heartwater endemic area, only a few animals survived (Collins *et al.* 2003). This experiment was repeated with an alternative strategy of using a prime-boost system, priming with a DNA vaccine and boosting with recombinant protein (Pretorius *et al.* 2008). Improved cellular immunity was indicated by increased INF-γ production, compared to sheep immunised with DNA vaccine alone. Animals immunised using this prime-boost strategy were immune to needle-derived challenge but not against field challenge (Pretorius *et al.* 2008).

1.5.3 Chemotherapy and chemoprophylaxis

For many years three babesiacides, quinuronuim sulphate (Ludobal®, Bayer Ltd), amicarbalide isothionate (Diampron®, May and Baker Ltd) and diminazine aceturate (Berenil®, Hoechst Ltd) were available in many countries for the treatment of bovine babesiosis (Vial & Gorenflot 2006). In the 1970s, a fourth drug, imidocarb dipropionate was introduced (Imizol®; Schering-Plough), and it rapidly became the drug of choice for the treatment of babesiosis (McHardy & Simpson 1974; Vial & Gorenflot 2006). Quinuronium and amicarbalide were withdrawn because of safety issues and diminazine, which is widely used in the tropics as both a babesiacide and a trypanocide, was withdrawn in Europe for marketing reasons (Vial & Gorenflot 2006). Imidocarb dipropionate is the

only babesiacide that consistently clears the host of parasites when used at dosages above 1 mg/kg (Lewis *et al.* 1981; McHardy & Simpson 1974). Treatment of *Ba. bigemina* infections with imidocarb can completely cure the animal of the infection but leave it susceptible to re-infection (McHardy & Simpson 1974; Vial & Gorenflot 2006). It can also induce complete clearance of *Ba. bovis*, but in some cases a second dose or a slightly increased dose may be desirable. Imidocarb is an effective chemoprophylactic that will prevent clinical infection for up to two months, but allows mild sub-clinical infection to occur as the blood drug levels decrease, resulting in an element of chemotherapeutic protection against disease while immunity develops (Kuttler 1975; Kuttler & Johnson 1986). Imidocarb has also been reported to affect the *Babesia* parasites in the tick vector, e.g. *Ba. Bovis*-infected *Bo. annulatus* ticks apparently lost their infectivity when placed on animals recently treated with imidocarb as their progeny failed to transmit the infection (Kuttler 1975). In contrast, in a similar experiment with *Ba. bigemina* in *B. decoloratus*, ticks remained infected following imidocarb treatment (Gray & Potgieter 1981). Oxytetracycline does not have a good chemotherapeutic activity against babesiosis. However when given 1 to 2 days before exposure, the drug has been reported to lengthen the incubation period of *Ba. bigemina* (Kuttler & Johnson 1986). The chemotherapeutic activity of diminazine is less than that of imidocarb although both drugs have an inhibitory effect on frozen *Ba. bovis* and *Ba. bigemina* attenuated vaccines (Combrink *et al.* 2002; Kuttler & Johnson 1986).

Four chemical products are known to have an effect against *Theileria parva* infections: tetracyclines, halofuginone, parvaquone and buparvaquone. The effect of halofuginone is unreliable during the incubation period of the disease, as it is active only against the schizont stage of the parasite (Peregrine 1994) and moderate to severe recrudescence has been reported following treatment of ECF with this drug (Kiltz & Humke 1986; Morgan & McHardy 1982). Tetracyclines have a very limited effect when used to treat clinical ECF (Peregrine 1994) and are mostly used as prophylactics in the treatment and infection method (Radley *et al.* 1975a). Parvaquone and buparvaquone are hydroxynaphthaquinone derivatives with improved anti-theilerial effectiveness compared to halofuginone based on recovery rate, recrudescence of infections and therapeutic indices (McHardy *et al.* 1983; McHardy *et al.* 1985; Morgan & McHardy 1982). Parvaquone is marketed as Clexon or Parvexon, buparvaquone is marketed as Butalex and halofuginone as Terit (D'Haese *et al.* 1999). Buparvaquone is reported to have a greater anti-theilerial activity than parvaquone (McHardy *et al.* 1985) although in a more recent study, Muraguri *et al.* (1999) did not find any significant difference in efficacy between the two drugs. Treating ECF using a

combination of parvaquone and frusemide (a diuretic drug) has been found to greatly improve recovery in ECF cases which manifest pulmonary signs (Mbwambo *et al.* 2002; Musoke *et al.* 2004). Buparvaquone has also been found to have prophylactic activity against both *T. parva* and *T. annulata* in cattle for five days when given at a dose of 5 mg/kg (Wilkie *et al.* 1998). Although buparvaquone and parvaquone can effectively reduce losses associated with clinical ECF, their high cost prohibits prompt and wide-scale use among small-scale and resource-poor farmers (D'Haese *et al.* 1999; Muraguri *et al.* 1999).

Drugs that are effective in the treatment of bovine anaplasmosis are imidocarb dipropionate, tetracyclines and enrofloxacin (Coetzee *et al.* 2006; Guglielmone *et al.* 1996; Kocan *et al.* 2000). Imidocarb is effective in the treatment of anaplasmosis at a dosage of 3 mg/kg (McHardy & Simpson 1974). However, this dose level results in the sterilisation of *Babesia* infections and possibly renders animals susceptible to babesiosis on re-exposure (De Wall 2000). Cattle treated with imidocarb, even at higher dosage, are not cleared of *Anaplasma* infections and may remain persistently infected (Adams & Todorovic 1974; McHardy & Simpson 1974). Oxytetracycline and doxycycline are effective in the treatment of acute anaplasmosis provided treatment is initiated early in the course of the disease (Kuttler & Simpson 1978). A number of authors reported elimination of *A. marginale* from carrier animals using oxytetracycline (Magonigle *et al.* 1975; Roby *et al.* 1978; Swift & Thomas 1983). However, Coetzee *et al*. (2005 and 2006) were not able to clear *A. marginale* infections from cattle using different oxytetracycline treatment regimes. Enrofloxacin is the newest drug to be found effective in the treatment of severe anaplasmosis (Coetzee & Apley 2006; Guglielmone *et al.* 1996). However this drug is also not able to eliminate *A. marginale* infections in cattle (Coetzee *et al.* 2006; Coetzee & Apley 2006). Tetracyclines are the only drugs currently available for the treatment of heartwater (Peregrine 1994).

1.6 Diagnosis of tick-borne diseases

1.6.1 Microscopic examination

Available methods for the diagnosis of TBDs include blood smear examination, serological tests and DNA-based assays. A comparison of these methods in terms of their sensitivity, specificity, costs and throughput is shown in Table 1.1. Blood smear examination is often considered to be the standard technique for routine diagnosis of TBDs. It is less time consuming than most other methods and is relatively inexpensive (Salih *et al.* 2007). The drawbacks of this method are that the accuracy of diagnosis relies on the training and

Table 1.1 *Comparison* of methods for detection of TBD pathogens of cattle

Table shows comparison of the sensitivity, specificity, cost and throughput of the methods used in the diagnosis of TBDs of cattle. + = very low, ++ = low, +++ = high and ++++ = very high.

Table 1.1 Comparison of methods for detection of TBD pathogens of cattle

Method	Sensitivity	Specificity	Cost	Throughput
PCR	++++	++++	++++	++++
Blood smear	+	+	+	++
IFAT	++	++	++	++
ELISA	+++	++	++	+++

experience of the laboratory personnel, and it is considered to have low sensitivity and specificity. Studies of infection rates of *Babesia* parasites in the haemolymph of *Boophilus* ticks has shown that lack of standardisation in the amount of haemolymph used to produce each smear can result in smears of different thickness and this can affect the sensitivity of the assay (Quintao-Silva *et al.* 2007). Since the amount of blood used to prepare a smear in most cases is not standardised, this observation is also relevant to microscopic examination of blood smears. Another problem of microscopic diagnosis is that differentiation of parasite species based solely on morphology is difficult, and confusion may arise if mixed species infections occur (Almeria *et al.* 2001).

1.6.2 Serological methods

Many serological methods standardised for the diagnosis of TBDs have been employed in epidemiological field studies (Billiouw *et al.* 1999; Jongejan *et al.* 1988; Swai *et al.* 2005). Serological tests have a number of limitations that are common to all assays based on the detection of antibodies, i.e. issues of stability, sensitivity, specificity and the objectivity of reading the results. Some serological techniques may be too cumbersome for testing large numbers of samples, e.g. fluorescent antibody test (IFAT). They are often unable to differentiate recovered animals with sterile immunity, carrier animals and clinical cases (Bose *et al.* 1995; Goff *et al.* 2008). In addition, antibody cross-reactions have been reported among closely related parasitic species (Edelhofer *et al.* 2004; Salih *et al.* 2007). Serological tests are also unable to detect some chronically infected animals (De Wall 2000). Incorrect interpretation of test results may result in inappropriate control measures being indicated. Some serological diagnostic techniques like the indirect fluorescent-antibody test (IFAT) are tedious, subjective and of low throughput (Kiltz *et al.* 1986; Salih *et al.* 2007). Crude parasite preparations are often used as a source of antigen leading to lack of reproducibility. Because most tick-borne parasites develop almost exclusively inside erythrocytes, such preparations frequently contain considerable amounts of host cell contaminants that reduce the performance of the assay and increase the non-specific background (Bose *et al.* 1995). To overcome this problem, recombinant antigen preparations that are free from host proteins have been used (Katende *et al.* 1998; McGuire *et al.* 1991). These antigens are relatively cheap to produce and batch-to-batch variations are minimal, however some recombinant antigens suffer from the drawback of being strain-specific (Bose *et al.* 1995).

1.6.3 DNA based methods

The application of DNA-based tests to study the epidemiology and diagnosis of TBDs is still incipient, but the desirable characteristics of high sensitivity and specificity have been verified by several authors (Figueroa *et al.* 1993a; Figueroa *et al.* 1993b; Salem *et al.* 1999). Available DNA-based techniques for the detection of TBDs include the final time PCR methods (which include standard PCR, Southern blotting and reverse line blot (RLB)), real time PCR (qPCR) and the isothermal amplification methods (Criado-Fornelio 2007). The cost of DNA-based diagnostic techniques are relatively high when compared to serological and microscopic methods. This makes the wide-scale use of these methods among the resource-poor veterinary services not a viable prospect. Any efforts aimed at finding ways of reducing these costs will aid in their wide-scale use in resource-poor countries.

1.6.3.1 LAMP

Among the isothermal amplification methods, only loop-mediated isothermal amplification (LAMP) has been used in the diagnosis of TBDs (Guan *et al.* 2008; Iseki *et al.* 2007; Salih *et al.* 2008b). LAMP is a novel DNA amplification method, which employs continuous strand-displacement DNA synthesis primed by a specially designed set of target-specific primers (Notomi *et al.* 2000). The basic LAMP method uses the *Bst* DNA polymerase and two primer pairs. One of the characteristics of LAMP is its ability to synthesis an extremely large amount of DNA. Accordingly, a large amount of by-product, the pyrophosphate ion, is produced, yielding a white precipitate of magnesium pyrophosphate in the reaction mixture. The presence of this white precipitate allows easy detection of nucleic acid amplified by the LAMP method. Alternatively, gel electrophoresis may be used to visualise amplified DNA (Criado-Fornelio 2007; Notomi *et al.* 2000). The sensitivity of LAMP has been reported to be higher than that of conventional nested PCR for detection of *Ba. bovis* and *Ba. bigemina* (Iseki *et al.* 2007). The sensitivity of LAMP has also been reported to be higher than that of conventional PCR, but equal to that of RLB, in the detection of *T. annulata* in Sudan (Salih *et al.* 2008b). However, it should be noted that the Sudanese experiment did not use a nested PCR strategy. Another advantage of the LAMP is that it does not require a PCR machine as amplification is undertaken at a single temperature, further simplifying equipment needs.

1.6.3.2 Real time PCR

Real time PCR (qPCR) is based on the detection of fluorescence produced by a reporter molecule which increases as the reaction proceeds. This occurs due to the accumulation of the PCR product with each amplification cycle. These fluorescent reporter molecules include dyes that bind to double stranded DNA (e.g. SYBR® Green) or sequence-specific probes (e.g. Molecular beacons or FRET® and TaqMan® probes). qPCR facilitates the monitoring of the reaction as it progresses. Since no post-PCR processing is required, both resource and operator time are saved. Additionally, due to the fact that automated fluorescence reading provides monitoring of the presence of PCR products, there is no need to open the reaction tubes, thus avoiding contamination with previously amplified products. A number of qPCR assays for the detection of TBDs with sensitivities comparable to conventional nested PCR have been developed and evaluated (Carelli *et al.* 2007; Kim *et al.* 2007; Sibeko *et al.* 2008). Despite these advantages, the use of qPCR on a wide scale is limited due to the high cost of equipment (Criado-Fornelio 2007).

1.6.3.3 Reverse line blotting

The reverse line blot (RLB) assay combines PCR with hybridisation of amplified products to one or several species-specific oligonucleotide probes bound to a support membrane. Compared to a standard Southern blot, the probe is not in solution but linked to a membrane (Tait & Oura 2004). An important advantage of this technique is that several probes are usually combined so that well-characterised species can be discriminated and identified, while related genotypes distinct from reference strains, within a pathogen species can still be detected (Criado-Fornelio 2007). This method has been used in the study of TBD pathogens derived from both ticks and cattle hosts since the 1990s (Bekker *et al.* 2002; Gubbels *et al.* 1999; Oura *et al.* 2004b). The main drawback with the RLB technique is that it requires specialised equipment and the production of membranes with immobilised oligonucleotides that limits its use in developing countries.

1.6.3.4 Standard PCR

Most of the DNA based methods employed in the diagnosis of TBDs are based on standard PCR techniques. An extensive review of the PCR protocols that have been developed and used in the study of TBDs was provided by Lew and Jongensen (2005) and Criado-Fornelio (2007). These reviews show that PCR diagnosis of TBDs has been based mainly on the amplification of fragments of the 18S and 16S rRNA gene from piroplasm and rickettsial parasites respectively. The sensitivity of a PCR assay can be increased several fold by performing a nested PCR, where two sets of amplification primers are used (Haqqi

et al. 1988). One set of primers is used for the first round of amplification, then sites located within the first product are subjected to a second round of amplification with another set of primers that are specific for an internal sequence amplified by the first primer set. This process not only improves the sensitivity of the assay, but also confirms the specificity of the first round product (Tang *et al.* 1997). Unlike serological tests, PCR is sensitive, specific and is able to detect current infections. Another advantage of PCR is that assays which are able to detect more than one tick-borne pathogen simultaneously can be developed (Bekker *et al.* 2002; Figueroa *et al.* 1993b). Such tests have the advantage in that they not only reduce the cost and time of performing the assay, but also allow study of the epidemiology of several TBDs that can occur concurrently in the same animal.

1.7 Population genetics

1.7.1 Introduction

Sexual recombination occurring in natural parasite populations plays a significant role in generating genetic diversity. Understanding the levels of genetic diversity in parasite populations is important in the application of chemotherapy and/or vaccine-based disease control strategies. *Babesia bovis* and *Ba. bigemina* populations have previously been studied using PCR-based amplification of antigen genes. Such studies have been mostly directed towards analysing the nature of polymorphism at a single locus and relating it to the structure and function of the encoded product (Fisher *et al.* 2001; Suarez *et al.* 2000; Wilkowsky *et al.* 2008). Though studying variation in parasite populations using antigen genes (which are potentially under selective pressure) may be of practical importance in development of subunit vaccines, such studies provide little information about the genome-wide variation in the natural parasite populations, which are better explored using multi-locus based typing schemes. To date, no studies have been undertaken to investigate the population structure of *Ba. bovis* and *Ba. bigemina*. Moreover, many of the population studies based on antigen encoding loci were focussed on developing molecular markers for investigating vaccine breakthroughs, following vaccination with attenuated *Ba. bovis* and *Ba. bigemina* vaccines (Berens *et al.* 2005; Dalrymple 1993; Lew *et al.* 1997a).

1.7.2 Studies in bovine *Babesia* parasites

Three different PCR assays were developed and compared for their ability to differentiate ten independent Australian *Ba. bovis* attenuated vaccine lines and cryo-preserved field isolates (Lew *et al.* 1997b). These comprised the BvVA1 gene PCR, the Bv80 gene PCR and random amplification of polymorphic DNA (RAPD) PCR. All three methods revealed

considerable genetic diversity among the Australian *Ba. bovis* isolates. The ability of the BvVA1 and BV80 PCR amplification to discriminate different parasite stocks was greatly increased when used in combination with Southern blot analysis of the BvVa1 repeat region. The BvVA1 PCR amplification was able to discriminate isolates that could not be differentiated using the Bv80 assay alone. Two isolates which could not be discriminated using the BvVA1 amplification were differentiated by Bv80 amplification profile or the BvVA1 Southern blot assay. The RAPD method was able to discriminate each of the isolates, though interpretation of the results from this analysis was often difficult in comparison to the Bv80 and the BvVA1 PCR assays.

A *Bsp*MI-based PCR restriction analysis assay (PRA)(more commonly called PCR-RFLP) has also been used as a molecular marker system for *Ba. bovis* (Wilkowsky *et al.* 2008). This assay is based on alleles of the merozoite surface antigen (msa-2a and -2b) that belong to a gene family encoding surface-exposed antigens. A variable degree of polymorphism between isolates was shown among five Argentinian, one USA and four Mexican isolates used in the study (Berens *et al.* 2005). The msa-2a/b PRA was able to discriminate isolates from different countries but also isolates from the same region of Argentina (Wilkowsky *et al.* 2008). In another study, phylogenetic analysis of the msa and ssrRNA gene sequences also showed that the Mexican *Ba. bovis* isolates clustered separately from the Australian ones, although not according to regions of origin within the country (Genis *et al.* 2008).

A molecular epidemiological study of *Babesia bigemina* isolates from five geographical regions of Brazil was performed using random amplification of polymorphic DNA (RAPD), repetitive extra-genic palindromic elements-polymerase chain reaction (REP-PCR), and enterobacterial repetitive intra-genic consensus sequences-polymerase chain reaction (ERIC-PCR) (Madruga *et al.* 2002). A dendrogram constructed using similarity coefficients among isolates showed two main clusters one of which incorporated a sub-cluster. The Western and mid-Western isolates showed the greatest genetic diversity, while the South-Eastern and Southern isolates showed the least diversity. These differences were attributed to cattle breed differences, ecological conditions, management and distinct *Boophilus microplus* populations.

While RAPD, PRA and the other methods described above are able to discriminate between different *Babesia* genotypes, they have a number of shortcomings that make their use difficult. The fingerprints generated by RAPD and PRA are sometimes difficult to interpret and may not be reproducible. Banding patterns can be further complicated by the

presence of more than one genotype and this renders interpretation difficult (Lew *et al.* 1997b). The Bv80 and BvVA1 gene PCR assays described by Lew *et al*. (1997b) were easier to interpret than the RAPD method. The Bv80 PCR assay had less discriminatory power than the BvVA1 assays. However, the BvVA1 PCR, although the more sensitive of the two, requires standardisation of input DNA, which may not be possible when working with field samples. In all the studies described above, no population genetic analyses of *Ba. bovis* or *Ba. bigemina* populations were undertaken, perhaps in part due to the small sample sizes used in some studies and difficulties in providing a genetic interpretation of data generated by the assays. The primary significance of the results from the studies described above is the confirmation that genetic polymorphism exists in *Ba. bigemina* and *Ba. bovis*. However, the basis for this polymorphism, the role of genetic exchange and the impact of diversity on disease epidemiology and control was not investigated. To perform such important studies, more suitable molecular tools and assays require to be developed.

1.7.3 Studies in other bovine apicomplexan parasites

Population analysis of *T. annulata* has been carried out using a panel of ten micro- and mini-satellite markers (Weir *et al.* 2007). Cloned and uncloned isolates of *T. annulata* maintained as cell lines, from Turkey, Tunisia and Sudan were PCR amplified using these markers. PCR products were separated using capillary electrophoresis and the DNA fragment sizes were determined relative to a ROX-labelled size standard that allows resolution of up to 1 bp. The predominant allele for each sample was used to create multi-locus genotypes (MLG), which were then used for population genetic analysis. *T. annulata* has a haploid genome and this was confirmed by the cloned isolates giving a single allele at each locus, while uncloned isolates comprised a multiplicity of genotypes, consistent with the findings of previous studies (Ben Miled *et al.* 1994; Shiels *et al.* 1986). High levels of genetic diversity were found both within and between countries, with estimated heterozygosities ranging from 0.86 within central Tunusia to 0.93 in south-Western Turkey. Moderate genetic differentiation, based on F_{ST} values and Nei's genetic distance were obtained between the three countries. Lower F_{ST} values were obtained when Tunisian and Turkish samples were analysed separately, and the observed genetic differentiation positively correlated with geographical distance between sampling sites. In order to determine whether genetic exchange was occurring in these populations, the level of linkage disequilibrium between pairs of loci was measured using the standard index of association (I^S_A). Pooling all the samples from the three countries resulted in I^S_A value significantly greater than zero, indicating linkage disequilibrium. Values of I^S_A not significantly greater than zero were obtained when Turkish and Tunisian population were

analysed separately. These results indicate that the isolates from the three countries are geographically sub-structured with limited or no genetic exchange among them. At the same time, the study shows that genetic exchange does occur in parasite populations within each country (Turkey and Tunisia), although the number of Turkish samples was too low for a firm conclusion to be drawn.

Studies of *T. parva* have mainly been focused on identifying diversity in field isolates, but after the publication of the genome sequence, microsatellite markers were developed enabling the analysis of the population genetics of this species. A molecular epidemiological study of *T. parva* was conducted using a combination of RFLP techniques (Geysen *et al.* 1999). Using Southern blots with restriction digested DNA (RFLP-DNA) probed with '*Tpr'* (*Theileria parva* repeat) locus and telomere probes, Zambian isolates were shown to be relatively homogenous, contrasting with the high level of heterogeneity observed in a previous Kenyan study (Bishop *et al.* 1997; Conrad *et al.* 1987). RFLP-PCR for the three loci (*PIM, p104* and *p150*) revealed that *PIM* was the most polymorphic and could be used to differentiate isolates from two geographical areas in Zambia; *p104* and *p15*0 were monomorphic for these isolates. Additionally the results indicate a homogenous, epidemic structure where clonal expansion of one of the introduced vaccine components dominated the population.

Following the publication of the *T. parva* genome sequence (Gardner *et al.* 2005), a panel of 11 micro- and 49 mini-satellite polymorphic markers was identified (Oura *et al.* 2003). Subsets of these markers have subsequently been used to analyse the population structure of *T. parva* in Kenya and Uganda. In Uganda (Oura *et al.* 2005), three geographically separate populations (Lira, Mbarara and Kayunga) were sampled. To overcome the problem of a high proportion of samples containing multiple genotypes, potentially making it impossible to determine the predominant genotype directly, samples were taken solely from animals aged between 3 and 9 months because the majority of the samples contained a single predominant allele at each locus. Samples taken from these areas were genotyped using twelve micro- and mini-satellite markers that are evenly dispersed across the four chromosomes. High levels of genetic diversity were observed within each of the three provinces with very few MLGs common among them. There was limited genetic differentiation between Lira and Mbarara populations. In contrast, moderate differentiation was observed between Lira and Kayunga populations, and between Mbarara and Kayunga. The Mbarara population showed evidence of sub-structuring with a sub-group of six MLGs that were very dissimilar to any of the other groups. When these six MLGs were omitted

from the analysis, little or no genetic differentiation was observed among the three populations. When the Lira, Mbarara and Kayunga populations were combined as one population, linkage disequilibrium (LD) was observed, which could be ascribed to geographical or genetic isolation. Interestingly, LD was still observed when each population was analysed separately. However, when the identical isolates from Lira were treated as one, LD disappeared, suggesting an epidemic structure in this group. Linkage equilibrium (LE) was also demonstrated in the Mbarara population when both the sub-group and the set of identical isolates were removed from the analysis. The Kayunga population remained in LD even after treating isolates with similar MLGs as one group, suggesting infrequent genetic exchange in this population. However, the importance of this study was the demonstration that genetic exchange was occurring at a significant level.

In the Kenyan study (Odongo *et al.* 2006), thirty six samples were genotyped using a panel of 30 micro-and mini-satellite markers (Oura *et al.* 2003). Similar to the Ugandan study (Oura *et al.* 2005), high genetic diversity was observed in the Kenyan *T. parva* populations. A similar lack of geographical sub-structuring as reported for Uganda was revealed. However, analysis of allele association at all pair-wise combinations of loci indicated significant LD both when all samples were considered as one population and when each area was analysed separately. These data suggest that the level of genetic exchange is limited in contrast with the results from the Lira and Mbarara populations in Uganda. Other possible cause of LD in this study could have been that the samples were isolated over a range of time points (1968 – 2005). Some samples were isolated during vaccine trials and the majority were cultured before genotyping. Thus either temporal sub-structuring or selection during *in vitro* expansion, could have, in part, accounted for the observed LD.

1.7.4 Evidence of genetic exchange and recombination in the vector

Population genetic analysis of *T. parva* (Oura *et al.* 2005) and *T. annulata* (Weir *et al.* 2007) have shown that genetic exchange occurs frequently. These studies provide strong evidence that a sexual stage does exist in the life-cycle of these parasite species as high levels of recombination between loci suggest frequent crossing-over during meiosis. More recently, micro- and mini-satellite genotyping of cloned *T. parva* parasite lines derived from mixed isolates after transmission through ticks has provided evidence for both recombination and allelic assortment. Analysis of loci encoding antigens recognised by cytotoxic T-cells has provided evidence for allelic assortment generating parasites with

different antigen repertoires, highlighting recombination as a mechanism for generating antigenic diversity (Katzer *et al.* 2006).

Morphological observation of different developmental stages and DNA measurement of *Ba. bigemina* in the gut of *Bo. microplus* ticks indicates that sexual reproduction, i.e. fusion of gametes resulting in the formation of zygotes followed by meiotic division, takes place in the tick (Gough *et al.* 1998; Mackenstedt *et al.* 1995). Additionally, sequence analysis of the msa-2 genes of *Ba. bovis* and the RAP-1 gene of *Ba. bigemina* suggests that genetic recombination may be the means by which variant antigen types are generated in these parasites (Berens *et al.* 2005; Hotzel *et al.* 1997). Berens *et al.* (2007) investigated whether recombination between virulent *Ba. bovis* strains contributed to variable merozoite surface antigen (VMSA) diversity. They co-infected cattle with two antigenically divergent strains of *Ba. bovis* which were then acquired by *Bo. microplus* ticks and transmitted to susceptible cattle. They found that both cattle and ticks could support virulent *Ba. bovis* co-infections through all phases of the parasite's life-cycle. However, recombination of the VMSA genes was not confirmed, suggesting that intragenic recombination of VMSA alleles may not be a frequent event (Berens *et al.* 2007) and may require selection. Moreover, testing for intragenic recombination at a single locus may not be an ideal approach.

1.8. Objectives of the work in this thesis

1.8.1 Objective I: Development of PCR for the diagnosis of tick-borne diseases

The high cost associated with PCR is the main reason preventing its wide-spread use in poorly resourced veterinary laboratories. Although a number of PCR assays are available for the diagnosis of TBDs in cattle, none of these is able to detect multiple species infections (more than 2) in one assay. Such a PCR assay would greatly reduce the costs associated with PCR and RLB and make it affordable for most laboratories in developing countries. The specific objective of this work was therefore to develop a sensitive and specific multiplex PCR assay, based on 18s and 16s rRNA genes that can diagnose *T. parva, T. taurotragi, T. mutans, Ba. bovis, Ba. bigemina, A. marginale* and *E. ruminantium* in a single assay.

1.8.2 Objective II: Investigation of the epidemiology of TBDs in Zambia

The Zambian environment provides a multi-tick species ecology where TBDs can co-infect cattle causing considerable losses to the livestock industry. However, information on the relative importance of these diseases across the country is either inadequate or entirely absent, and this has had a negative impact on the design and implementation of diseases control programs. The aim of this work was therefore to use the PCR assay described in section 1.8.1 to undertake a detailed analysis of the epidemiology of TBDs (theileriosis, babesiosis and anaplasmosis) in Eastern and Central Zambia. Specifically, the work presented in this section was aimed at determining:

- The prevalence of *T. parva, T. mutans, T. taurotragi, Ba. bigemina*, Ba. bovis*, A. marginale* and *E. ruminantium* in Lusaka, Central and Eastern provinces of Zambia
- The impact of infection with these parasites across the three provinces using the reduction of PCV as a measure of impact
- Risk factors associated with infections of these parasites in cattle across the three provinces
- The interactions among these 7 parasites
- Tick burdens on cattle across the three provinces

1.8.3 Objective III: Determination of the underlying population structure of *Ba. bovis* and *Ba. bigemina*

As outlined in section 1.7.2, a number of studies have shown that *Ba. bovis* and *Ba. bigemina* parasites in several countries comprise highly genetically diverse populations. However, most of these studies were carried out using either very few isolates or with methods which made genetic interpretation of the data difficult. As a result, no population genetic analysis of these parasites has ever been undertaken. In order to gain insight into the population structure of these parasites in the field, a study employing appropriate polymorphic genetic markers was required. A panel of mini- and micro-satellite markers similar to those employed in *T. annulata* (Weir *et al.* 2007) and *T. parva* (Oura *et al.* 2005) populations, given the availability of the genome sequences for both parasites could be readily developed. Therefore, the objectives of the research presented in this section of the thesis were to:

- Develop two panels of genetic markers that are specific for multi-locus genotyping of *Ba. bovis* and *Ba. bigemina* isolates

- Determine the population structure of *Ba. bovis* in two geographically distinct countries: Zambia and Turkey

- Determine the population structure of *Ba. bigemina* in Eastern and Central Zambia

Specifically, the work set out in this section aims at answering the following questions for each parasite species:

- What is the underlying population structure with reference to sexual recombination?

- Is there geographical sub-structuring within (for both parasites species) and between countries (*Ba. bovis*)?

- Does host phenotype (region, age, sex, breed, management, province and PCV) influence genetic diversity?

- How does the population structure of the two *Babesia* parasite species compare in Zambia?

CHAPTER TWO

Development of PCR assays for the diagnosis of tick-borne diseases

2.1 Introduction

2.1.1 Background

Available methods for the diagnosis of TBDs include blood smear examination, serological tests and DNA-based assays. As described in Section 1.6, blood smear examination and serological tests have serious drawbacks,which may lead to misdiagnosis and the implementation of inappropriate disease control measures. The advent of the polymerase chain reaction (PCR) (Saiki *et al.* 1988) has allowed the development of sensitive and specific diagnostic assays for the detection of numerous pathogens. PCR-based techniques allow the detection of parasites at low parasitaemia while discriminating various species of co-infecting agents (Oura *et al.* 2004a; Shayan & Rahbari 2005). By including several pairs of primers, multiple target loci may amplify and this technique is termed multiplex PCR (Elnifro *et al.* 2000; Quintao-Silva *et al.* 2007).

Some of the successful applications of multiplex PCR include the differentiation of medically important *Candida* species in humans (Arancia *et al.* 2009), typing for virulence in *Pasteurella multocida (Atashpaz et al. 2009)*, and typing of bovine diarrheoa viruses in cattle (Gilbert *et al.* 1999). Despite its successful use in other fields, there currently are no optimised multiplex PCR protocols for the diagnosis of TBDs in cattle. The only such assay was described by Figueroa *et al.* (1993) for the detection of *Ba. bovis, Ba. bigemina* and *A. marginale* in latently infected cattle. However, this assay involved a hybridisation step and the hybridisation signal in the mixed-infection samples was weak and occasionally difficult to discern from the background. Multiplex PCR offers potential savings in cost, time and effort without compromising test utility and does not require the expensive equipment and membranes needed for RLB (Gubbels *et al.* 1999). The high cost associated with PCR is one of the reasons limiting its use in developing countries where TBDs of cattle are prevalent. Therefore, it would be highly desirable to combine the diagnostic tests for several pathogen species in a single multiplex PCR assay.

The most frequently used gene targets for the development of diagnostic PCR for tick-transimitted protozoan and rickettsial pathogens are the 18S and 16S rRNA genes

respectively (Criado-Fornelio 2007; Lew *et al.* 1997b). This is because of their ubiquity, suitable size and the availability of a very large set of sequences from many organisms, coupled with regions of low and high sequence divergence between species. (Van de & De Wachter 1997). Although the rickettsia are reported to only have one 16S rRNA gene per genome (Collins *et al.* 2005), the number of 18S rRNA genes in tick – transmitted protozoan parasites of cattle ranges between 2 and 4 copies per genome (Brayton *et al.* 2007; Criado *et al.* 2006). Another gene that has frequently been used in the development of diagnostic PCR of TBDs is cytochrome b. The cytochrome b gene shows regions of both high and low sequence divergence between species and is not subject to diversifying selection (Escalante *et al.* 1998). It is universally found in mitochondrial DNA and *Babesia* parasites are reported to have over 100 copies of this gene per genome (Salem *et al.* 1999). The low evolutionary rate of the rRNA and cytochrome b genes makes them ideal for the development of diagnostic PCR. The β-tubulin gene has also been previously used for species discrimination of parasitic species (Caccio *et al.* 2000; Zamoto *et al.* 2004). Zamoto *et al*. (2004) reported developing a diagnostic PCR assay for *Babesia* parasites that had similar discriminatory power to that of the rRNA-based PCR. Therefore this gene can be used as a good alternative for the development of a multiplex PCR.

2.1.2 Objectives

The objectives of the work described in this chapter were to:

- **Develop sensitive and specific PCR assays that can be used to diagnose seven TBDs of cattle, namely *T. parva*, *T. mutans*, *T. taurotragi*, *Ba. bigemina*, *Ba. bovis*, *A. marginale* and *E. ruminantium***

- **Compare the sensitivities of the PCR assays developed above for the diagnosis of *Ba. bovis* and *Ba. bigemina***

2.2 Material and Methods

2.2.1 Parasite material

The *Ba. bovis* DNA stocks used in the development of PCR assays were *Ba. bovis* Mexico (Erp *et al.* 1978; Smith *et al.* 1978), *Ba. bovis* Kwanyangwa (Taylor & McHardy 1979) and *Ba. bovis* Lismore (Kahl *et al.* 1982). The *Ba. bigemina* DNA stocks were *Ba. bigemina* Mexico (Vega *et al.* 1985), *Ba. bigemina* Muguga (Posnett *et al.* 1998) and *Ba. bigemina* Zaria (Leeflang & Ilemobade 1977). The *E. ruminantium* Welgevonden stock was from South Africa (Jongejan *et al.* 1991). All the above parasite DNA preparations

were provided by the Centre for Tropical Veterinary Medicine (CTVM) in Edinburgh where *Babesia* parasites were maintained as blood stabilates, cryopreserved in liquid nitrogen, and cultured *in vitro* on several occasions (Passos *et al.* 1998). *A. marginale* DNA was kindly provided by Katherine M. Kocan of Oklahoma State University, USA. The origin and source of the DNA stocks of *T. parva* Muguga, *T. parva* Marikabuni, *T. mutans* and *T. taurotragi* are described in Oura *et al*. (2003).

2.2.2 Cloning and sequencing of the *T. taurotragi* β-tubulin gene

A segment of the β-tubulin gene of *T. taurotragi* was PCR amplified using the primers and conditions described by Caccio *et al*. (2000). The resulting PCR product was separated by gel electrophoresis in TAE buffer (0.04M Tris, 0.4mM EDTA, pH adjusted to 7.7 - 8.8 with glacial acetic acid) on a 1.5 % agarose gel which was pre-stained with ethidium bromide for visualisation of amplicons. Amplicon sizes were determined relative to a 100 bp ladder. The cloning reaction for the obtained PCR product and transformation procedure were carried out according to the user manual for TOPO TA cloning® Kit. Briefly, 1 µl PCR product was gently mixed with the vector and incubated for five minutes at 23 ^{o}C. Then 2 µl of the mixture were added to chemically competent *E. coli* cells, mixed gently and incubated on ice for 30 minutes. The cells were transformed by heat shocking at 42 ^{o}C for 30 seconds. 250 µl of SOC media (20 g Bacto Tryptone, 5g Bacto Yeast Extract, 2ml of 5M NaCl, 2.5 ml of M KCl, 10 ml of 1M $MgCl_2$, 10ml of 1M $MgSO_4$, 20ml of 1M glucose in 1 litre of distilled water) was added and the reaction incubated at 37 ^{o}C in an orbital shaker at 200 rpm for 1 hour. Transformed bacteria (50 µl) were spread on selective plates containing 50 µg/ml ampicillin and incubated at 37 ºC overnight. Selected colonies were grown in Luria Bertani broth (10g Tryptone, 5g Yeast extract and 5g Sodium chloride in 1 litre of distilled water; pH 7.2) containing ampicillin overnight, before plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. Plasmid DNA was quantified using a spectrophotometer at 260 nm wavelength, and 2 µg was sent in duplicate, for sequencing by Eurofins MWG Operon under a commercial contract.

2.2.3 Sequence alignment

Genbank accession numbers for the 18S rRNA gene sequences used in the primer design were LO2366 for *T. parva*, L19082 for *T. taurotragi*, AFO78815 for *T. mutans*, L31922 for *Ba. bovis* and X59604 for *Ba. bigemina.* 18S rRNA gene sequences for *T. annulata* (FJ426369) and *Ba. divergens* (EU182594) were also included in the sequence alignment to ensure that the designed primers could not amplify these parasite DNA. The accession

number for the 16S rRNA gene sequences were M60313.1 for *A. marginale* and X62432.1 for *E. ruminantium*. The accession numbers for the β-tubulin gene sequences were AJ28924 for *Ba. bovis*, DQ104522 for *Ba. bigemina* and XM759937 for *T. parva*. The β-tubulin gene sequence for *T. taurotragi* was derived as outlined above (Section 2.2.2). The accession number for the gene sequence used in the development of the cytochrome b gene PCR was AF109354 for *Ba. bigemina,* and the sequence of *Ba. bovis* cytochrome b gene was obtained from GeneDB. Sequences for each gene were aligned using Clustal X software (Thompson *et al.* 1997). The alignments enabled the design of species-specific PCR primers for each parasite allowing amplification of different sized fragments from each species that could be differentiated following agarose gel electrophoresis.

2.2.4 PCR

For each reaction, PCR was carried out in a total volume of 20 µl. For all the primary reactions 1 µM of each primer, 1 µM custom PCR master mix (Thermo Scientific), 2 µl DNA template and 1 U *Taq polymerase* (Applied Biosystems) were used. The thermocycler conditions for the β-tubulin gene external PCR were: 94 °C for 4 minutes, 30 cycles of 94 °C for 50 seconds, 55 °C for 30 seconds, 72 °C for 1 minute and a final extension period at 72 °C for 5 minutes. Those for the cytochrome b PCR assay were similar to the β-tubulin conditions except the annealing temperature was 49 °C. The thermocycler conditions for the 18S/16S rRNA gene PCR were as described previously (Bekker *et al.* 2002).

The PCR mixture for the semi-nested 18S/16S rRNA and the nested cytochrome b genes species-specific reaction were similar to the primary one, except that 2 µl of a 1:100 dilution of the primary PCR product was used as template. In the 18S/16S rRNA gene PCR reaction, the same universal forward primer was used for primary and secondary reactions while the reverse primers were species-specific. The semi-nested species-specific β-tubulin multiplex PCR incorporated 2 µl of a 1:100 dilution of the primary PCR product, 1 µM each of the *T. parva* and *Ba. bovis* species-specific primers and the universal reverse primer, 0.15 µM of the *Ba. bigemina* species-specific primer, 1 µM custom PCR mix and 1 U *Taq* polymerase. The thermocycler conditions for 18S/16S rRNA gene PCR were: 94 °C for 4 minutes, 30 cycles of 94 °C for 50 seconds, 68 °C for 30 seconds, 72 °C for 1 minute and a final extension period of 72 °C for 5 minutes. The thermocycler conditions for the β-tubulin and cytochrome b gene PCR were similar to those of the 18S/16S RNA gene except the annealing temperatures were set at 58 °C and 55 °C respectively. The PCR

products were separated on 2 % agarose gels as described in Section 2.2.2. Amplicon sizes were determined relative to the 100 bp ladder.

2.2.5 Determination of the sensitivity of the PCR assays

To determine the sensitivity of the *Ba. bigemina* and *Ba. bovis* PCR assays serial dilutions (1 %, 0.1 %, 0.05 %, 0.01 %, 0.005 %, 0.001 %, 0.0005 % and 0.0001 % parasitaemia), generated from infected blood of known parasitaemia diluted in phosphate buffered saline (PBS), were spotted on Flinders Technology Associates (FTA) filters (Rajendram *et al.* 2006). These filters were kindly provided by Dr Shkap (Kimron Veterinary Institute, Israel). To provide template for PCR amplification, a disc of 2 mm in diameter was cut from the FTA blood spot using a clean Harris Micro-punch™ (Whatman) and placed in an Eppendorf tube. The discs were washed three times with 200 µl of FTA purification reagent (Whatman) and twice with 200 µl of 10 mM Tris, 1 mM EDTA (pH 8.0) buffer for five minutes at room temperature, as per the manufacturer's instructions. The discs were then left to dry at room temperature before use in PCR.

2.3 Results

2.3.1 Development of semi-nested PCR for 18S rRNA gene of *Theileria/Babesia* and 16S rRNA gene of *Ehrlichia/Anaplasma*

2.3.1.1 Primer design

The 18S rRNA gene sequences for *T. parva*, *T. taurotragi*, *T. mutans*, *T. annulata*, *Ba. Bigemina, Ba. bovis* and *Ba. divergens*, and the 16S rRNA gene sequences for *A. marginale* and *E. ruminantium* were aligned. This allowed design of internal primers that were specific for each parasite gene sequence (shown in Figures 2.1 and 2.2 respectively), and predicted to amplify gene sequence fragments whose sizes could be easily be distinguished on agarose gel electrophoresis. The *Theileria* / *Babesia* universal (genus specific) primers (Table 2.1) were predicted to amplify fragment sizes of 385 to 429 bp depending on the parasite species, while the species specific-primers (Table 2.1) were predicted to amplify fragments whose sizes were 224 bp, *T. parva*; 243 bp, *T. taurotragi*; 258 bp, *T. mutans*; 278 bp *Ba. bovis* and 359 bp for *Ba. bigemina*. The *Ehrlichia / Anaplasma* universal PCR primers were predicted amplify a fragment of 430 bp, while the internal species specific primers were predicted to amplify fragments of 335 bp for *A. marginale* and 201 for *E. ruminantium* (Table 2.1). The primers used in the 18S rRNA primary PCR reaction, i.e. *Theileria/Babesia* genus-specific primers were as described previously (Oura *et al.* 2004a).

Figure 2.1 18S rRNA sequence alignment

The 18S rRNA gene sequences of *T. parva, T. taurotragi, T. mutans, T. annulata, Ba. divergens, Ba. bigemina* and *Ba. bovis* were aligned using Clustal software and then the *Theileria* / *Babesia* genus (universal) and species-specific primers were designed as shown in the shaded areas. Yellow is the universal forward primer, green are the species-specific primers with the respective parasites shown on the left side. Blue is the universal reverse primer.

Figure 2.1 18S rRNA sequence alignment

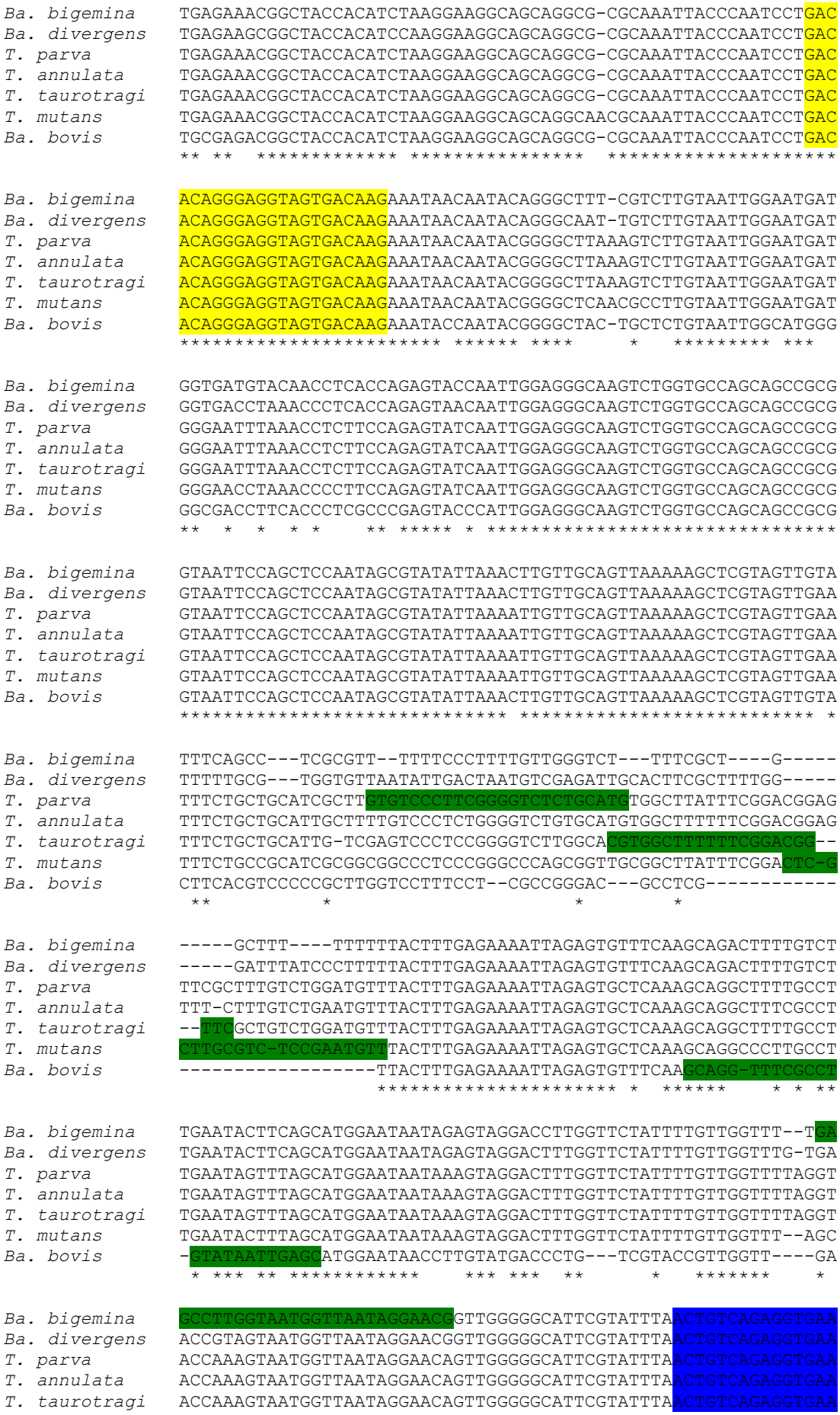


Figure 2.1 18S rRNA sequence alignment (continued)

```
T. mutans       GCCAAAGTAATGGTTAATAGGAACAGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAA
Ba. bovis       CTTTGGGTAATGGTTAATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAA
                ****************** **************** *  ***************

Ba. bigemina    ATTCTTAGATTTGTTAAAGACGAACCACTGCGAAAGCATTTGCCAAGGACGTTTTCATTA
Ba. divergens   ATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGACGTTTTCATTA
T. parva        ATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTA
T. annulata     ATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCTTTA
T. taurotragi   ATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTA
T. mutans       ATTCTTAGATTTGTTAAAGACGAACCACTGCGAAAGCATTTGCCAAGGATGTTTTCATTA
Ba. bovis       ATTCTTAGATTTGTCGATGACGCACGACTGCGAAAGCATTTGCCAAGGACGCTTCCATTA
                ************** * **** ** ********************** * ** * ***
```

Figure 2.2 16S rRNA sequence alignment

The 16S rRNA gene sequences of *E. ruminantium* and *A. marginale* were aligned using Clustal software and then the *Anaplasma* / *Ehrlichia* genus (universal) and species-specific primers were designed as shown in the shaded areas. Yellow is the universal forward primer, green are the species-specific primers with the respective parasites shown on the left side. Blue is the universal reverse primer.

Figure 2.2 16S rRNA sequence alignment

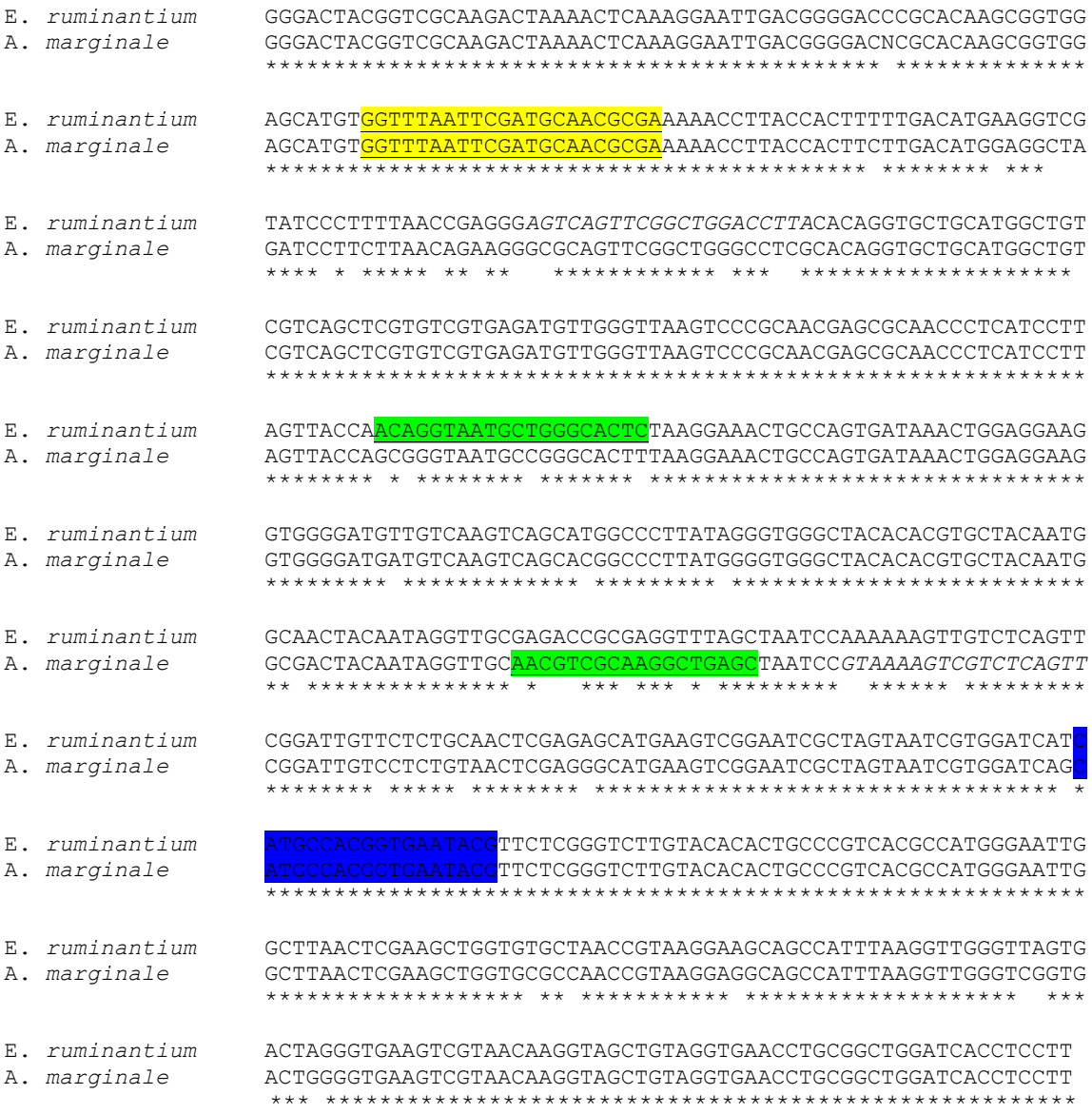


Table 2.1 18S and 16S rRNA primer sequences

Primer sequences and expected amplicon sizes for the 18S and 16S rRNA gene semi-nested PCR assay for *Theileria/Babesia* and *Anaplasma / Ehrlichia* pathogens respectively. The primer lengths, GC content and the nearest neighbour and salt adjusted melting temperatures are also shown. The assays were designed to maximise the difference in amplicon sizes between parasite species.

Table 2.1 18S and 16S rRNA primer sequences

Parasite species	Primer sequence (5′ to 3′)	Amplicon size (bp)	Primer length	% GC	TM [1] (nearest neighbour) (°C)	TM [2] (salt adjusted) (°C)
Theileria/Babesia universal	F: GACACAGGGAGGTAGTGACAAG R: CTAAGAATTTCACCTCTGACAGT	385 - 429	22 23	55 39	54 52	64 59
T. parva	R: CATGCAGAGACCCCGAAGGGACAC	224	24	63	61	71
T. taurotragi	R: GAACCGTCCGAAAAAAGCCACG	243	22	55	57	64
T. mutans	R: AACATTCGGAGACGCAAGCGAG	258	22	55	60	64
Ba. bovis	R: GCTCAATTATACAGGCGAAACCTGC	278	25	48	59	66
Ba. bigemina	R: CGTTCCTATTAACCATTACCAAGGCTCA	359	28	43	59	67
Anaplasma/Ehrlichia universal	F: GGTTTAATTCGATGCAACGCGA R: CGTATTCACCGTGGCATG	430	22 18	45 56	56 50	60 56
Anaplasma spp	R: GCTCAGCCTTGCGACGTT	335	18	61	55	58
E. ruminantium	R: GAGTGCCCAGCATTACCTGT	201	20	55	53	60

The length, GC content and melting temperatures (T_m) (both nearest neighbour and salt adjusted) for each primer were calculated using the computer program OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html)(Kibbe 2007). The primers ranged in length from 18 to 28 nucleotides, while the GC content ranged from 43 % to 63 %. The nearest neighbour T_ms ranged from 50 to 61 ^oC, while the salt adjusted ones ranged from 56 to 71 ^oC (Table 2.1). All primers were tested for specificity against online databases using BLAST searches to verify the absence of similarity to other pathogen species.

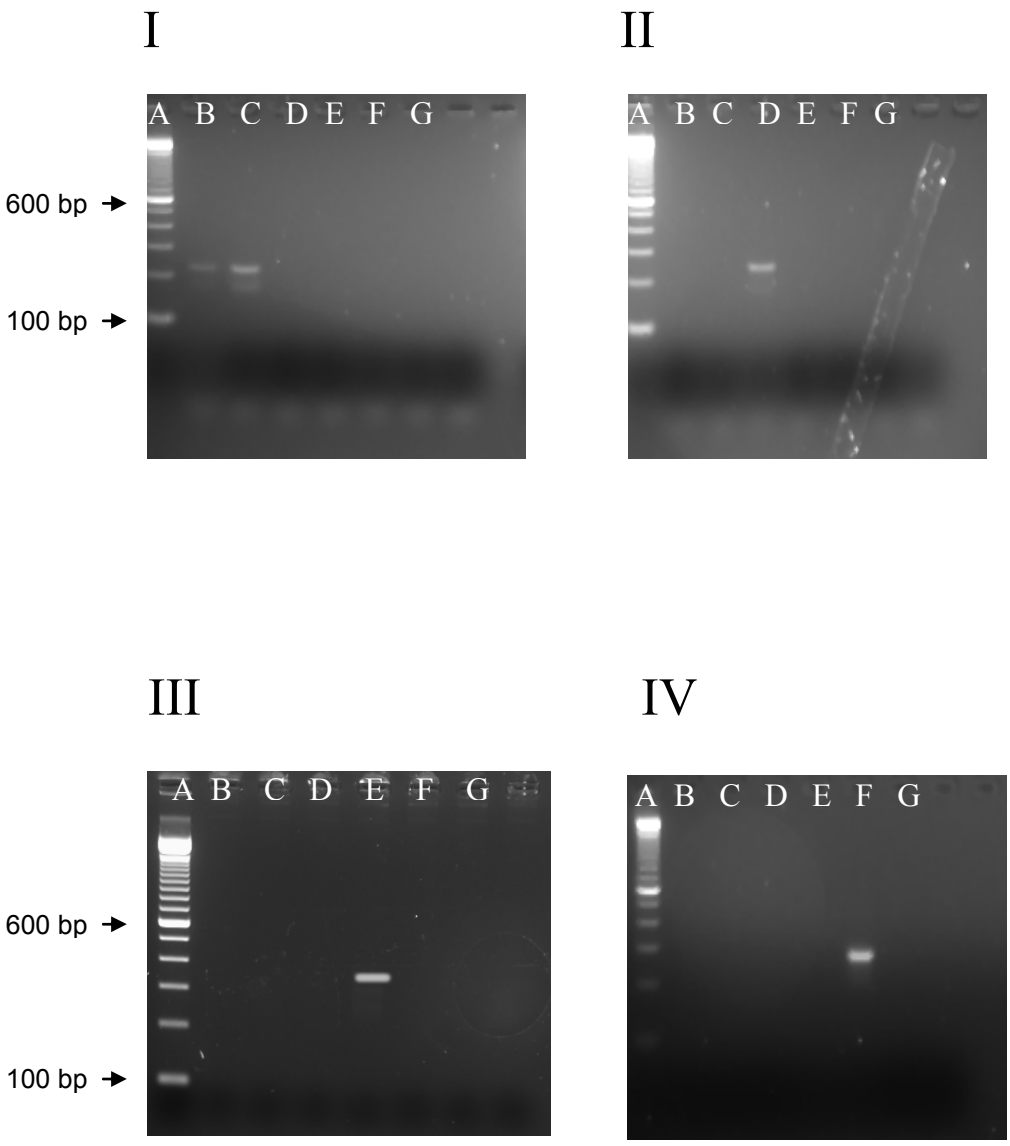
2.3.1.2 PCR amplification

The initial aim of the study was to develop a semi-nested multiplex PCR that could detect and differentiate seven tick-transmitted pathogens i.e. *T. parva, T. taurotragi, T. mutans, Ba. bigemina, Ba. bovis, A. marginale* and *E. ruminantium* simultaneously. The primary PCR reactions generated amplicons of expected sizes from all the DNA preparations of different parasite species (results not shown). The primary amplicons were used as the template for the secondary species-specific reactions and when the PCR reactions were run for each parasite species individually, products of expected sizes were observed, as shown in Figure 2.3 for *T. parva, T. taurotragi, Ba. bovis* and *Ba. bigemina* and Figure 2.4 for *E. ruminantium* and *A. marginale*. Thus these results showed specific amplification for each species with amplicon sizes of 224 bp (*T. parva*), 243 bp (*T. taurotragi*), 278 bp (*Ba. bovis*), 359 bp (*Ba. bigemina*), 335 bp (*A. marginale*) and 201 bp (*E. ruminantium)*. To test whether all these species could amplify in a single multiplex PCR reaction, DNA templates of *T. parva, T. taurotragi, Ba. bovis, Ba. bigemina, E. ruminantium*, *T. annulata* and *B. divergens* were mixed and PCR amplified with two pairs of universal primers for *Theileria/Babesia* and *Anaplasma/Ehrlichia*. The amplicons generated were then subjected to a second round of semi-nested multiplex PCR using species specific-primers and one universal primer in a single reaction following which the products separated by agarose gel electrophoresis. The resulting gel image is shown in Figure 2.5. While species-specific products were amplified when single species DNA were used as template (lanes D to H), the combined templates did not give desired results (Lanes B and C). *Ba. bigemina* and *E. ruminantium* could not amplify despite several adjustments which were made to the primer mixture concentrations and annealing temperatures. The multiplex PCR reaction also resulted in non-specific bands which made conclusive identification of some expected bands difficult. The species specificity of the amplification was shown by the lack of amplification of both *T. annulata* and *B. divergens* (Figure 2.5). Unfortunately, as shown in Figure 2.1, the alignment of 18S RNA sequences revealed no additional regions of

Figure 2.3 *Theileria* spp and *Babesia* spp PCR amplicons

These images shows PCR products of *T. parva, T. taurotragi, Ba. bigemina* and *Ba. bovis* generated using the 18S rRNA gene species-specific semi-nested PCR assays for the respective parasites. All the assays were species-specific.

Figure 2.3 *Theileria* spp and *Babesia* spp PCR amplicons

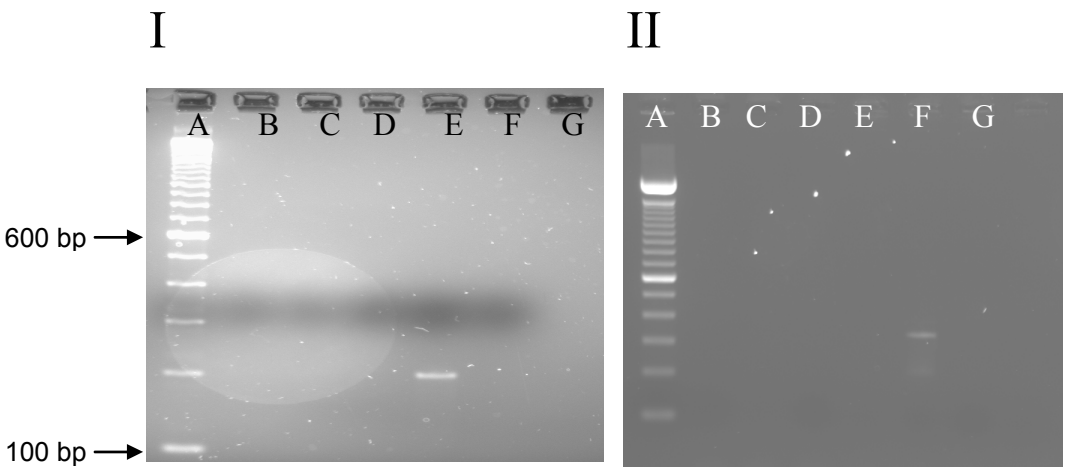


(I) *T. parva*, (II) *T. taurotragi*, (III) *Ba. bigemina* and (IV) *Ba. bovis* semi-nested species-specific PCR . Lanes: A, 100 bp ladder; B, *T. parva* muguga; C, *T. parva* marikabuni; D, *T. taurotragi*; E, *Ba. bigemina*; F, *Ba. bovis* and G, negative control.

Figure 2.4 *E. ruminantium* and *Anaplasma* spp PCR amplicons

These images show the PCR products of *E. ruminantium* and *A. marginale* generated using the 16S rRNA gene species-specific semi-nested PCR assays for the respective parasites. All the assays were species-specific.

Figure 2.4 *E. ruminantium* and *Anaplasma* spp PCR amplicons

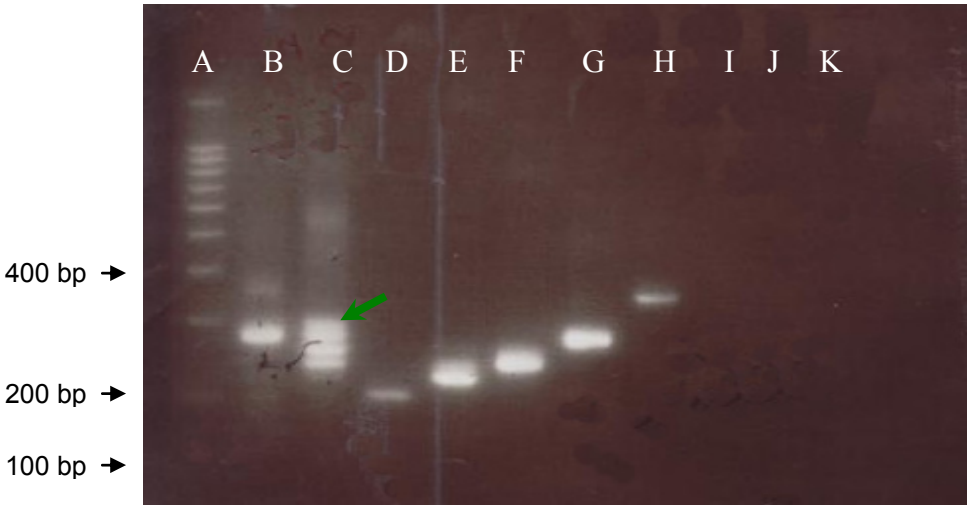


(I) *E. ruminantium* and (II) *Anaplasma spp* semi-nested PCR. Lanes A, 100 bp ladder; B, *T. parva*; C, *Ba. bigemina*; D, *Ba. bovis*; E. *E. ruminantium*; F, *A. marginale* and G, negative control.

Figure 2.5 18S / 16S rRNA genes semi-nested multiplex PCR

This image represents PCR products of *T. parva, T. taurotragi, Ba. bigemina,* Ba. bovis *and E. ruminantium* generated using the 18S and 16S rRNA gene species-specific semi-nested multiplex PCR assays. Expected products were obtained from single species DNA (Lanes D to H). For the 1:1000 mixed species DNA templates (Lane B) only a product corresponding to the size of *Ba. bovis* amplicon can be seen, while in 1:100 dilution, *Ba. bigemina* and *E. ruminantium* did not amplify, and a non-specific band can be seen (green arrow). The assay was species-specific as it did not amplify *T. annulata* and *B. divergens*.

Figure 2.5 18S / 16S rRNA genes semi-nested multiplex PCR



Lanes A, 100 bp ladder; B, 1:1000 dilution of primary PCR DNA mixture; C, 1:100 dilution of primary PCR DNA mixture; D, *E. ruminantium*, E. *T. parva*; F, *T. taurotragi*; G, *Ba. bovis*; H, *Ba. bigemina*; I, *T. annulata*; J, *Ba. divergens* and K, negative control.

sequence variation between the parasite species that would have allowed design of alternative primers for multiplex PCR. Therefore, the objective of developing a semi-nested multiplex PCR that would detect all seven pathogens using this locus was not pursued further. However, using equi-molar primer concentrations, a semi-nested duplex PCR of *A. marginale* and *E. ruminantium* amplified products of expected sizes, as shown in Figure 2.6. This assay was developed by amplifying a mixture of *A. marginale* and *E. ruminantium* DNA in the primary reaction. Then, 1:100, 1:1,000 and 1:10,000 dilutions were made from the primary product and these were amplified using a mixture of the species-specific primers at equi-molar concentration (1 μM each) in the secondary PCR. To ensure that each PCR reaction was species-specific, *T. parva* and *Ba. bovis* DNA were included in the reaction (Lanes G and H) and these were not amplified. The ability of the *A. marginale* species-specific PCR to differentiate between *A. marginale* and *A. centrale* was tested. It was found out that this PCR assay could not differentiate between the two species (results not shown). Therefore, this assay is from here onwards referred to as the *Anaplasma* spp PCR.

2.3.2. Development of semi-nested multiplex PCR for β-tubulin gene of *T. parva*, *Ba. bigemina* and *Ba. bovis* and a semi-nested PCR for *T. taurotragi*

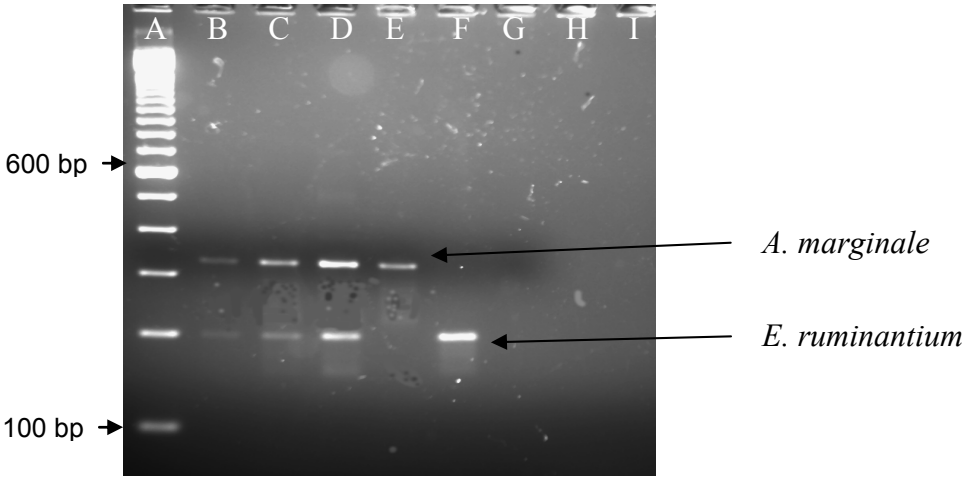
2.3.2.1 Primer design

The β-tubulin gene has previously been used in the discrimination of parasitic species (Caccio *et al.* 2000), and on this basis it was considered to be an alternative target for development of a semi-nested multiplex PCR. Nucleotide sequences were aligned to allow design of primers that could species-specifically amplify fragments of the β-tubulin gene for *T. parva, T. taurotragi, Ba. bovis* and *Ba. bigemina* (Figure 2.7). Degenerate forward and reverse universal *Theileria*/*Babesia* primers that can amplify fragments of 364 to 458 bp depending on the species, from the β-tubulin gene have been described previously (Caccio *et al.* 2000). Within this region, species-specific internal primers capable of amplifying fragments of different sizes that could be discriminated by agarose gel electrophoresis were designed as shown in Figure 2.7. The species-specific sequence fragment sizes predicted to amplify by these primers were 325 bp (*T. parva)*, 395 bp (*Ba. bigemina*), 274 bp *(Ba. bovis)* and 293 bp (*T. taurotragi)*. Primer specificity was tested against online databases using BLAST searches to verify the absence of similarity to other pathogen species. The sequences of the designed primers are shown in Table 2.2.

Figure 2.6 *E. ruminantium* and *Anaplasma* spp duplex PCR amplicons

This image represents PCR products of *A. marginale* and *E. ruminantium* generated using the 16S rRNA gene species-specific semi-nested duplex PCR assays. Expected products were obtained from both mixed species template DNA (Lanes B to D) and single species DNA (Lanes E and F). The assay was species-specific, as it did not amplify *Ba. bovis* and *T. parva*.

Figure 2.6 *E. ruminantium* and *Anaplasma* spp duplex PCR amplicons



Lanes A, 100 bp ladder; B, 1:10,000 dilution of *A. marginale* and *E. ruminantium* DNA mixture; C, 1:1000 dilution of *A. marginale* and *E. ruminantium* DNA mixture; D, 1:100 dilution of *A. marginale* and *E. ruminantium* DNA mixture; E, *A. marginale*; F, *E. ruminantium* and G, *Ba. bovis*; H, *T. parva*; I, negative control.

Figure 2.7 ß-tubulin sequence alignment

The β-tubulin gene sequences of *T. parva, T. annulata, T. taurotragi, Ba. bigemina* and *Ba. bovis* were aligned using the Clustal software to allow degenerate *Theileria* / *Babesia* genus (universal) and species-specific primers to be designed as shown in the shaded areas. The primer shaded yellow is the universal forward, green are the species-specific primers with the respective parasites shown on the left side. Blue is the universal reverse primer.

Figure 2.7 ß-tubulin sequence alignment

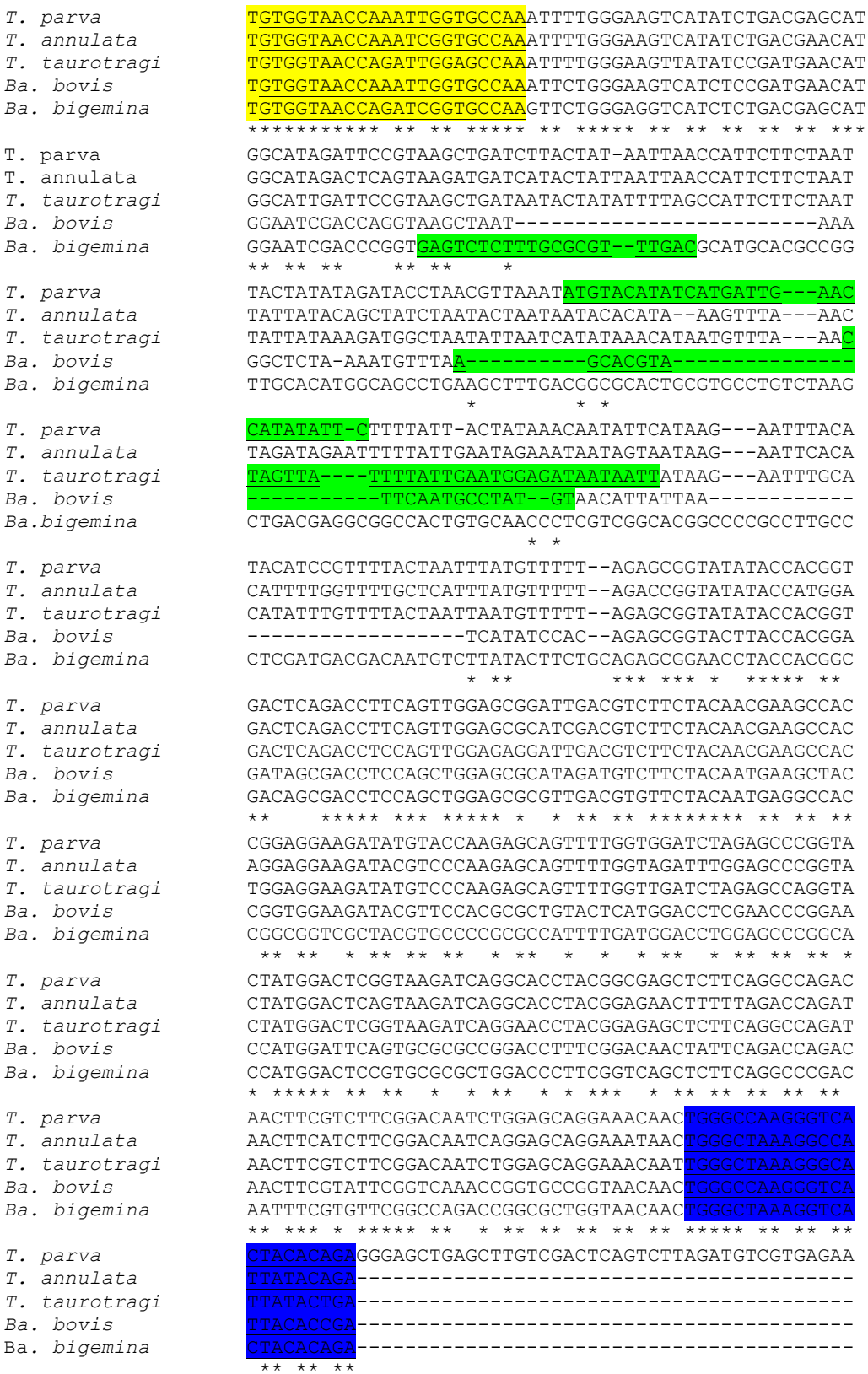


Table 2.2 β-tubulin primer sequences

Primer sequences and expected amplicon sizes for the β-tubulin gene semi-nested PCR assay for *Theileria/Babesia* pathogens. The assay was designed to maximise the difference in amplicon sizes between parasite species.

Table 2.2 β-tubulin primer sequences

Parasite species	Primer sequence (5′ to 3′)	Amplicon size (bp)
Babesia/*Theileria* universal	F: TGTGGTAACCAGATYGGWGCCAA R: TCNGTRTARTGNCCYTTRGCCCA	364 - 458
T. parva	F: ATGTACATATCATGATTGAACCATATATTC	325
Ba. bigemina	F: GAGTCTCTTTGCGCGTTTGAC	395
Ba. bovis	F: AGCACGTATTCAATGCCTATGT	274
T. taurotragi	F: CTAGTTATTTTATTGAATGGAGATAATAATT	293

2.3.2.2 PCR amplification

Fragments ranging from 364 bp to 458 bp were amplified with the universal primers (results not shown). Dilutions of the primary PCR products obtained from DNA representing each parasite were made and used as template in the species-specific semi-nested multiplex PCR assays. Each species-specific PCR assay was performed individually in the first instance to determine if it could amplify a product of the expected size and was specific for the expected parasite species, before being included in the semi-nested multiplex PCR. Each species-specific PCR assay was species-specific and amplified fragments of expected sizes (results not shown). The primary PCR product generated from a parasite DNA mixture of *T. parva, T. taurotragi*, *Ba. bovis* and *Ba. bigemina* DNA was diluted to 10^{-2}, 10^{-3} and 10^{-4} to determine the dilution that would be appropriate for use in the multiplex PCR. The multiplex PCR was carried out using species-specific primers for each parasite and one universal primer, and the resulting gel image is shown in Figure 2.8. Species-specific bands were obtained from both reactions containing a mixture of parasite DNA (Lanes B, C, and D), and those with single-species DNA (Lanes E, F and G). The bands representing *Ba. bovis* (Lane E) and *Ba. bigemina* (Lane G) appear fainter than that representing *T. parva* (Lane F). This is because lower DNA dilutions were used for these parasites and so does not represent differences in the sensitivities of the primers used. The species-specific fragment sizes amplified were 325 bp (*T. parva)*, 274 bp (*Ba. bovis)* and 395 bp (*Ba. bigemina*) (Figure 2.8). The 10^{-2} dilution of the DNA mixture gave the clearest bands. The semi-nested multiplex PCR was species-specific as it did not amplify *B. divergens* and *T. taurotragi*. Unfortunately, the primer for *T. taurotragi* did not amplify in the multiplex PCR reaction (results not shown) and was not included in the PCR reactions described in Figure 2.8. They were however species-specific and able to amplify *T. taurotragi* when used in a uniplex PCR reaction as shown in Figure 2.9.

2.3.3 Development of nested PCR for *Ba. bovis* and *Ba. bigemina* cytochrome b gene

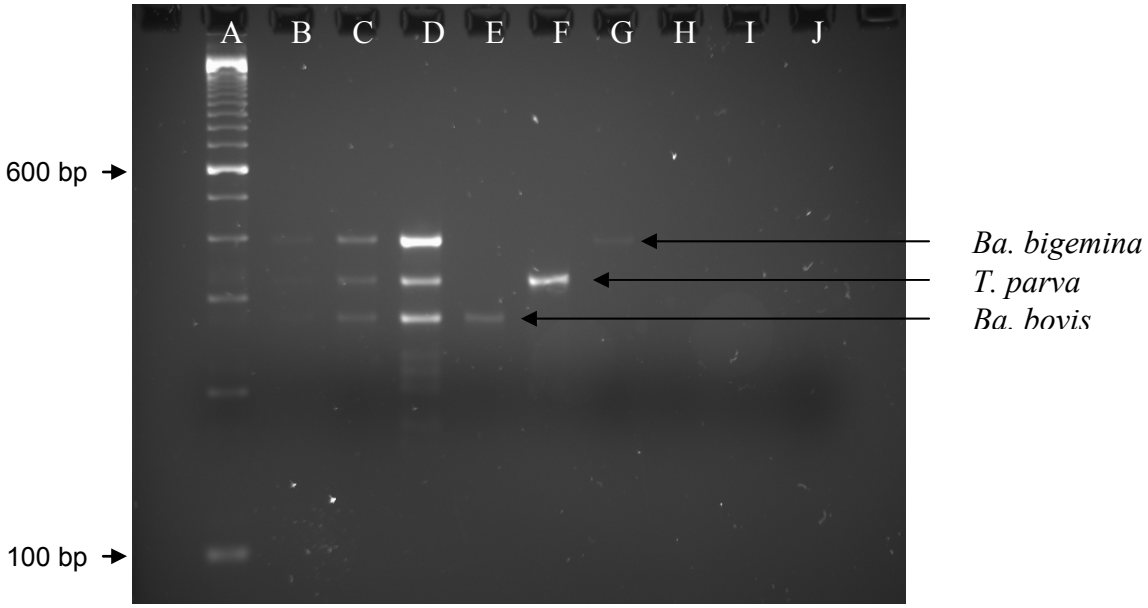
2.3.3.1 Primer design

The rational for developing this assay was that the cytochrome b gene of *Babesia* is reported to have over 100 copies per genome (Salem *et al.* 1999) and therefore a PCR assay developed from this gene is likely to be more sensitive that that from the 18S rRNA gene. The cytochrome b gene sequences of *Ba. bovis* and *Ba. bigemina* were aligned as shown in Figure 2.10. The degenerate genus-specific *Babesia* primers used in the primary reaction were those described by Salem *et al*. (1999). Within this region, separate

Figure 2.8 ß-tubulin semi-nested multiplex PCR amplicons

PCR products of *T. parva, Ba. bigemina, and Ba. bovis* generated using the β-tubulin gene species-specific semi-nested multiplex PCR assay. Expected products were obtained from both mixed DNA templates (Lanes B to D) and single species DNA (Lanes E to G). The difference in the intensities of the bands in lanes E, F and G is a reflection of the different dilutions of template DNA used for each parasite species and not the sensitivities of the primers.

Figure 2.8 ß-tubulin semi-nested multiplex PCR amplicons

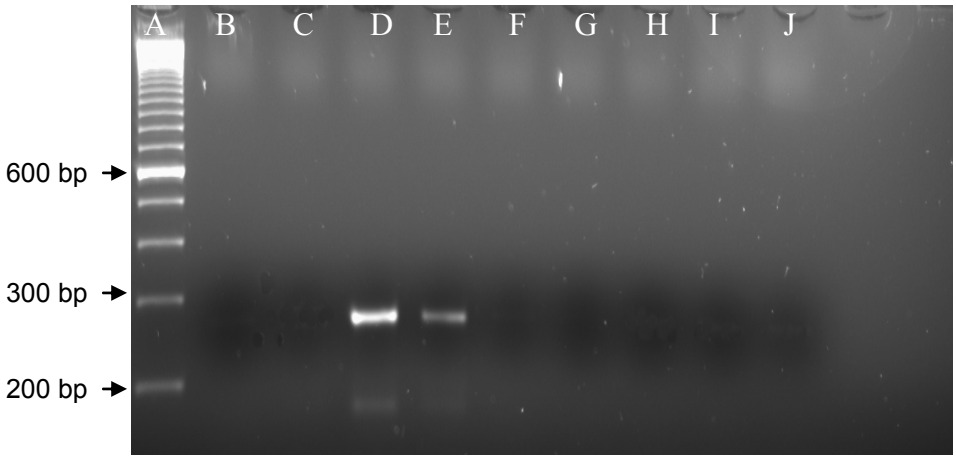


Lanes A, 100 bp ladder; B 10^{-4} dilution of DNA mixture; C, 10^{-3} dilution of DNA mixture; D, 10^{-2} dilution of DNA mixture; E, *Ba. bovis;* F, *T. parva;* G, *Ba. bigemina;* H, *Ba. divergens;* I, *T. taurotragi;* J, negative control.

Figure 2.9 *T. taurotragi* species-specific PCR amplicons

PCR products of *T. taurotragi* generated using the β-tubulin gene species-specific semi-nested PCR assay. The assay was species-specific as it did not amplify non-*T. taurotragi* DNA.

Figure 2.9 *T. taurotragi* species-specific PCR amplicons

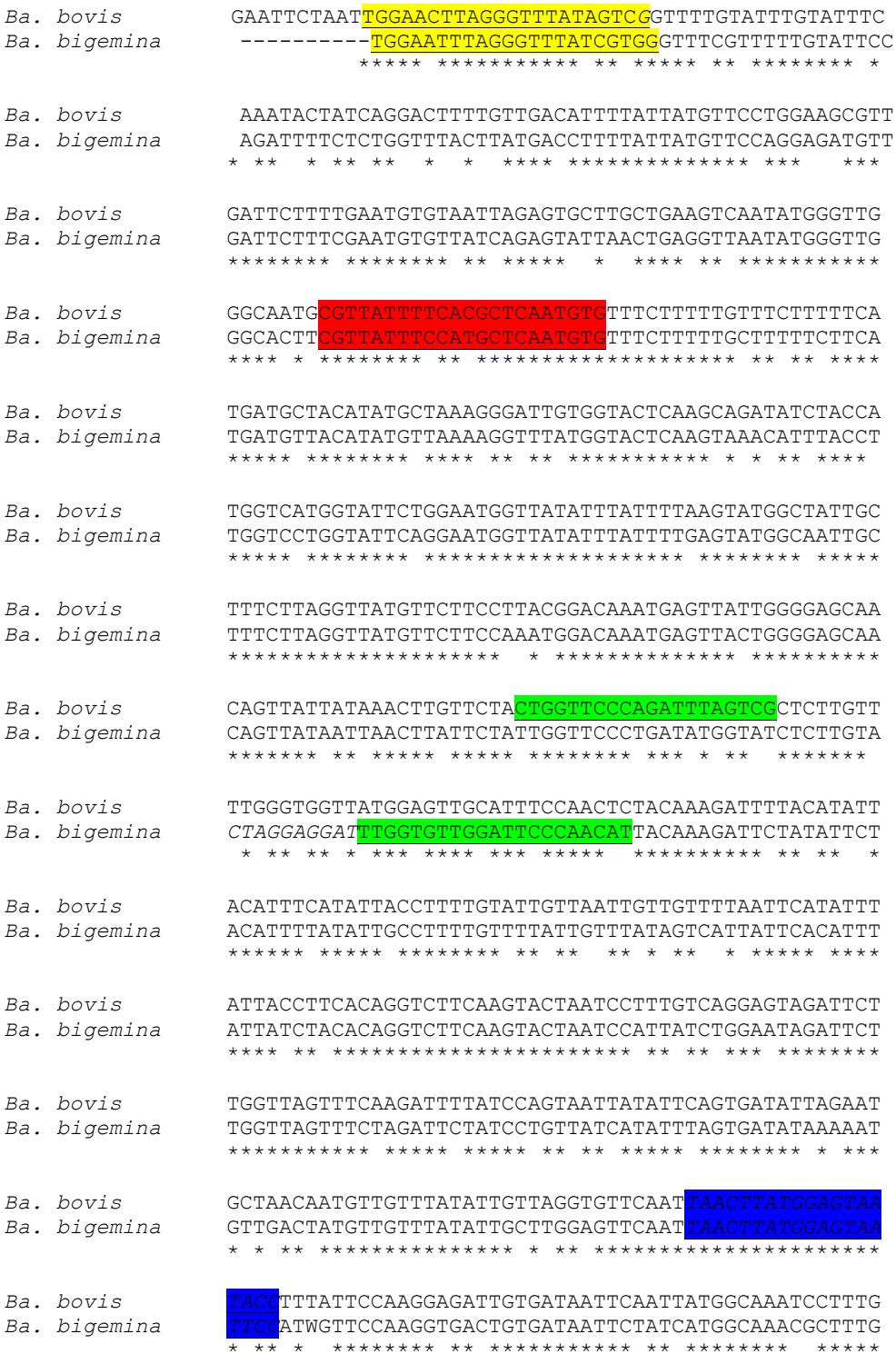


Lane A, 100 bp ladder; B, *T. parva*; C, *A. marginale*; D, *T. taurotragi* (1:100 dilution); E, *T. taurotragi* (1:1000 dilution); F, *Ba. bigemina*; G, *Ba. bovis*; H; *E. ruminantium*; I, *Ba. divergens*; J, Negative control.

Figure 2.10 Cytochrome b sequence alignment

Cytochrome b gene sequences of *Ba. bovis* and *Ba. bigemina* were aligned using Clustal software to allow degenerate Babesia genus (universal) and species-specific primers to be designed as shown in the shaded areas. Yellow is the external universal forward primer, red is the internal universal forward primer, green are the species-specific reverse primers with the respective parasites shown on the left side. Blue is the external universal reverse primer.

Figure 2.10 Cytochrome b sequence alignment



Ba. bigemina and *Ba. bovis* species-specific reverse primers and a single degenerate forward primer that could amplify both parasites, were designed as shown in Figure 2.10. The species-specific fragment sizes predicted to amplify by these internal primers were 235 bp for *Ba. bovis* and 274 bp for *Ba. bigemina*. The sequences of the designed primers are shown in Table 2.3.

2.3.3.2 PCR amplification

The *Babesia* genus-specific degenerate primers amplified a 644 bp fragment from both *Ba. bovis* and *Ba. bigemina* (results not shown). A dilution of the primary PCR product was made and used in the species-specific nested PCR reaction. To demonstrate that the nested PCR assay was species-specific, individual primary and nested PCR reactions were carried out using DNA from three *Ba. bovis* strains, three *Ba. bigemina* strains, *T. parva, T. taurotragi, A. marginale*, *E. ruminantium* and bovine lymphocytes. The *Ba. bovis* species-specific PCR amplified 235 bp fragments while, the *Ba. bigemina*-specific PCR amplified 274 bp fragments (Figure 2.11)**.** Both assays were species-specific and did not amplify from non-*Babesia* templates.

2.3.4 Sensitivity of the *Ba. bovis* and *Ba. bigemina* PCR assays

To test and compare the sensitivities of the *Babesia* species 18S rRNA gene, β-tubulin gene and cytochrome b gene PCR assays, serial dilutions of blood infected with *Ba. bovis* and *Ba. bigemina* were obtained from Israel on FTA filters. For all target genes tested and for both parasite species, the primary PCR reaction could amplify a specific product from DNA representing parasitaemia as low as 0.01 % (results not shown). This sensitivity was increased by at least a 100-fold by the nested PCR assays, as they could detect a parasitaemia of 0.0001 % for both parasite species (Figure 2.12). This corresponded to the most dilute FTA preparation available and therefore it was impossible to determine which of the three PCR assays was more sensitive for detection of bovine babesiosis. Moreover, the method of preparing the dilutions was not ideal, as diluting blood with PBS is likely to reduce the concentration of PCR inhibitors, and may therefore have increased the apparent sensitivity of the assays, relative to a low parasitaemia in undiluted blood.

In a further attempt to determine the sensitivity of the assays based on the three different genes, ten Zambian cattle blood samples on FTA filters were used. These samples had not been characterised in terms of the species of parasites present and it was anticipated that they could be used to compare the relative sensitivities of the PCR assays for *T. parva,*

Table 2.3 Cytochrome b primer sequences

Primer sequences and expected amplicon sizes of the cytochrome b gene nested PCR assay for *Babesia* parasites. The assays were designed to maximise the difference in amplicon sizes between parasite species. The forward primer for both parasites is the same.

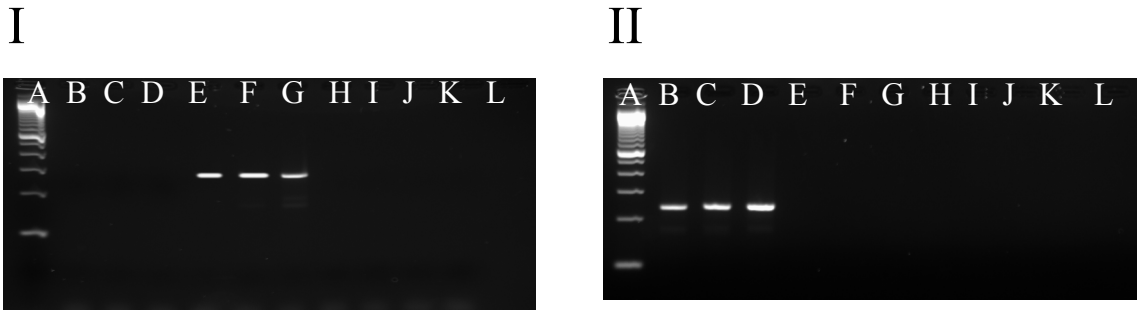
Table 2.3 Cytochrome b primer sequences

Parasite species	Primer sequence (5′ to 3′)	Amplicon size (bp)
Babesia universal	F: TGGAAYTTAGGGTTTATMGTSG R: GGWATTACTCCATAAGTTA	644
Ba. bovis	F: CGTTATTTYCAYGCTCAATGTG R: CGACTAAATCTGGGAACCAG	235
Ba. bigemina	F: CGTTATTTYCAYGCTCAATGTG R : ATGTTGGGAATCCAACACCAA	274

Figure 2.11 Cytochrome b PCR amplicons

These images represent PCR products of *Ba. bigemina* and *Ba. bovis* generated using the cytochrome b gene species-specific nested PCR assays for respective parasites. Specific PCR products were obtained for each assay and they were all species-specific as they did not amplify non-*Babesia* DNA.

Figure 2.11 Cytochrome b PCR amplicons



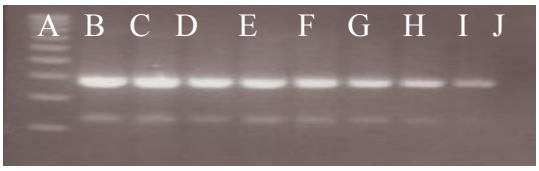
(I) *Ba. bigemina* and (II) *Ba. bovis* Cytochrome b gene nested PCR. Lanes A, 100 bp ladder; B, *Ba. bovis* mexico; C, *Ba. bovis* kwanyangwa; D, *Ba. bovis* lismore; E, *Ba. bigemina* zaria; F, *Ba. bigemina* muguga; G, *Ba. bigemina* gonen; H, *T. taurotragi*; I, *A. marginale*; J, *E. ruminantium*; K, bovine DNA; L, control

Figure 2.12 Sensitivities of *Ba. bovis* and *Ba. bigemina* PCR assays based on serial dilutions spotted on FTA cards

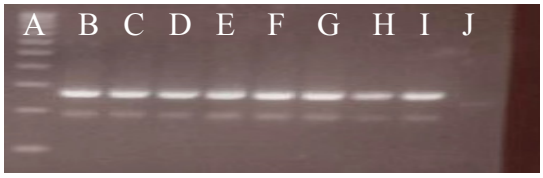
Sensitivity of the *Babesia* species β-tubulin, 18S rRNA and the cytochrome b gene nested PCR assay, using serial dilutions of infected blood spotted on FTA filters. All the assays amplified the lowest dilution of DNA tested, representing a parasitaemia of 0.0001 %.

Figure 2.12 Sensitivities of *Ba. bovis* and *Ba. bigemina* PCR assays based on serial dilutions spotted on FTA cards

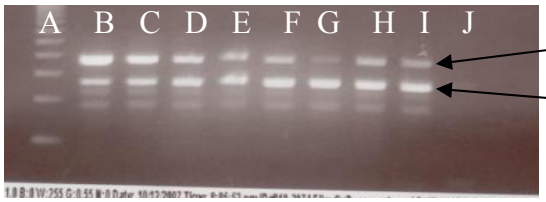
AI: β-tubulin, *Ba. bigemina*



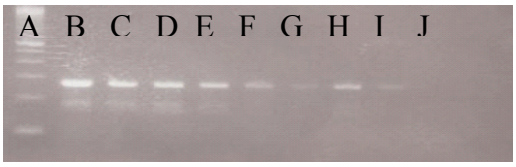
AII: β-tubulin, *Ba. bovis*



AIII: β-tubulin Duplex PCR



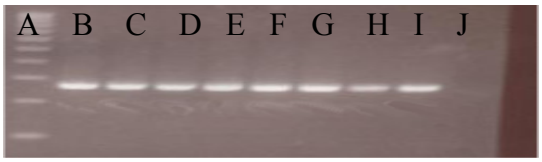
BI: Cytochrome b, *Ba. bigemina*



BII: Cytochrome b, *Ba. bovis*

CI : 18S rRNA, *Ba. bigemina*

CII : 18S rRNA, *Ba. bovis*



Lanes A, 100 bp ladder; B, 1%; C, 0.1%; D, 0.05%; E, 0.01%; F, 0.005%; G, 0.001%; H, 0.0005%; I, 0.0001% and J, Negative control.

T. taurotragi, Ba. bovis and *Ba. bigemina*. All the blood samples had previously been screened by microscopy and were negative, thus they were known to be of low parasitaemia. It was postulated that the assay which detected the highest number of positive samples would be the most sensitive. Assays based on the nested 18S rRNA and the cytochrome b genes both detected the same number of *Ba. bovis* positive samples (6/10). Additionally, the 18S rRNA PCR detected 6/10 positive samples for both *T. parva* and *T. taurotragi*. The β-tubulin PCR assay only detected 2/10 positive *Ba. bovis* samples and 3/10 positive samples for *T. parva* or *T. taurotragi*. None of the samples were found to be positive for *Ba. bigemina* (Table 2.4). All the samples that were positive using the β-tubulin gene PCR were also positive using the 18S RNA gene PCR. The samples that were positive for *Ba. bovis* using the cytochrome b gene PCR were the same ones found positive on the 18S rRNA gene PCR. The β-tubulin semi-nested multiplex PCR gave similar results to the individual species-specific semi-nested PCR, indicating that they had similar sensitivities. For this gene however, PCR amplification of DNA preparations on FTA filters gave several non-specific bands, which were most likely due to amplification of bovine DNA (results not shown). In conclusion, the β-tubulin PCR assay was the least sensitive of the three while the limit of the sensitivities of the 18S rRNA and cytochrome b genes could not be resolved.

2.4 Discussion

Accurate and up-to-date epidemiological data is important if TBDs are to be controlled in most developing countries where these diseases are endemic. This requires sensitive, specific and cost-effective methods capable of detecting causative pathogens in cattle hosts and tick vectors. In this chapter several nested PCR assays are described that can be used to detect tick-borne pathogens of cattle based on the 18S and 16S rRNA genes, the β–tubulin gene and the cytochrome b gene. The initial aim of this work was to develop a semi-nested multiplex PCR, based on the 18S and 16S rRNA gene that could simultaneously detect *T. parva*, *T. taurotragi*, *T. mutans*, *Ba. bigemina*, *Ba. bovis*, *A. marginale* and *E. ruminantium*. However, this was not achieved due to the number of non-specific amplicons resulting from some PCR reactions and the failure of some of the target templates to amplify.

When 18S and 16S rRNA gene species-specific PCR assays were performed individually, specific bands were observed following electrophoresis on ethidium bromide stained agarose gels. However, when these PCR assays were incorporated into a multiplex assay, non-specific bands were generated, which made conclusive detection of expected bands

Table 2.4 Comparison of sensitivities of the PCR assays

To compare the relative sensitivities of the assays based on three genes, cattle blood samples collected and stored on FTA filters were utilised. The β-tubulin gene assays were the least sensitive of the three. The cytochrome b and 18S rRNA gene assays showed similar sensitivity.

Table 2.4 Comparison of sensitivities of the PCR assays

Parasite species	Number of samples tested	Number of isolates which successfully amplified (%)		
		18S RNA	Cytochrome b	ß-tubulin
T. parva	10	6 (60)	-	3 (30)
T. taurotragi	10	6 (60)	-	3 (30)
Ba. bovis	10	6 (60)	6 (60)	2 (20)
Ba. bigemina	10	0	0	0

difficult. The presence of many primer pairs in the multiplex reaction mixture may have increased the chance of spurious amplification, primarily because of formation of primer dimers (Brownie *et al.* 1997). These non-specific products may amplify more efficiently than the desired target, consuming reaction components and impairing the efficiency of annealing and extension. The success of specific PCR amplification depends on the rate at which primers anneal to their target and the rate at which annealed primers are extended (Elnifro *et al.* 2000). Factors preventing optimal annealing rates include poorly designed primers, sub-optimal buffer composition and incorrect annealing temperature. The extension rate of a specific primer-target hybrid depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs) and the nature of the target DNA (Elnifro *et al.* 2000; Henegariu *et al.* 1997). The constituents, dNTPs and enzyme used in this multiplex PCR were the same as those used in the uniplex PCR, and in the β–tubulin gene multiplex PCR which worked well, and thus it is unlikely that these reagents were responsible for the non-specific amplification. Alteration of the PCR conditions and constituent concentrations did not improve the specificity of the reactions. Moreover, alteration of these constituents in multiplex PCR relative to those described in the uniplex resulted in little, if any, improvement in the sensitivity and specificity of the test. Furthermore, it is known that increasing the concentration of these components may increase the likelihood of mis-priming with subsequent production of spurious non-specific products (Elnifro *et al.* 2000). It is likely that the main cause of the observed non-specific products was related to properties of the PCR primers. However, all the primers used in this study had GC contents ranging from 43 to 63 % and their nucleotide length ranged from 20 to 28, which should have enabled similar amplification efficiencies for their respective targets (Cha & Thilly 1993; Dieffenbach *et al.* 1993).

The *Ba. bigemina* and the *E. ruminantium* templates did not amplify in the multiplex PCR. Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a well recognised phenomenon in multiplex PCR (Polz & Cavanaugh 1998). Two distinct processes are understood to skew template-to-product ratios based on theoretical modelling of PCR: PCR selection and PCR drift (Wagner *et al.* 1994). PCR drift is a bias assumed to be due to stochastic fluctuations in the interaction of PCR reagents, particularly in the early cycles, which could arise due to the presence of very low template concentrations (Dieffenbach *et al.* 1993); variations in thermal profiles of a thermocycler, resulting in unequal ramping temperatures; or simple experimental errors. PCR selection on the other hand is defined as a mechanism which inherently favors the amplification of

certain templates due to the properties of the target; the target's flanking sequences or the entire genome. These properties include inter-regional differences in GC content, leading to preferential denaturation; higher binding efficiencies because of rich GC primers; differential accessibility of targets within genomes, due to secondary structures; and the gene copy number within the genome (Elnifro *et al.* 2000; Polz & Cavanaugh 1998). Of the five species-specific primers used in the multiplex PCR in this experiment, the *Ba. bigemina* primer had the lowest GC content and this could have resulted in lower amplification efficiency for this primer. Amplification biases that are strongly dependent on choice of primer, and to a lesser extent on the templates have been described, where some primer pairs with high amplification efficiency resulted in template saturation while other primer pairs produced products independent of starting product concentrations (Suzuki & Giovannoni 1996). The relatively low GC content of the *Ba. bigemina* primer compared to the other species-specific primers may have resulted in insufficient priming affinity for the target sequence (Polz & Cavanaugh 1998), resulting in non-amplification. Another important parameter known to affect PCR amplification is the annealing temperature (Elnifro *et al.* 2000; Henegariu *et al.* 1997). Although there was a lot of variation in the T_ms of the primers used in this assay, the primers that failed to amplify (*Ba. bigemina and E. ruminantium*) did not have the lowest T_ms. Therefore, it is unlikely that the annealing temperatures could have been responsible for the failure of these primers to amplify. Because of the requirement of the amplified PCR products to be of different sizes so that they can be discriminated by agarose gel electrophoresis, the 18S rRNA genes for *Theileria/Babesia* parasites did not offer many options in the design of alternative primers.

The β-tubulin multiplex PCR was able to detect *T. parva, Ba. bigemina* and *Ba. bovis*. However the *T. taurotragi* species-specific primer did not amplify in this PCR. The *T. taurotragi* β-tubulin gene sequence has a very low GC content and consequently the primer designed to amplify this segment of the gene was also very low in GC content. As explained for the *Ba. bigemina* primer, this could have resulted in poor binding efficiency leading to failure to amplify the target sequence. Conversely, the *Ba. bigemina* species-specific primer had the highest GC content, making it the most efficient primer in the reaction, requiring a primer concentration of only 0.15 μM in the multiplex PCR reaction. A previous study has demonstrated that more efficiently amplified loci can negatively affect the yield of product from less efficiently amplified loci (Markoulatos *et al.* 2002). The concentration of this more efficient primer was reduced to allow all the loci to compete favourably for the limited supply of enzyme and nucleotides from the pool of

reactants. When the β-tubulin PCR assays were used to amplify parasite DNA from field samples on FTA cards, it resulted in a number of non-specific bands which could have been due to amplification of host DNA. This PCR assay would therefore give unreliable results when used for field studies.

The serial dilutions obtained from Israel on FTA cards were used to determine the sensitivity of each *Babesia* PCR assay. Since all the assays were able to amplify each parasite species down to the concentration corresponding to the lowest parasitaemia, the issue of which of the assays was more sensitive was unresolved. The use of PBS to experimentally dilute blood samples to generate a dilution series meant that the amount of PCR inhibitors would be relatively lower compared to field samples. Therefore, the results obtained may not be an accurate reflection of the sensitivities that would be obtained if the assays were used on field samples. To resolve the issue of relative sensitivity of the three assays, 10 blood samples spotted on FTA cards of unknown parasitaemia and which were all negative on microscopic examination, were used to determine which of the three assays was most sensitive. The β-tubulin gene PCR assay was the least sensitive, while the 18S and cytochrome b gene PCR assays identified the same number of *Ba. bovis*, *T. taurotragi* and *T. parva* parasites from the samples (Table 2.4), and the agreement between these assays was 100 %. The ability of the assays to detect parasite DNA from samples that were microscopically negative renders them ideal for use in epidemiological studies where it is important to detect both clinical and sub-clinical cases. One factor known to increase the sensitivity of a PCR assay is the copy number of the target gene in the genome. The 18S rRNA gene in *Ba. bovis* and *Ba. bigemina* is reported to have 3 copies per genome (Brayton *et al.* 2007; Salem *et al.* 1999) while the two parasites have only one copy of cytochrome b gene per mitochondrial DNA. However, the number of mitochondria per genome of *Babesia* parasites is unknown. Salem *et al.* reported finding over 100 copies of the cytochrome b gene per genome in *Ba. bovis* and *Ba. bigemina* and higher sensitivity of a cytochrome b PCR assay over those of the 18S rRNA assay (Salem *et al.* 1999). However, it is important to note that the cytochrome b assay reported by Salem *et al.* involved a single PCR plus a hybridisation method while the 18S rRNA assay used a nested PCR protocol. Studies where the sensitivity of PCR assays was independent of the copy number of the target gene have been reported (Martins *et al.* 2008; Oura *et al.* 2004a). Martins *et al*. described developing a babesipsin (a single copy gene) hot-start PCR assay that was more sensitive than the 18S rRNA nested PCR. This was attributed to the use of a hot-start DNA polymerase and the longer babesipsin primers. In the present study, the optimum PCR assay was not identified. Given that the sensitivities of both the 18S rRNA

and the cytochrome b PCR assays were comparable and sufficient to detect non clinical carrier cases, the assay with the greatest specificity would appear to be the one of choice for deployment in the field. In this case, the cytochrome b assay would be better suited for field use, as it showed more specificity in this study and has been reported to be more sensitive in previous studies (Salem *et al.* 1999).

CHAPTER THREE

Epidemiological analysis of tick-transmitted diseases of cattle in Central and Eastern Zambia

3.1 Introduction

3.1.1 Background

The most important TBDs of cattle in Zambia are East coast fever (ECF) caused by *T. parva*, babesiosis caused by *Ba. bovis* and *Ba. bigemina*, anaplasmosis caused by *A. marginale* and heartwater caused by *E. ruminantium* (Makala *et al.* 2003). Despite the considerable economic losses to the livestock industry associated with these diseases, information on their epidemiology in Zambia is either inadequate or completely absent. When such information is available, it is often obsolete and based upon serological tests or microscopic examination of blood smears, diagnostic techniques whose shortcomings are well-documented (Bose *et al.* 1995). Since the Zambian environment supports a multi-vector ecology (Pegram *et al.* 1986), mixed infections of these pathogens in natural populations of cattle are likely to be commonplace. This provides an opportunity to study how interaction among pathogens impacts on the epidemiology of TBD and such information is important in the design and implementation of disease control programmes. This chapter documents a study on the epidemiology of tick-borne pathogens of cattle in the Central, Lusaka and Eastern Provinces of Zambia.

3.1.2 Epidemiology of TBDs in Zambia

Bovine babesiosis and anaplasmosis are among the most important diseases of livestock in Zambia. Two species of *Babesia, Ba. bovis* and *Ba. bigemina* are of economic importance in cattle (Jongejan *et al.* 1988; Makala *et al.* 2003). *Ba. bovis* is transmitted solely by *Bo. microplus* while *Ba. bigemina* is transmitted by both *Bo. microplus* and *B. decoloratus,* with the latter species having a wider distribution in the country. Another vector of *Ba. bigemina*, *R. evertsi evertsi* occurs throughout the country (Pegram *et al.* 1986; Pegram & Banda 1990), although the significance of this tick as a vector of *Ba. bigemina* in the field is unknown. Infections caused by *Ba. bigemina* are distributed more extensively in the country than those caused by *Ba. bovis,* and this has been attributed to a wider range of vectors of *Ba. bigemina* (Jongejan *et al.* 1988). Using the indirect fluorescent antibody test (IFAT), Jongejan *et al.* (1988) found antibodies against *Ba. bigemina* in samples from

cattle throughout the country. The distribution of cattle with antibodies against *Ba. bovis* followed that of the vector *B. microplus,* which was limited to the Eastern and Northern parts of the country. Unexpectedly, they also found low numbers of *Ba. bovis* serologically positive cattle in Central and Southern Provinces, areas where *Bo. microplus* was not known to occur. In the same study, but using the card agglutination test (CAT), Jongejan *et al.* found antibodies to *Anaplasma* spp in cattle serum sampled from throughout the country. When they re-tested the same samples using ELISA, they revealed sero-prevalence rates which were 1.5 to 2.3 fold greater than those obtained using CAT.

The main vector of *T. parva* in Zambia is *R. appendiculatus* and in arid areas, the closely related *R. zambeziensis* transmits the disease (Berkvens *et al.* 1995; Berkvens *et al.* 1998; Mulumba *et al.* 2000). ECF is widely distributed in Southern, Eastern, Central, Lusaka, Northern and Copperbelt Provinces of the country (Makala *et al.* 2003). Although the ECF epidemiological situation in both the Southern and Eastern Provinces has been described as endemically unstable (Billiouw *et al.* 2002; Mulumba *et al.* 2000), fundamental differences in the transmission of the disease have been observed between the provinces (Berkvens *et al.* 1995; Billiouw *et al.* 1999; Billiouw *et al.* 2005; Mulumba *et al.* 2000). In the Eastern Province, the uni-modal rainfall pattern and year-to-year variation in rainfall result in restricted activity of *R. appendiculatus* instars, fluctuations in tick phenology (i.e. life cycle stages and how their occurrence is influenced by seasonal and interannual variations in climate) and *T. parva* transmission. Adult tick activity invariably peaks during the rains and is associated with the highest ECF incidence with nymphal transmission of *T. parva* to cattle apparently less important (Billiouw *et al.* 1999; Billiouw *et al.* 2002). Second periods of activity of both adult and nymphal instars (Berkvens *et al.* 1998) are pronounced only when the climate is suitable and when they occur, they ensure a more sustained and efficient transmission of *T. parva.* This second wave of adult tick activity also plays a key role in the dynamics of prolonged outbreaks of the disease in epidemic areas (Billiouw *et al.* 1999). The epidemiology of the disease in the Southern part of the country is more complex with nymphal transmission being more important in years of below average rainfall (Mulumba *et al.* 2000) and adult transmission being more important in years of above average rainfall (Mulumba *et al.* 2001). No second peak of adult tick activity has been reported in the Southern part of the country (Speybroeck *et al.* 2002). Years of below average annual rainfall in the regions result in periods of reduced transmission in which the susceptible cattle population increases. Consequently, it is doubtful whether a state of endemic stability of this disease in the country can be reached in the near future (Billiouw *et al.* 1999).

Other species of *Theileria* known to infect cattle in Zambia are *T. mutans*, *T. taurotragi* and *T. velifera* (Jongejan *et al.* 1986b; Musisi *et al.* 1984). *T. mutans* is a primary parasite of the Cape buffalo (*Syncerus caffer*) but is also infective for cattle (Paling *et al.* 1981; Young *et al.* 1977). *T. taurotragi* is a parasite of eland (*Taurotragus oryx*) in which it can sometimes produce fatal infections (Grootenhuis *et al.* 1980). Although *T. mutans* and *T. taurotragi* are considered non-pathogenic in cattle, anaemia and disease syndromes characterised by high morbidity and low mortality have been attributed to these two parasites in Botswana (Binta *et al.* 1998). Pathogenic strains of *T. mutans* have also been isolated in Kenya and Zambia (Irvin *et al.* 1972; Musisi *et al.* 1984; Young *et al.* 1978). *T. taurotragi* is considered to be one of the causes of cerebral theileriosis (de Vos *et al.* 1981). *T. velifera* and *T. taurotragi* share the same vector as *T. parva* while *T. mutans* is transmitted by *Amblyomma* ticks. Distinguishing these parasites from *T. parva* under field conditions is very important and this cannot be achieved easily by microscopic examination.

Heartwater, caused by *E. ruminantium,* affects domestic and wild ruminants in Zambia (Makala *et al.* 2003). The ticks *A. hebraeum* and *A. variegatum* are the vectors of heartwater in Zambia (Jongejan *et al.* 1989; Pegram *et al.* 1986; Pegram & Banda 1990) and the disease occurs throughout the country (Makala *et al.* 2003). Heartwater mainly affects cattle, although outbreaks in small ruminants have been reported (Ahmadu *et al.* 2004). The incidence of the disease is not associated with presence of exotic and cross-breeds, but is mainly seen in areas where regularly dipped cattle are in close proximity to indigenously kept cattle that receive no acaricide treatment and also in areas where game and cattle co-graze (Makala *et al.* 2003).

TBDs in Zambia are mainly controlled by the regular treatment of cattle with acaricides and treating clinical cases with appropriate anti-parasitic drugs where possible. ECF is also controlled by vaccination using the Infection and Treatment method (Radley *et al.* 1975a), with local parasite strains being employed. Vaccination against ECF is carried out in the Southern and Eastern Provinces only. However, even in areas where vaccination is practised, ECF epidemics still occur and the epidemiological situation fails to meet the criteria for endemic stability (Billiouw *et al.* 1999). No vaccines for the other TBDs are currently available in the country. Acaricide application among the traditional farmers is often irregular and most of the farms lack proper facilities to adequately treat their cattle.

Most of the epidemiological information described above was obtained by examination of Giemsa-stained blood smears and serological tests whose limitations are well known (Bose

et al. 1995). It has been more than twenty years since any comprehensive studies on the epidemiology of babesiosis and anaplasmosis in Zambia have been conducted and there is no evidence of epidemiological studies on heartwater in cattle ever having been undertaken. There is also no information on the prevalence of *T. parva* in Eastern and Central Zambia. With extensive movement of cattle and frequent changes in Governmental disease control policies (Chilonda *et al.* 1999), it is likely that most of the published data is no longer valid. Therefore new studies, using more sensitive and specific diagnostic methods, are needed to determine the current epidemiological situation of these diseases in the country.

Among the key determinants of the epidemiology of TBDs are the distribution and abundance of tick vectors; the presence, abundance and movements of cattle and the resistance of the host to both the tick and TBD pathogens (Bakheit & Latif 2002; Bock *et al.* 1997; Norval *et al.* 1988). In a study to determine potential risk factors that are associated with the distribution of *T. parva* and *T. annulata* infections in cattle in Sudan, Salih *et al.* (2007) found that location, management systems and animal age were risk factors for *T. annulata* infections in Northern Sudan, while location and season were risk factors for *T. parva* infections in Southern Sudan. Swai *et al.* (2005) conducted a cross-sectional study to determine spatial and management factors associated with exposure to TBDs in smallholder dairy cattle in two regions of Tanzania. The study found that sero-prevalence to all the tick-borne diseases increased significantly with age and that animals pasture-grazed in the three months prior to the start of the sampling period were significantly more likely to be sero-positive for *Theileria* spp and *Babesia* spp. They also found that risk factors specific to individual farms were more important than those common to the local neighborhood. From these published reports, it is evident that determinants of these diseases vary from region to region and in some cases, local (farm level) factors maybe more important than those common to the whole area. To date, no studies have been undertaken to determine and quantify risk factors associated with tick-borne diseases in Zambia. An understanding of these factors is important in the design and implementation of effective TBD control programmes.

Another important aspect of the TBDs epidemiology that remains largely unexplored is how mixed infections occurring in a single host affect disease epidemiology and host fitness. Variations in severity of clinical presentations, pathogenicity, and response to therapy are often associated with co-infection with different pathogens (Belongia 2002; Graham *et al.* 2005; Sasanelli *et al.* 2009; Thompson *et al.* 2001). An experimental

examination of *Plasmodium*-helminth co-infection demonstrated that mice infected with *Listomosoides simodontitis* and *P. chabaudi chabaudi* lost more body mass and red blood cell density than mice infected with *Plasmodium* alone (Graham *et al.* 2005). Critically, for a given level of parasitaemia, co-infected animals exhibited severe clinical signs compared to animals infected only with *P. chabaudi chabaudi*. Co-infections may also be associated with a reduction in the pathology of a disease. For example, Pinchbeck *et al.* (2008) observed that co-infection with *T. vivax* and *T. congolense* appeared to cause less reduction in PCV in working donkeys in the Gambia, compared to those infected with *T. congolense* alone.

Associations between co-infecting organisms may be positive or negative. A positive association is one where being infected by one pathogen increases the chances of being infected by another, while the opposite denotes a negative association (Dib *et al.* 2008). A positive association between parasites may be due to identical ecological needs, as is the case with the deer-associated zoonoses of Lyme disease, babesiosis and ehrlichiosis in humans (Thompson *et al.* 2001), or by one parasite increasing the susceptibility of the host to infection by another (Graham *et al.* 2007). A negative association may be due to direct interference of the parasites with each other, either indirectly through competition for resources or via the immune system (Telfer *et al.* 2008). In a country such as Zambia where mixed infections of tick-transmitted pathogens are likely to be common, it's important to understand the associations among the various TBDs and the effect these associations could have on the host-parasite interactions. Such associations not only affect the epidemiology of the diseases, but also the outcome of therapeutic regimes and therefore should be taken into consideration when designing integrated disease control programs and evaluating treatment options.

3.1.3 Objectives

The aim of the work presented in this chapter was to carry out an epidemiological investigation of TBDs in three provinces of Zambia (Central, Lusaka and Eastern) with the following specific objectives:

1. **Determine the prevalence of TBDs in Central, Lusaka and Eastern Provinces of Zambia in both the dry and wet seasons**

2. Determine and quantify effects of the various risk factors associated with the occurrence of TBDs in cattle in these provinces

3. To investigate interactions among co-infecting TBD pathogens in the three provinces

4. Investigate the quantitative effects of mixed and single infections together with other confounding variables on PCV in apparently healthy carrier cattle in the three provinces

5. Determine the relative abundance of ticks and the factors that influence the observed tick burdens on cattle in the three provinces

3.2 Materials and methods

3.2.1 Study area

The study was conducted in three Provinces (Eastern, Lusaka and Central) of Zambia. The location of each of the three provinces and the distribution of the sampling sites are shown in Figure 3.1. The Eastern Province borders Malawi to the East and Mozambique to the South and covers an area of 69,000 km^2, about 9 % of Zambia's total territory. It is divided into eight districts: Chipata, Chama, Lundazi, Chadiza, Mambwe, Nyimba, Katete and Petauke. This study was conducted in the latter two districts. The plateau of the Eastern Province has a flat to gently rolling landscape with altitudes ranging from 900 to 1200 m. The vegetation is miombo woodland dominated by tree species such as *Braychystegia* and *Julbernadia* (Van Den Bossche P. & De Deken 2002). The livestock production system is predominantly traditional with goats, sheep and pigs roaming freely in the vicinity of the villages. Cattle are usually herded, but grazing patterns differ between seasons (Van Den Bossche P. & De Deken 2002). According to the 2007 livestock census, a total number of 306,668 cattle (Angoni breed), 1,779,322 goats (mainly Small East African breed), 18,053 sheep and 256,908 pigs were present in the province (Anon 2007).

The Central province is located centrally in Zambia (Figure 3.1) and shares borders with all the other eight provinces of the country. The province covers an area of about 94,395 km^2, which is about 12.5 % of the total Zambian territory. It is divided into six districts, Serenje, Kabwe, Kapiri Mposhi, Mukushi, Mumbwa and Chibombo, and this study was conducted in the latter two districts. The altitude of this region varies from 1100 to 1200 m above sea level and the annual rainfall ranges from 800 to 1000 mm (Chipanshi 1989). The topography consists of gently undulating terrain and the vegetation is dominated by dry miombo woodland (Chidumayo 1987). Both commercial and traditionally managed livestock enterprises are found in the Central province. There are

Figure 3.1 Map of Zambia showing the location of the three provinces and sampling sites within each province

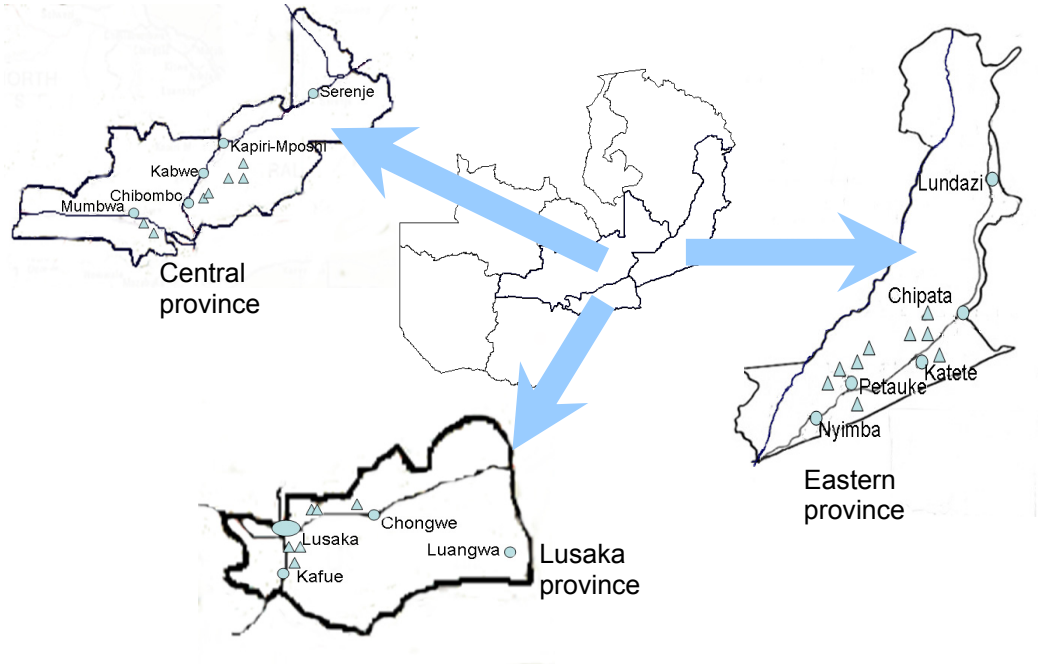


Figure legend

Borders

Roads

Towns

Sampling sites

about 663,878 cattle, 2,739 sheep, 39,889 goats and 16,093 pigs in the province (Anon 2007).

Lusaka province (Figure 3.1) shares borders with Zimbabwe and Southern Province to the South, Central province to the West and North and Eastern Province to the East. It covers a total area of about 21,898 km^2 and is divided into four districts namely Luangwa, Lusaka, Kafue and Chongwe. Samples were collected from Lusaka, Chongwe and Kafue. The mean average rainfall and vegetation are similar to that of the Central Province. The number of commercially reared cattle is greater than that found in the traditional sector in this province and as of 2007, the province had 3,933 cattle, 4,479 sheep, 5,139 goats and 18,981 pigs (Anon 2007).

3.2.2 Sample collection

Cattle blood samples were collected from the selected districts of Eastern, Central and Lusaka provinces (Figure 3.1) in the dry season (June to September 2007) and wet season (December 2007 to March 2008). Attempts were made to collect samples from the same areas in both seasons. However, heavy rainfall rendered some of the dry season sampling areas inaccessible during the rainy season and alternative sampling areas close by were sought. Both statistical and non-statistical considerations were taken into account in when choosing the sample size. The formula for measuring the characteristic of a population in terms of a proportion was used (Eng 2003). Based on previous studies (Ahmadu *et al.* 2004; Jongejan *et al.* 1988), a conservative average prevalence of TBDs in the three provinces was taken to be around 20 %, with a 95 % confidence interval of ± 10 %. From this, the minimum number of animals required from each province in the dry season was calculated to be 62. However, because of the need to have a sufficient number of *Babesia* parasites for population genetic analysis and the greater likelihood of getting both *Babesia* species in Eastern province than in the other provinces, more samples were collected from this province than the other provinces. In the dry season, a total of 637 cattle were sampled and of these, 422 were from Eastern Province, 151 from Central province and 65 from Lusaka province. Because of the expectation that the number of positive animals in the wet season was going to be significantly higher than in the dry season, the number of samples collected in the wet season was reduced to 349 and of these, 211 were from Eastern Province, 82 from Lusaka and 56 from Central Province. Up to 10 % of cattle in each herd or group of cattle were randomly sampled (10 % sampling fraction) ensuring that all age groups and both sexes were represented. Blood was collected in EDTA tubes and was obtained from either the coccygeal or the jugular vein, depending on the technique used to

restrain cattle. 125 µl of each blood sample was spotted on FTA Classic cards ™ (Whatman) and left to dry for one hour before being stored until required for PCR. The remainder of the blood was transported on ice in cooler boxes to the laboratory. In the laboratory, the packed cell volume (PCV) was measured for each sample collected. At the time of blood sampling, information on sex, breed, age, source and vaccination status of the cattle against East Coast fever was collected. Additionally, information on the type of farm management (whether commercial or traditional) and the frequency and method of tick control employed was recorded. The tick burden (adult and nymph) on each animal was assessed as: none seen, few (1 to 20 ticks), moderate (21 – 50 ticks) and abundant (> 50 ticks).

3.2.3 Preparation of samples and PCR amplification

To provide template DNA for PCR, blood samples on FTA cards were prepared as described in Section 2.2.5. The 18S rRNA and 16S rRNA gene semi-nested PCR assays for *Theileria/Babesia* and *Anaplasma/Ehrlichia* respectively (detailed in Chapter Two) were utilised. For each set of samples analysed, a positive control (containing DNA of the test species) and negative control (containing PCR reagents but no template DNA) were included.

3.2.4 Data analysis

Data was initially manipulated on Microsoft Excel before being exported to SPSS version 15.0 (SPSS Inc, USA). The chi-square or the Fisher's Exact tests were used to test for association among categorical variables while the one-way analysis of variance (ANOVA) was used to compare means of continuous variables. In order to assess the effect of infection with different tick-borne pathogenic species on PCV, whilst allowing for the effect of other confounding variables (age, gender, breed, province, tick burden, tick-control method, tick control frequency and farming system) multivariable regression, using the Generalise Linear Model in SPSS was used. In the initial model, both the main effects and the two-way interaction terms of the variables were tested. In the final model, only those variables and interactions terms that had significant effects were included. Type III sum of squares (partial sums of squares, where each effect is adjusted for every other effect) were used to test the significance of each fixed effect specified in the model. The adequacy of the fitted models was checked using residual and probability plots.

The step-wise binary logistic regression model (Hosmer & Lemeshow 2000) was used to determine predictors (risk factors) of being PCR positive for each of the five tick-borne

pathogens under study (i.e. *Ba. bovis, Ba. bigemina, T. parva, Anaplasma* spp and *E. ruminantium*). The Logit link function reported the coefficient, *p value*, odds ratio (OR) and 95 % lower and upper confidence interval values for the OR. The OR is a measure of effect size, describing the strength of association or non-independence between two binary data values (Viera 2008). An OR of 1 implies that the event is equally likely in both groups. An OR greater than one implies that the event is more likely in the first group. An OR less than one implies that the event is less likely in the first group.

The ordinal regression model (Hosmer & Lemeshow 2000) was used to determine which set of variables had a significant effect on tick burden observed on cattle in the wet season using the negative log log as a link function. This link function was used because it gives a good model fit when the cumulative probabilities for lower scores are high and the approach to 1 is slow, as was the case with the data under consideration. It relates to the probability (p) of success (values between 0 and 1) to a predictor variable x. It is defined as $p = \exp[-\exp(-x)]$ (or equivalently $x = -\log(-\log p)$) where log is the natural logarithm function and exp is the base of the natural logarithm (Chen & Hughes 2004). Unlike the logit link function, the negative log log does not directly measure the odds ratio. The signs of the regression coefficients (estimates) were used to interpret the model. A positive regression coefficient indicated that there was a positive relationship between the explanatory variable and the ordinal outcome. A negative regression coefficient indicated that there was a negative relationship between the explanatory variable and the ordinal outcome (Chen & Hughes 2004). All statistical tests were considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 Prevalence of tick-borne pathogens in the dry and wet seasons in Central and Eastern Zambia

TBDs pathogens under study were detected in all three provinces, with variations in abundance observed between the seasons and the provinces. The prevalence of tick-borne pathogens in Central and Eastern Zambia in the two seasons under study are shown in Table 3.1 together with the Chi-square (and where this test was not appropriate, the Fisher's Exact test) *p* values for the seasonal changes in prevalence. In Eastern Province, the prevalence of each of the pathogens were significantly higher in the wet season than in the dry season. In both seasons in this province, *T. mutans* was the most prevalent, followed by *Anaplasma* spp while *Ba. bigemina* was the least prevalent.

Table 3.1 Prevalence (%) of tick-borne pathogens of cattle for the dry and wet season in Central and Eastern Zambia

Prevalence of tick-borne disease (TBD) pathogens in cattle in Eastern, Central and Lusaka Provinces, together with the *p* values (Chi-square and where this was not appropriate, the Fisher's Exact test) for the change in prevalence between the dry and wet seasons are shown. The 'Overall' section shows combined prevalences of TBD pathogens in the three provinces. Overall, the prevalence of TBD pathogens was higher in the wet season than in the dry season. *p* values marked * were significant.

Table 3.1 Prevalence (%) of tick-borne pathogens of cattle for the dry and wet season in Central and Eastern Zambia

	Eastern province			Central province			Lusaka province			Overall		
	Dry season	Wet season	*p value*	Dry season	Wet season	*p value*	Dry season	Wet season	*p value*	Dry Season	Wet season	*p value*
n	422	211		151	56		64	82		637	349	
Ba. bovis	10.90	32.70	< 0.001*	4.67	1.78	0.686	32.81	9.76	< 0.001*	11.62	22.35	< 0.001*
Ba. bigemina	0.24	11.85	< 0.001*	1.32	0.00	1.00	0.00	14.63	< 0.001*	0.47	10.60	< 0.001*
T. parva	22.04	30.33	0.023 *	25.83	14.29	0.078	14.06	40.24	< 0.001*	22.14	30.09	0.006 *
T. taurotragi	21.33	36.49	<0.001 *	23.18	17.86	0.410	28.13	37.80	0.219	22.45	33.81	< 0.001 *
T. mutans	48.82	83.89	< 0.001*	66.89	39.29	<0.001*	70.31	74.39	0.584	55.26	74.50	< 0.001*
Anaplasma spp	40.76	63.51	< 0.001*	76.83	48.21	<0.001*	62.50	52.44	0.223	51.49	58.45	0.036 *
E. ruminantium	5.45	18.96	< 0.001*	37.75	33.93	0.613	45.31	29.27	0.045*	17.11	23.78	0.011 *

In Lusaka Province (Table 3.1), all the parasites had higher prevalences in the wet season than in the dry season except *Ba. bovis*, *E. ruminantium* and *Anaplasma* spp which had lower prevalences in the wet season than in the dry season, although the differences for *Anaplasma* spp, *T. mutans* and *T. taurotragi* were not statistically significant. Similar to the situation in the Eastern Province, *T. mutans* was the most prevalent in all the seasons followed by *Anaplasma* spp. The drop in the seasonal prevalences of *E. ruminantium* and *Ba. bovis* (Table 3.1) in the wet season may have been due to the change in sampling sites, as the roads to farms where samples were collected in the dry season had become impassable.

The situation in the Central Province was very different to that in Eastern and Lusaka Provinces with all parasite species showing lower prevalences in the wet season than in the dry season. This drop in prevalence was statistically significant only for *T. mutans* and *Anaplasma* spp (Table 3.1), and this is surprising given that ticks were more abundant on cattle in the wet season than in the dry season (See section 3.3.7). The reasons for the drop in pathogen prevalence in this province are unknown, but it is possible that some of the farmers may have treated their cattle with anti-parasitic drugs immediately prior to sample collection. It is understood that a proportion of farmers tend to treat their animals with long-acting tetracycline when the risk from TBD is high (Geoffrey Mainda, personal communication).

When the prevalences of TBD pathogens were compared across the provinces using the Chi-square test and where this test was not appropriate, the Fisher's exact test, the differences were not consistent across the two seasons. The prevalence of *Ba. bovis* was significantly higher in Lusaka Province than in Eastern and Central Provinces in the dry season ($p < 0.001$) while in the wet season, it was significantly higher in Eastern Province than in Lusaka and Central Provinces ($p < 0.001$). For *Ba. bigemina*, no significant differences were observed in prevalences, between the three provinces in the dry season ($p = 0.252$). However, in the wet season, significant differences were observed between the provinces ($p = 0.004$). The prevalence of this parasite was highest in Lusaka and lowest in Central Province with no positive cases of *Ba. bigemina* being detected in Central Province in the wet season. There were no significant differences in *T. parva* prevalences among the three provinces in the dry season ($p = 0.180$). However, in the wet season, the prevalence of *T. parva* was significantly different between the provinces ($p = 0.004$). Lusaka province had the highest while Central province showed the lowest prevalence. There was equally no significant difference in the prevalence of *T. taurotragi*

among the three provinces in the dry season ($p = 0.601$), while the prevalence of this disease was significantly different between the provinces in the wet season ($p < 0.001$). The prevalence of *T. mutans* was significantly lower in Eastern Province than in the other two provinces in the dry season ($p < 0.001$) but significantly higher in the wet season in this province than in Lusaka and Central province. The prevalence of this parasite in the wet season was significantly higher in Lusaka province than in Central Province ($p < 0.001$). The prevalence of *Anaplasma* spp was significantly different among the three provinces in both seasons ($p < 0.001$) with the prevalence being highest in Central Province and lowest in Eastern Province in the dry season. In the wet season, the trend was reversed. The prevalence of *E. ruminantium* was significantly lower in the Eastern Province in both seasons compared to the other two provinces ($p < 0.001$ for the wet season and $p = 0.027$ for the dry season).

When the data from each of the provinces were combined (Table 3.1), all the parasites showed an increase in prevalence in the wet season and this was significant for *Ba. bovis* ($p = 0.040$), *Ba. bigemina* ($p < 0.001$) and *T. mutans* ($p = 0.007$). The seasonal changes in prevalence for *T. parva* ($p = 0.201$), *T. taurotragi* ($p = 0.067$), *Anaplasma* spp ($p = 0.321$) and *E. ruminantium* ($p = 0.243$) were not significant although they were consistently higher in the wet season than in the dry season. The parasite with the highest change was *Ba. bigemina*, whose prevalence increased from 0.5 % in the dry season to 10.6 %, representing a change of over 21-fold. This increase in the prevalence of all the parasites was likely due to the greater abundance of ticks in the wet season (Section 3.3.7). The high humidity, high temperatures and the vegetation in the wet season made the environment conducive to the survival of the various tick vectors.

Among the provinces under study, ECF vaccination is only carried out in Eastern Province. To determine whether vaccination with live parasites increased the number of PCR positive cattle for *T. parva* in this province, prevalence rates were compared between vaccinated and unvaccinated cattle. Of the cattle examined, 43 (20.38 %) and 41 (9.79 %) were reported to have been vaccinated against ECF in the wet and dry seasons respectively. Out of these vaccinated cattle, only 15 (34.88 %, wet season) and 7 (17.07 %, dry season) were found to be PCR positive for *T. parva* (Table 3.2). There was no significant difference in prevalence of *T. parva* between vaccinated and non-vaccinated cattle over the two seasons ($p = 0.463$ in wet season and $p = 0.553$ in dry season) in this region. This may be an indication that the carrier status induced by the vaccine strain is short-lived.

Table 3.2 Number of ECF vaccinated cattle that were PCR positive for *T. parva* in Eastern Province

The number of cattle (with percentages in brackets) that were PCR positive for *T. parva* in ECF vaccinated and non-vaccinated cattle in the wet and dry seasons in Eastern province are shown. No significant differences were found in prevalence of *T. parva* between ECF vaccinated and non-vaccinated cattle ($p = 0.463$ for the dry season and $p = 0.553$ for wet season).

Table 3.2 Number of ECF vaccinated cattle that were PCR positive for *T. parva* in Eastern Province

Season	Vaccination status	*T. parva* (PCR)		Total
		Negative (%)	Positive (%)	
Wet	Not Vaccinated	119 (70.80)	49 (29.20)	168
	Vaccinated	28 (65.12)	15 (34.88)	43
Dry	Not vaccinated	292 (77.25)	86 (22.75)	378
	Vaccinated	34 (82.93)	7 (17.07)	41

3.3.2 Age-specific prevalences of tick-borne pathogens in the dry and wet seasons

The relative amount and severity of a tick-borne disease among different age groups of a cattle population is a good indicator of the epidemiological status of the disease in that population. The epidemiology of TBDs varies between states of endemic stability, where most calves encounter the causative pathogen early in life, and clinical disease is rare or restricted to calves, to endemic instability, where very few calves come in contact with the causative pathogen early in life and clinical disease is common even in older cattle. To determine the relative amounts of infection in the different age groups of cattle in Central and Eastern Zambia, the age-specific prevalences of each of the TBD pathogens under study were calculated for cattle in the age groups 1 to 12, 13 to 24, 25 to 48 and >48 months old for both the dry and the wet season; the results are shown in Figures 3.2 and 3.3 respectively. The Chi-square test was used to determine whether the prevalences between the age groups were significantly different. In the dry season (Figure 3.2), the prevalences of *T. parva* ($p < 0.05$) and *T. taurotragi* ($p < 0.001$) were significantly lower in 1 to 12 months old calves than in older cattle. However, in the wet season (Figure 3.3), there was no significant difference in the prevalence of the two parasites among the age groups. Both *T. parva* and *T. taurotragi* are transmitted by the same vector, *R. appendiculatus.* This seasonal shift in the epidemiological status of the diseases is in agreement with the change in the abundance of the vector tick between the two seasons. There is an increase in the abundance of *R. appendiculatus* ticks, transmitting the parasite to all the susceptible cattle during the wet season while the numbers in the dry season decline (Pegram *et al.* 1986). Therefore, all age groups are equally exposed to the parasites in the wet season, while most of the calves born in the dry season are not. This scenario might indicate some degree of endemic stability to the disease in the wet season, although the reported reported relatively low prevalence (30 %) does not support this.

There was also a shift in the epidemiological status of *T. mutans* and *E. ruminantium* among the age groups between the two seasons. Both *E. ruminantium* and *T. mutans* are transmitted by *Amblyomma* ticks. In the dry season (Figure 3.2), no significant differences were observed in the prevalence of the two pathogens among the different age groups. However, in the wet season (Figure 3.3), cattle 1 to 12 months old had significantly lower prevalences of *T. mutans* ($p < 0.001$) and *E. ruminantium* ($p < 0.05$) than cattle in the older age groups. This observed seasonal change in the epidemiological state of these two parasites can be attributed to the vector. However, one would expect the opposite to be true given that *Amblyomma* ticks are more prevalent on cattle in the rainy season than in the dry

Figure 3.2 Dry season age-specific prevalences of tick-borne pathogens in Central and Eastern Zambia

Age-specific prevalences of TBD pathogens in Central and Eastern Zambia in the dry season. Prevalences of *T. parva* ($p < 0.050$) and *T. taurotragi* ($p < 0.001$) were significantly lower in 1 to 12 months old calves than in older cattle. No significant differences in prevalences of TBD among the age groups were detected for the other pathogens.

Figure 3.2 Dry season age-specific prevalences of tick-borne pathogens in Central and Eastern Zambia

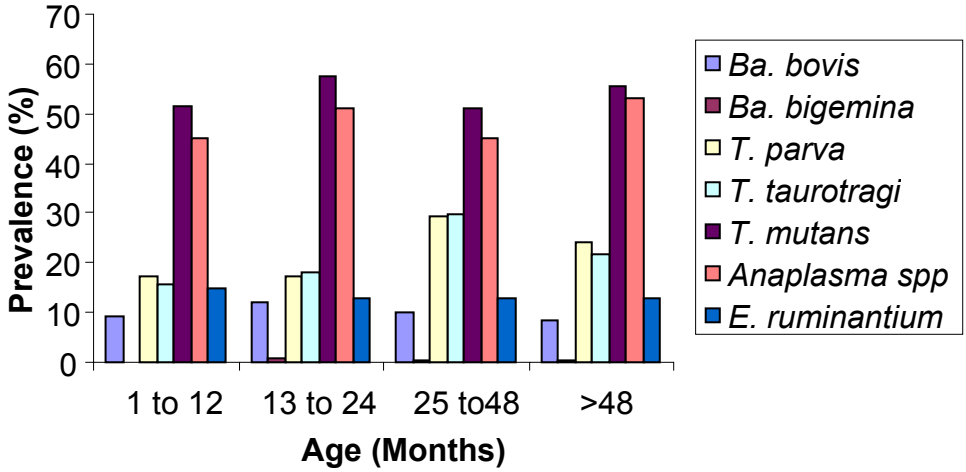
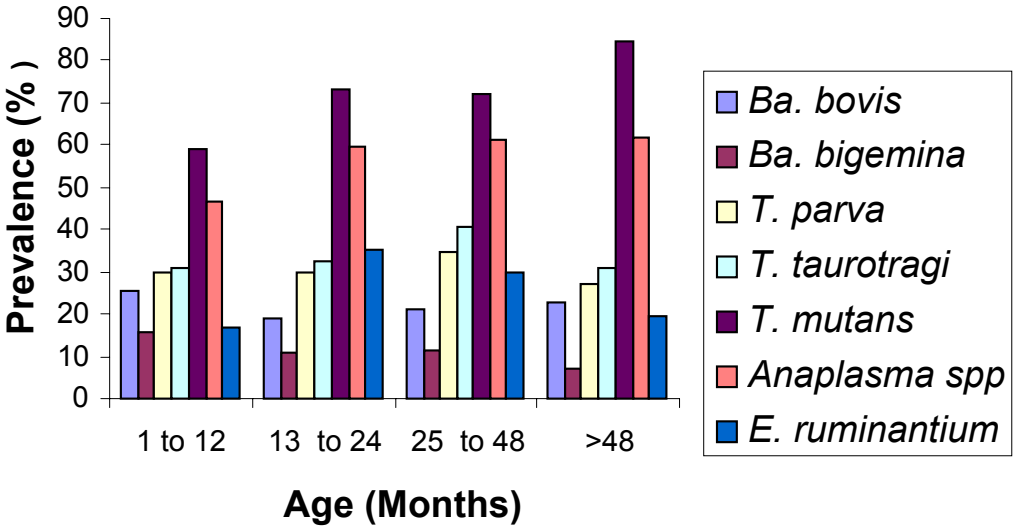


Figure 3.3 Wet season age-specific prevalences of tick-borne pathogens in Central and Eastern Zambia

Age-specific prevalences of TBD pathogens in Central and Eastern Zambia in the dry season. Prevalences of *T. mutans* ($p < 0.001$) and *E. ruminantium* ($p < 0.050$) were significantly lower in 1 to 12 months old calves than in older cattle. No significant differences in prevalences of TBD among the age groups were detected for the other pathogens.

Figure 3.3 Wet season age-specific prevalences of tick-borne pathogens in Central and Eastern Zambia



season (Pegram *et al.* 1986). There were no significant differences in prevalences observed among different age groups for the other pathogens in either season.

3.3.3 Mixed infections

In both seasons, high numbers of mixed infections were observed in cattle. Frequencies of occurrence for each TBD pathogen combination in the dry and wet seasons are shown in Tables 3.3 and 3.4 respectively. In the dry season (Table 3.3), mixed infections were detected in a total of 337 samples (52.9 %), with 38 different combinations of species and a maximum of six pathogens detected in one animal. There were 140 (22 %) single infections detected in the dry season. *T. parva* was detected as a single infection in four samples (0.6 %), *Ba. bovis* in four samples (0.6 %), *E. ruminantium* in five samples (0.8 %), *Anaplasma* spp in 62 samples (9.7 %), *T. taurotragi* in three samples (0.5 %) and *T. mutans* in 62 samples (9.7 %). In the wet season (Table 3.4), mixed infections were detected in a total of 242 samples (69.3 %) with 47 different combinations of species and a maximum of seven pathogens being detected in a single animal. There were fewer single infections detected in the wet season (41 samples (11.7 %)) than in the dry season. *T. mutans* was detected as a single infection in 32 samples (9.1 %), *Anaplasma* spp in seven samples (0.02 %) and *Ba. bovis* and *T. taurotragi* in one sample each. *T. parva* was never detected as single infection in the wet season (Table 3.4). For both seasons, the mean number of co-infecting pathogens decreased with the age of cattle, although this difference was not significant (data not shown, $p = 0.080$ for the dry season and $p = 0.291$ for the wet season.

3.3.4 Frequencies of joint occurrence of tick-borne pathogens in the dry and wet seasons in Central and Eastern Zambia

The results above show that different tick-transmitted pathogens in Zambia can occur within the same locality, providing an opportunity for them to interact with each other in cattle hosts, either positively or negatively. The understanding of such interactions is important because they not only affect the outcome of therapeutic interventions but also the severity and epidemiology of tick-transmitted diseases. To determine whether two species of pathogens occurring in one cattle host are associated with each other positively or negatively, the observed and expected frequencies of joint occurrence were compared. The expected frequency of joint occurrence of two pathogens was calculated as the product of the individual observed frequencies of the two pathogens under consideration divided by n, the total number of cattle. If the observed frequency was greater than the expected, then the association is positive, if it is less than the expected, the association is negative. A

Table 3.3 Parasite combinations in the dry season in Central and Eastern Zambia

Frequency of single and mixed infections in cattle in the dry season. Only 161 cattle were found not to be infected with any of the TBD pathogens under study, while 140 were infected with only a single pathogen. The remainder of the cattle were infected with two or more TBD pathogens.

Table 3.3 Parasite combinations in the dry season in Central and Eastern Zambia

Parasite species	Frequency	
	No.	%
Totally negative	161	25.24
T. parva	4	0.63
Ba. bovis	4	0.63
E. ruminantium	5	0.78
Anaplasma spp	62	9.72
T. taurotragi	3	0.47
T. mutans	62	9.72
Ba. bovis + T. mutans	7	1.10
Ba. bovis + A. marginale	1	0.16
T. parva + T. mutans	13	2.04
T. parva + Anaplasma spp	7	1.10
T. taurotragi + T. mutans	13	2.04
T. taurotragi + Anaplasma spp	6	0.94
T. mutans + Anaplasma spp	60	9.40
T. mutans + E. ruminantium	1	0.16
Anaplasma spp + E. ruminantium	22	3.45
Ba. bovis + T. parva + T. mutans	2	0.31
Ba. bovis + T. taurotragi + T. mutans	3	0.47
Ba. bovis + T. taurotragi + Anaplasma spp	2	0.31
Ba. bovis + T. mutans + Anaplasma spp	10	1.57
Ba. bovis + Anaplasma spp + E. ruminantium	1	0.16
T. parva + T. taurotragi + T. mutans	21	3.29
T. parva + T. taurotragi + Anaplasma spp	3	0.47
T. parva + T. mutans + Anaplasma spp	16	2.51
T. parva + Anaplasma spp + E. ruminantium	3	0.47
T. taurotragi + T. mutans + Anaplasma spp	19	2.98
T. taurotragi + Anaplasma spp + E. ruminantium	1	0.16
T. mutans + Anaplasma spp + E. ruminantium	32	5.02
Ba. bovis + T. parva + T. taurotragi + T. mutans	9	1.41
Ba. bovis + T. parva + T. mutans + Anaplasma spp	4	0.63
Ba. bovis + T. parva + T. mutans + E. ruminantium	1	0.16
Ba. bovis + T. mutans + Anaplasma spp + E. ruminantium	8	1.25
Ba. bovis + T. taurotragi + T. mutans + Anaplasma spp	3	0.47
Ba. bigemina + T. parva + T. mutans + Anaplasma spp	1	0.16
T. parva + T. taurotragi + T. mutans + Anaplasma spp	21	3.29
T. parva, T. taurotragi + Anaplasma spp + E. ruminantium	1	0.16
T. parva + T. mutans + Anaplasma spp + E. ruminantium	7	1.10
T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	7	1.10
Ba. bovis + T. parva + T. taurotragi + T. mutans & Anaplasma spp	10	1.57
Ba. bovis + T. parva + T. taurotragi + T. mutans + E. ruminantium	1	0.16
Ba. bovis + T. parva + T. mutans + Anaplasma spp + E. ruminantium	1	0.16
Ba. bovis + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	4	0.63
Ba. bigemina + T. parva + T. taurotragi + T. mutans + Anaplasma spp	2	0.31
T. parva + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	11	1.72
Ba. bovis + T. parva + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	3	0.47
Total	638	100.00

Table 3.4 Parasite combinations in the wet season in Central and Eastern Zambia

Frequency of single and mixed infections in cattle in the dry season. Only 66 cattle were found not to be infected with any of the TBD pathogens under study, while 41 were infected with only a single pathogen. The remainder of the cattle were infected with two or more TBD pathogens.

Table 3.4 Parasite combinations in the wet season in central and eastern Zambia

Parasites	Frequency	
	No.	%
Totally negative	66	18.91
Ba. bovis	1	0.29
Anaplasma spp	7	2.01
T. taurotragi	1	0.29
T. mutans	32	9.17
Ba. bovis + T. parva	1	0.29
Ba. bovis + T. mutans	4	1.15
Ba. bigemina + T. mutans	2	0.57
T. parva + T. taurotragi	3	0.86
T. parva + T. mutans	3	0.86
T. taurotragi + T. mutans	8	2.29
T. mutans + Anaplasma spp	37	10.60
Anaplasma spp + E. ruminantium	5	1.43
Ba. bigemina + T. taurotragi + T. mutans	1	0.29
B.bigemina + T. mutans + Anaplasma spp	2	0.57
Ba. bovis + B.bigemina + T. mutans	1	0.29
Ba. bovis + T. parva + T. mutans	4	1.15
Ba. bovis + T. taurotragi + T. mutans	4	1.15
Ba. bovis + T. mutans + Anaplasma spp	10	2.87
T. parva + T. taurotragi + T. mutans	6	1.72
T. parva + T. taurotragi + Anaplasma spp	1	0.29
T. parva + T. mutans + Anaplasma spp	7	2.01
T. parva + T. mutans + E. ruminantium	1	0.29
T. parva + Anaplasma spp + E. ruminantium	1	0.29
T. taurotragi + T. mutans + Anaplasma spp	13	3.72
T. taurotragi + T. mutans + E. ruminantium	1	0.29
T. mutans + Anaplasma spp + E. ruminantium	22	6.30
Ba. bovis + T. parva + T. taurotragi + T. mutans	2	0.57
Ba. bigemina + T. mutans + Anaplasma spp + E. ruminantium	2	0.57
Ba. bovis + T. parva + T. mutans + Anaplasma spp	1	0.29
Ba. bovis + T. mutans + Anaplasma spp + E. ruminantium	7	2.01
Ba. bovis + Ba. bigemina + T. taurotragi + T. mutans	2	0.57
Ba. bovis + T. taurotragi + T. mutans + Anaplasma spp	4	1.15
Ba. bigemina + T. parva + T. mutans + Anaplasma spp	1	0.29
T. parva + T. taurotragi + T. mutans + Anaplasma spp	17	4.87
T. parva + T. taurotragi + Anaplasma spp + E. ruminantium	3	0.86
T. parva + T. mutans + Anaplasma spp + E. ruminantium	7	2.01
T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	6	1.72
Ba. bovis + Ba. bigemina + T. mutans + Anaplasma spp	2	0.57
Ba. bovis + T. parva + T. taurotragi + T. mutans + Anaplasma. spp	9	2.58
Ba. bovis + Ba. bigemina +T. parva + T. taurotragi + T. mutans	2	0.57
Ba. bovis + T. parva + T. mutans + Anaplasma spp + E. ruminantium	1	0.29
Ba. bigemina + T. parva + T. mutans + Anaplasma spp + E. ruminantium	1	0.29
Ba. bovis + Ba. bigemina + T. mutans + Anaplasma spp + E. ruminantium	3	0.86
Ba. bovis + Ba. bigemina +T. taurotragi + T. mutans + Anaplasma spp	1	0.29
Ba. bigemina + T. parva +T. taurotragi + T. mutans + Anaplasma spp	3	0.86
T. parva + T. taurotragi + T. mutans +Anaplasma spp + E. ruminantium	9	2.58
Ba. bovis + T. parva + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	5	1.43
Ba. bovis + Ba. bigemina + T. parva + T. taurotragi + T. mutans + Anaplasma spp	8	2.29
Ba. bigemina + T. parva + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	2	0.57
Ba. bovis + Ba. bigemina + T. parva + T. taurotragi + T. mutans + Anaplasma spp + E. ruminantium	7	2.01
Total	349	100

significant association ($p \leq 0.05$, tested by Chi-squared test or the Fisher's exact test) indicates that the frequency of joint occurrence is greater than what would be expected by chance alone. The observed and expected frequencies of joint occurrence of tick-borne pathogens are shown in Table 3.5 and Table 3.6 for the dry and wet seasons, respectively. Results from the dry season shows that all the pathogens under study were positively associated with each other except for *Ba. bigemina* and *E. ruminantium*. Apart from *Ba. bigemina* whose associations could not be statistically tested because of the very low prevalence in the dry season, all the other piroplasms were significantly associated with each other. There was also a significant association between the two rickettsiae, *Anaplasma* and *E. ruminantium*. None of the protozoan parasites were significantly associated with *E. ruminantium* and only *T. parva* and *T. mutans* were significantly associated with *Anaplasma* spp. A similar trend was observed in the wet season. However, compared to the dry season results, two differences were observed in the results for the wet season (Table 3.6). Firstly, there was no significant association between *T. mutans* and the other piroplasms. Secondly, with the increased prevalence of *Ba. bigemina*, statistically significant associations between this parasite and the other piroplasms were observed except with *T. mutans*.

3.3.5 Determination of predictors for being PCR positive for tick-borne pathogens in Central and Eastern Zambia

A step-wise binary logistic regression model was used to determine risk factors (predictors) of an animal being positive on PCR to *T. parva*, *Ba. bigemina*, *Ba. bovis*, *Anaplasma* spp and *E. ruminantium* in the wet season. Logistic regression examines the relationship between one or more predictor variables and a binary response. It examines how the probability of an event changes as the predictor variable changes and obtains maximum likelihood estimates of the parameters using an iterative-reweighted least squares algorithm. Risk factors under consideration included PCR positivity to the six other tick-borne pathogens under study, farm management system, tick control method, frequency of tick control, province, tick burden, age, breed type and sex and, for *T. parva*, vaccination status. Criteria used in determining whether the model adequately fitted the data were a non-significant Hosmer and Lemeshow Test ($p > 0.05$) and a significant Omnibus Test of Model Coefficients ($p < 0.05$). All five models were found to fit the data adequately. Analysis using SPSS revealed a number of models in the output from which the final model, which met the set criteria, was always selected. The results are shown in

Table 3.5 Frequency of joint occurrence of tick-borne pathogens in cattle in the dry season

Frequencies of expected and observed joint occurrence of TBD pathogens are shown together with *p value* (Chi-square and where not appropriate, the Fisher's Exact test) of the differences between the two frequencies. *p* values for associations involving *Ba. bigemina* were not calculated because of the low prevalence of this parasite in the dry season. Significant associations are marked *.

Table 3.5 Frequency of joint occurrence of tick-borne pathogens in cattle in the dry season

Parasite combination	Frequency of joint occurrence (count)		Chi square *p value*
	Observed	Expected	
Ba. bovis + *Ba. bigemina*	0	0	-
Ba. bovis + *T. parva*	31	16	0.037*
Ba. bovis + *T. taurotragi*	35	17	0.016*
Ba. bovis + *T. mutans*	66	41	0.015*
Ba. bovis + *Anaplasma* spp	47	38	0.369
Ba. bovis + *E. ruminantium*	19	13	0.371
T. taurotragi + *T. parva*	82	32	< 0.001*
T. parva + *T. mutans*	123	78	< 0.001*
T. mutans + *T. taurotragi*	127	79	< 0.001*
Anaplasma spp + *E. ruminantium*	101	56	< 0.001*
T. Parva + *Anaplasma* spp	90	72	< 0.001*
T. mutans + *Anaplasma* spp	219	181	< 0.001*
Ba. bigemina + *T. taurotragi*	2	1	-
T. parva + *Ba. bigemina*	3	1	-
Ba. bigemina + *T. Mutans*	3	2	-
Ba. bigemina + *Anaplasma* spp	3	2	-
Ba. bigemina + *E. ruminantium*	0	1	-
T. parva + *E. ruminantium*	28	24	0.670
T. taurotragi + *Anaplasma* spp	93	32	0.095
T. taurotragi + *E. ruminantium*	28	24	0.670
T. mutans + *E. ruminantium*	76	60	0.170

Table 3.6 Frequency of joint occurrence of tick-borne pathogens in cattle in the wet season

Frequencies of expected and observed of joint occurrence of TBD pathogens together with *p* value (Chi-square and where not appropriate, the Fisher's Exact test) of the differences between the two frequencies in cattle in the wet season. Significant associations are marked *.

Table 3.6 Frequency of joint occurrence of tick-borne pathogens in cattle in the wet season

Parasite combination	Frequency of joint occurrence (count)		Chi square *p value*
	Observed	Expected	
Ba. bovis + *Ba. bigemina*	26	9	0.005*
Ba. bovis + *T. parva*	40	24	0.036*
Ba. bovis + *T. taurotragi*	44	27	0.033*
Ba. bovis + *T. mutans*	77	59	0.085
Ba. bovis + *Anaplasma* spp	58	46	0.202
Ba. bovis + *E. ruminantium*	23	19	0.633
T. taurotragi + *T. parva*	77	36	< 0.001*
T. parva + *T. mutans*	96	78	0.115
T. mutans + *T. taurotragi*	110	88	0.065
Anaplasma spp + *E. ruminantium*	81	49	0.001*
T. Parva + *Anaplasma* spp	83	61	0.040*
T. mutans + *Anaplasma* spp	187	152	0.010*
Ba. bigemina + *T. taurotragi*	26	14	0.049*
T. parva + *Ba. bigemina*	24	12	0.040*
Ba. bigemina + *T. mutans*	40	30	0.200
Ba. bigemina + *Anaplasma* spp	32	23	0.390
Ba. bigemina + *E. ruminantium*	8	10	0.633
T. parva + *E. ruminantium*	37	25	0.110
T. taurotragi + *Anaplasma* spp	88	69	0.085
T. taurotragi + *E. ruminantium*	33	28	0.509
T. mutans + *E. ruminantium*	74	62	0.253

Tables 3.7 to 3.11. The confidence intervals for some estimates in the models are very wide, indicating the degree of uncertainity in the estimates.

The results of the binary regression model (Table 3.7) indicate that, cattle which were PCR positive for *Ba. bovis* were more than eight times (C.I = 3.51 – 20.57) more likely to be PCR positive for *Ba. bigemina* than those that were negative for *Ba. bovis (p < 0.001).* Although age was statistically a significant predictor, its effect on being positive for *Ba. bigemina* was minimal (OR = 0.98 (C.I = 0.97 – 0.99), *p = 0.036*). The province from which the sample was taken had a significant effect on being positive for *Ba. bigemina* (*p = 0.016*). Cattle from Eastern province were 0.22 (C.I = 0.10 – 0.65) times less likely to be PCR positive for *Ba. bigemina* than those from Lusaka Province (*p = 0.001*). Cattle breed type, sex, management system, frequency and method of tick control and tick burden were not predictors of being PCR positive for *Ba. bigemina*.

Cattle on commercially managed farms were 21 (C.I = 1.75 – 253.63) times more likely to be PCR positive for *Ba. bovis* than those under a traditional management regime (*p = 0.020*) (Table 3.8). Being PCR positive for *T. mutans*, *T. parva* or *Ba. bigemina* increased the probability of being PCR positive for *Ba. bovis* by a factor of 12.48 (C.I = 2.26 – 69.09), 2.07 (C.I = 1.09 – 3.91) and 9.16 (C.I = 3.49 – 24.06) respectively. Cattle in Eastern Province were 186 (C.I = 9.53 – 3639.13) times more likely to be positive on PCR for *Ba. bovis* than those from Central Province (*p < 0.001*). Spraying as a method of tick control decreased the risk of being positive for *Ba. bovis* by a factor of 0.03 (C.I = 0.001 – 0.30) compared to cattle which were dipped (*p < 0.001*). Breed type, age, sex, frequency of tick control and tick burden were not significant predictors of being PCR positive for *Ba. bovis*.

Being PCR positive for *Ba. bovis*, *T. taurotragi* or *Anaplasma* spp increased the risk of being PCR positive for *T. parva* by a factor of 2.31 (C.I = 1.17 – 4.56), 11.08 (C.I = 6.15 – 19.97) and 3.06 (C.I = 1.59 – 5.88) respectively (Table 3.9). Cattle on commercial farms were twelve (C.I = 1.43 – 99.01) times more likely to be PCR positive for *T. parva* than those under traditional husbandry (*p = 0.020*). Angoni breed-type cattle were eight (C.I = 1.59 – 40.23) times more likely to be positive on PCR for *T. parva* than the Tonga breed-type *(p = 0.001*). Province, method and frequency of tick control, tick burden, ECF vaccination status, age and sex were found not to be significant predictors of being PCR positive for *T. parva.*

Table 3.7 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *Ba. bigemina* in cattle in Central and Eastern Zambia

*Age, province and PCR positivity to *Ba. bovis* were significant predictors of being PCR positive for *B.bigemina*.

Table 3.7 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity to *Ba. bigemina* in cattle in Central and Eastern Zambia

Independent variables	Coefficient	*p value*	Odds ratio (OR)	95% C.I. for OR	
				Lower	Upper
Age	-0.01	*0.040**	0.98	0.97	0.99
Province		*0.016**			
Eastern Province	-1.51	*0.001**	0.22	0.10	0.65
Central Province	18.56	*0.997*	0.00	0.00	0.00.
Lusaka [(a)]	_	_			
Ba. bovis	2.14	*< 0.001**	8.49	3.51	20.57
Constant	-38.07	*1.000*	0.00		

(a) = reference category, C.I = confidence interval

Table 3.8 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *Ba. bovis* in cattle in Central and Eastern Zambia

*Farming system, tick control method, province and being PCR positive for *T. mutans, T. parva* and *Ba. bigemina* were significant predictors of being PCR positive for *Ba. bovis*.

Table 3.8 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity to *Ba. bovis* in cattle in Central and Eastern Zambia

Independent variables	Coefficient	*p value*	Odds Ratio (OR)	95% C.I. for OR	
				Lower	Upper
Commercial farms	3.05	*0.020**	21.05	1.75	253.63
Traditional farms [(a)]					
Spraying	-3.49	*<0.001**	0.030	0.00	0.30
Dipping [(a)]					
Province		*<0.001**			
Eastern Province	5.22	*<0.001**	186.23	9.53	3639.13
Lusaka Province	-0.58	*0.672*	0.56	0.04	7.85
Central [(a)]					
T. mutans	2.52	*<0.001**	12.48	2.26	69.09
T. parva	0.73	*0.030**	2.07	1.09	3.91
Ba. bigemina	2.22	*<0.001**	9.16	3.49	24.06
Constant	-5.30	*<0.001**	0.01		

(a) = reference category, C. I. = confidence interval

Table 3.9 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity to *T. parva* in cattle in Central and Eastern Zambia

Farming system, breed type and PCR positivity to *Ba. bovis*, *T. taurotragi* and *Anaplasma* spp were significant predictors of being PCR positive for *T. parva* in central and eastern Zambia.

Table 3.9 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity to *T. parva* in cattle in Central and Eastern Zambia

Independent variable	Coefficient	*p value*	Odds ratio (OR)	95% C.I. for OR	
				Lower	Upper
Ba. bovis	0.84	*0.002**	2.31	1.17	4.56
Anaplasma spp	1.12	*< 0.001**	3.06	1.59	5.88
T. taurotragi	2.41	*< 0.001**	11.08	6.15	19.97
Commercial farms	2.48	*0.020**	11.89	1.43	99.01
Traditional farms (a)					
Breed type		*0.003**			
Angoni	2.08	*0.001**	7.99	1.59	40.23
Cross	-1.67	*0.150*	0.19	0.02	1.80
Friesian	0.01	*0.982*	1.01	0.42	2.44
Tonga (a)					
Constant	-3.11	*< 0.001*	0.05		

(a) = reference category, C.I. = confidence interval

Cattle on which ticks were controlled every week were 0.19 (C.I = 0.16 – 2.13) times less likely to be PCR positive for *Anaplasma* spp than those cattle on which ticks were occasionally controlled ($p < 0.001$), while commercial farms were 3.10 (C.I = 1.13 – 8.49) times more likely to be *Anaplasma* spp positive by PCR than those under traditional husbandry ($p = 0.030$) (Table 3.10). Being PCR positive for *E. ruminantium*, *T. mutans* and *T. parva* was associated with an increased risk of being positive for *Anaplasma* spp by a factor of 90.68 (C.I = 18.25 – 450.45), 8.46 (C.I. = 3.81 – 18.79) and 2.23 (C.I = 1.14 – 4.36) respectively. Method of tick control, breed type, age, sex, tick burden and province were not significant predictors of being PCR positive for *Anaplasma* spp.

Angoni breed-type of cattle were 0.16 (C.I = 0.07 – 0.37) times less likely to be PCR positive for *E. ruminantium* than the Tonga breed-type ($p < 0.001$), while being PCR positive for *Anaplasma* spp increased the risk of being positive for *E. ruminantium* by a factor of 79.20 (C.I = 17.93 – 349.91) ($p < 0.001$) (Table 3.11). Province, sex, age, frequency and method of tick control, tick burden and farming system were not significant predictors of being PCR positive for *E. ruminantium*.

3.3.6 Effect of infections with multiple tick-borne pathogens and other variables on PCV in Central and Eastern Zambia

The PCV of cattle in Central and Eastern Zambia was measured in both the dry and the wet season. The mean PCV was higher in the dry season (33.00 %) than in the wet season (30.10 %) and this difference was statistically significant ($p < 0.001$). To determine whether the number of co-infecting pathogens had a significant effect on PCV in cattle, the PCV of samples corresponding to the number of different co-infecting pathogens was compared using the one-way ANOVA for both the wet and the dry season. For both seasons, the mean PCV reduced with an increase in the number of co-infecting pathogens and this pattern was more evident in the dry season (Tables 3.12 and 3.13). In the dry season, cattle with five co-infecting pathogens had significantly lower PCV than those with two or no co-infecting pathogens ($p = 0.040$); in the wet season, the difference observed was not statistically significant ($p = 0.090$). A multivariant linear regression model was used to investigate which tick-borne pathogens (single and mixed infections) had a significant effect on PCV in the dry season while allowing for the effect of other variables. All parasite combinations with frequencies of five and above were included in the analysis. The results of the multivariant linear regression are shown Table 3.14. No single infections were found to be associated with a significant reduction in PCV, while two different combinations of pathogens were found to have a significant effect on PCV. A combination

Table 3.10 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *Anaplasma* spp in cattle in Central and Eastern Zambia

*Frequency of tick control, farming system and PCR positivity for *E. ruminantium*, *T. mutans* and *T. parva* are significant predictors of being PCR positive for *Anaplasma spp*.

Table 3.10 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *Anaplasma* spp in cattle in Central and Eastern Zambia.

Independent variable	Coefficient	*P value*	Odds Ratio (OR)	95% C.I. for OR	
				Lower	Upper
Frequency of tick control		*<0.001**			
Every two weeks	-0.53	*0.420*	0.59	0.16	2.13
Weekly	-1.66	*<0.001**	0.19	0.09	0.42
Occasionally (a)					
Commercial farm	1.13	*0.030**	3.10	1.13	8.49
Traditional farm (a)					
E. ruminantium	4.51	*<0.001**	90.68	18.25	450.45
T. mutans	2.14	*<0.001**	8.46	3.81	18.79
T. parva	0.80	*0.023**	2.23	1.14	4.36
Constant	-1.64	*<0.001**	0.20		

(a) = reference category , C.I. = confidence interval

Table 3.11 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *E. ruminantium* in cattle in Central and Eastern Zambia

*Cattle breed type and PCR positivity to *Anaplasma* spp were significant predictors of being PCR positive for *E. ruminantium*.

Table 3.11 Maximum likelihood estimates of binary logistic model of factors for prediction of PCR positivity for *E. ruminantium* in cattle in Central and Eastern Zambia

Independent variable	Coefficient	*p value*	Odds Ratio (OR)	95% C.I. for OR	
				Lower	Upper
Breed type		*<0.001**			
Angoni	-1.82	*< 0.011**	0.16	0.07	0.37
Cross	-20.84	*1.000*	0.00	0.00	
Friesian	-0.73	*0.153*	0.48	0.18	1.30
Tonga [(a)]					
Anaplasma spp	4.37	*< 0.001**	79.20	17.93	349.91
Constant	-3.43	*<0.001**	0.032		

(a) = reference category, C.I. = confidence interval

Table 3.12 Mean PCV according to number of co-infections in the dry season

The relationship between the number of co-infecting pathogens and mean PCV in cattle in the dry season is shown together with the 95 % confidence interval of the mean PCV and the range of PCV values for each number of co-infections. The one-way analysis of variance (ANOVA) showed that cattle with two or no infections had significantly higher mean PCV than those with five co-infecting pathogens ($p = 0.040$).

Table 3.12 Mean PCV according to number of co-infections in the dry season

Number of co-infections	n	Mean PCV (%) (SD)	95% C.I. for mean PCV (%)		PCV Range (%)	
			Lower	Upper	Mini	Max
0	156	33.6 (5.5)	32.8	34.5	16	47
1	133	33.2 (5.4)	32.3	34.1	19	47
2	122	33.5 (5.1)	32.6	34.4	18	46
3	102	32.6 (5.9)	31.4	33.7	12	50
4	57	32.4 (5.3)	31.0	33.8	20	47
5	24	29.8 (5.3)	27.6	32.0	17	40
6	2	32.0 (4.2)	-6.2	70.1	29	35
Total	595	33.0 (5.5)	32.60	33.48	12	50

n = number of cattle, C.I. = confidence interval, SD = standard deviation

Table 3.13 Mean PCV according to number of co-infections in the wet season

The relationship between the number of co-infecting pathogens and PCV in cattle in the wet season is shown together with the 95 % confidence interval of the mean PCV and the range of PCV values for each number of co-infections. Although uninfected cattle had higher mean PCV than those with two or more co-infections, the results of the one-way ANOVA showed that these differences were not significant ($p = 0.090$).

Table 3.13 Mean PCV according to number of co-infections in the wet season

Number of co-infections	n	Mean PCV (%) (SD)	95% C.I. for Mean PCV (%)		PCV Range (%)	
			Lower	Upper	Mini	Max
0	64	32.4 (6.3)	30.87	34.00	16	42
1	41	29.7 (5.5)	27.95	31.42	17	44
2	63	30.8 (5.0)	29.53	32.03	15	46
3	74	29.2 (5.6)	27.84	30.45	11	40
4	54	29.2 (5.2)	27.83	30.66	18	41
5	29	28.8 (6.4)	26.32	31.20	17	40
6	15	29.6 (5.2)	26.71	32.49	25	47
7	7	29.9 (7.7)	22.72	36.99	19	40
Total	347	30.1 (5.7)	29.47	30.68	11	47

n = number of cattle, C.I = confidence interval, SD = standard deviation

Table 3.14 Multi-variant linear regression model of the effect of infection with different tick-borne pathogens and other significant confounding factors on the PCV of cattle in Central and Eastern Zambia

Age, multiple infections of *T. parva*, *T. mutans* and *Anaplasma* spp, multiple infection of *T. parva*, *T. mutans*, *T. taurotragi*, *Anaplasma* spp and *E. ruminantium* or being abundantly infested with ticks (more than 50 ticks / animal) were associated with significant drop in mean PCV in carrier cattle in the dry season in central and eastern Zambia. An interaction between being abundantly infested with ticks (>50 ticks / animal) and multiple infections of *T. parva, T. taurotragi, T. mutans, Anaplasma* spp and *E. ruminantium* was indicated.

Table 3.14 Multi-variant linear regression model of the effect of infection with different tick-borne pathogens and other significant confounding factors on the PCV of cattle in Central and Eastern Zambia

Variable	Coefficient	*p value*	95% C.I. for coefficient	
			Lower	Upper
Constant	22.38	<0.001*	17.01	27.74
Age (months)	-0.03	<0.001*	-0.04	-0.02
T. parva + *T. mutans* + *Anaplasma* spp	-5.52	0.001*	-8.83	-2.20
T. parva + *T. taurotragi* + *T. mutans* + *Anaplasma* spp + *E. ruminantium*	-7.32	0.001*	-11.56	-3.07
Abundant (>50 ticks/animal)	-14.44	0.011*	-25.52	-3.37
Abundant (>50 ticks/animal) * *T. parva* + *T. taurotragi* + *T. mutans* + *Anaplasma* spp + *E. ruminantium*	-21.03	<0.001*	-32.78	-9.28

C.I. = confidence interval

of *T. parva*, *T. mutans*, *T. taurotragi*, *Anaplasma* spp and *E. ruminantium* caused an average reduction in PCV of 7.32 % points ($p = 0.001$), while that of *T. parva*, *T. mutans* and *Anaplasma* species caused an average reduction of 5.52 % points ($p = 0.001$). Cattle that were heavily infested with ticks (more than 50 ticks / animal) showed a reduction of 14.44 % points in PCV on average ($p < 0.001$). There was a significant interaction between having more than 50 ticks per animal and being infected with *T. parva*, *T. mutans*, *T. taurotragi*, *Anaplasma* spp and *E. ruminantium.* After allowing for the effect of other variables, the PCV was reduced by 0.03 % points for every one month increase in age ($p < 0.001$). Cattle breed-type, the method and frequency of tick control, farming system and province did not have a significant effect on PCV. The validity of the model (i.e. whether it met the assumptions of linearity, constant variance and normality) was checked using residual and probability plots (data not shown).

3.3.7 Tick burden on cattle in the wet and dry season in Central and Eastern Zambia

The percentage of cattle in each of the four tick burden categories for the dry and wet seasons are shown in Tables 3.15 and 3.16 respectively. In both seasons, ticks were observed even on some of the cattle on which tick control was practised intensively, indicating that tick control, even in commercial herds, was incomplete. In the dry season (Table 3.15), tick burdens were not assessed for those cattle which were dipped in Central Province because of non-compliance by the farmers. Of the cattle that were sprayed to control ticks in this province, no ticks were seen on 50.00 % of them while 29.59 % had a few ticks, 18.37 % had a moderate tick burden and 2.04 % had an abundant infestation of ticks. In Lusaka Province all the sampled cattle in the dry season came from farms that sprayed to control ticks. Over 64 % of cattle in this province had no ticks seen on them while 19.35 % had a few ticks, 12.90 % had a moderate tick burden and 3.26 % had an abundant tick burden. In Eastern Province, no tick control was practised on a proportion of cattle sampled (16.6 %) and those cattle had significantly higher tick burdens than those that were sprayed ($p < 0.001$). For cattle that were sprayed, over 48 % had no ticks on them while 37.50 % had few ticks, 12.22 % had a moderate tick burden and 1.42 % were abundantly infested with ticks. For those cattle on which no tick control was practised, less than 6 % had no ticks, 67.14 % had few ticks, 27.14 % had moderate tick burdens and none were abundantly infested with ticks (Table 3.15). Tick burdens on cattle were significantly higher in the Eastern Province than in Lusaka and Central Provinces ($p = 0.048$).

Table 3.15 Percentage of cattle in each tick burden category in the three provinces grouped according to the method of tick control (dry season)

The tick burden on cattle in the dry season in the three provinces was calculated with cattle grouped according to method of tick control. The chi-square test was used to test for significant differences in tick burdens among the provinces and tick control methods in Eastern Province. Cattle in Eastern Province had a significantly higher tick burden than those from Central and Lusaka Provinces ($p = 0.048$). Cattle that were sprayed in Eastern province had fewer tick burdens than those on which no tick control was practiced ($p < 0.001$).

Table 3.15 Percentage of cattle in each tick burden category in the three provinces grouped according to the method of tick control (dry season)

Province	Tick control method	n	Tick burden (%)			
			None seen	Few	Moderate	Abundant
Central	Spraying	98	50.00	29.59	18.37	2.04
Eastern	None	70	5.71	67.14	27.14	0.00
	Spraying	352	48.86	37.50	12.22	1.42
	Total (Eastern)	422	41.71	42.42	14.69	1.18
Lusaka	Spraying	31	64.52	19.35	12.90	3.26
Total		551	44.46	38.84	15.25	1.45

n = number of cattle

Table 3.16 Percentage of cattle in each tick burden category in the three provinces grouped according to the method of tick control (Wet season)

Tick burdens on cattle in the wet season in the three provinces were calculated with cattle grouped according to method of tick control. The chi-square test was used to determine whether significant differences existed in tick burdens between the provinces and between tick control methods in the provinces. Cattle in the Eastern province had significantly higher tick burdens than those from Central and Lusaka Province ($p < 0.001$). Cattle in Lusaka Province showed a significantly lower tick burdens than those in Central Province ($p = 0.020$). In Central province, cattle which were dipped showed significantly lower tick burdens than those that were sprayed ($p = 0.002$). In Lusaka Province there was no significant difference in tick burdens between cattle that were dipped and those that were sprayed.

Table 3.16 Percentage of cattle in each tick burden category in the three provinces grouped according to the method of tick control (Wet season)

Province	Tick control method	n	Tick burden (%)			
			None seen	Few	Moderate	Abundant
Central	Spraying	24	20.83	29.16	29.16	20.83
	Dipping	32	59.38	21.88	18.75	0.00
	Total (Central)	56	42.86	25.00	23.21	8.93
Eastern	Spraying	211	0.47	46.92	31.28	21.33
Lusaka	Spraying	41	73.17	19.51	7.32	0.00
	Dipping	41	78.05	12.20	4.88	4.88
	Total (Lusaka)	82	75.61	15.85	6.10	2.44
Total		349	24.93	36.10	24.07	14.90

n = number of cattle

In the wet season (Table 3.16) all the farms from which samples were collected in the three provinces controlled ticks on their cattle. In Central province 43 % of cattle were sampled from farms that used spraying as a tick control method while the remainder of the cattle were from farms that used dipping as a tick control method. For cattle that were sprayed, 20.83 % had no ticks seen on them, 29.16% had few ticks, 29.16 % had a moderate tick burden and 20.83 % were abundantly infested with ticks. Of the cattle that were dipped, 59.38 % had no ticks seen on them, 21.88 % had few ticks, 18.75 % had moderate tick burdens and no cattle were abundantly infested with ticks. Cattle that were dipped in this province had significantly fewer ticks that those that were sprayed ($p = 0.002$). In Eastern province, spraying was the only tick control method used and less than 1 % of the cattle sampled had no ticks seen on them, 46.92 % had a few ticks, 31.28 % were moderately infested and 21.33 % were abundantly infested. In Lusaka Province the proportion of cattle on which dipping and spraying were used as tick control methods was the same (Table 3.16). Of the cattle that were sprayed, 73.17 % had no ticks seen on them, 19.51 % had a few ticks, 7.32 % were moderately infested and none of the cattle was abundantly infested with ticks. For the cattle that were dipped, 78.05 % had no ticks seen on them, 12 % had a few ticks, 5 % were moderately infested and another 5 % were abundantly infested with ticks. There was no significant difference in tick burden between those cattle that were dipped and those that were sprayed ($p = 0.291$) in this province. Table 3.17 shows the number of cattle in each of the tick burden categories in traditionally and commercially reared cattle in Lusaka province. There was no significant difference in tick burden between cattle from commercial farms and those from farms where traditional husbandry was practised in Lusaka Province ($p = 0.101$). When all the cattle from all three provinces were combined, cattle that had been dipped had significantly fewer ticks than those that were sprayed ($p < 0.001$), and cattle from Eastern Province had significantly higher tick burdens than those from the other two provinces ($p < 0.001$). When the two seasons were compared, cattle had a significantly higher tick burden in the wet season than in the dry season ($p < 0.001$).

3.3.8 Determination of risk factors associated with tick-burden in the wet season in Central and Eastern Zambia

The ordinal logistic regression model was used to determine which of the risk factors were strongly associated with each of the tick burden categories in the wet season. The goodness of fit *p value* for the model was 1.00 and the null hypothesis that the location parameters are the same across the responses categories ($p = 0.700$) was accepted. Results of the ordinal regression model (Table 3.18) indicate that cattle that were dipped ($p < 0.001$) and

Table 3.17 Tick burden according to farming system in the wet season in Lusaka Province

The percentage of cattle in each of the tick burden categories in commercial and traditional cattle are shown for Lusaka province in the wet season. No significant difference was found in tick burden between commercially and traditionally reared cattle in this province in the wet season (Fisher's Exact test, $p = 0.102$).

Table 3.17 Tick burden according to farming system in the wet season in Lusaka Province

Farming system	n	Tick burden (%)			
		None seen	Few	Moderate	Abundant
Traditional	21	66.67	14.29	9.52	9.52
Commercial	61	78.69	16.39	4.92	0

n = number of cattle. Fisher's Exact test, $p = 0.102$

Table 3.18 Risk factors associated with tick burden on cattle in the wet seas0on in Central and Eastern Zambia based on the ordinal regression model

Dipping cattle as a method of tick control and commercial farming were significantly associated with lower tick burdens while occasionally controlling ticks on cattle (frequency of tick control) was significantly associated with higher tick burdens on cattle.

Table 3.18 Risk factors associated with tick burden on cattle in the wet season in Central and Eastern Zambia based on the ordinal regression model

Variable		Estimate	*p value*	95% C.I. for estimate	
				Lower	Upper
Threshold	None seen	-0.80	0.171	-1.94	0.35
	Few	0.90	0.132	-0.26	2.05
	Moderate	2.10	<0.001	0.92	3.28
Age		0.00	0.730	-0.00	0.01
Tick Control method	Dipping	-1.65	<0.001*	-2.45	-0.85
	Spraying (a)				
Sex	Female	-0.01	0.931	-0.30	0.28
	Male (a)				
Breed type	Angoni	-0.15	0.816	-1.45	1.16
	Cross	0.71	0.151	-0.26	1.68
	Friesian	0.65	0.364	-0.73	2.03
	Tonga (a)				
Farming system	Commercial	-2.35	<0.001*	-3.93	-0.77
	Traditional (a)				
Province	Central	0.27	0.55	-0.62	1.17
	Eastern	0(a)			
	Lusaka (a)				
Frequency of tick control	Every two weeks	-0.57	0.149	-1.34	0.20
	Occasionally	0.85	0.017*	0.13	1.57
	Weekly (a)				

(a) = reference category, C.I. = confidence interval

cattle in commercial farms ($p < 0.001$) were significantly associated with lower tick burdens while cattle exposed to infrequent tick control measures were significantly associated with a higher burden of ticks ($p = 0.020$).

3.4 Discussion

3.4.1 Prevalence

Tick-borne diseases are an important constraint to livestock production in tropical and sub-tropical regions (Jongejan & Uilenberg 2004). However, information on their distribution, abundance and the factors that affect their occurrence is either inadequate or entirely lacking in most of these countries. For example, in Zambia, the only epidemiological studies of anaplasmosis and babesiosis that have been reported previously were conducted more that two decades ago. Moreover, most of the epidemiological studies on TBD carried out in Zambia to date have utilised the IFA test (Billiouw *et al.* 1999; Billiouw *et al.* 2002; Fandamu *et al.* 2005; Jongejan *et al.* 1988) and therefore studies using more specific, sensitive and reproducible laboratory tests are currently required. The justification for this study was the paucity of epidemiological data on TBD pathogens in the country. The results presented in this chapter show that all the TBDs investigated are prevalent in the three provinces. *T. mutans* and *Anaplasma* spp are the most prevalent tick-borne pathogens of cattle in Central and Eastern Zambia, with *Ba. bigemina* being the least prevalent. *T. parva*, *Ba. bovis*, *Ba. bigemina, Anaplasma marginale* and *E. ruminantium* are known to cause diseases of economic importance in cattle in Zambia and most of sub-Saharan Africa (Makala *et al.* 2003; Minjauw & McLeod 2003). The importance of *T. mutans* and *T. taurotragi* in terms of causing morbidity and mortality in cattle in Zambia is unknown.

The prevalence of *Ba. bovis* obtained in this study in Central Zambia in both seasons was higher than that observed by Jongejan *et al*. (1988), who reported prevalences ranging from 1.8 to 3 %. However, the prevalence of this parasite in the current study in the Eastern Province is lower than that reported by Jongejan *et al*. who described sero-prevalence rates of between 43.3 % and 45.5 % in this province. The prevalences of *Ba. bigemina* obtained in this study were also lower than those reported by Jongejan *et al*. (1988) who described sero-prevalence rates between 40 % and 57.6 % in both Central and Eastern Zambia. The *Ba. bigemina* and *Ba. bovis* prevalences observed in the current study are also lower than the sero-prevalence rates reported in small holder dairy cattle in Kenya (Maloo *et al.* 2001) and for *Ba. bigemina* they are lower than those reported in smallholder dairy cattle in Tanzania (Swai *et al.* 2005). Although the difference in the prevalence

between the two Zambian studies may indicate a change in the epidemiological situation of these parasites over the years, the different diagnostic methods used may account for much of the observed variation. The PCR method used in this study detects animals that are carriers of the disease while the IFAT method used by Jongejan *et al*. does not distinguish between carrier animals and immune animals that may have cleared the infection. The serological response to *Babesia* infection has been reported to remain high long after parasites are apparently cleared from the host (Salem *et al.* 1999). IFAT also has low specificity and cross-reactions between *Babesia* species have been reported (Bose *et al.* 1995; Edelhofer *et al.* 2004). In addition, the use of FTA cards in providing template DNA for use in PCR may also reduce the sensitivity of the PCR assay. It was observed in this study that when saponin lysates were prepared from the same samples and re-analysed using the same PCR method, the number of *Babesia* positive samples increased (data not shown).

An interesting finding from this study is the higher number of *Ba. bovis* cases that were detected in Central Zambia. *Ba. bovis* was first reported in Central Zambia in 1986 (Jongejan *et al.* 1986a). The increase in prevalence indicates that this parasite is becoming endemic in this part of the country and this may be due to the uncontrolled movement of cattle. It would therefore be of interest to determine the distribution of *B. microplus*, the vector of *Ba. bovis*, in Central Zambia. In this study prevalences of *Ba. bigemina* close to zero were reported in all the three provinces in the dry season and this increased to over 10 % in the wet season. *Boophilus* ticks, which transmit this parasite, were observed on cattle in both the dry and wet season, although the tick numbers were lower in the dry season. Other studies have also reported the year-round presence of *Boophilus* ticks on cattle in Zambia with monthly fluctuations in abundance (Berkvens *et al.* 1998; Pegram *et al.* 1986). The observed differences in the prevalence of *Ba. bigemina* between the dry and wet season may indicate higher transmission intensity of the parasite in the wet season, probably due to reduced tick numbers in the dry season and/or differences in infection rates in ticks found in the two seasons. This difference in prevalence between the seasons may also be due to the inability of the *Ba. bigemina* parasite to survive the high temperatures and low humidity found in Zambia in the dry season while in the tick. Persistent high temperatures (above 37 ^{o}C) have been reported to inhibit development of or eliminate *Ba. bigemina* infections in ticks (Kocan 1995). Therefore, the parasite may be transmitted to susceptible cattle mainly in the wet season, and during the dry season, the ensuing parasitaemia is cleared by the immune system to levels below the detection limit of PCR (Salem *et al.* 1999).

T. parva is an important disease of cattle in Zambia, causing high morbidity and mortality (Nambota *et al.* 1994). The disease is prevalent in all the provinces of Zambia except Western, Luapula and North-Western Provinces (Makala *et al.* 2003). However, there are no published figures on the prevalence of this disease in Central and Eastern Zambia. The prevalences obtained in this study in Central and Eastern Zambia are higher than the sero-prevalences reported in the Southern Province (Fandamu *et al.* 2005), but lower than those reported in Kenya, Tanzania and Rwanda (Bazarusanga *et al.* 2007; Maloo *et al.* 2001; Swai *et al.* 2005). In this study, it was found that there was no significant difference in the prevalence of *T. parva* between ECF immunised and non-immunised cattle in Eastern Province. Fandamu *et al* (2005) also found that immunisation against ECF in Southern Province of Zambia did not have a significant effect on the prevalence of *T. parva*. This is surprising given that animals that become infected with *T. parva* are believed to remain carriers for a considerable time during their life (Kariuki *et al.* 1995). These results suggest that the carrier state induced by the strains used to vaccinate cattle against ECF in Zambia does not persist for long periods. Further studies, similar to those carried out in Uganda (Oura *et al.* 2007) are required to determine the length of time the carrier status is induced post-vaccination.

Different epidemiological states of ECF, related to the seasonal abundance of *R. appendiculatus* ticks on cattle (Berkvens *et al.* 1998; Pegram *et al.* 1986) were observed in this study. The prevalence of *T. parva* in young animals was significantly lower than that found in older cattle in the dry season, while no significant differences in prevalence between age groups were observed in the wet season. Studies of ECF epidemics in the Eastern province indicate that waves of ECF challenge in cattle coincide with peaks of adult *R. appendiculatus* abundance which occurs in the rainy season (December to March) and occasionally in the cold dry season (May to July) (Billiouw *et al.* 2002). The time of sampling in the wet season coincided with the wet season transmission peak, while the dry season samples were collected at a time of low adult tick activity. This indicates that most of the calves born in the dry season may not encounter the disease until the rainy season when there is a peak in abundance of vector ticks. A similar trend in the epidemiology of *T. taurotragi*, which shares the same vector, *R. appendiculatus,* was observed.

The prevalences of anaplasmosis revealed in this study are higher than those obtained using the card agglutination test (CAT) (Jongejan *et al.* 1988), but lower than those reported using the ELISA test in the same study. The prevalence of anaplasmosis reported in this study is higher than that was reported in Sudan, where a sero-prevalence of 37.8 %

was revealed using ELISA (Salih *et al.* 2008a). Although heartwater is a relatively important disease of livestock in Zambia (Anon 2007; Makala *et al.* 2003) there have been no previous studies on the epidemiology of this disease in Zambian cattle. In the current study, higher prevalences of the disease were reported in Central Zambia than in the Eastern Province (Table 3.1), though these were lower than the sero-prevalences of 40 % reported previously in goats (Ahmadu *et al.* 2004).

Taken together, the low prevalences of *T. parva, Ba. bigemina, Ba. bovis* and *E. ruminantium* (see Table 3.1) may indicate the existence of a large pool of susceptible cattle through which a disease outbreak could spread if appropriate epidemiological parameters were met. That is to say, an unstable state of endemicity, which favors the occurrence of disease outbreaks, exists for all the diseases caused by these pathogens in Zambia. It should be noted, however, that being PCR negative does not indicate that an animal is susceptible to infection, as it may have sterile immunity to the disease, or the levels of parasitaemia may be beneath the detection limit of the assay used. The prevalences of *T. mutans* and *Anaplasma* were high in all the provinces and both seasons indicating an endemically stable state of these pathogens may exist in all three provinces. The high prevalences of *T. mutans* over the other pathogens are in agreement with those reported in Uganda (Oura *et al.* 2004a) and Sudan (Salih *et al.* 2008a). This may indicate that cattle in these parts of Africa are exposed to a high and continuous challenge with *T. mutans* or that this species is harboured for long periods at detectable levels post-infection. Alternatively, these results may simply suggest that cattle are more susceptible to *T. mutans* infection than to any other TBD pathogen.

3.4.2 Predictors of PCR positivity for TBD pathogens

Knowledge of risk factors associated with TBDs is an important pre-requisite in the design and implementation of effective control strategies. An understanding of these risk factors and the associations among TBD pathogens is also a good aid for clinical diagnosis, treatment choice and determining the potential of a particular disease to spread. In this study, the logistical regression model was used to identify risk factors associated with being PCR positive for *T. parva*, *Ba. bigemina*, *Ba. bovis*, *Anaplasma* spp and *E. ruminantium* in cattle in Central and Eastern Zambia in the wet season. Significant risk factors associated with being PCR positive for *Ba. bovis* were found to be the farming system, tick control method, the province and being PCR positive for *Ba. bigemina, T. mutans* or *T. parva*. Cattle in commercial farms were more at risk of being PCR positive for *Ba. bovis* than those under traditional management. This could be a reflection of the

management system in commercial farms, which leaves the cattle naïve to infection by this parasite. An additional explanation is that commercial establishments favour the use of taurine breeds of cattle which are known to have a high susceptibility to *Ba. bovis* (Bock *et al.* 1997; Bock *et al.* 1999b). The positive association between *Ba. bovis* and *Ba. bigemina* may be due to the fact that the two parasites share the same vector species, *B. microplus*. This tick is able to acquire and transmit these two parasites together (Quintao-Silva *et al.* 2007). Although *Ba. bigemina* can be transmitted by other vectors in Zambia, there is evidence than *Bo. microplus* is spreading into new areas and replacing *Bo. decoloratus* which only transmits *Ba. bigemina* (Berkvens *et al.* 1998; Jongejan *et al.* 1988; Pegram *et al.* 1986). Cattle in the Eastern province were more at risk of being PCR positive for *Ba. bovis* than those in the Central Province (odds ratio (OR) = 186). This is because *Bo. microplus* is widespread in most areas of the Eastern Province while the tick was introduced into Central Zambia a few decades ago and its population is still establishing (Berkvens *et al.* 1998; Jongejan *et al.* 1988; Pegram *et al.* 1986). Consequently cattle in the Eastern Province are more exposed to this parasite.

Significant predictors of being PCR positive for *Ba. bigemina* were the age of the animal, the province and being PCR positive for *T. parva* and *Ba. bovis*. Although the Odds Ratio was close to one, older animals were significantly less likely to be PCR positive for *Ba. bigemina* than younger ones. This is in agreement with the Ugandan study (Magona *et al.* 2008) where it was found that age was negatively associated with sero-conversion to *Ba. bigemina*. Although an inverse age resistance to *Ba. bigemina* exists, with young animals possessing innate resistance while older animals are fully susceptible (Jongejan *et al.* 1988; Mahoney *et al.* 1973), the constant presence of ticks means that the chance of previous exposure to babesiosis in Zambia increases as the animals gets older. As a result, older animals may have developed strong immunity that keeps the parasitaemia lower than the PCR detection limit.

Significant predictors of being PCR positive for *T. parva* included the breed of cattle, farming system, and being PCR positive for *Anaplasma*, *T. taurotragi* and *Ba. bovis*. Angoni cattle were more likely to be PCR positive for *T. parva* than Tonga cattle (OR = 7.99). There is, however, no information on the relative resistance of the two breed-types of cattle to *T. parva* infection. Therefore, the increased risk of infection in Angoni cattle over the Tonga cattle may either be due to Angoni cattle being more susceptible to *T. parva* infection or there may be differences in the virulence of *T. parva* strains found in the two regions. The increased risk of *T. parva* infection in commercially reared cattle is

perhaps a reflection of the management practises which leave these animals susceptible to infection by this parasite and also the high susceptibility to infection of the taurine breeds of cattle found in such establishments (Bakheit & Latif 2002). Oura *et al.* (2004) also reported higher prevalences of *T. parva* in cross-bred compared to indigenous cattle in Uganda, despite the cross-bred cattle being sprayed weekly with acaricides. Significant positive association between *T. parva* and *Anaplasma* spp, *Ba. bovis* and *T. taurotragi* were found in the present study. These pathogens were also significant predictors of PCR positivity for *T. parva*. The association between *T. parva* and *T. taurotragi* may be explained by the fact that both parasites are transmitted by the same vector tick *R. appendiculatus*. However, the positive relationship between *T. parva* and the other two pathogens, *Ba. bovis* and *Anaplasma* spp could be due to two reasons, the first being that their vectors share the same habitat. The second reason could be that *T. parva* is an immuno-suppressive disease and, as such, it renders the animals more susceptible to infection by *Ba. bovis* and *Anaplasma* spp

Based on the strong evidence of *T. parva* infection being associated with co-infection with other TBD agents, one should consider treatment for concurrent infections whenever an animal is diagnosed with East Coast Fever.

Significant predictors of PCR positivity to *Anaplasma* spp included the frequency of tick control, farming system and being PCR positive for *E. ruminantium*, *T. mutans* or *T. parva*. *Anaplasma* was the only TBD pathogen for which frequency of tick control was a significant predictor. That is to say, cattle on which ticks were controlled weekly were less likely to be PCR positive for *Anaplasma* spp than cattle on which ticks were controlled occasionally (OR = 0.19). Anaplasmosis has many vectors including several ticks spp and biting flies and it is likely that weekly application of acaricides acted as repellant, preventing these insects from transmitting the disease, even when the acaricides are not strong enough to kill the ticks. As was found for *Ba. bovis* and *T. parva*, cattle on commercial farms were at greater risk of being PCR positive for *Anaplasma* spp than those under traditional management. The positive association between *Anaplasma* spp and *E. ruminantium*, *T. mutans* and *T. parva* suggests that environmental conditions that are conducive for the transmission of *Anaplasma* to cattle are also supportive of the vectors of these other pathogens.

Significant risk factors for PCR positivity for *E. ruminantium* were the breed of cattle and being PCR positive for *Anaplasma* spp. Angoni cattle, which are predominantly found in the Eastern Province, were 0.16 times less likely to be PCR positive for *E. ruminantium*

than Tonga cattle. Currently, there is no information on the relative susceptibility of the various traditional breeds of cattle to TBDs in Zambia. Additionally, the positive association between *E. ruminantium* and *T. mutans* was not significant despite these parasites being transmitted by the same tick species. This might indicate that the infection rates of these parasites differ in the ticks. The observed lack of significant association between the two pathogens could also be because *T. mutans* predominantly infects red blood cells while *E. ruminantium* is mostly found in endothelial cells. Therefore, fewer *E. ruminantium* pathogens are likely to be found in circulating blood as compared to *T. mutans* making the *E. ruminantium* PCR less sensitive.

3.4.3 Effect of multiple TBD pathogen infections on PCV

In both seasons, more mixed infections of TBD pathogens than single infections were observed in cattle in Central and Eastern Zambia. The mean PCV in cattle was significantly higher in the dry season than in the wet season, despite the improved nutritional status of cattle in the wet season. Seasonal differences in PCV may have been due to the increase in the number of parasites affecting cattle in the wet season. During the course of this study, it was observed there were more ticks, haemopathogens and other biting flies feeding on cattle in the wet season. In a study in Ghana, Bell-sakyi *et al.* (2004) found that the mean PCV of cattle with no detectable parasite infections was significantly higher than that of cattle infected with one or more parasites. This is in agreement with the results of this study, where a negative relationship was observed between the number of co-infecting organisms and the mean PCV. This relationship was found to be statistically significant for the dry season but not for the wet season. This could be because the low tick burdens and other blood-sucking ecto-parasites found on cattle in the dry season made this relationship more visible. All the samples collected in the dry season were from apparently healthy cattle. Therefore these results demonstrate that, even in the carrier state, the presence of multiple tick-borne pathogens is associated with significant reductions in PCV in Zambian cattle.

A multivariant linear regression model was used to determine which combinations of parasites were significantly associated with reductions in PCV, while controlling for the effect of other confounding variables. Combinations of multiple infections associated with significant reductions in PCV in cattle were *T. parva, T. mutans* and *Anaplasma* spp which caused a reduction of 5.52 % points and *T. parva, T. taurotragi, T. mutans, Anaplasma* spp and *E. ruminantium* which caused a reduction of 7.32 % points in PCV. Anaemia is a consistent feature associated with the pathology of anaplasmosis. Although anaemia is not

the main clinical feature associated with ECF, other studies have found that *T. parva* infections can cause a significant reduction in PCV (Magona *et al.* 2008; Maxie *et al.* 1982; Mbassa *et al.* 1994). Equally, although they are generally considered to cause milder disease, infection with *T. mutans* and *T. taurotragi* have been associated with anaemia in cattle (Binta *et al.* 1998; Musisi *et al.* 1984). Anaemia is also one of the main clinical features associated with *E. ruminantium* infections in cattle (van Amstel *et al.* 1987). Mixed infections involving *Babesia bovis* were not associated with significant reductions in PCV. This could be because, in a carrier state, *Babesia* parasites are maintained at very low parasitaemia, causing very little destruction of red blood cells. Two other factors found to cause significant decreases in PCV were the age of the cattle and the presence of an abundant infestation of ticks (i.e. more than 50 ticks / animal). The reduction in PCV with age supports the findings of a study in southwest Ethiopia where it was reported that cattle of nine years of age or older had significantly lower PCV than three year old cattle (Rowlands *et al.* 1995). Heavy infestations with ticks are known to reduce productivity of cattle. Previous studies on the economic impact of ticks in Zambia found that infestation of cattle with 50 - 120 adult *Amblyomma variegatum* ticks caused significant reductions in live weight gains (Pegram *et al.* 1989). In this study it was found that cattle hosting more than 50 ticks showed a reduction in PCV of 14.44 % points compared to the average. These results are in agreement with those obtained from Gobora zebu cattle in Gambia where a negative correlation was found between tick burden and PCV (Mattioli *et al.* 1995).

3.4.4 Tick burdens on cattle

In this study, some ticks were found on cattle on which intensive tick control was practised, indicating that tick control was incomplete. This is in agreement with findings of a study carried out in Tanzania where *R. appendiculatus* and *Boophilus* ticks were observed on zero-grazed cattle that were subjected to high frequencies of acaricide application (Ogden *et al.* 2005). In the present study, three reasons can be advanced for incomplete tick control on these cattle. The first being that most farmers do not have proper cattle handling facilities and therefore they are unable to adequately restrain animals during the acaricide spraying process, resulting in a failure to cover the entire body with acaricide. During the course of sample collection, we were able to witness a situation where a farmer confined his animals to a corner of the kraal and just sprayed their backs. The second reason may be that the concentration of acaricides used by the farmers was sub-optimal. Some of the traditional farmers do not have sufficient financial resources to buy enough acaricide to adequately treat their herds. Consequently, such farmers

deliberately apply under-strength acaricide mixtures so that all the cattle in the herd are treated. This represents ill-advised attempts to economise on the use of acaricides and reflects the low literacy level of the resource-poor farmers. Such practises have been cited as one of the contributing factors to the development of acaricide resistance in ticks in Tanzania (Ogden *et al.* 2005). The third reason could be that some of the ticks have developed resistance to the acaricides being used. The generation of acaricide-resistant ticks is a recognised problem associated with wide-scale use of these chemicals (George *et al.* 2004). Strains of tick species that were resistant to a number of acaricides have previously been reported in Zambia (Luguru *et al.* 1984).

Over both seasons, higher burdens of ticks were found on cattle in the Eastern Province than in the other two provinces under study. This could be because a proportion of farmers in this province did not control ticks on their animals in the dry season. For those who sprayed to control ticks on their cattle, the frequency and efficiency of spraying was insufficient to completely control ticks. Most of the farmers sprayed their animals monthly or only intermittently, when the number of ticks on their cattle became unacceptably high. This is evidenced by the high percentage of abundantly infested cattle and the low percentage of cattle that had no ticks on them in the wet season, despite all the farmers reporting that they controlled ticks on their animals. Conversely, farmers in Lusaka province were highly efficient at controlling ticks regardless of whether they dipped or sprayed their animals with acaricides. This is evidenced by the low proportion of cattle that were abundantly infested with ticks and the high proportion of cattle that did not harbour any ticks. However, the consistently higher proportion of ticks on cattle in the Eastern Province was not reflected in differences in prevalence of TBD pathogens among the provinces. Some parasite species were significantly higher in the Eastern Province while others were significantly higher in the two provinces with lower tick burdens (Table 3.1). These differences may be a reflection of the relative abundances of the various tick species found on cattle in the three provinces or the different infection rates of TBD pathogens in tick species between the provinces and the two seasons. The relative abundance of the various tick species found on cattle in this study were not quantified and therefore it is not possible to relate the observed seasonal fluctuations in TBD pathogen prevalences to the seasonality and infestation rates of specific vectors. However, previous studies on the seasonality and infestation rates of ticks on cattle in Zambia show that *A. variegatum* and *R. appendiculatus* possess similar life-cycles with adults observed on cattle from October to December and December to April respectively (Pegram *et al.* 1986). Higher prevalences of *T. parva, T. taurotragi, T. mutans* and *E. ruminantium* which are transmitted by these

ticks were recorded in the wet season in this study, and this coincides with the reported increase in abundance of their vectors.

The logistic regression model of the wet season data demonstrated that tick burdens on cattle in Central and Eastern Zambia were significantly related to management practices. Cattle that were dipped to control ticks and those that were on commercial farms were associated with lower tick burdens than those that were sprayed and those on traditionally managed farms respectively, while controlling for other factors. This indicates that dipping is a more effective method of tick control than spraying probably due to the whole animal being immersed in the acaricide suspension. Despite its effectiveness, almost 5 % of the cattle that were dipped in Lusaka Province were abundantly infested with ticks.

This study also shows that despite being associated with a lower tick burden, cattle in commercial farms are still more at risk of being infected with specific TBD pathogens (*Ba. bovis, T. parva* and *Anaplasma* spp) than cattle under traditional farm management. This further confirms the high susceptibility of the breed types of cattle found in these establishments to TBD and also that these diseases can be transmitted by only a few ticks feeding on cattle. These results also indicate that intensive application of acaricides is not enough to prevent transmission of disease. Ochanda *et al*. (1988) demonstrated that under an ambient temperature above 23 ^{o}C, ticks can transmit *T. parva* in the field within 24 h of attachment.

Cattle on which ticks were infrequently controlled were associated with higher tick burdens. Most of the farmers in the Eastern Province infrequently controlled ticks on their cattle, and this may be why tick burdens in this province were always higher than those found in Central Zambia. The sex of the animal, age, breed-type and the province were not significant predictors of tick burden on cattle. This is contradictory to a Tanzanian study (Ogden *et al.* 2005) where age and agro-ecological zone were identified as significant risk factors of tick burdens on cattle. This difference could perhaps be because in the Tanzanian study the analysis was done individually for each tick species while in the present study all ticks were grouped together. This could have masked the effect of the province (spatial) on tick burdens in Zambia.

CHAPTER FOUR

Population genetic analysis of *Babesia bovis* and *Ba. bigemina*

4.1 Introduction

4.1.1 Bovine babesiosis in Turkey and Zambia

Turkey extends from 36° to 42° N and from 26° to 45° S. It is roughly rectangular in shape, is a massive 1,660 kilometres wide and is considered a transcontinental country bridging Europe and Asia. Turkey has six distinct geographic and climatic regions ranging from the high Anatolian plateau of rolling steppe and mountain ranges to the Aegean region centred on Izmir and Aydın as shown in Figure 4.1. The latter has been described as a breadbasket, with low hills and high mountains forming fertile valleys full of rich alluvial soils. Bovine babesiosis in Turkey is distributed throughout the country with *Bo. annulatus* being the main tick vector (Aydin & Bakirci 2007). Both *Ba. bovis* and *Ba. bigemina* occur in the country although their distributions are reported to differ. *Ba. bovis* can be found throughout Turkey while *Ba. bigemina* does not occur in the Western region of the country (H. Bilgic, personal communication). The epidemiology of bovine babesiosis in Zambia is discussed in Section 3.1.2

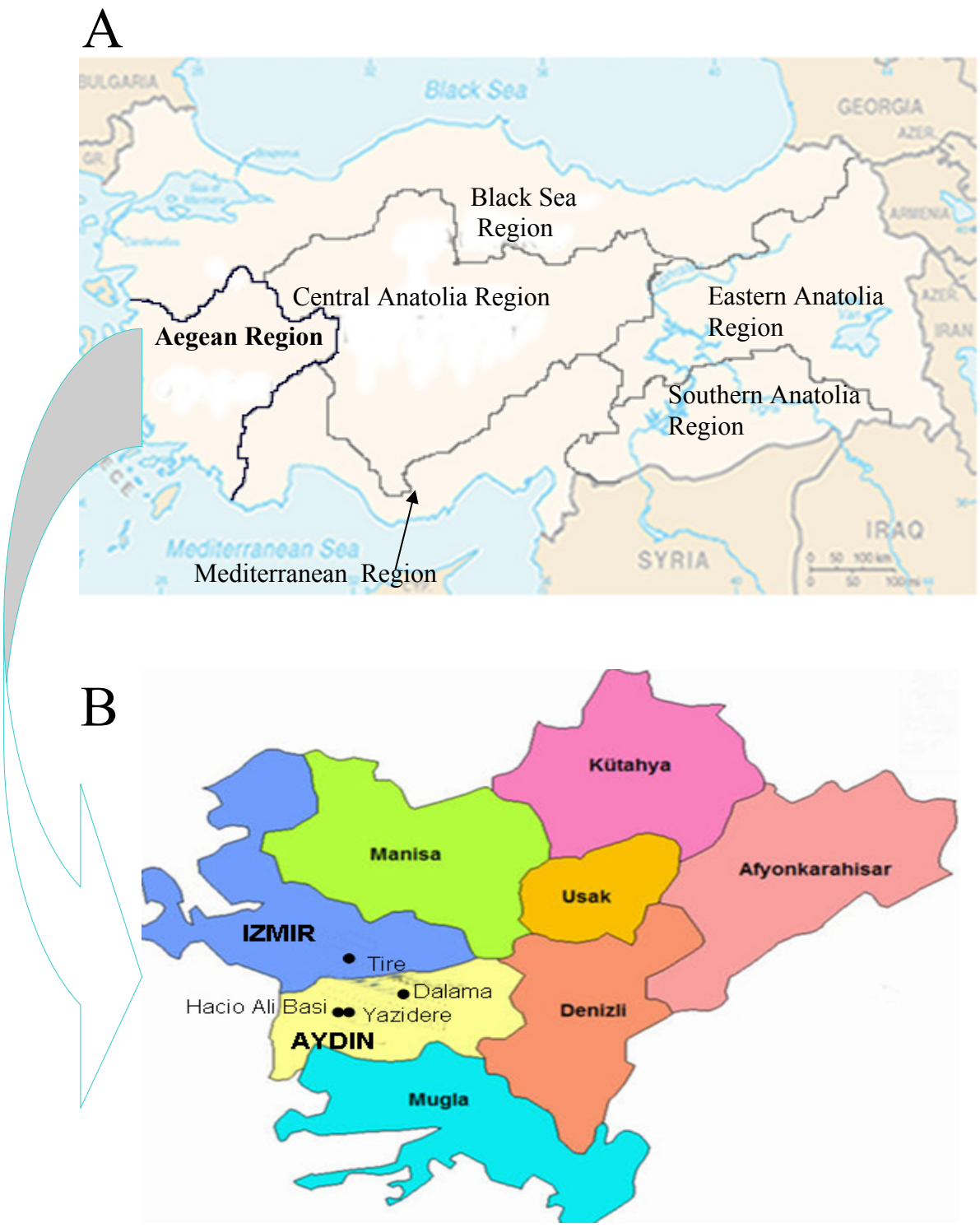
4.1.2 The genomes of *Ba. bovis* and *Ba. bigemina*

Babesia bovis and *Ba. bigemina* have a haploid genome with a brief diploid phase in the tick when the zygotes are formed (Mackenstedt *et al.* 1995). The genome of *Ba. bovis* was sequenced at Washington State University using a clone of the virulent Texas strain (Brayton *et al.* 2007). A combined clone-by-clone and whole genome shotgun approach was used to obtain the complete genome sequence for this organism. The project was carried out in three phases: phase one was end sequencing of a Bacterial Artificial Chromosome (BAC) library, phase two was whole genome shotgun sequencing to 8X coverage and phase three was gap closure (www.vetmed.wsu.edu/research_vmp/babesia-bovis/). The nuclear genome of *Ba. bovis* is estimated to be 8.2 Mb, arranged in four chromosomes ranging in size from 1.25 to 2.62 Mb (Brayton *et al.* 2007). The genome of *Ba. bovis* (8.2 Mb) is similar in size to that of *T. parva* (8.3 Mb) (Gardner *et al.* 2005) and *T. annulata* (8.35 Mb) (Pain *et al.* 2005), the smallest apicomplexan genomes sequenced to date. The *T. parva* and *Ba. bovis* genomes are remarkably similar at a structural level and

Figure 4.1 Map of Turkey showing the sampling sites

Map A shows the six main regions in Turkey. Map B is a magnification of the Aegean region showing Izmir (shaded blue) and Aydın (shaded yellow). The dark dotted areas in Izmir and Aydın denote the sample collection sites.

Figure 4.1 Map of Turkey showing the sampling sites



despite several chromosomal rearrangements, synteny is extensively conserved. In contrast, *P. falciparum,* which shares similar clinical and pathological features to *Ba. bovis* shows major differences in genome size, chromosome number, and gene complement and synteny between these species is limited to microregions (Brayton *et al.* 2007). Like other members of the apicomplexa, *Ba. bovis* has an apicoplast genome of about 33 kb which displays similarities in size, gene content and order to those of *Eimeria tenella*, *P. falciparum*, *T. parva* and *Toxoplasma gondii*. *Ba. bovis* also contains a 6 kb linear mitochondrial genome with genes for Cytochrome b and c polypeptides arranged identically to those of *Theileria* spp, but different to those of *P. falciparum* (Brayton *et al.* 2007).

A virulent Australian strain of *Ba. bigemina* is being sequenced at the Sanger Institute, Cambridge, in collaboration with CSIRO Livestock Industries, Australia. The nuclear genome of *Ba. bigemina* is estimated to be 10 Mb in size, distributed over four chromosomes, and a whole genome shotgun sequencing approach (to 8-fold coverage) has been undertaken (www.sanger.ac.uk/Projects/B_bigemina/). The project is currently at the assembly stage.

4.1.3 Population structure

A fundamental question regarding the biology of parasitic protozoa is the impact of different mating systems on the population structure and the epidemiology of these transmissible diseases (Tibayrenc & Ayala 2002). A variety of population structures has been demonstrated in apicomplexan parasites ranging from clonal in *Toxoplasma gondii* (Sibley & Ajioka 2008) to panmixia (mating randomly) in *Cryptosporidium parvum* (Mallon *et al.* 2003a). Population structures vary, not only between species but also within a single species in different ecological situations. For example, a worldwide survey of diverse *P. falciparum* populations using microsatellite markers found strong linkage disequilibrium in six out of nine populations (Anderson *et al.* 2000). High genetic variation and panmixia prevailed in places of high transmission (Africa), while the opposite was true in places of low transmission (South America). There is also evidence to suggest that the genetic structure of this parasite might be related to the severity of the disease. Analysis of *P. falciparum* isolated from Thai-Myanmar suggested that the genetic structure of the parasite population in patients with severe malaria was panmictic while linkage disequilibrium was observed in patients with uncomplicated malaria (Susomboon *et al.* 2008). Although most population geneticists have accepted the model of a population structure of *P. falciparum* that is related to transmission intensity (Susomboon *et al.* 2008),

other studies have indicated that regions with high transmission are not panmictic and show evidence of inbreeding with linkage disequilibrium (Razakandrainibe *et al.* 2005), and inbreeding in the absence of detectable linkage disequilibrium (Paul *et al.* 1995). However, it is accepted that an obligate sexual stage exists in *P. falciparum* (Cowman & Crabb 2005; Talman *et al.* 2004) and that the resulting genetic exchange plays an important role in generating genetic diversity.

Multi-locus genotyping of *T. parva* isolates from Uganda (Oura *et al.* 2005) and *T. annulata* from Turkey and Tunisia (Weir *et al.* 2007) has provided evidence that genetic exchange occurs frequently in these parasite populations and this is discussed in detail in section 1.7.3. To date, no population genetic analyses of *Ba. bigemina* and *Ba. bovis* have been undertaken. However, a number of studies have demonstrated high levels of diversity in these parasites, both within and between countries (Genis *et al.* 2008; Madruga *et al.* 2002; Wilkowsky *et al.* 2008). It is known that an obligate sexual phase does occur in the life cycle of *Babesia* parasites (Gough *et al.* 1998; Mackenstedt *et al.* 1995). To determine whether high levels of genetic recombination are the primary mechanism for generating genetic diversity in *Ba. bigemina* and *Ba. bovis*, a panel of suitable markers are required for each parasite species so that a formal population genetic analysis can be undertaken.

4.1.4 Mini- and micro-satellite genotyping

Micro-and mini-satellite genotyping provides a convenient method for genetic studies of *Ba. bovis* and *Ba. bigemina*. Such markers have been successfully used in the genotyping of a number of apicomplexan parasites (Anderson *et al.* 2000; Oura *et al.* 2005; Weir *et al.* 2007). Mini-satellite markers are composed of tandem repeats of short DNA motifs (7-24 bp), which are generally subject to a very high frequency of mutation in motif copy number (Jeffreys *et al.* 1988). This has facilitated the development of various mini-satellite-based DNA fingerprinting and DNA profiling systems that have been used for forensic purposes as well as genetic markers for linkage analysis and examination of population structure (Bois & Jeffreys 1999). Similar to mini-satellites, micro-satellites are neutral polymorphic markers that have a shorter period length of between 2 to 6 base pairs. Micro-satellites owe their variability to a high rate of mutation by slipped strand mis-pairing (slippage) during DNA replication. The rate of such mutations is significantly greater than the rate of base substitutions and may vary between 10^{-6} to 10^{-2} per generation (Schlotterer 2000). Mutations may also occur during meiotic crossing over (Blouin *et al.* 1996), although this mechanism is more usually associated with the higher level of diversity observed in mini-satellites. The mechanism of DNA slippage can occasionally

lead to incorrect amplification from micro-satellite loci if it occurs early in a PCR reaction, resulting in fragments of spurious size. Both mini-satellites and micro-satellites are considered to be abundant and widespread across eukaryotic genomes (Tautz & Renz 1984), as evidenced by the *T. parva* and *T. annulata* markers. In the study by Oura *et al*. (2003) and Weir *et al*. (2007) primers were designed to the sequences flanking micro-satellites to permit PCR amplification. PCR products were separated either using electrophoresis on agarose gels (Oura *et al.* 2005) or by capillary electrophoresis (Weir *et al.* 2007). For each system, markers were able to document extensive diversity of *T. parva* and *T. annulata* parasite populations. Capillary electrophoresis represents an improved method for separating and identifying PCR products at a very high resolution (Weir 2006). The availability of the published and unpublished genome sequences for *Ba. bovis* (Brayton *et al.* 2007) and *Ba. bigemina* (Sanger Institute) makes it possible to develop such a genotyping system for these important parasites of livestock.

4.1.5 Objectives of the chapter

The aim of the work presented in this chapter was to identify, characterise and utilise a panel of polymorphic micro- and mini-satellite markers to analyse diversity in field populations of *Ba. bovis* and *Ba. bigemina*. These markers were then used to answer the following specific questions relating to the population genetics of *Ba. bovis* (Zambian and Turkish) and *Ba. bigemina* (Zambian) parasite populations:

1. **What is the population structure of these two parasite populations?** Using standard population genetic techniques, the hypothesis that both *Ba. bovis and Ba. bigemina* have a panmictic population structure was formerly tested.

2. **Do genotypes of these parasites derived from widely separated geographical locations comprise a single or multiple distinct populations**? *Ba. bovis* samples from Zambia and Turkey and *Ba. bigemina* samples from Zambia were analysed to test whether geographical sub-structuring could be detected.

3. **How do host characteristics relate to multiplicity of infection in individual animals?** Formal statistical tests were used to determine whether the number of genotypes observed in individual animals were significantly associated with host factors.

4. **How does the population structure of *Ba. bovis* compare to that of *Ba. bigemina* in Zambia?**

4.2 Materials and methods

4.2.1 Parasite material and DNA preparation

For the initial screening of markers, five stocks of *Ba. bovis* were used: *Ba. bovis* Lismore (Australia), *Ba. bovis* Kwanyangwa (South Africa), *Ba. bovis* M07 (Mexico), *Ba. bovis* Mexican (Mexico) and *Ba. bovis* (unknown origin). Four *Ba. bigemina* stocks were used in the initial screening and these were *Ba. bigemina* Australia, *Ba. bigemina* Zaria (Nigeria), *Ba. bigemina* Muguga (Kenya) and *Ba. bigemina* Mexico (Mexico). The Australian *Ba. bigemina* stock is the genome strain. DNA stocks represented parasites isolated from widely separated geographical areas that were selected in order to maximise the chance of detecting polymorphism.

The distribution of sampling sites in Turkey is shown in Figure 4.1. A total of 44 Turkish isolates were available and these included 11 from Tire in Izmir region and 30 from Aydın region (11 from Dalama, 14 from Haci Ali Obasi and 5 from Yazidere). The details of three remaining samples could not be traced. These isolates were collected in 2004 and were kindly provided by Dr T. Karagenç of the Department of Parasitology, Adnan Menderes University, Aydın.

The distribution of the Zambian sampling sites is shown in Figure 3.1 (Chapter Three). The Zambian isolates were collected in the wet season between December 2007 and March 2008 (Section 3.2). From the analysis of samples collected earlier in the dry season (Section 3.2), it was established that the parasitaemia of *Ba. bovis* and *Ba. bigemina* was very low, and that blood spotted on FTA cards would not provide sufficient parasite material for genotyping. To overcome this problem, blood samples collected for genotyping were prepared as saponin lysates as follows: for each sample 2 microfuge tubes were filled with 400 µl of blood and 1 ml lysis buffer (0.22 % NaCl, 0.015 % saponin, 1 mM EDTA, pH 7.5) was added to each tube. The tube contents were mixed and then centrifuged at 10,000 xg for 3 minutes and the supernatant discarded. The pellets in each tube were then re-suspended in 0.75 ml lysis buffer and the contents of the 2 tubes were combined. The tubes were again centrifuged and the supernatant discarded. This process was repeated until the pellet was clear of haemoglobin. The pellet was then re-suspended in 100 µl of 50 mM KCl, 10 mM tris-HCl pH 8.0, 0.5 % tween and 100 µg proteinase K per ml. The tubes were incubated in a water bath at 56 ^{o}C for 2 hours after which they were immediately stored at –20 ^{o}C. The lysates were boiled for ten minutes and a 1 in 5 dilution was made for use as PCR template. This method of processing the samples increased the

number of PCR positive samples from 78 to 89 for *Ba. bovis* and from 37 to 58 for *Ba. bigemina* over those detected on FTA filters. Of the 89 *Ba. bovis* positive samples, 2 were from Central, 18 from Lusaka and 69 from Eastern provincs. For the *Ba. bigemina* samples, 1 was from Central, 25 from Lusaka and 32 from Eastern province.

4.2.2 Identification of tandemly repeated sequences

The genome sequences of *Ba. bovis* and *Ba. bigemina* were screened with the tandem repeat finder program (Benson 1999). Repeat motifs of up to 500 bp were identified using stringent parameters for identifying matches, mismatches and indels in the sequences.

4.2.3 PCR amplification

Primers were designed to the unique sequence flanking each repeat region and used in a semi-nested PCR to test for amplification and polymorphism of DNA from the panel of stocks. DNA preparations were PCR amplified in a total reaction volume of 20 µl. For the primary reaction 1 µM of each primer, 1 µM custom PCR master mix (Thermo Scientific), 2 µl DNA template and 1 U *Taq polymerase* (Applied Biosystems) were used. The thermocycler conditions were 94 ^{o}C for 2 minutes, 30 cycles of 94 ^{o}C for 50 seconds, 50 – 60 ^{o}C for 50 seconds, 72 ^{o}C for 1 minute and a final extension period of 5 minutes at 72 ^{o}C. The PCR mixture and thermocycler conditions for the secondary reactions were similar to the primary one, except 2 µl of a 1:100 dilution of the primary reaction was used as template. The details of the markers, including the repeat motifs, primer sequences and annealing temperatures and chromosome/contig location are shown in Table 4.1 for *Ba. bovis* and Table 4.2 for *Ba. bigemina*. During the initial screening for polymorphism, amplicons were separated by electrophoresis on 2 % agarose gels pre-stained with ethidium bromide. Gels were photographed under ultra-violet trans-illumination and the size of each PCR product was determined with reference to a 100 bp DNA ladder. To facilitate high resolution genotyping of field isolates, a fluorescently labelled internal primer was incorporated into the secondary PCR reaction. The products were separated by capillary electrophoresis using the commercial service at the University of Dundee. Each fragment size was determined relative to a Rox-labelled size standard, which allowed resolution of 1 bp differences. For all loci and DNA preparations, the fragment size (i.e. peak position) was determined to two decimal places. Analysis of the distribution of the fragment sizes facilitated the creation of 'fixed bins' of variable sizes to score alleles. Multiple products from a single PCR reaction indicated a mixture of genotypes, allowing for the generation of a total 'fingerprint' representing the entire population of genotypes within each sample. The data from the capillary electrophoresis output also provided a

Table 4.1 Characteristics of *Ba. bovis* polymorphic markers

Eight markers located across all four chromosomes were selected for population genetic studies of *Ba. bovis*. For the primers, *Int. F.* refers to the internal forward primer used in the secondary PCR reaction, *Ext. F.* refers to an external primer used in the primary PCR reaction and *Rev*. refers to a reverse primer used in both the primary and secondary PCR reaction. Repeat copy number refers to copy number of the repeat motif in the Texas, genome strain of *Ba. bovis*. For the annealing temperatures, *Int*. refers to the annealing temperature for the secondary PCR reaction and *Ext*. refers to the annealing temperature for the primary PCR reaction.

Table 4.1 Characteristics of *Ba. bovis* polymorphic markers

Name	PCR primers 5′ – 3′	Chromosome	Repeat copy number	Consensus repeat sequence	Amplicon size range (bp)	Annealing Temp (ºC)
Bbv5	*Int. F: GCATTAAGGTGTTGACTACCT* *Rev: CTTAAGTTTCTGGTCGGATATG* *Ext .F: GTGGATTTCAATGCTGTCACG*	1	18.5	AT	108 – 406	Ext.: 52 Int. : 52
Bbv19	*Int. F: CTTAAGCATAACCGACGCAGCTAG* *Rev: CGTTACAAGTTCGTCTCGGTTG* *Ext. F: CAGTGTGCCAAAGTAGTACA*	4	4.6	AAGAAGTGAAG	113 – 393	Ext.: 53 Int. : 53
Bbv20	*Int. F: GTGCCGAAGACGGTGAAACAC* *Rev: ATGAGATGTGCTTTCGTGTTCGTC* *Ext. F: CAAACAAGTATGTTGGACAC*	4	11	GAA	102 – 419	Ext.: 52 Int. : 52
Bbv25	*Int. F: GATGTGGCATAGCATTGTCAAAC* *Rev: TCTACGTAAGGGGCAGGATAACG* *Ext. F: GAACGTCTTGTCTCATTCATTGC*	4	20	AT	114 – 492	Ext.: 52 Int. : 52
Bbv26	*Int. F: CTGTTGAGTGGTATCTGTTATCC* *Rev: GGTTGGCTAACATCAGTGGT* *Ext. F: CTAGTACTCAAGGAAACAGTG*	1	4.7	AGAAAC	106 – 515	Ext.: 55 Int. : 52
Bbv50	*Int. F: ATCCAACCCATCATACATCGCT* *Rev: CAGAACTCAATTGAAACCGCAG* *Ext. F: GAGAAGCATGTCTAAATGTGATC*	2	16.5	TA	104- 251	Ext.: 55 Int. : 55
Bbv52	*Int. F: GTCCTTCGGATGGTTCACATGATG* *Rev: CACAGTGAACGCATTTGACTAC* *Ext. F: GGTTGTATCAGTGCGTGGTAT*	2	13	AT	155 – 461	Ext.: 55 Int. : 55
Bbv58	*Int. F: GTGGAATCCCTTGTCTGCATTGCAG* *Rev: GTAGTCGGCGGTGTAATATTTGTTG* *Ext. F: GTCCAATTCGAAGGTGGTGAGCTG*	3	6.3	CAC	154 – 211	Ext.: 57 Int. : 55

Table 4.2 Characteristics of *Ba. bigemina* polymorphic markers

Nine markers were selected for population genetic studies of *Ba. bigemina*. For the primers, *Int. F.* refers to the forward primer used in the secondary PCR reaction, *Ext. F.* refers to a forward primer used in the primary PCR reaction, *F.* refers to a forward PCR primer used in both the primary and secondary PCR reactions, *Int. R.* refers to a reverse primer used in the secondary PCR reaction, *Ext. R.* refers to a reverse primer used in the primary PCR reaction and *Rev*. refers to a reverse primer used in both the primary and secondary PCR reaction. Repeat copy number refers to the copy number of repeat motif in the Australian genome strain of *Ba. bigemina*. For the annealing temperatures, *Int*. refers to the annealing temperature for the secondary PCR reaction and *Ext.* refers to the annealing temperature for the primary PCR reaction.

Table 4.2 Characteristics of *Ba. bigemina* polymorphic markers

Name	PCR primers 5′ – 3′	Contig	Repeat copy number	Consesus repeat sequence	Amplicon range size (bp)	Annealing Temperature (°C)
Bbg2	*Ext. F: GCTGTACATATCAGGTCACGCGCAC* *Int. F: CACTGATGGCAGCGAGTGTC* *Rev: CTCACCAATGACCGTGTTCG*	413	4	ACCGCAGGA	149 - 282	Ext.: 55 Int.: 55
Bbg7	*Ext. F: CGAAGGTACGTTATAGGTCG* *Int. F: GACTTGCCTTCCGAGGATTCG* *Rev: CGTCGGACGACTCCTCGGAG*	235	11.7	GAT	147 - 280	Ext.: 55 Int.: 57
Bbg9	*Ext. F: CAGCCTCATTCATGGCGGAG* *Int. F: CTCAACAACATCGTCAACACCGTCG* *Rev: GAAGAGGTTACACCCGACAC*	28	15.7	TCA	117 - 291	Ext.: 55 Int.: 55
Bbg13	*Ext. F: GACGTTGGCGATACCAGAACTCAC* *Int. F: CAGACAAGATCCTCGCACGTCA* *R GTTAGTACCAACAACCGGGTGAC*	27	25.8	CCTCAG	105 - 383	Ext.: 57 Int.: 55
Bbg14	*F: CTTCCGTTGTAGGCGGTACTG* *Int. R: CGATGGCTTCTCACAAGTACATAC* *Ext. R: CTGAGTTGGCAGTGGTGGATG*	341	20.7	ATC	155 - 305	Ext.: 57 Int.: 55
Bbg18	*F: CGATGTCCAAGTTGCTGAGGTG* *Int. R: GACTCCATGGTCGACGATGGAAC* *Ext. R: CTGCGCTAAGGACAACTCTGAG*	509	12.7	CCTCAG	171 - 271	Ext.: 57 Int.: 54
Bbg23	*Ext. F: CACGTCATTGGCGTTGGTGTTC* *Int. F: GAATCTTTGGCGTCTTTTGTGTCCTTC* *Rev: GTCAACGATATGGCATTGGAAGAC*	353	9.2	TTCGTTTCT	154 - 318	Ext.: 55 Int.: 55
Bbg34	*Ext. F: CTACAACCAAATCGCATGCTTC* *Int. F:CTGGGCAGACAGGTAATCATGTCGC* *Rev: TTGTCGCCGATTCCATCAATGTCCT*	342	23	ATG	173 - 309	Ext.: 57 Int.: 54
Bbg42	*Ext. F: CTGACACCCTTCGCATCTTTGCAG* *Int. F: GATCACTCTCATCGTTCTTCCTCG* *Rev: GAGGAGGACGAAGAAGCAGATGC*	341	16.9	TCTTCATCC	154 - 384	Ext.: 55 Int. 54

semi-quantitative measurement of the abundance of each allele, allowing the predominant allele to be identified. This information was then used to generate a multi-locus genotype (MLG) representing the most abundant genotype in each sample.

4.2.4 Data analysis

Similarity comparison of MLGs was undertaken using an allele sharing co-efficient (Bowcock *et al.* 1994) in the Excel Microsoft-microsatellite toolkit (Park 2001). For similarity analysis, Principal Component Analysis (PCA) was used. PCA is a powerful statistical procedure for identifying patterns in multi-dimensional data, which has been applied in diverse fields such as facial recognition and digital image compression. PCA is used to reduce the number of dimensions in a data set while retaining those characteristics of the dataset that contribute most to its variance. Essentially, a mathematical procedure transforms a number of potentially correlated variables into a reduced number of uncorrelated variables called principle components. The objective of the analysis was to identify underlying trends within a dataset. The technique has been successfully applied in the analysis of MLGs of *T. annulata* (Weir 2006). The Microsoft Excel plug-in software, 'Genalex6' (http://www.anu.edu.au/BoZo/GenAlEx/) (Peakall & Smouse 2006) was used to construct a similarity matrix and perform PCA on sets of MLG data. Population genetic analysis for the estimation of F-statistics was performed using Genepop DOS versions 3.3/3.4 (http://genepop.curtin.edu.au/genepop_op6.html).

To determine whether genetic distance correlated with geographical distance, the Mantel test (Fortin 2002) was employed. This approach overcomes the problem of analysing spatially auto-correlated data, which occurs when observations are not independent of one another, because of their arrangement in space. Spatial auto-correlation violates the assumption of independence of observations which is a serious concern for traditional parametric tests. When an association is detected, it may be interpreted in one of three ways: 1) there is no significant spatial auto-correlation and the observed association really exists, 2) the degree of spatial auto-correlation is significant and is introducing spurious associations, or 3) both the degree of spatial auto-correlation and the observed association are significant. The Mantel test, which is non-parametric, allows one to distinguish among these three cases by assessing the extent of spatial auto-correlation among subjects. During the time of sample collection, the longitude and latitude co-ordinates for each sampling sites were obtained. These were used to estimate the geographical distances in kilometres, between sampling sites in each country using the GPS Visualizer program (http://www.gpsvisualizer.com/calculators). Then tri-matrices of genetic distances (Nei

1978) and geographical distances were created for *Ba. bigemina* and *Ba. bovis* isolates from each country and these were used to undertake a Mantel test using the GenAlEx program.

The null hypothesis of linkage equilibrium was tested using LIAN (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl), which also calculated the standard index of association (I_S^A) (Haubold & Hudson 2000), a quantification of linkage equilibrium/disequilibrium. Linkage equilibrium (LE) is characterised by statistical independence of alleles across all loci under investigation. LIAN tests for this independent assortment by initially determining the number of loci at which each pair of MLGs differs. From the distribution of mis-match values, a variance V_D is calculated. This value is compared to the variance expected for LE, which is termed V_e. The null hypothesis that $V_D = V_e$ is tested by a Monte Carlo computer simulation. The software returns 95 % confidence limit, L denoted L_{MC}. When V_D is found greater than L, the null hypothesis is disproved and linkage disequilibrium is indicated.

To test the hypothesis that the *Ba. Bigemina* population was genetically sub-structured, the computer program STRUCTURE (Pritchard *et al.* 2000) was used to identify the most probable number of sub-populations or clusters (K) in the dataset. The method aims at delineating clusters of individuals on the basis of their genotypes at multiple loci using a Bayesian approach. The programme fits the data by introducing population structure and attempts to find population groupings that (as far as possible) are not in disequilibrium, but are undergoing random mating (Pritchard *et al.* 2000). The estimated log probability of the data Pr(X/K) for each value of K is given, allowing the estimation of a likely number of sub-populations or clusters. A quantification of how likely each individual is to belong to each group is also given, allowing the assignment of individuals to sub-populations. The 'no admixture' model was chosen with the option of 'no correlation' of allele frequencies between populations. Ten independent simulations were carried out at each value of K (K = 1 to 10). The length of 'burning in' period was set at 100,000 with the number of Markov chain Monte Carlo (MCMC) iterations, after burning, of 1,000,000. LnP(D) was calculated by first computing the log likelihood of the data at each step of the MCMC, then the average of these values was computed and half their variance was subtracted from the mean. This gave a value referred to as L(K). The 'true' number of sub-populations was identified as the value of K that returned the highest L(K) value (Evanno *et al.* 2005; Pritchard *et al.* 2000).

4.3 Results

4.3.1 Population genetic analysis of *Ba. bovis*

4.3.1.1 Identification and evaluation of markers

The tandem repeat finder program identified 11,064 repetitive sequences using the defined input parameters. However, a large number of these repeated sequences either overlapped partially or completely and variant motif forms were identified at the same locus. An *in silico* filtration process was used to identify a manageable subset of loci that could be tested using the panel of five stocks. The process involved discarding repeat regions greater than 500 bp in length and those flanking sequences that were not ideal for primer design, e.g sequences with excessive GC content. The remaining sequences were ranked based on the fidelity of the repeat within each region (> 70 % fidelity) and the number of repeats. 63 top loci were identified (Table 4.3) comprising 49 micro-satellites and 14 mini-satellites. Primers were designed to the unique sequence flanking each of the 63 satellite loci and used to amplify the DNA from the panel of five stocks. Since *Ba. bovis* is haploid, only a single band was expected to be seen for each of the DNA stocks under consideration. For a locus to be considered a suitable marker, it had to amplify from at least four out of the five DNA stocks, be polymorphic, give single bands and not amplify from non-*Ba. bovis* DNA stocks (i.e. *Ba. bigemina, T. parva, T. mutans, T. taurotragi, A. marginale* and bovine DNA*)* as shown in Table 4.3. Out of the 63 loci, only eleven met the stated criteria. Seven of the loci did not amplify any of the five DNA stocks while the remainder either amplified less than four of the stocks, produced multiple bands or were not polymorphic (Table 4.3.). The eleven markers that met the criteria were further tested for their ability to amplify a set of Zambian field isolates and to demonstrate polymorphism. Three of the markers (Bbv16, Bbv48 and Bbv49) did not show polymorphism among the Zambian isolates and were not used to genotype the Zambian and Turkish isolates. Figure 4.2 shows a gel picture of marker Bbv26, an example of one of the markers that met the set criteria. This marker was able to amplify from all the *Ba. bovis* stocks under consideration (Lanes B to F), and the amplicon size for *Ba. bovis* Kwanyangwa (Lane C) was different from the others. The marker was species-specific because it did not amplify from the other non-*Ba. bovis* DNA samples (Lanes G to L). In total, eight markers (Bbv5, Bbv19, Bbv20, Bbv25, Bbv26, Bbv50, Bbv52 and Bbv58) were identified (Table 4.1). All these markers were polymorphic and were able to amplify from the panel of test DNA. On this basis, these markers were used for the population genetic analysis.

Table 4.3 Criteria employed in selecting *Ba. bovis* markers

This table illustrates the criteria used to select the markers for genotyping *Ba. bovis* isolates from Zambia and Turkey. For a marker to be considered suitable, it had to amplify at least four out of the five *Ba. bovis* reference stocks, produce single bands, be species-specific and polymorphic. The markers highlighted in green satisfied all these criteria. Those highlighted in blue were polymorphic for the five stocks used for screening of the markers but did not show polymorphism with the Zambian isolates.

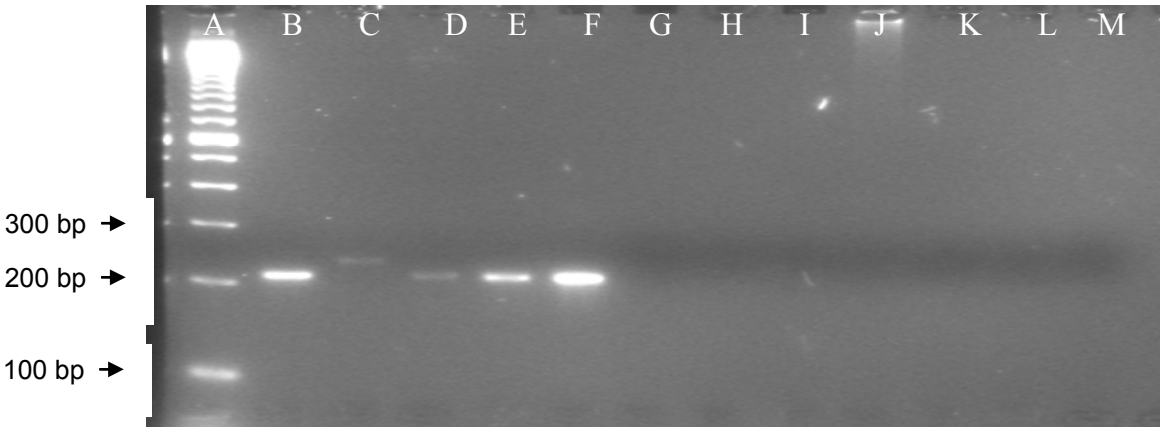
Table 4.3 Criteria employed in selecting *Ba. bovis* markers

Marker	Motif length (bp)	Repeat copy number	Genome predicted amplicon size (bp)	Proportion of stocks amplified	Single bands / species-specific	Polymorphic
BBV1	6	16.7	243	5/5	no/yes	yes
BBB2	9	4.3	243	5/5	no/yes	yes
BBV3	3	12.7	259	5/5	yes/yes	no
BBV4	2	18	205	5/5	yes/no	yes
BBV5	2	18.5	235	5/5	yes/yes	yes
BBV6	9	4	254	2/5	yes/yes	no
BBV7	9	4.4	288	5/5	yes/yes	no
BBV8	10	4	356	3/5	no/Yes	yes
BBV9	3	8.3	219	0/5	-	-
BBV10	3	8	284	0/5	-	-
BBV11	2	16.5	311	3/5	yes/yes	yes
BBV12	7	4	295	5/5	no/yes	yes
BBV13	9	4.2	223	5/5	yes/yes	no
BBV14	9	4	281	2/5	yes/no	no
BBV15	12	4.4	302	3/5	yes/Yes	yes
BBV16	12	4.8	238	5/5	yes/Yes	yes
BBV17	3	9	332	0/5	-	-
BBV19	12	4.6	276	5/5	yes/yes	
BBV20	3	11	231	5/5	yes/yes	yes
BBV21	14	4.4	280	5/5	yes/yes	no
BBV22	3	8	311	0/5	-	-
BBV23	3	10	353	0/5	-	-
BBV24	2	19	369	5/5	yes/yes	no
BBV25	2	20	316	4/5	yes/yes	yes
BBV26	6	4.7	226	5/5	yes/yes	yes
BBV27	3	6.7	277	5/5	yes/yes	no
BBV28	6	6.3	339	5/5	yes/yes	no
BBV29	21	11.4	447	5/5	no/yes	yes
BBV30	21	7.3	352	2/5	yes/yes	yes
BBV31	5	5.8	270	5/5	yes/yes	no
BBV32	3	8.7	271	5/5	yes/yes	no
BBV33	3	8	270	0/5	-	-
BBV34	3	6.7	116	2/5	yes/no	yes
BBV35	3	6.7	273	0/5	-	-
BBV36	3	6.7	283	5/5	yes/yes	no
BBV37	3	6	245	3/5	yes/yes	no
BBV38	3	6.7	269	5/5	yes/yes	no
BBV39	3	7.7	295	4/5	yes/yes	no
BBV40	3	7	247	5/5	yes/yes	no
BBV41	6	4.8	229	4/5	yes/yes	no
BBV44	6	4	235	4/5	yes/yes	no
BBV45	3	6.7	191	5/5	yes/yes	no
BBV46	11	5	227	2/5	no/yes	yes
BBV47	3	8	215	1/5	yes/yes	-
BBV48	3	8.7	234	4/5	yes/yes	yes
BBV49	3	6	255	5/5	yes/yes	yes
BBV50	2	20	173	5/5	yes/yes	yes
BBV51	2	11.5	180	5/5	yes/yes	no
BBV52	2	13	243	5/5	yes/yes	yes
BBV53	3	7.3	185	0/5	-	-
BBV54	3	9	215	5/5	yes/yes	no
BBV55	3	6.7	156	5/5	yes/yes	no
BBV56	3	6.7	238	5/5	yes/yes	no
BBV57	3	7	179	5/5	yes/yes	no
BBV58	3	6.3	216	4/5	yes/yes	yes
BBV59	3	6.7	240	5/5	yes/yes	no
BBV60	4	5	166	2/5	yes/yes	no
BBV61	4	5.3	131	5/5	yes/yes	no
BBV62	4	5.3	164	5/5	yes/yes	no
BBV63	4	5.3	178	5/5	yes/yes	no

Figure 4.2 Example of agarose gel electrophoresis for marker BbvV26

This image represents PCR products generated using the Bbv26 primer set on the panel of DNA samples used to screen all of the *Ba. bovis* markers. An aliquot of the PCR product from each reaction was loaded onto a 2 % agarose gel and separated by electrophoresis. Lane A contains a 100 bp ladder used as a reference to determine the size of the amplicon in each lane; Lanes B to F contain the *Ba. bovis* stocks used in screening the markers. Lanes G to L contains non-*Ba. bovis* DNA and M was the negative control. This marker was polymorphic, gave single bands and did not amplify non-*Ba. bovis* DNA.

Figure 4.2 Example of agarose gel electrophoresis for marker BbvV26



Lane A, 100 bp ladder; B, *Ba. bovis* Lismore; C, *Ba. bovis* Kwanyangwa; D, *Ba. bovis* (Unknown strain); E, *Ba. bovis* Mexico; F, *Ba. bovis* MO7; G, *T. parva* Muguga; H, *T. taurotragi*, I, *T. annulata*; J, *Ba. bigemina* Muguga; K, Bovine DNA; L, *A. marginale* and M, negative control.

Capillary electrophoresis was utilised to analyse amplicons from the field isolates from Zambia and Turkey. A Rox-labeled Mapmarker® 1000 size-standard was used to determine the amplicon sizes. An example of an electropherogram generated using Peak Scanner® software is shown in Figure 4.3 and represents the amplified product of a Zambian field isolate using the Bbv5 primer set. This clearly shows three discrete blue peaks, the positions of which are interpolated from the distribution of the size standard. Three alleles of 208, 251 and 331 bp in size were identified, with the 208 bp allele being the predominant one by virtue of its greater area under the peak. The size standards are represented by the red peaks. All eight markers showed polymorphism between stocks isolated from different countries, as well as among stocks isolated from the same region. All the markers demonstrated the ability to detect multiple alleles in the same sample with Bbv25 being the most polymorphic. The eight markers were distributed across all four chromosomes (Table 4.1) and none were closely physically linked. Markers Bbv5 and Bbv26 were on chromosome 1 and were separated by a distance of 471 kb. Bbv50 and Bbv52 were on chromosome 2 and were separated by a distance of 920 kb. Bbv19, Bbv20 and Bbv25 were on chromosome 4. Bbv19 and Bbv25 were separated by a distance of 437 kb, while Bbv19 and Bbv20 were separated by a distance of 964 kb. Only Bbv58 was on chromosome 3.

4.3.1.2 Allelic diversity of the markers

The eight markers were used to genotype DNA preparations of field samples from Zambia and Turkey, using capillary electrophoresis to determine the amplicon sizes. All the markers were polymorphic for both Zambian and Turkish samples. The allelic variation and the diversity of the markers in each *Ba. bovis* population is shown in Table 4.4. Across all isolates the maximum number of alleles identified by each marker in a single sample ranged from five for Bbv58 to ten for Bbv25, with a mean range of 2.30 for Bbv20 to 2.86 for Bbv25 and Bbv52. The number of different alleles identified by each marker in the total Zambian population ranged from six for Bbv58 to 41 for Bbv25 while in the Turkish population, it ranged from six for Bbv58 to 16 for Bbv50. Overall, Bbv25 was the most polymorphic marker, identifying 48 different alleles in the Zambian and Turkish *Ba. bovis* populations, while Bbv58 was the least polymorphic identifying only nine alleles in the two populations. None of the markers could amplify from all the samples. Bbv5, Bbv20, Bbv52 and Bbv58 each failed to amplify from one sample, Bbv26 failed to amplify from two samples while Bbv19, Bbv25 and Bbv50 failed to amplify from three samples each. However, at least one marker was amplified from each sample. The maximum number of markers that failed to amplify from the same sample was two, suggesting that failure to

Figure 4.3 Example of Peak Scanner™ analysis for *Ba. bovis* isolates

This image represents a trace, or electrophoretogram, generated by Peak Scanner™ analysis and depicts fluorescently-labelled PCR products. This particular trace was produced using the Bbv5 primer set with a DNA template from a Zambian isolate containing a mixture of three genotypes. The horizontal axis represents fragment sizes while the vertical axis represents units of fluorescent intensity. Blue peaks represent the labeled PCR product, while red peaks indicate the Mapmarker® 1000 size standards. The sizes of the alleles represented by the three blue peaks are interpolated from their position with respect to the size standards. The predominant allele, in this case 208 bp, is defined as the peak with the greatest area under the curve.

Figure 4.3 Example of Peak Scanner™ analysis for *Ba. bovis* isolates

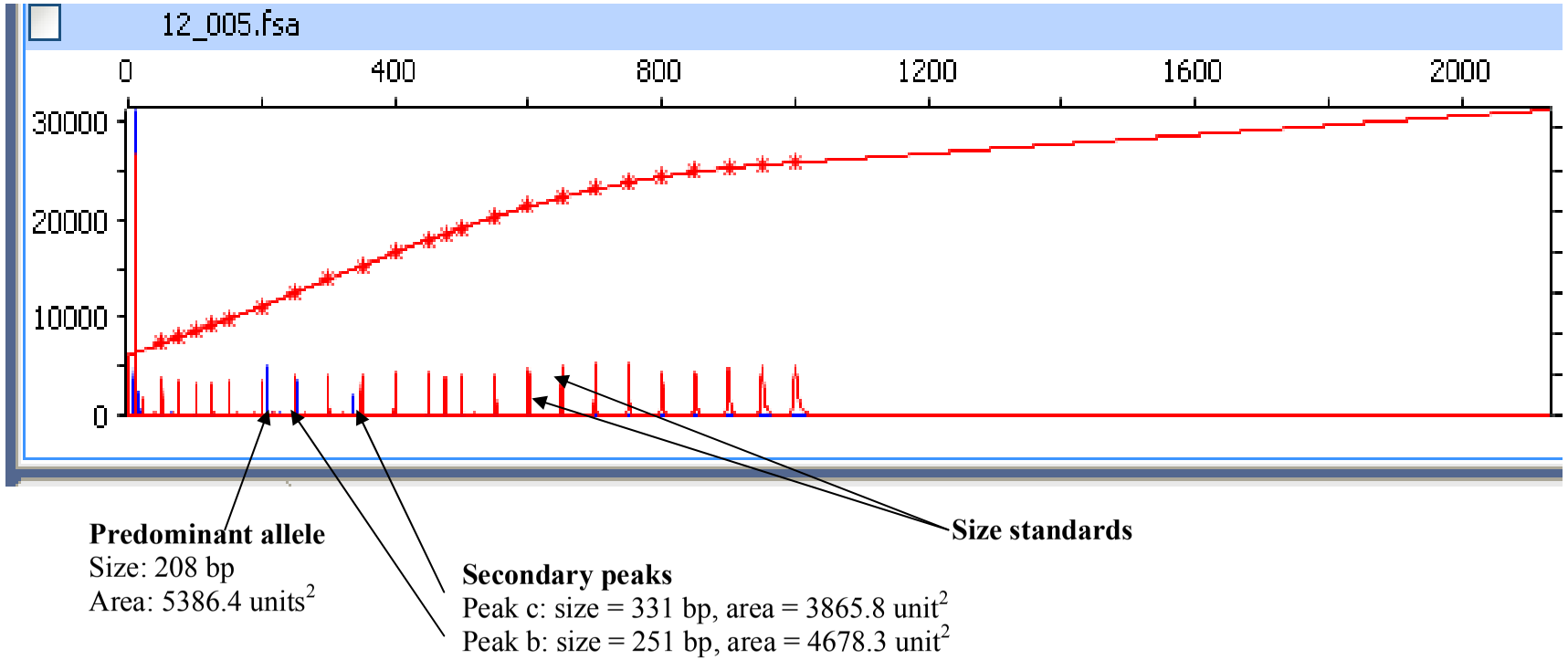


Table 4.4 Allelic variation in Zambian and Turkish *Ba. bovis* isolates

All samples were genotyped using the panel of eight markers. The minimum and maximum number of alleles detected at each locus within a sample was determined across all eight markers for each sample that amplified. The number of alleles represented in each population was calculated, taking into account the most abundant and all the minor alleles present in each sample from that country. Gene diversity was calculated for each marker and is equivalent to estimated heterozygosity.

Table 4.4 Allelic variation in Zambian and Turkish *Ba. bovis* isolates

		Number of samples	Bbv5	Bbv19	Bbv20	Bbv25	Bbv26	Bbv50	Bbv52	Bbv58
Alleles within each sample	Minimum		1	1	1	1	1	1	1	1
	Maximum		9	7	7	10	7	6	7	5
	Mean		2.53	2.43	2.30	2.86	2.35	2.70	2.86	2.5
	No amplification		1	3	1	3	2	3	1	1
Alleles Within population	Zambia	80	15	19	23	41	33	21	19	6
	Turkey	40	14	10	9	15	9	16	12	6
	Overall	120	25	25	27	48	37	32	26	9
Gene diversity	Zambia	80	0.767	0.762	0.905	0.968	0.866	0.877	0.861	0.662
	Turkey	40	0.892	0.786	0.845	0.906	0.806	0.882	0.874	0.709

amplify was due to polymorphism at the primer binding site. In total, nine Zambian samples and four Turkish samples failed to amplify at least at one locus. The average number of alleles per locus was 22.13 in the Zambian population and 11.38 in the Turkish population. Although there was variation in gene diversity from marker to marker, the values for Turkish and Zambian populations for each marker were very similar (Table 4.4). This suggests that gene diversity is an inherent characteristic of the markers and, to an extent, is independent of the parasite population.

Multi-locus genotypes (MLGs) were constructed using the predominant allele present at each locus and used to generate a dataset for population genetic analysis. MLGs for samples that failed to amplify from at least one locus were not constructed. Each sample had a unique MLG with the greatest identity between any two samples being six out of eight allelic markers. The predominant allele frequencies were determined for the Zambian and Turkish populations and high levels of diversity were observed within each country as shown in Figure 4.4. Many of the markers showed a considerable number of rare alleles, many of which were private to either Zambia or Turkey. For example, of the 25 alleles that were identified by Bbv19, 21 were private. Of these private alleles, 15 were from Zambia, while the remainder were from the Turkish populations. For six out of eight markers (Bbv5, Bbv20, Bbv25, Bbv26, Bbv50 and Bbv52) private alleles were detected with a frequency greater than 0.15 in one or both populations, suggesting sub-structuring. There were more private alleles detected in the Zambian population than in the Turkish one, probably reflecting the large sample size from the former population. Examination of the different allele frequencies within each population showed that for more than 50 % of the markers (Bbv5, Bbv19, Bbv20, Bbv50 and Bbv58), there were at least two alleles with a frequency greater than 0.15. Two further markers (Bbv25 and Bbv26) in the Turkish population had more that two alleles with a frequency of 0.15, while one (Bbv52) in the Zambian population also showed such a feature (Figure 4.4).

4.3.1.3 Similarity analysis

Due to the high level of diversity within *Ba. bovis* and the large number of mixtures of genotypes present in individual samples, a MLG was constructed for each sample representing the combination of predominant alleles identified at each locus. It was hypothesized that the population structure of *Ba. bovis* is panmictic and that sexual recombination occurs at a sufficiently high rate so that discrete genotypes are not stable over time. The mixture of genotypes ingested by ticks feeding on a cattle host represents a sub-population, which has the ability to sexually recombine in the tick gut. Hence the

Figure 4.4 Allele frequencies of Turkish and Zambian *Ba. bovis* isolates

The frequency of each allele in the Zambian and Turkish population was determined as a proportion of the total for each marker using the predominant allele in each sample. These histograms were directly generated from the multi-locus genotype data.

Figure 4.4 Allele frequencies of Turkish and Zambian *Ba. bovis* isolates

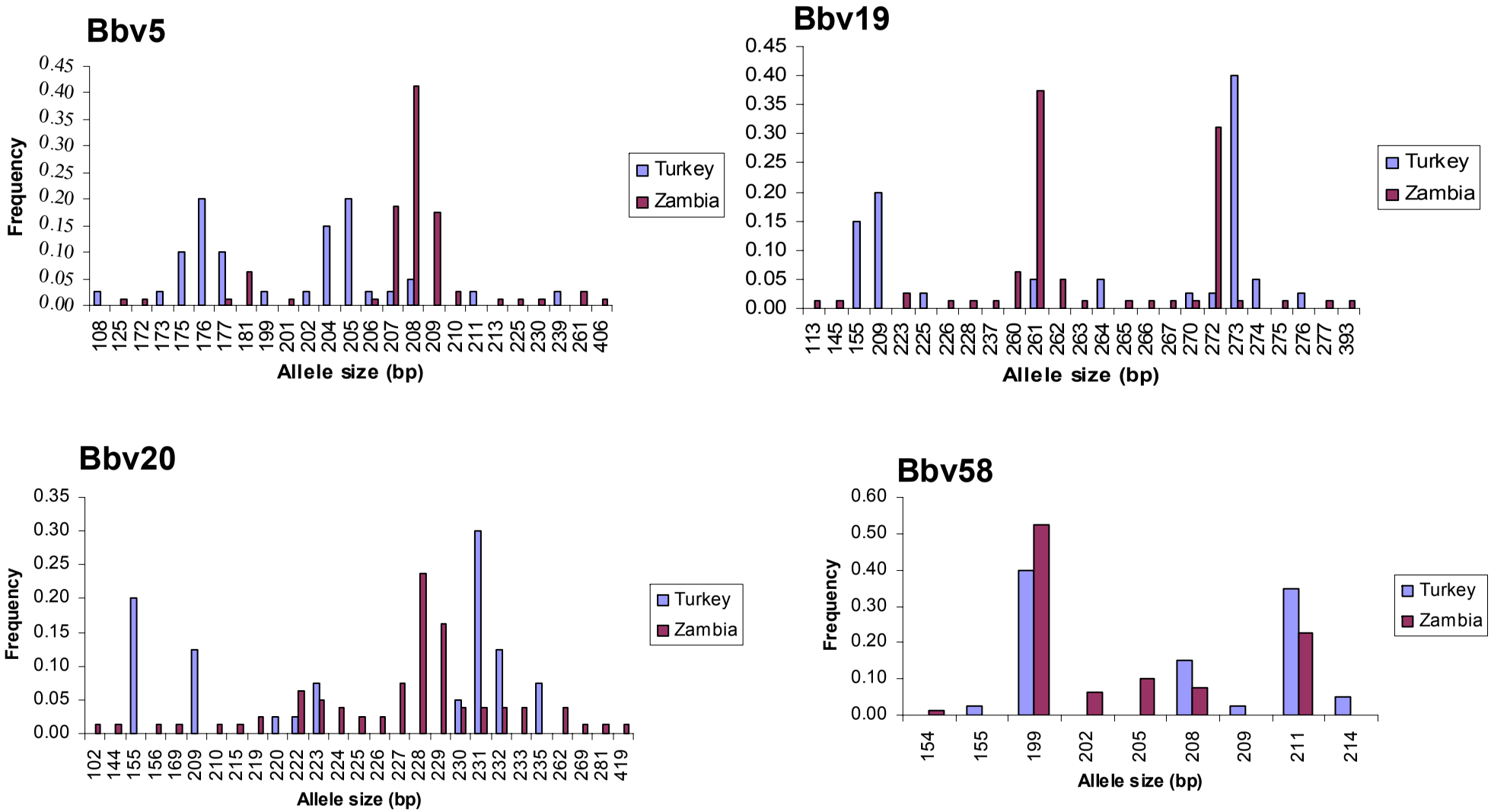


Figure 4.4 Allele frequencies of Turkish and Zambian *Ba. bovis* isolates (continued)

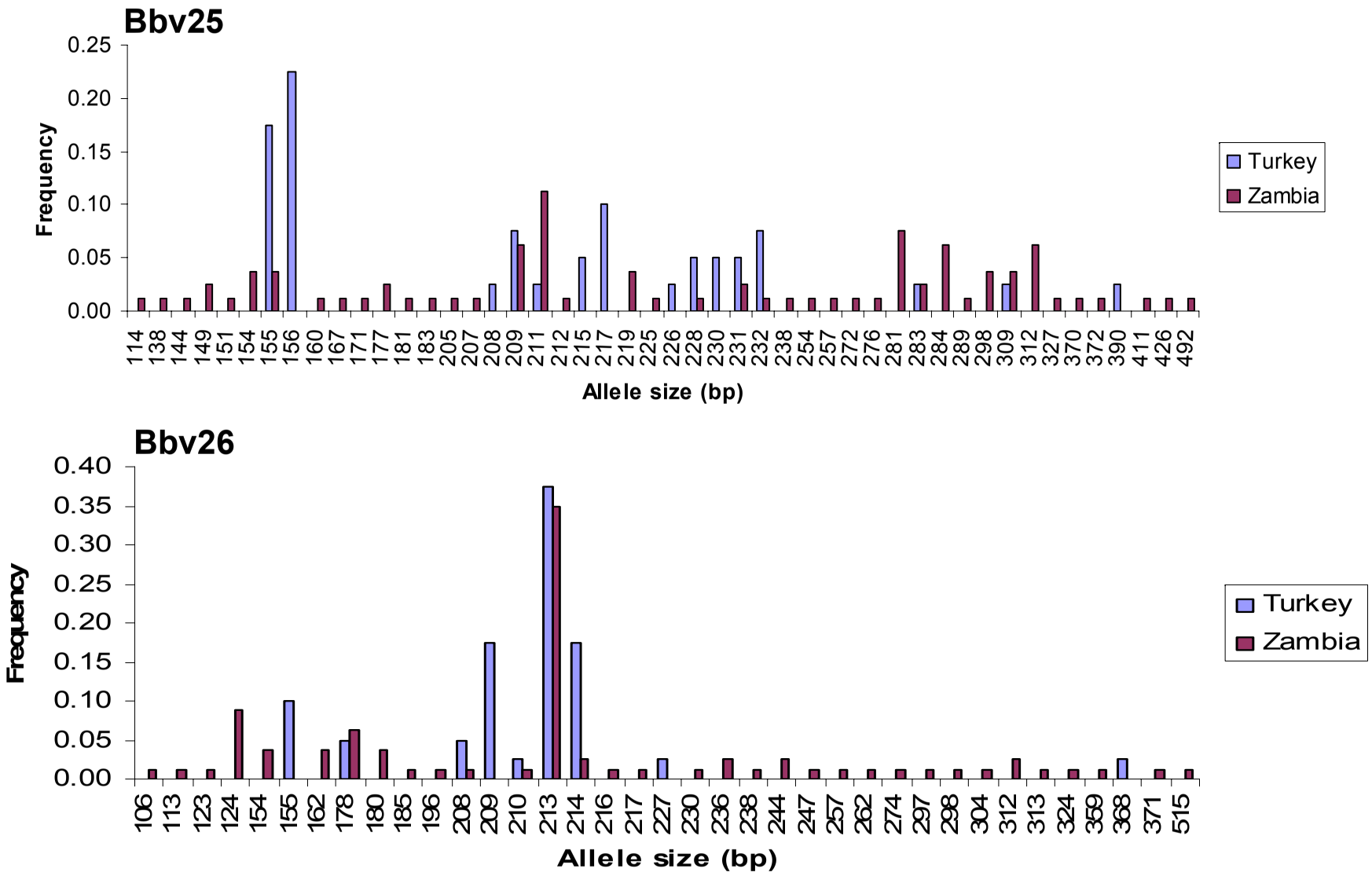
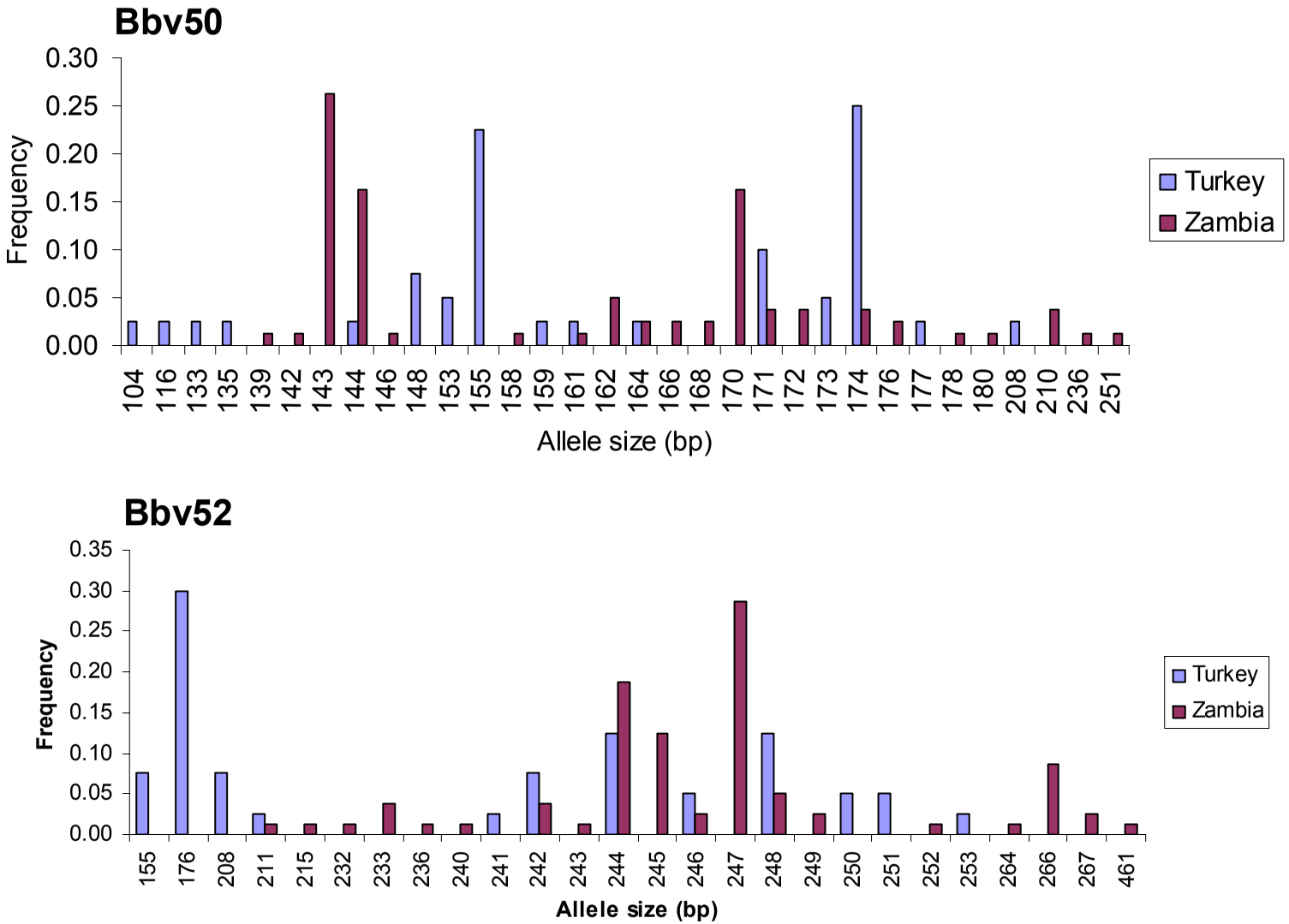


Figure 4.4 Allele frequencies of Turkish and Zambian *Ba. bovis* isolates (continued)



predominant allele represents a product of the most abundant recombining genotypes circulating in that population. On this basis, the MLG, i.e. the combination of predominant alleles for each sample, can be viewed as a representative sample and therefore these data were used to undertake PCA between and within countries. The PCA for the Zambian and Turkish isolates are illustrated in Figure 4.5 and isolates from each country can be seen to cluster separately. For the PCA representing the Zambian population (Figure 4.6), the Eastern province isolates were widely distributed over the whole diagram. In contrast, the Lusaka province genotypes clustered on the lower side of the second axis (Figure 4.6) and they may therefore represent a subset of genotypes from Eastern Province. This is consistent with the view that *Ba. bovis* has been spreading into Central Zambia from the Eastern Province due to uncontrolled cattle movements. PCA of the Turkish isolates (Figure 4.7) shows that the *Ba. bovis* population in this country is divided into two distinct sub-populations, suggesting that these are genetically isolated. The isolates from Tire (Izmir region) and those from Dalama (Aydın) clustered together, while those from Yazidere and Haci Oli basi (Aydın region) form a second cluster.

4.3.1.4 Diversity and Population sub-structuring

High levels of genetic diversity were observed for both Zambian and Turkish populations and within each region in these countries. Estimated heterozygosities for each country and region are shown in Table 4.5. Similar values were obtained for within each country, ranging from 0.733 to 0.821 in Izmir and Aydın regions of Turkey respectively to 0.766 to 0.830 in Lusaka and Eastern Provinces of Zambia respectively. Estimated heterozygosities for each marker in the combined Turkish and Zambian *Ba. bovis* populations are shown in Table 4.6. These ranged from 0.684 for Bbv58 to 0.968 for Bbv25. The lower number of genotypes per locus in the Turkish population (11.38) compared to that in the Zambian population (22.23) probably reflects its small sample size rather than a lower diversity (Table 4.5).

To test the hypothesis that the population in each country was genetically isolated, genetic differentiation in each country was assessed by calculating Wright's fixation index, i.e. F_{ST} values (Weir & Cockerham 1984). The calculated F_{ST} values are shown in Table 4.5. Moderate genetic differentiation ($F_{ST} = 0.094$) was obtained between Turkish and Zambian populations. The ability of each marker to differentiate between Zambian and Turkish isolates was evaluated and the F_{ST} values are shown in Table 4.6. Values close to zero indicated lack of differentiation and Bbv58 was the only marker with an F_{ST} value that was not significantly greater than zero ($p = 0.070$). The other seven markers had F_{ST} values

Table 4.5 Population genetic analysis of *Ba. bovis*

Standard population tests were conducted on parasite samples representing (a) different countries and (b) areas within Zambia and Turkey. The ‘mean number of genotypes per locus was calculated as the mean value for the number of alleles detected at each of the eight loci. Structured combinations of populations were pooled to test for linkage disequilibrium. Variance of mismatch values (V_D) were compared to values of L (the upper confidence limits of Monte Carlo simulations) and where $V_D > L$, linkage disequilibrium (LD) was indicated. When $L > V_D$ the null hypothesis of linkage equilibrium (LE) was not disproved. F_{ST} were used to measure genetic differentiation between and within the countries.

Table 4.5 Population genetic analysis of *Ba. bovis*

Population compared	n	H_e	Mean number of genotypes/locus	I^S_A	V_D	L	*p value*	Linkage	F_{ST}	*p value*
Between Countries										
Zambia	80	0.834	22.13	0.0742	1.0283	0.8664	<0.001	LD	0.094	0.001
Turkey	40	0.837	11.38							
Within Zambia										
Eastern	62	0.829	18.88	-0.0003	1.0413	1.1230	0.50	LE	0.067	0.001
Lusaka	16	0.766	7.13							
Within Turkey (regions)										
Izmir	10	0.733	4.75	0.1295	2.0146	1.1571	< 0.001	LD	0.098	0.001
Aydın	27	0.822	9.00							
Within Turkey (Town)										
Dalama	9	0.694	3.50	0.0031	1.5720	1.8661	0.408	LE	-0.026	0.794
Tire	10	0.733	4.75							
Within Turkey (Town)										
Hacio Ali Basi	13	0.726	5.50	0.0399	1.7937	1.8068	0.060	?	0.020	0.280
Yazidere	5	0.625	2.88							

n = number of samples, H_e = estimated heterozygosity, I^S_A= standard index of association, VD = mismatch variance (linkage analysis), L = upper 95 % confidence limits of Monte Carlo simulation (linkage analysis), LD = linkage disequilibrium, LE = linkage equilibrium

Figure 4.5 Principal component analysis of Zambian and Turkish *Ba. bovis* isolates

Principal component analysis was performed on the multi-locus genotype data representing the Zambian and Turkish populations. The first two principal axes generated by this analysis are presented, demonstrating sub-structuring between isolates from each country. The proportion of the variation in the dataset explained by each axis is indicated in parenthesis.

Figure 4.5 Principal component analysis of Zambian and Turkish *Ba. bovis* isolates

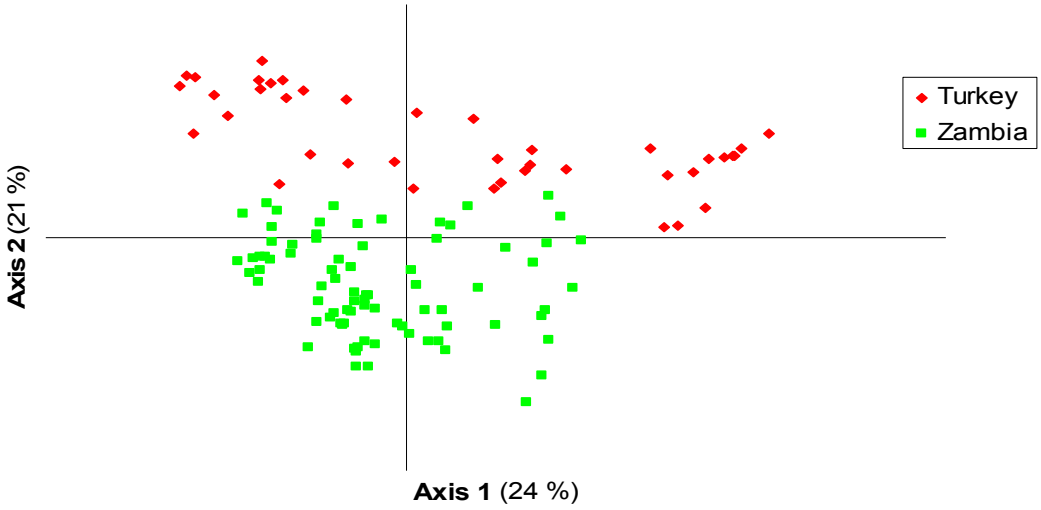


Figure 4.6 Principal component analysis of Zambian *Ba. bovis* isolates

Principal component analysis was performed on the multi-locus genotype data representing Eastern, Lusaka and Central Province isolates from Zambia. The first two principal axes generated by this analysis are presented, demonstrating a degree of sub-structuring between isolates from each province. The proportion of the variation in the dataset explained by each axis is indicated in parenthesis.

Figure 4.6 Principal component analysis of Zambian *Ba. bovis* isolates

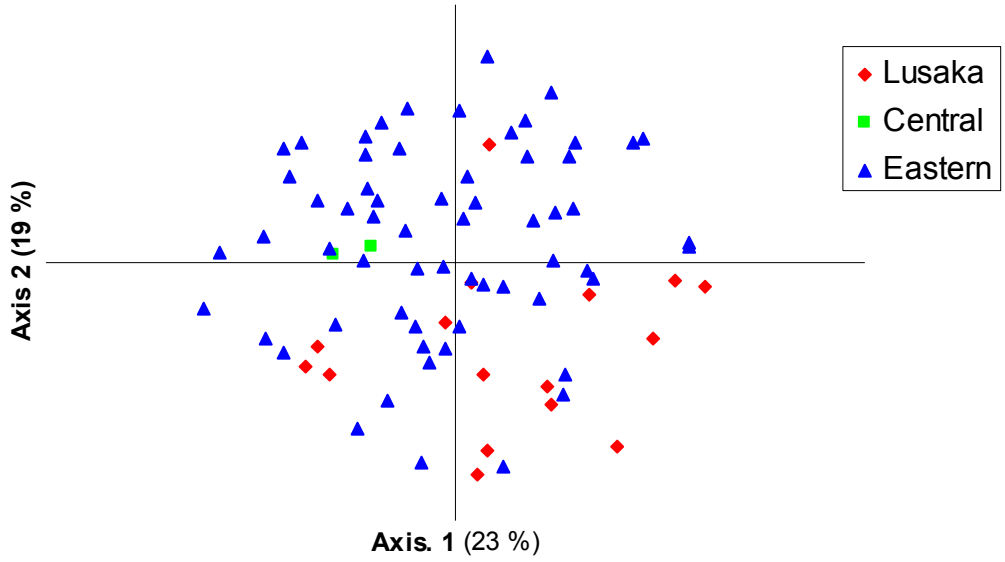


Figure 4.7 Principal component analysis of Turkish *Ba. bovis* isolates

Principal component analysis was performed on the multi-locus genotype data representing the Aydın (Dalama, Hacio Ali Basi and Yazidere) and Izmir (Tire) regions of Turkey. The first two principal axes generated by this analysis are presented, demonstrating sub-structuring between isolates from each region. The proportion of the variation in the dataset explained by each axis is indicated in parenthesis.

Figure 4.7 Principal component analysis of Turkish *Ba. bovis* isolates

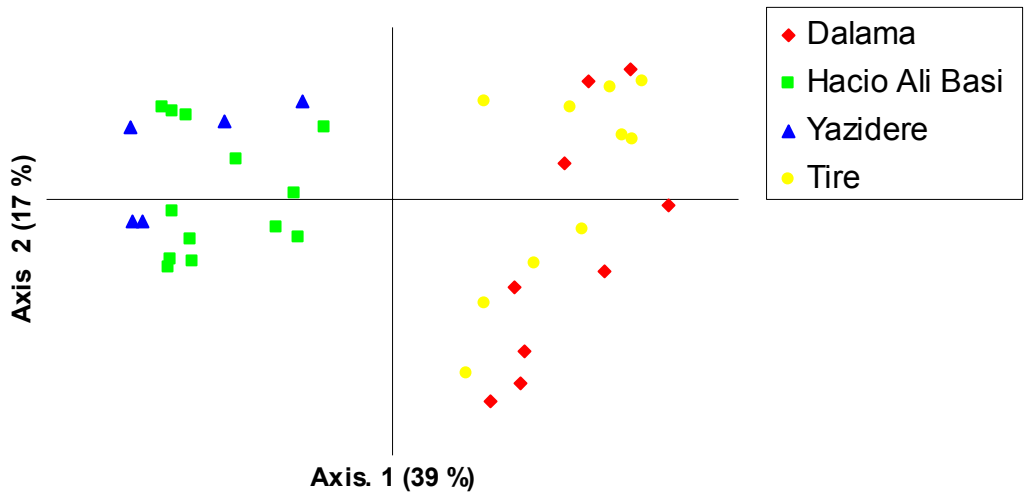


Table 4.6 *Ba. bovis* marker indices of genetic diversity and differentiation

The genetic diversity of each marker and its ability to differentiate between populations of *Ba. bovis* in Zambia and Turkey are shown in this table. The F_{ST} values ranged between 0.0211 (little genetic differentiation) for Bbv58 to 0.2009 for Bbv19 (great genetic differentiation). Except for Bbv58, all the markers had F_{ST} values significantly greater than zero. All the markers showed high diversity ranging from 0.6843 for Bbv58 to 0.968 for Bbv25.

Table 4.6 *Ba. bovis* marker indices of genetic diversity and differentiation

Marker	H_e	F_{ST}	*p* value
Bbv5	0.864	0.151	0.010
Bbv19	0.853	0.201	0.010
Bbv20	0.930	0.103	0.010
Bbv25	0.968	0.044	0.010
Bbv26	0.857	0.030	0.020
Bbv50	0.924	0.104	0.010
Bbv52	0.910	0.101	0.010
Bbv58	0.684	0.021	0.070
Overall	0.874	0.094	0.010

H_e = Estimated heterozygosity, F_{ST} is a measure of genetic differentiation

significantly greater than zero with the greatest value being 0.201 for Bbv19, indicating significant genetic differentiation. Moderate genetic differentiation was also identified within each country. The genetic differentiation between Aydın and Izmir in Turkey was 0.098 while between Lusaka and Eastern provinces in Zambia, it was 0.068 (Table 4.5).

From the PCA analysis, it is clear that the Zambian population shows limited or no geographical sub-structuring, whereas the Turkish population separated into two discrete sub-populations. Based on this, one would predict that in the Zambian population, there would be limited correlation between geographical and genetic distance (Nei 1978), and that such a correlation in the Turkish population would be evident. To confirm this prediction, the Mantel test was used and the results are shown in Table 4.7. The results show that genetic distance between isolates was positively correlated with geographical distance in both countries. In Turkey, geographical distance accounted for almost 22 % of the variation observed in genetic distance and the genetic distance increased by a factor of 0.014 for every 1 km increase in geographical distance. Although a significant positive association between genetic and geographical distance existed in Zambia, the slope of the regression line (0.0006) and the coefficient of determination ($R^2 = 0.01$) (Table 4.7) were quite low for this relationship to be of biological significance; i.e., the percentage of the variation in genetic distance explained by geographical distance is very low and one would have to sample sites separated by a long distance before any change in genetic distance between isolates could be detected.

4.3.1.5 Genetic analysis of *Ba. bovis* populations

The principal MLGs representing stocks from the populations of Eastern and Central Zambia and Izmir and Aydın in Turkey were analysed using standard techniques (see materials and methods) to measure heterozygosity, linkage equilibrium and population differentiation and the results are shown in Table 4.5. Since *Ba. bovis* is haploid and heterozygosity can not be observed directly, the estimated heterozygosity (H_e) was calculated from the data set. The estimated heterozygosity from each region was generally high, ranging from 0.73 in Izmir, Turkey to 0.83 in Eastern Zambia (Table 4.5).

In order to test whether the parasite populations from the two countries are comprised of a single randomly mating population with a high degree of genetic exchange, the level of linkage equilibrium between pairs of loci was measured using the standard index of association (I^S_A). A value close to zero or negative indicates a randomly mating (panmictic) population while values significantly greater than zero are obtained for non-panmictic populations. To test the null hypothesis of panmixia, the variance of the pair-

Table 4.7 Correlation between genetic distance and geographical distance (Mantel test)

The Mantel test was used to determine whether a correlation existed between genetic and geographical distance in *Ba. bovis* populations in Zambia and Turkey. Significant positive correlations were observed in both countries.

Table 4.7 Correlation between genetic distance and geographical distance (Mantel test)

	Slope	R^2	*p value*
Within Zambia	0.0006	0.01	0.009
Within Turkey	0.014	0.218	0.010

R^2 = coefficient of determination (i.e. a measure of the proportion of the variance in genetic distance explained by geographical distance)

wise differences (V_D) and L, the 95 % confidence interval for V_D relative to the null hypothesis, are calculated. These parameters can be compared to determine if there is linkage disequilibrium (LD). When V_D is less than L, the population is found to be in linkage equilibrium (LE) and the null hypothesis of panmixia is accepted. When V_D is greater than L, the population is non-panmictic and a degree of LD exists. When the Zambian and Turkish populations were analysed as a single population, an I^S_A value significantly greater than zero was obtained and V_D was greater than L, indicating that these populations were in LD (Table 4.5). The observed LD in the combined populations could have been due to the fact that the populations are geographically sub-structured. To test this hypothesis, the I^S_A was re-calculated for each population separately. A negative I^S_A value was obtained for the Zambian population while that of the Turkish population remained in LD. This indicated that there was frequent genetic exchange in the Zambian *Ba. bovis* population. The observed LD in the Turkish population could be explained by three non-mutually exclusive hypotheses: 1) the population is further sub-structured into genetically distinct populations; 2) the population has an epidemic structure, where asexual expansion of certain genotypes masks underlying random mating and 3) genetic exchange could be occurring infrequently. There were no duplicate genotypes in the Turkish population and this argued against the existence of an epidemic population structure. When the I^S_A was recalculated for each of the two Turkish sub-populations shown in the PCA diagram (Figure 4.7), a value close to zero was obtained for each population (Table 4.5) indicating LE. In the case of the Dalama and Tire sub-population, there is clear evidence for genetic exchange as the value for the I^S_A is very low and L is greater than V_D, while for Haci Ali Basi and Yazidere sub-population, the value is low and L is just greater than V_D giving a low probability that the data fit the null hypothesis of panmixia. However, the conclusions about these two sub-populations need to be treated with caution as the sample size in each case is very low.

4.3.1.6 Multiplicity of infection in individual cattle

Every isolate genotyped in this study represented a mixed infection, with several alleles identified at one or more loci. The mean number of alleles for the eight loci was calculated for each isolate, including those where some loci could not amplify, to provide an index value that represented the multiplicity of infection within each isolate. A summary of the multiplicity of infection with respect to the country of isolation is presented in Table 4.8. The multiplicity of infection in Zambia ranged from 2.25 in Central province to 2.92 in Eastern Province with a mean of 2.67 for the whole country. In Turkey, the multiplicity of infection ranged from 1.84 in Aydın region to 2.23 in Izmir with a whole country mean of

Table 4.8 Multiplicity of infection in Zambian and Turkish *Ba. bovis* populations

The multiplicity of infection was estimated for each isolate by calculating the mean number of alleles present at each of the eight loci, providing an index of the number of genotypes present in each sample. A mean value for this measurement was calculated for the Zambian and Turkish populations and for several sub-populations within each country. Additionally, the standard deviation (SD) and the minimum and maximum values for this index of multiplicity were calculated for each group. *The independent sample t-test was used to compare the multiplicity of infection between the two countries and it was significantly higher in the Zambian population than in the Turkish population ($p < 0.001$).

Table 4.8 Multiplicity of infection in Zambian and Turkish *Ba. bovis* populations

Country/Region	n	Multiplicity of infection			
		Mean	SD	Min	Max
Zambia	**89**	**2.67***	**0.67**	**1.50**	**4.75**
Eastern Province	69	2.92	0.68	1.50	4.75
Lusaka Province	18	2.84	0.58	2.13	4.13
Central Province	2	2.25	0.18	2.13	2.38
Turkey	**41**	**2.19***	**0.46**	**1.13**	**3.25**
Aydın region	30	1.84	0.42	1.13	2.43
Izmir Region	11	2.23	0.46	1.75	3.25

n = number of samples, SD = standard deviation

2.04. The multiplicity of infection was significantly higher in the Zambian isolates than the Turkish isolates ($p < 0.001$).

The variation in multiplicity of infection between the countries may reflect differences in parasite epidemiology such as transmission intensity. This may in turn be related to variation in the level of tick infestation on cattle and / or variation in the rate of *Ba. bovis* infections in ticks, and this is pertinent given that the vectors of the parasite in the two countries are different. Information on *Ba. bovis* infection rates in ticks and the infestation rates of *Boophilus* ticks on cattle in each country was not available in this study. Therefore, it was impossible to establish the extent to which these two factors could influence the multiplicity of infection in individual animals. Host related factors may also be responsible for explaining some of the variation in multiplicity of infection observed between and within countries. On this basis, the relationship between age (in months), sex, breed type, province, tick burden, farming system and PCV, and the multiplicity of infection were investigated for the Zambian isolates using either the one-way ANOVA or the independent sample t-test. For PCV, a cut-off point of 25 % was used, where values less than or equal to 25 % were considered to be sub-normal and those above 25 % were considered to be within the normal range. For the Turkish isolates, the host factors investigated were age (in months), sex, region and breed. Summaries of the multiplicity of infection in relation to the host factors for the Zambian and Turkish isolates are shown in Table 4.9 and Table 4.10, respectively. For the Zambian isolates the multiplicity of infection ranged from 2.42 in 1 to 12 month olds to 3.21 in cattle over 48 months old. Age was found to be significantly associated with multiplicity of infection ($p < 0.001$). The other factors, (province, tick burden, sex, farming system, breed type and PCV) were not found to be significantly associated with multiplicity of infection (Table 4.9). For the Turkish isolates (Table 4.10), the multiplicity of infection ranged from 1.83 in 1 to 12 month olds to 2.36 in cattle older than 48 months. Similar to Zambia, age was found to be significantly associated with multiplicity of infection ($p < 0.001$). In Aydın region, the multiplicity of infection was 1.84 while in Izmir it was 2.23. Izmir had a significantly higher multiplicity of infection than Aydın region ($p = 0.015$). Breed type and sex were not found to be significantly associated with multiplicity of infection.

A multivariant linear regression model was used to determine the magnitude and direction of the relationship between the host factors and multiplicity of infection (i.e. whether they could be used to predict multiplicity of infection in individual animals). The results of the regression analysis are shown in Table 4.11 and Table 4.12 for the Zambian and Turkish

Table 4.9 Multiplicity of infection according to host attributes in the Zambian isolates

The table shows a summary of the multiplicity of infection in relation to host factors in the Zambian *Ba. bovis* population. For PCV, a cut off point of 25 % was used, where a figure below 25 % was considered to be sub-normal. Either the one way ANOVA or t-test was used to determine which of these factors was significantly associated with multiplicity of infection in individual animals. Only age was found to be significantly associated with multiplicity of infection ($p < 0.001$).

Table 4.9 Multiplicity of infection according to host attributes in the Zambian isolates

Host attribute	Category	n	Multiplicity of infection		
			Mean	SD	*p value*
Age (months)	1 to 12	18	2.43	0.35	< 0.001
	3 to 24	6	2.63	0.76	
	24 to 48	31	2.86	0.66	
	>48	34	3.21	0.64	
Province	Eastern	69	2.92	0.68	0.641
	Lusaka	18	2.84	0.64	
Tick Burden	No tick	16	2.75	0.69	0.167
	Few	39	2.77	0.57	
	Moderate	23	3.08	0.67	
	Abundant	11	3.11	0.88	
Sex	Male	43	2.91	0.72	0.776
	Female	46	2.87	0.62	
Farming System	Traditional	73	2.92	0.67	0.469
	Commercial	16	2.78	0.60	
Breed Type	Commercial	14	2.73	0.61	0.334
	Local	75	2.92	0.67	
PCV	≤ 25 %	19	2.90	0.64	0.927
	>25 %	70	2.89	0.68	

n = number of samples, SD = standard deviation

Table 4.10 Multiplicity of infection according to host attributes in the Turkish isolates

The table shows a summary of the multiplicity of infection in relation to the host factors in the Turkish Ba. bovis population. Either the one way ANOVA or t-test was used to determine which+- of these factors were significantly associated with multiplicity of infection in individual animals. Age ($p < 0.001$) and region ($p = 0.015$) were found to be significantly associated with multiplicity of infection.

Table 4.10 Multiplicity of infection according to host attributes in the Turkish isolates

Host attribute	category	n	Multiplicity of infection		
			Mean	SD	*p value*
Age (months)	1 to 12	4	1.83	0.29	< 0.001
	13 to 24	17	1.67	0.29	
	25 to 48	9	1.96	0.49	
	> 48	11	2.36	0.46	
Region	Aydın	30	1.84	0.42	0.015
	Izmir	11	2.23	0.46	
Sex	Male	3	1.88	0.18	0.853
	Female	38	1.94	0.47	
Breed	Local	4	2.13	0.45	0.466
	Commercial	37	1.92	0.47	

n = number of samples, SD = standard deviation

Table 4.11 Predictors of multiplicity of infection in the Zambian *Ba. bovis* isolates

Multivariant linear regression was used to determine host factors that were significant predictors of the multiplicity of infection in cattle in Zambia. Only age was found to be a significant predictor of the multiplicity of infection ($p < 0.001$), i.e. the multiplicity of infection increased by a factor of 0.01 for every one month increase in age.

Table 4.11 Predictors of multiplicity of infection in the Zambian *Ba. bovis* isolates

Predictor	Coefficient	SE	*p value*	95 % CI of coefficient	
				Lower	Upper
Constant	2.440	0.528	< 0.001	1.389	3.491
Age (months)	0.010	0.002	0.000	0.005	0.014

SE = standard error of the coefficient

Table 4.12 Predictors of multiplicity of infection in the Turkish *Ba. bovis* isolates

Multivariant linear regression was used to determine host factors that were significant predictors of the multiplicity of infection in cattle in Turkey. Only age was found to be a significant predictor of the multiplicity of infection. ($p < 0.001$), i.e. the multiplicity of infection increased by 0.01 for every one month increase in age.

Table 4.12 Predictors of multiplicity of infection in the Turkish *Ba. bovis* isolates

Variable	Coefficient	SE	*p value*	95 % C.I. for coefficient	
				Lower	Upper
(Constant)	1.90	0.270	0.000	1.35	2.456
Age (Months)	0.01	0.002	0.000	0.01	0.020

SE = standard error of the coefficient, C.I. = confidence interval

isolates, respectively. In both countries, only age was found to be a significant predictor of multiplicity of infection ($p < 0.001$). Although the region, in Turkey, was significantly associated with multiplicity of infection, it was not found to be a significant predictor. The multiplicity of infection increased by a factor of 0.01 for every one month increase in age of cattle in the two countries (Tables 4.11 and 4.12). There were no interactions between variables in either population. The effect of age on multiplicity of infection partially explains the observed difference in multiplicity of infection between the two countries. The mean age (48 months) of Zambian cattle sampled in this study was higher than that of the Turkish cattle (42 months).

4.3.2 Population genetic analysis of *Ba. bigemina*

4.3.2.1 Identification and evaluation of markers

The genome sequence of *Ba. bigemina* is currently being assembled and is estimated to be 10 Mb in size, distributed across four chromosomes. 627 contigs were obtained from the Sanger Institute and screened using the tandem repeat finder program to identify repetitive sequences with the same parameters as those described for *Ba. bovis*. In contrast to *Ba. bovis,* only 194 repetitive sequences were identified with very little overlap evident among the repeat motifs. An *in silico* filtration process similar to that used for *Ba. bovis* was employed to identify a manageable subset of loci that could be tested for polymorphism, specificity and whether they amplified a single locus. The top 42 loci comprised 11 mini- and 31 micro-satellites. Primers were designed to regions flanking the repeat regions and these loci were screened against a panel of DNA from four stocks of *Ba. bigemina*. A similar criteria to that used for *Ba. bovis* was employed in assessing the suitability of each loci. The only difference was that, to be selected, a marker had to amplify from all four DNA stocks. Six of the primer pairs failed to amplify from any of the four test DNA stocks (Table 4.13). The majority of the markers were either not polymorphic, amplified non-*Ba. bigemina* DNA stocks, did not represent a single locus or failed to amplify from all the stocks. From the original forty two loci, three mini- and six micro-satellite markers (Bbg2, Bbg7, Bbg9, Bbg13, Bbg14, Bbg18, Bbg23, Bbg34 and Bbg42; Table 4.2) met the stated criteria, and these were used for genotyping Zambian *Ba. bigemina* isolates. Figure 4.8 represents an image of gel electrophoresis analysis for Bbg9, one of the markers that met the set criteria. This marker was able to amplify all the four *Ba. bigemina* DNA stocks (Lanes B to E) and it did not amplify from non - *Ba. bigemina* DNA (Lanes F to K). Amplicons of differing sizes can be seen on the figure, indicating that the marker was polymorphic. Bbg2 was located on contig 413 (481 kb in size), Bbg7 was on contig 235

Table 4.13 Criteria employed in selecting *Ba. bigemina* markers

This table shows the criteria used to select the markers for genotyping *Ba. bigemina* isolates from Zambia. For a marker to be considered suitable, it had to amplify all four *Ba. bigemina* reference stocks, produce single bands after agarose gel electrophoresis of PCR product, be species specific and polymorphic. The nine markers highlighted in green satisfied these criteria.

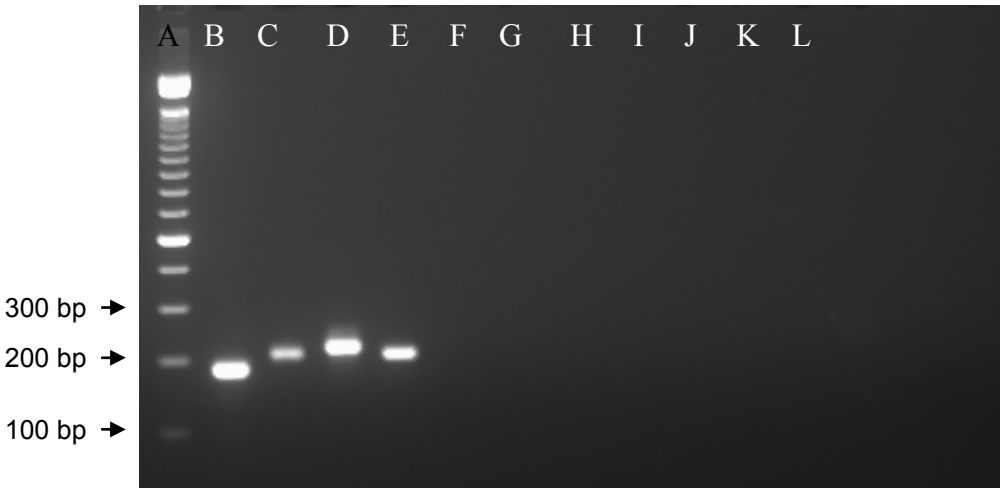
Table 4.13 Criteria employed in selecting *Ba. bigemina* markers

Marker	Motif length (bp)	Repeat copy number	Genome predicted amplicon size (bp)	Proportion of stocks *Ba. bigemina* amplified	Single bands / species-specific	Polymorphic
Bbg1	10	3.5	237	0/4	-	-
Bbg2	9	4	345	4/4	yes/yes	yes
Bbg3	3	11.3	350	0/4	-	-
Bbg4	3	11.3	343	4/4	yes/no	no
Bbg5	3	11.4	345	4/4	yes/no	no
Bbg6	3	11.7	353	3/4	yes/yes	Yes
Bbg7	3	11.7	343	4/4	yes/yes	Yes
Bbg8	3	14.7	312	4/4	yes/yes	no
Bbg9	3	47	191	4/4	yes/yes	yes
Bbg10	3	55	368	2/4	yes/yes	no
Bbg11	3	69	23	2/4	no/yes	no
Bbg12	8	27.5	325	0/4	-	-
Bbg13	6	25.8	481	4/4	yes/yes	yes
Bbg14	3	20.7	434	4/4	yes/yes	yes
Bbg15	3	19.7	247	3/4	yes/no	yes
Bbg16	3	14.7	266	4/4	yes/yes	yes
Bbg17	3	14.7	270	1/4	yes/yes	-
Bbg18	6	12.7	274	4/4	yes/yes	Yes
Bbg19	3	10.7	429	1/4	yes/yes	-
Bbg20	6	10.3	274	1/4	yes/yes	-
Bbg21	3	10.3	287	4/4	no/no	yes
Bbg22	3	10.3	227	4/4	no/no	yes
Bbg23	9	9.2	477	4/4	yes/yes	yes
Bbg24	6	6.5	367	4/4	no/no	no
Bbg25	5	6.2	305	4/4	no/no	no
Bbg26	5	5.2	214	4/4	no/no	no
Bbg27	9	4.2	363	0/4	-	-
Bbg28	10	4.1	257	4/4	no/no	no
Bbg29	9	3.7	249	2/4	yes/no	no
Bbg30	9	3.3	287	3/4	yes/yes	no
Bbg31	9	3.1	207	0/4	-	-
Bbg32	9	3.1	347	4/4	yes/no	no
Bbg34	3	23	296	4/4	yes	yes
Bbg35	3	14.7	202	2/4	n/yes	yes
Bbg36	6	9.5	342	3/4	yes/no	yes
Bbg37	3	8.7	272	4/4	yes/no	no
Bbg38	4	8.3	238	2/4	yes/yes	no
Bbg39	6	12.7	342	3/4	yes/yes	4
Bbg40	6	5.3	330	4/4	yes/yes	no
Bbg41	9	9.2	335	4/4	no/yes	no
Bbg42	9	16.9	395	4/4	yes/yes	Yes
Bbg43	2	20.5	302	0/4	-	-

Figure 4.8 Example of agarose gel electrophoresis for marker Bbg9

This image represents PCR products generated using the Bbg9 primer set on the panel of DNA samples used to screen all of the *Ba. bigemina* markers. An aliquot of PCR product from each reaction was loaded onto a 2 % agarose gel and separated by electrophoresis. Lane A contains a 100 bp ladder used as a reference to determine the size of the amplicon in each lane; Lanes B to E contains the *Ba. bigemina* stocks used in screening the markers. The *Ba. bigemina* in lane B is the Australian genome stock and it gave the expected fragment size of 191 bp. Lanes F to K contain non-*Ba. bigemina* DNA and L was the negative control. This marker was polymorphic, gave single bands and did not amplify non-*Ba. bigemina* DNA.

Figure 4.8 Example of agarose gel electrophoresis for marker Bbg9



Lane A, 100 bp ladder; B, *Ba. bigemina* Australia; C, *Ba. bigemina* Zaria; D, *Ba. bigemina* Muguga; E, *Ba. bigemina* Mexico; F, *Ba. bovis* Mexico; G, *Ba. bovis* Lismore; H, bovine DNA; I, *Ba. divergens*; J, *T. taurotragi*; K, *T. parva* marikabuni; L, negative control.

(143 kb), Bbg9 was on contig 28 (12 kb), Bbg13 on contig 27 (17 kb), Bbg18 was on contig 509 (146 kb), Bbg23 was on contig 353 (88 kb), Bbg34 was on contig 342 (48 kb) and Bbg14 and Bbg42 were on contig 341 (319 kb) and were separated by distance of more than 80 Kb.

Capillary electrophoresis was utilised to analyse amplicons from the Zambian field isolates. A Rox-labeled GS500® size standard was used to determine the amplicon sizes. An example of an electrophoretogram generated using Peak Scanner ® software is shown in Figure 4.9 and represents the products generated from an isolate amplified using Bbg7 primer set. Three alleles of 214 bp, 242 bp and 260 bp (blue peaks) are identified, with 214 bp being the predominant one by virtue of its greater area under the peak. The size standard is represented by the orange peaks. All selected markers showed polymorphism among isolates from the two regions of Zambia as well as isolates from within the same region.

4.3.2.2 Allelic diversity of the markers

The nine selected markers were used to amplify *Ba. bigemina* field isolates from Lusaka and Eastern provinces of Zambia using semi-nested PCR assays with amplified products separated using capillary electrophoresis. The characteristics of each marker and the primers used in the amplification are detailed in Table 4.2. All the markers were found to be polymorphic. The number of alleles identified for each marker in a single sample and the two populations from Eastern and Lusaka Provinces are shown in Table 4.14. The maximum number of alleles identified by each marker in one single sample ranged from four for Bbg18 to eight for Bbg42. The mean number of alleles identified at each locus in a single sample ranged from 1.58 for Bbg18 to 2.73 for Bbg42. The number of alleles identified by each marker in the Eastern province population ranged from four for Bbg18 and Bbg23 to 17 for Bbg42, while in the Lusaka province population it ranged from four for Bbg23 to 15 for Bbg42. The number of alleles identified by each marker in the whole Zambian *Ba. bigemina* population ranged from 5 for Bbg23 and Bbg2 to 24 for Bbg42. These figures were lower than those obtained for the Ba. bovis markers using isolates obtained from the same areas. Only six samples failed to amplify by the full range of markers. Markers Bbg9, Bbg13, Bbg18 and Bbg42 each failed to amplify one of the samples while, Bbg14 failed to amplify two samples.

The predominant allele (determined as described for *Ba. bovis*) was used to create a MLG for each sample. Each sample had a unique MLG and the closest identity between any pair of samples was eight out of nine allelic markers. The allelic frequencies of *Ba. bigemina*

Figure 4.9 Example of Peak Scanner™ analysis of *Ba. bigemina* isolate

This image represents a trace, or electrophoretogram, generated by Peak Scanner™ analysis and depicts fluorescently-labelled PCR products. This particular trace was produced using marker Bbg7 primer set. The horizontal axis represents fragment sizes while the vertical axis on the left hand side represents units of fluorescent intensity. Blue peaks represent the labelled PCR product, while orange peaks indicate the GS500^{TM} size standards. The sizes of the alleles represented by the three blue peaks are interpolated from their position with respect to the size standards. The predominant allele, 214 bp is defined as the peak with the greatest area under the curve.

Figure 4.9 Example of Peak Scanner™ analysis of *Ba. bigemina* isolate

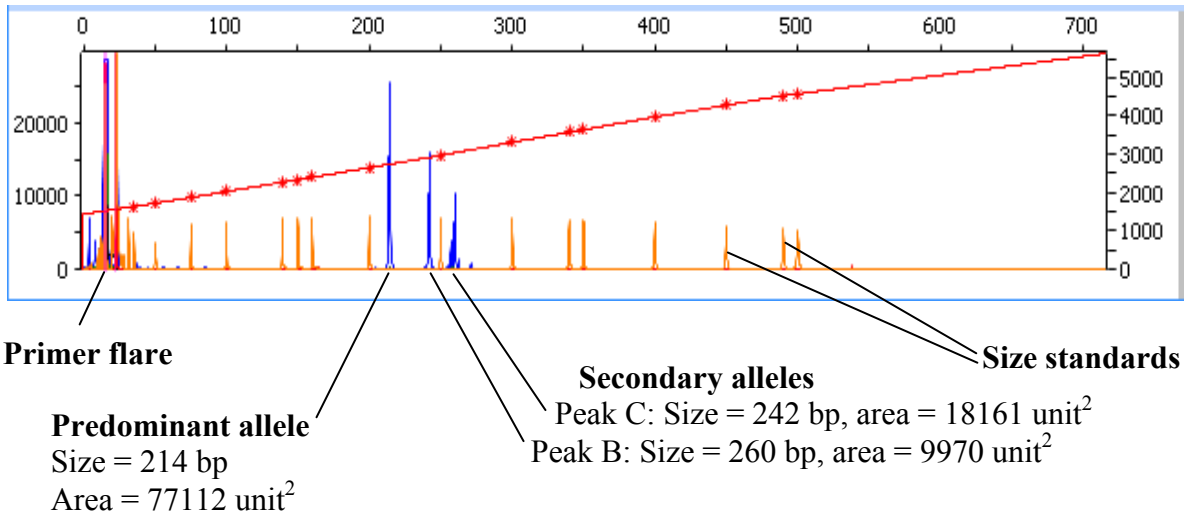


Table 4.14 Allelic variation in Lusaka and Eastern Province *Ba. bigemina* isolates

All samples were genotyped using the panel of nine markers. The minimum and maximum number of alleles detected at each locus within a sample was determined across all nine markers for each sample that amplified. The number of alleles represented in each population was calculated, taking into account the most abundant and all minor alleles present in each sample from that country. Gene diversity was calculated for each marker and is equivalent to estimated heterozygosity.

Table 4.14 Allelic variation in Lusaka and Eastern Province *Ba. bigemina* isolates

		n	Bbg2	Bbg7	Bbg9	Bbg13	Bbg14	Bbg18	Bbg23	Bbg34	Bbg42
Alleles within each sample	Minimum		1	1	1	1	1	1	1	1	1
	Maximum		5	5	6	5	6	4	5	7	8
	Mean		1.69	1.89	1.90	1.79	1.86	1.58	2.03	2.64	2.73
	No amplif.		0	0	1	1	2	1	0	0	1
Alleles within Population	Lusaka	26	7	13	9	6	12	7	4	9	15
	Eastern	32	10	11	11	9	13	4	4	13	17
	Overall	58	13	17	15	10	20	8	5	16	24
Gene diversity	Lusaka	26	0.858	0.877	0.822	0.880	0.899	0.345	0.726	0.905	0.923
	Eastern	32	0.796	0.903	0.845	0.770	0.950	0.657	0.714	0.885	0.948

n = number of samples

isolates based on MLGs, are shown in Figure 4.10. A number of markers had many private alleles, some of which were of high frequency. For example, of the 20 alleles identified by Bbg14, 15 were private alleles. Of these private alleles, seven were from Lusaka and eight from Eastern Provinces. Seven of the markers (Bbg2, Bbg7, Bbg9, Bbg13, Bbg18, Bbg14 and Bbg42) had at least one private allele with a frequency greater than 0.15 suggesting sub-structuring. Examination of the allele frequencies within each population showed that three of the markers (Bbg2, Bbg13 and Bbg23) had at least two alleles with a frequency greater than 0.15. Three markers (Bbg7, Bbg9, Bbg42) had such a feature only in the Eastern province population, while two markers had this in only Lusaka Province population (Figure 4.10).

4.3.2.3 Genetic analysis of *Ba. bigemina* populations

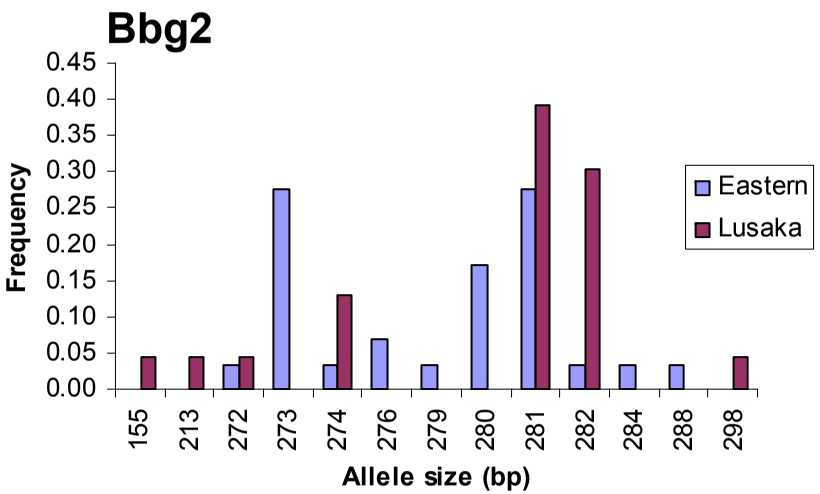
Standard techniques (see Materials and Methods section) were used to measure the estimated heterozygozities, LD and genetic differentiation and the values obtained are shown in Table 4.15. In order to test whether the Zambian *Ba. bigemina* population comprised a single randomly mating population with a high level of genetic exchange, the level of linkage equilibrium between pairs of loci was measured using the standard index of association (I^S_A). The same criterion as described for *Ba. bovis* was used in determining whether the population was panmictic or non–panmictic. I^S_A values significantly greater than zero were obtained whether isolates from Eastern and Lusaka provinces were analysed as a single population or separately (Table 4.15). When isolates from all the provinces were treated as one, an I^S_A value of 0.064, with a value of L significantly less than V_D ($p < 0.001$) was obtained. For the Eastern Province population, an I^S_A value of 0.055, with a value of L significantly less than V_D ($p < 0.001$) was obtained. The Lusaka Province Population also had a value of L that was significantly less than V_D ($p < 0.001$) and an I^S_A value of 0.078 (Table 4.15). Therefore, it is unlikely that geographical sub-structuring could account for the observed LD when the two populations were combined. This meant that either the populations were genetically sub-structured or had an epidemic population structure. However, the observation that there were no duplicate MLGs in the populations argues against the existence of an epidemic structure.

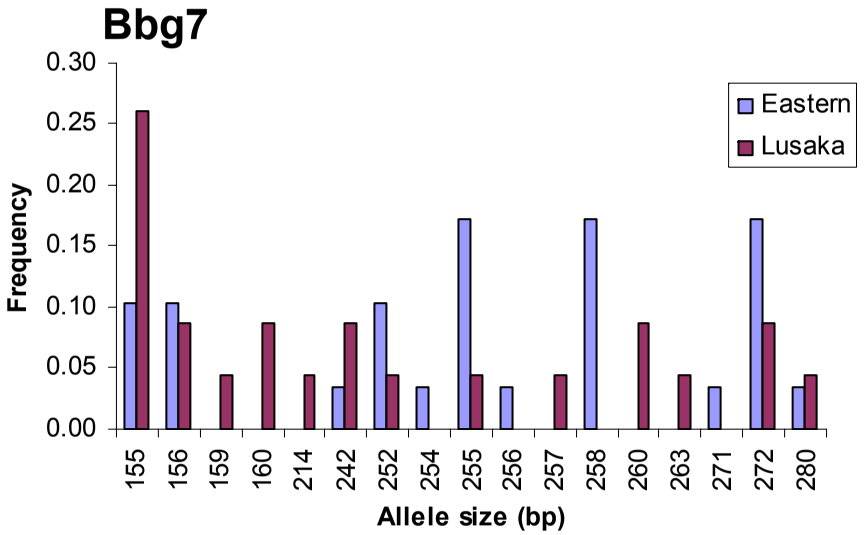
The computer program, STRUCTURE was used to test the hypothesis that genetic sub-structuring was the cause of the LD observed in the *Ba. Bigemina* population. The plot of L(K) vs K is shown in Figure 4.11 predicting the existence of five sub-populations. Figure 4.12 is an output from STRUCTURE, showing the proportion of genotypes in each predicted sub-population (A, B, C, D and E). The clusters are sorted according to colour

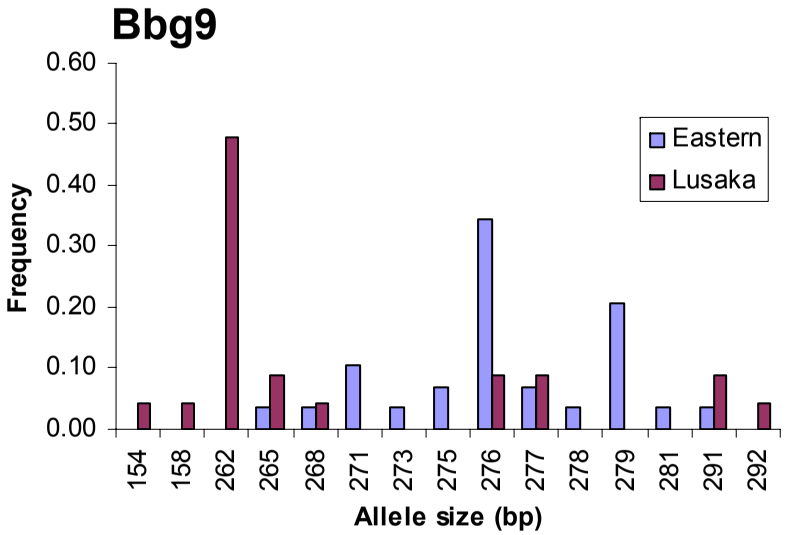
Figure 4.10 Allele frequencies of Zambian *Ba. bigemina* isolates

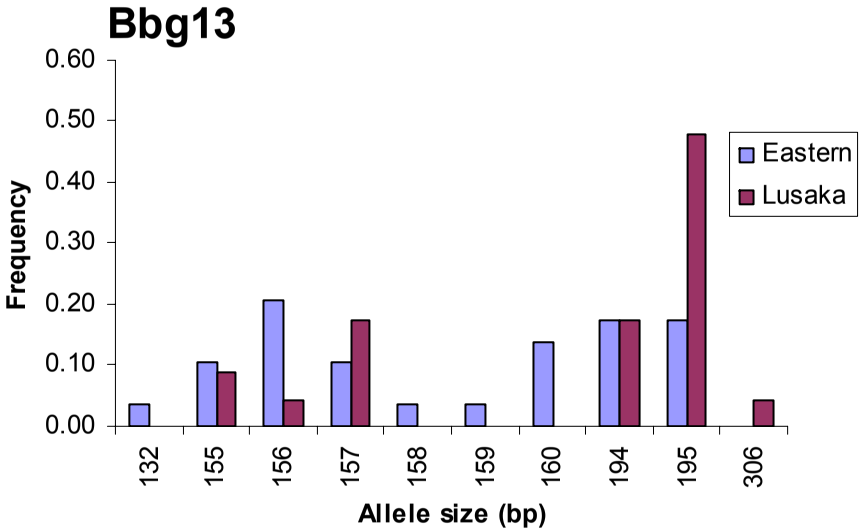
The frequency of each allele in the Eastern and Lusaka province population was determined as a proportion of the total for each marker using the predominant allele in each sample. These histograms were directly generated from the multi-locus genotype data.

Figure 4.10 Allele frequencies of Zambian *Ba. bigemina* isolates









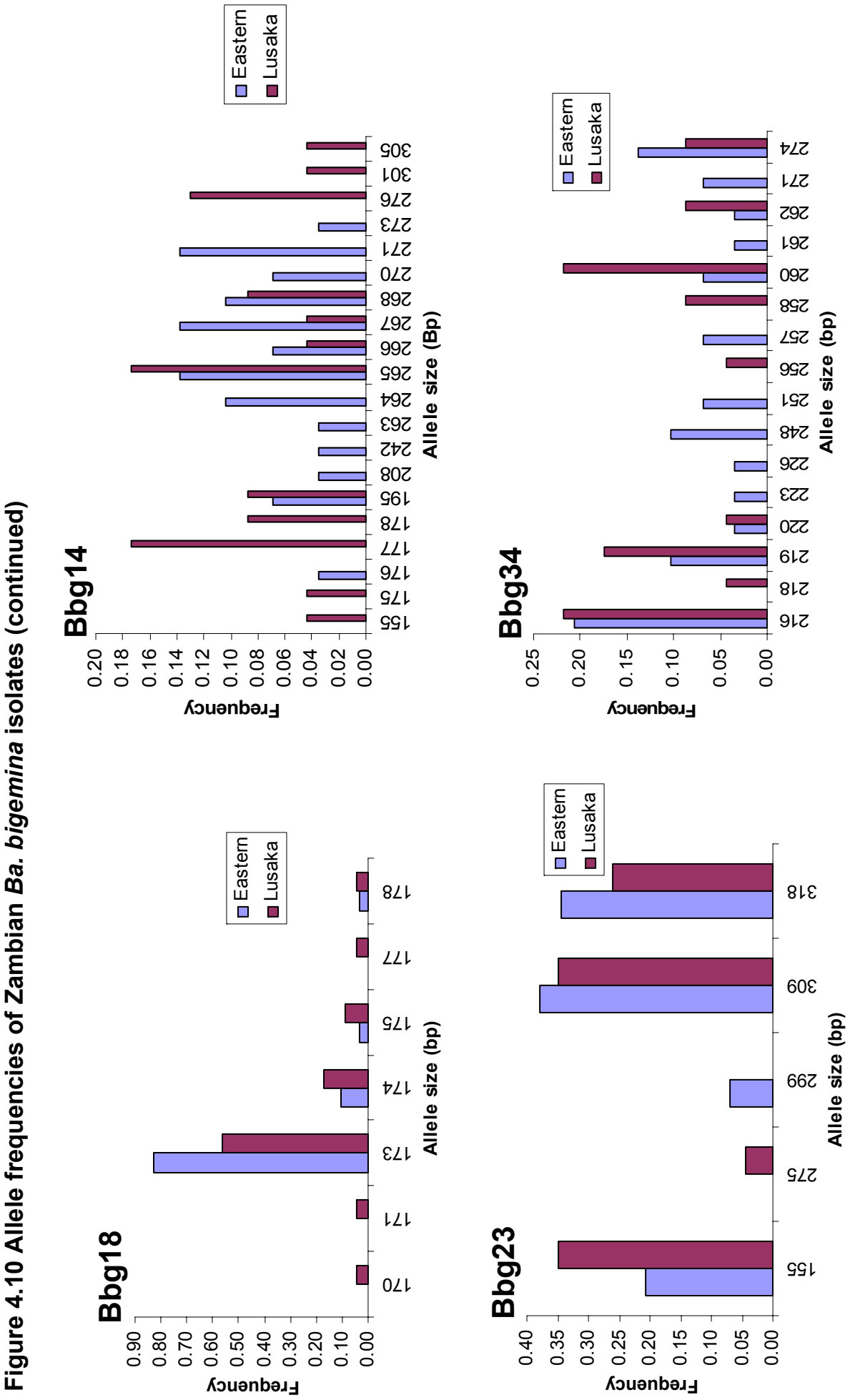


Figure 4.10 Allele frequencies of Zambian *Ba. bigemina* isolates (continued)

Figure 4.10 Allele frequencies of Zambian *Ba. bigemina* isolates (continued)

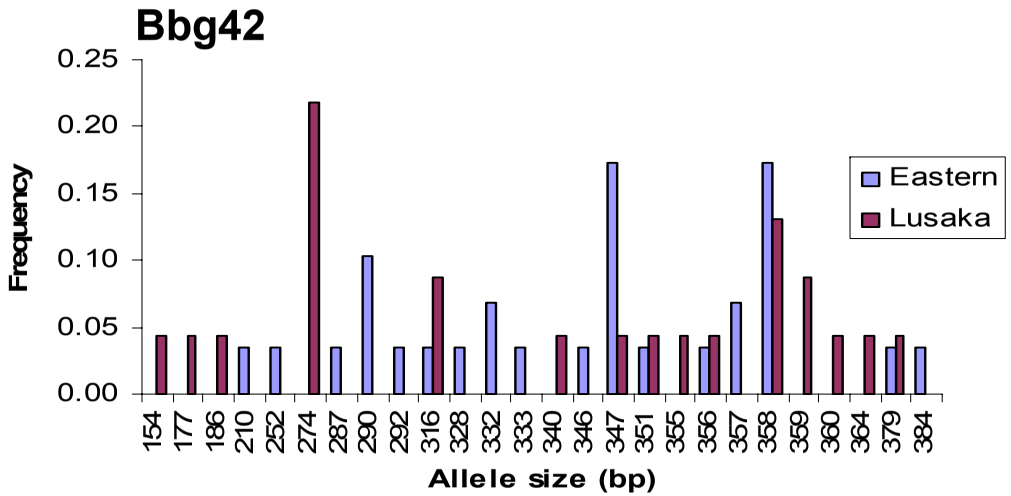


Table 4.15 Population genetic analysis of *Ba. bigemina*

Standard population tests were conducted on parasite samples representing isolates between and from within the two provinces of Zambia. The 'mean number of genotypes per sample' was calculated as the mean value for the number of alleles detected at each of the nine loci. Structured combinations of populations were pooled to test for linkage disequilibrium. Variance of mismatch values (V_D) were compared to values of L (the upper confidence limits of Monte Carlo simulations), and where $V_D > L$, linkage disequilibrium (LD) was indicated. When $L > V_D$ the null hypothesis of linkage equilibrium (LE) was not disproved. All the populations were in LD. F_{ST} values were used to measure genetic differentiation between the provinces.

Table 4.15 Population genetic analysis of *Ba. bigemina*

Population compared	n	H_e	Mean number of genotypes per sample	I^S_A	V_D	L	*p value*	Linkage	F_{ST}	*p value*
Across provinces	52	0.831	15.22	0.064	1.638	1.182	<0.001	LD	0.047	0.010
Within province										
Eastern	29	0.824	11.22	0.055	1.996	1.487	<0.001	LD	-	-
Lusaka	23	0.816	9.11	0.078	1.778	1.402	<0.001	LD		

n = number of samples, H_e = estimated heterozygosity, I^S_A= standard index of association, VD = mismatch variance (linkage analysis), L = upper 95 % confidence limits of Monte Carlo simulation (linkage analysis), LD = linkage disequilibrium

Figure 4.11 Plot of mean likelihood L(K) for each value of K

The value of LnP(D) was calculated for each step of the Markov chain Monte Carlo (MCMC). The average of this value for the 10 runs performed for each value of K, was computed and half the variance was subtracted from the mean to obtain a value called L(K). The 'true' number of sub-populations (K) was identified as the value of K that gave the highest L (K), which is 5 in this case.

Figure 4.12 Estimated population sub-structure (n = 58)

The clusters are sorted according to colours and the size of each block of colour represents the proportion of genotypes derived from each of the five clusters (Q) as detected by STRUCTURE. Red = A, Green = B, Blue = C, Yellow = D and Purple = E.

Figure 4.11 Plot of mean likelihood L(K) for each value of K

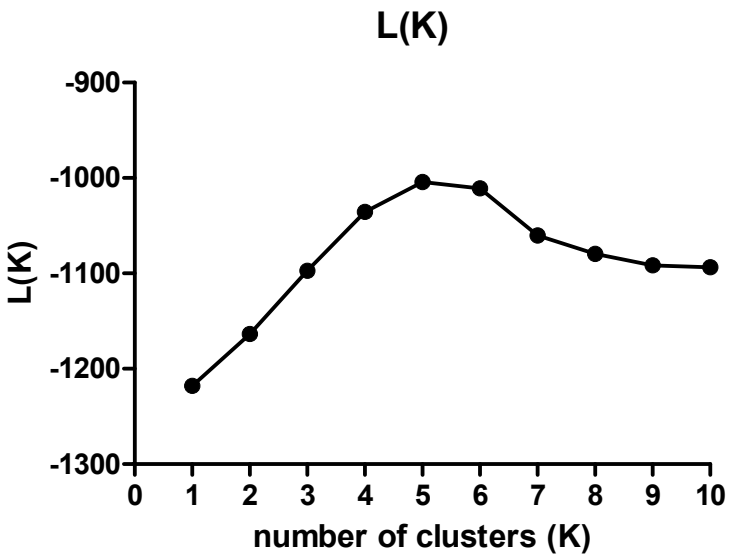


Figure 4.12 Estimated population sub-structure (n = 58)

and the size of each block of colour represents the proportion of genotypes in that cluster. The compositions of the five sub-populations are shown in Table 4.16. The sub-populations corresponded to 14 members in group A (1 from Eastern and 14 from Lusaka Province), 12 in group B (6 from Eastern and 6 from Lusaka Province), 7 in group C (3 from Eastern and 4 from Lusaka Province), 11 in group D (8 from Eastern and 3 from Lusaka Province) and 13 in group E (all from Eastern Province). Considerable genetic differentiation was observed between all the sub-populations, as shown by the F_{ST} values in Table 4.17. Unfortunately, the sample size of each sub-population was not large enough to determine whether any were in LE.

4.3.2.4 Diversity and population sub-structuring

Genetic differentiation between the two provinces was assessed using Wright's fixation index and moderate genetic differentiation (F_{ST} = 0.05) was observed between isolates from the two provinces (Table 4.15). The ability of each marker to differentiate between Eastern and Lusaka Province isolates was assessed and the F_{ST} values for each marker are shown in Table 4.18, together their estimated heterozygosities. All the markers displayed F_{ST} values significantly greater than zero ($p \leq 0.05$) except for Bbg42 and Bbg23. Bbg9 had the highest F_{ST} value of 0.114, which indicated a high level of genetic differentiation. High genetic diversity was observed in the two provinces with estimated heterozygosities of 0.816 in Lusaka province and 0.824 in Eastern Province (Table 4.15). The estimated heterozygosities for the markers when the two populations were combined ranged from 0.479 for Bbg18, which was the least diverse, to 0.951 for Bbg42 which was the most diverse (Table 4.18).

The Mantel test was used to determine whether a correlation between geographical and genetic distance existed and the results are shown in Table 4.19. A significant positive correlation was identified. However, similar to the Zambian *Ba. bovis* population, the coefficient of determination (R^2 = 0.03) and the slope of the regression line (0.001) were too low for this relationship to be of any biological significance.

4.3.2.5 Similarity analysis

The PCA for *Ba. bigemina* isolates was constructed and the first two principal axes are illustrated in Figure 4.13. 26 % of the variation was explained by axis 1, while 21 % was explained by axis 2. The PCA showed two main clusters which did not correspond to the province of origin. The smaller cluster, which comprised of ten isolates, five from each province is seen on the far left side of Axis 1. The remaining isolates formed a larger sub-

Table 4.16 Composition of each sub-population in each province

The computer program STRUCTURE was used to identify the most probable number of sub-populations in the Zambian *Ba. bigemina* population. The composition of each sub-population (A, B, C, D and E) in each province is shown

Table 4.17 Pair-wise F_{ST} values for each sub-population

The level of genetic differentiation between the five sub-populations was determined using F_{ST} values. Great genetic differentiation was observed between all combinations of sub-population.

Table 4.16 Composition of each sub-population in each province

Province / sub-population	A	B	C	D	E
Eastern	1	6	3	8	13
Lusaka	14	6	4	3	0
Total	15	12	7	11	13

.

Table 4.17 Pair-wise F_{ST} values for each sub-population

	A	B	C	D	E
A	0.000				
B	0.244	0.000			
C	0.174	0.288	0.000		
D	0.197	0.277	0.118	0.000	
E	0.143	0.255	0.213	0.188	0.000

Table 4.18 *Ba. bigemina* marker indices of genetic diversity and differentiation between isolates from Eastern and Lusaka provinces

The diversity of each marker and its ability to differentiate between populations of *Ba. bigemina* in Zambia are shown in this table. The F_{ST} values ranged between -0.0026 (no genetic differentiation) for Bbg23 to 0.1136 for Bbv19 (moderate genetic differentiation). Except for Bbg23 and Bbg42, all the markers had F_{ST} values significantly greater than zero. All the markers showed great genetic diversity ranging from 0.479 for Bbg18 to 0.951 for Bbg42.

Table 4.19 Correlation between genetic distance and geographical distance (Mantel test) for *Ba. bigemina* in Zambia

The Mantel test was used to determine whether a correlation existed between genetic and geographical distance in *Ba. bigemina* populations in Zambia, a significant positive correlation was observed.

Table 4.18 *Ba. bigemina* marker indices of genetic diversity and differentiation between isolates from Eastern and Lusaka provinces

Marker	F_{ST}	*p* value	H_e
Bbg2	0.093	0.030	0.842
Bbg7	0.066	0.010	0.922
Bbg9	0.114	0.010	0.885
Bbg13	0.080	0.010	0.839
Bbg14	0.026	0.050	0.942
Bbg18	0.076	0.020	0.479
Bbg23	-0.003	0.370	0.711
Bbg34	0.031	0.050	0.907
Bbg42	0.019	0.060	0.951

Table 4.19 Correlation between genetic distance and geographical distance (Mantel test) for *Ba. bigemina* in Zambia

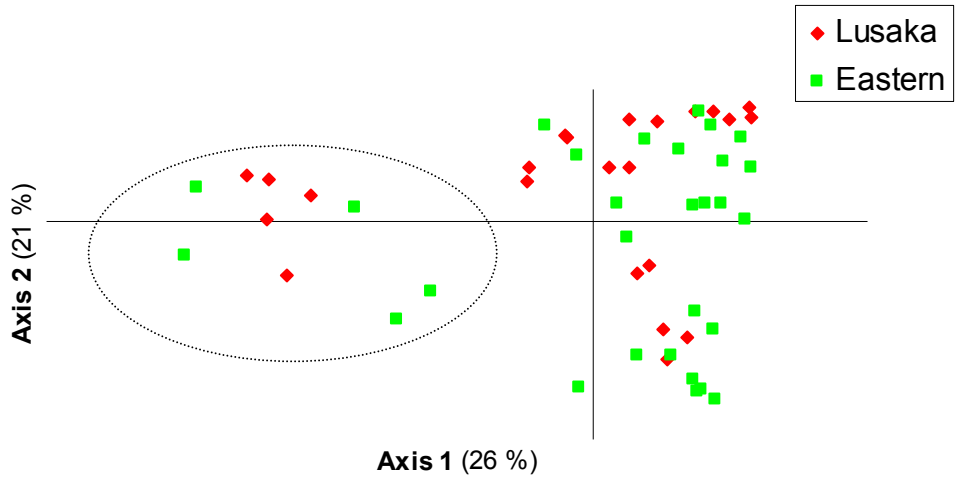
	Slope	R^2	*p* value
Within Zambia	0.001	0.030	0.010

R^2 = coefficient of determination (i.e. a measure of the proportion of the variance in genetic distance explained by geographical distance)

Figure 4.13 Principal component analysis of Zambian *Ba. bigemina* isolates

Principal component analysis was performed on the multi-locus genotype data representing the Eastern and Lusaka Provinces isolates of Zambia. The two principal axes generated by this analysis are presented, demonstrating a degree of sub-structuring of the population, although not according to provinces. The proportion of the variation in the dataset explained by each axis is indicated in parenthesis. The circle denotes the small sub-group composed of ten non-geographically distinct isolates.

Figure 4.13 Principal component analysis of Zambian *Ba. bigemina* isolates



group which, on closer examination, showed evidence of sub-structuring with a smaller sub-group below Axis 2. Another PCA (Figure 4.14) was constructed according to the sub-populations inferred by STRUCTURE and it showed good agreement with the earlier one. This further confirms that the observed LD in this parasite population was due to genetic sub-structuring and not due to asexual reproduction.

4.3.2.6 Multiplicity of infection

The one way ANOVA or the independent sample t-test was used to determine host factors that were significantly associated with the observed variation in multiplicity of infection between individual animals in Central and Eastern Zambia. A summary of the analysis are shown in Table 4.20, together with the *p* values computed for each variable. The multiplicity of infection ranged from 1.61 in 1 to 12 month old cattle to 2.23 in cattle more than 48 months old. Age was significantly associated with multiplicity of infection ($p < 0.001$). Female cattle had a significantly higher multiplicity of infection than male cattle (2.16 vs 1.86, $p = 0.010$). The other host factors (tick burden, province, farming system, breed type and PCV) were not found to be significantly associated with multiplicity of infection (Table 4.20). Multivariant linear regression was used to quantify the relationship between the host factors and the multiplicity of infection and the results are shown in Table 2.21. Only age was found to be a significant predictor of *Ba. bigemina* multiplicity of infection in cattle ($p < 0.001$). Although sex was found to be significantly associated with multiplicity of infection, it was not a significant predictor. Similar to the case for *Ba. bovis*, multiplicity of infection increased by a factor of 0.01 for every one month increase in age. There was no apparent interaction between variables.

4.4 Discussion

4.4.1 Population genetic analysis of *Ba. bovis*

4.4.1.1 Suitability of the markers

Marker based genetic studies are an invaluable way to investigate the basic biology of pathogens and the interplay between control strategies and the behaviour of the parasite at the population level. Mini- and micro-satellite markers have been extensively used in studying the epidemiology and population structures of many parasites (Anderson *et al.* 2000; Mallon *et al.* 2003b; Oura *et al.* 2005; Weir *et al.* 2007). In the present study, 1 mini- and 7 microsatellite markers were developed and successfully applied to analyse the population genetics of *Ba. bovis* in Zambia and Turkey. However, these markers were not without their limitations. For example, it is recognised that polymorphism in primer

Figure 4.14 Principal component analysis of Zambian *Ba. bigemina* isolates according the sub-populations inferred by STRUCTURE

Principal component analysis was performed on the multi-locus genotype according to the sub-populations inferred by STRUCTURE. There results shows that there was a good agreement between the two methods.

Figure 4.14 Principal component analysis of Zambian *Ba. bigemina* isolates according the sub-populations inferred by STRUCTURE

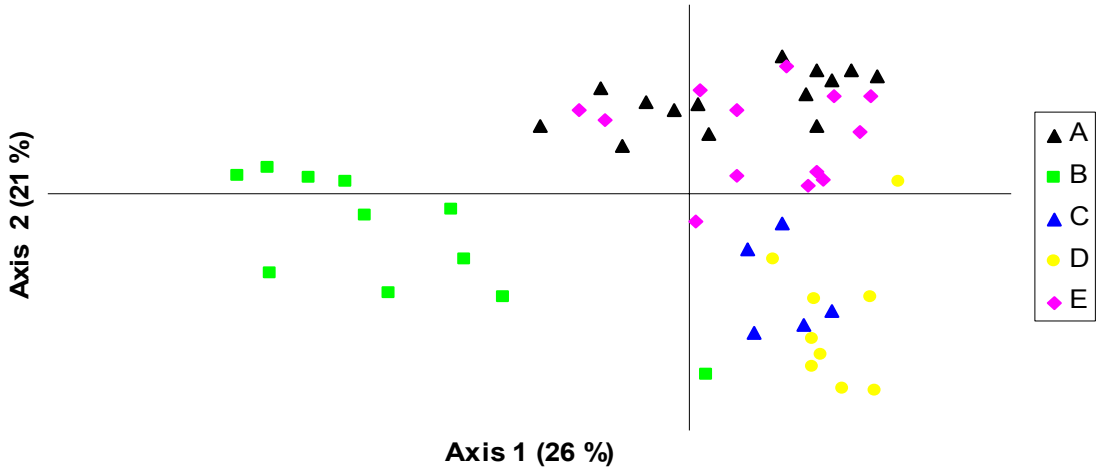


Table 4.20 Multiplicity of infection according to host attributes in the Zambian *Ba. bigemina* isolates

The table shows a summary obtained for the multiplicity of infection in relation to the host factors in the Zambian *Ba. bigemina* population. For PCV, a cut-off point of 25 % was used, where a figure below 25 % was considered to be sub-normal. Either the one way ANOVA or t-test was used to determine which of these factors were significantly associated with multiplicity of infection in individual animals. Only age was found to be significantly associated with multiplicity of infection ($p < 0.001$).

Table 4.20 Multiplicity of infection according to host attributes in the Zambian *Ba. bigemina* isolates

Host attribute	Category	n	Multiplicity of infection		*p value*
			Mean	SD	
Age (months)	1 to 12	13	1.61	0.35	0.001
	13 to 24	6	1.85	0.14	
	25 to 48	24	2.14	0.43	
	> 48	16	2.23	0.45	
Tick Burden	None	21	2.08	0.53	0.397
	Few	16	1.95	0.39	
	Moderate	15	1.91	0.40	
	Abundant	7	2.23	0.51	
Sex	Male	28	1.86	0.44	0.010
	Female	31	2.16	0.43	
Province	Lusaka	26	2.10	0.50	0.233
	Eastern	32	1.96	0.42	
Farming system	Commercial	19	2.10	0.47	0.370
	Traditional	40	1.98	0.45	
Breed	Commercial	19	2.10	0.47	0.475
	Traditional	40	1.98	0.45	
PCV (%)	≤ 25	15	1.90	0.36	0.252
	> 25	44	2.06	0.48	

n = number of samples, SD = standard deviation

Table 4.21 Predictors of multiplicity of infection in the Zambian *Ba. bigemina* isolates

The multivariant linear regression was used to determine which host factors were significant predictors of the multiplicity of infection in *Ba. bigemina* populations in Zambia. Only age ($p < 0.001$) was found to be a significant predictor of multiplicity of infection; i.e. multiplicity of infection increased by 0.01 for every one month increase in age.

Table 4.21 Predictors of multiplicity of infection in the Zambian *Ba. bigemina* isolates

Predictor	Coefficient	SE	*p value*	95 % CI of coefficient	
				Lower	upper
Constant	1.60	0.097	<0.001	1.40	1.80
Age	0.01	0.002	<0.001	0.01	0.01

SE = Standard error of the coefficient, CI = confidence interval

annealing sites may lead to failure to amplify PCR product resulting in 'null alleles'. In this study, none of the markers was able to amplify from every available sample. However, the number of samples that failed to amplify in the whole population was low (9.6 %) and this may be attributed to the rigorous screening process prior to the selection of markers. Furthermore, the method used to prepare the samples and the use of nested PCR are likely to have provided a level of sensitivity that ensured the majority of the samples could amplify by the markers.

The markers used in this study were ideal in that they were distributed across all four chromosomes and were physically unlinked. However, it is unknown whether these markers are neutral. If a particular marker were in close proximity to an antigen under selective pressure, the marker sequence would also be under selective pressure whether it was in a coding sequence or non-coding sequence, because of its proximity to a locus under selection. Therefore, although a marker may lie in a non-coding sequence, this does not necessarily indicate whether it is neutral or not.

All the markers used in this study showed high levels of genetic diversity and were able to genetically differentiate between Turkish and Zambian isolates, except for Bbv58 which had a F_{ST} value that was not significantly different from zero. Bbv58 was also the least diverse of all the markers used in this study.

4.4.1.2 Population genetic analysis

Studies on *T. parva* and *T. annulata* have revealed that most field isolates are composed of a mixture of genotypes (Oura *et al.* 2005; Weir *et al.* 2007) and this situation was similar to the findings for *Ba. bovis* and *Ba. bigemina* in this study. In the current study, no samples were found to have the same MLGs. This indicates that ticks feeding on cattle are likely to ingest a mixture of genotypes which will then recombine within the mid-gut of the vector as a result of genetic exchange. The high number of genotypes in individual samples, and in the population as whole, is likely to be an indication of high transmission intensity by the vector ticks *Bo. microplus* and *Bo. annulatus* in Zambia and Turkey, respectively, or the high number of ticks feeding on individual animals. Varying levels of genetic recombination have been reported in *Plasmodium* providing evidence for self-fertilisation in areas of low transmission intensity and increased levels of out-breeding in areas of high transmission (Anderson *et al.* 2000). The data presented in this study indicates that a high level of genetic recombination occurs in natural populations of *Ba. bovis*. Despite high levels of genetic recombination, the Zambian and the Turkish *Ba. bovis* populations are genetically distinct as shown by the PCA (Figure 4.7). However, *Ba. bovis* in Eastern and

Central Zambia appears to be a single randomly mating population and Zambian PCA (Figure 4.8) demonstrated that the isolates from Lusaka are a subset of those from Eastern province. This is consistent with the current understanding of the epidemiology of the parasite and the view that it has been spreading from Eastern parts of the country towards the west. The first cases of *Ba. bovis* infection in cattle in Central Zambia were reported in the late 1980s (Jongejan *et al.* 1986a; Jongejan *et al.* 1988) and, as indicated by the data in Chapter Three, this parasite appears to have become endemically established in this region. Cattle movement is thought to be responsible for the westward spread of both *Ba. bovis* and its vector. Although official figures may show that there is limited movement of cattle between these two regions (Anon 2007), there is significant anecdotal evidence that illegal movement of cattle in Zambia is extensive. It is also possible that the abundant wildlife population that exists between the two regions may aid in the spread of the tick vectors.

Based on the analysis of the Turkish samples, the *Ba. bovis* population representing Aydın and Izmir regions are composed of two discrete sub-populations. The isolates from Tire in Izmir and those from Dalama in Aydın formed a single population, while those from Hacio Ali Basi and Yazidere formed another sub-population based on the PCA. Both of these populations appear to be in linkage equilibrium, although the small sample size and the low p value for the latter population indicate that a larger sample size could reveal evidence of LD. The possible cause of this sub-division could be that, while there is cattle movement between Tire and Dalama and between Hacio Ali Basi and Yazidere, there no cattle movement between Tire / Dalama and Hacio Ali Basi / Yazidere. Therefore, there is no opportunity for genetic exchange to occur between the two sub-populations resulting in genetic isolation. There is little information in the published literature on the epidemiology of babesiosis in this part of Turkey. Another possible explanation for the observed sub-structuring could be the presence of cryptic biological speciation that prevents genetic exchange. However, this is unlikely given that *Ba. bovis* is not sub-structured as is the case in Zambia. In addition, according to Tibaryenc and Ayala (2002), in randomly mating parasites, genetic exchange is frequent enough to inhibit the appearance and persistence of discrete durable genetic sub-divisions. Regional genetic differentiation in Turkey was also supported by the strong evidence for a positive correlation between geographical and genetic distance, i.e. the large coefficient of determination and the steep slope of the regression line obtained by the Mantel test. This was in contrast to the situation in Zambia, where although a significant positive correlation between geographical and genetic distance was found, the percentage of genetic variation attributed to geographical distance was low.

4.4.1.3 Multiplicity of infection

A high number of mixed genotype infections was found in both countries. However the multiplicity of infection in individual animals in Zambia was significantly higher than that found in Turkey. Since the results of this study demonstrated a significant positive correlation between age of host and multiplicity of infection, this difference may be explained by the age difference of cattle sampled from each country with the mean age of the Zambian cattle being higher than those from Turkey. Such a situation is likely because once infected with *Ba. bovis*, cattle remain carriers for periods ranging from six months to several years (Bock *et al.* 2004a; Calder *et al.* 1996). Thus, older cattle will have been exposed to more tick bites and *Babesia* inoculations, and will consequently have more genotypes circulating in their blood. The lack of a correlation between multiplicity of infection and tick burden is counterintuitive considering that one would expect cattle with higher tick burdens to have more genotypes. However, this might be explained by the fact that the observed level of tick infestation at the time of sampling doesn't necessarily correlate with levels of previous tick challenge on cattle. The results in Chapter Three showed that some of the farmers inconsistently controlled ticks on their cattle, i.e. absence of ticks on an animal at a particular time does not indicate that the herd is consistently kept tick free. Therefore, a cross-sectional study such as this, where tick control history is unknown, is not ideal to investigate the relationship between tick numbers and the number of genotypes in individual cattle. Furthermore, the numbers of ticks on individual cattle in this study were only qualitatively evaluated (Section 3.2) and the species found on each animal were not identified. To an extent, this may also have masked the effect of tick burden on the multiplicity of infection.

4.4.2 Population genetic analysis of *Ba. bigemina*

4.4.2.1 Suitability of Markers

The nine markers used for the population genetic analysis of *Ba. bigemina* showed that they were polymorphic and species-specific. However, a small proportion of the samples (10 %) were not amplified by every marker. This was attributed to polymorphism at the primer sites. The assembly of the whole genome sequence of *Ba. bigemina* at the Sanger Institute is incomplete and ongoing and as a result, it was impossible to determine whether the developed markers were distributed across all four chromosomes. Nevertheless, except for one pair of markers, all the others were on different contigs and the two markers on the same contig were separated by a sufficient physical distance, which made it unlikely that they were genetically linked

4.4.2.2 Population genetic analysis

Similar to *Ba. bovis*, no samples were found to have duplicate MLGs indicating considerable genetic diversity in the Zambian *Ba. bigemina* population. This was evidenced by the high estimated heterozygosities which ranged from 0.816 in Lusaka Province to 0.824 in Eastern Province. However, linkage disequilibrium (LD), defined as the non-random association of alleles at two or more loci, was observed, regardless of whether the data from each province were analysed as one population or separately. The existence of a high number of genotypes in individual animals and the high genetic diversity observed in this parasite population argues against a clonal or epidemic population structure being responsible for the observed LD. The most probable reason for linkage disequilibrium was that the *Ba. bigemina* population in Zambia was genetically sub-structured. This hypothesis was investigated using computer modeling and five sub-populations were deduced from the dataset. F_{ST} measurements indicated that there was a high level of genetic differentiation between the five sub-populations. Unfortunately, the resulting sample sizes of each of the sub-populations were too small to determine whether there was random mating within any of them.

The population structure identified for *Ba. bigemina* in Zambia was contrary to that of *Ba. bovis* population that was found to be panmictic across both provinces, indicating that there is considerable movement of cattle / ticks between the two provinces. Moreover, more than 50% of the *Ba. bigemina* isolates were from cattle that had *Ba. bovis* / *Ba. bigemina* concurrent infections (Table 3.6). The observed LD in the *Ba. bigemina* population could be due to the low prevalence (see Chapter 3) of this parasite which results in limited transfer of parasites between the two provinces, i.e. the low prevalence lowers the probability of an infected animal being moved. When infected cattle are moved, the transmission rate is low for sufficient genetic exchange, that would result in LE, to occur between the parasites from the two provinces. Furthermore, unlike *Ba. bovis*, *Ba. bigemina* transmission seems to occur mostly in the wet season. Therefore, most of the genetic exchange for *Ba. bigemina*, unlike that for *Ba. bovis*, occurs only in the wet season. In addition, the presence of more than one tick vector of *Ba. bigemina* in Zambia may also contribute to the observed sub-structuring. The Australian *Ba. bovis* vaccine strain has been found to contain more than one genotype, some of which are poorly transmitted by *B. microplus*, while the others are efficiently transmitted (Cowman *et al.* 1984; Kahl *et al.* 1983). It is therefore possible that there may be preferential transmission of the different genotypes by the different vectors of *Ba. bigemina* found in Zambia, although further sampling and analysis would be needed to confirm this.

Clear evidence of sub-structuring is shown by both the PCA and STRUCTURE analysis. However, the two methods use different approaches with the latter being able to detect population sub-groups that may not be evident by the former. The PCA is a distance-based method, which works by calculating a pair-wise distance matrix between every pair of individuals. The matrix is represented graphically by using a multi-dimensional scaling plot, giving clusters that can be visually identified (Pritchard *et al.* 2000). Conversely, STRUCTURE uses a model-based approach with the assumption that observations are randomly drawn from some parametric model. Inference of parameters corresponding to each cluster is then done jointly with inference for cluster membership of each individual using Bayesian methods (Evanno *et al.* 2005; Pritchard *et al.* 2000). Therefore, the distance based methods (PCA) are only loosely connected to statistical procedures that allow the identification of homogenous clusters of individuals and are thus more suitable for visual exploration of data (Evanno *et al.* 2005). In this study STRUCTURE was able to identify the five sub-groups in the population by maximising the probability of random mating within each group. As shown in Table 4.16, sub-population A contains genotypes almost exclusively from Lusaka Province while sub-population E consists of genotypes that are exclusively from Eastern Province, possibly representing the 'core' evidence of the populations in each province. The remaining three populations (B, C and D) could be the product of the low level of recombination between A and E, and as a result, are different from the two parental groups. To determine whether this is the correct interpretation of the data, a larger sample size, from both cattle and the vector ticks in the two provinces, would be required.

4.4.2.3 Multiplicity of infection

Similar to the situation for *Ba. bovis*, host age was found to be a significant predictor of the number of *Ba. bigemina* genotypes in an individual animal. A correlation between the number of genotypes and age of the host has also been observed in *T. parva* and *T. annulata* populations (Oura *et al.* 2005; Weir 2006).

4.4.3 Comparisons between *Ba. bovis* and *Ba. bigemina* in Central and Eastern Zambia

Ba. bigemina and *Ba. bovis* are two protozoan parasites that belong to the same family and both can be transmitted by *Bo. microplus* and *B. annulatus*. However, in sub-Saharan Africa, *Bo. decoloratus* and *R. evertsi* are additional vectors of *Ba. bigemina*. It has been known for some time that the two parasites cause different disease syndromes, with the pathology generated by *Ba. bovis* caused by over-production of pro-inflammatory

cytokines as well as the direct effect of red blood cell destruction. In contrast, the pathogenesis of *Ba. bigemina* is mainly due to the direct effect of red blood cell destruction (Bock *et al.* 2004a; Brown & Palmer 1999). By obtaining *Ba. bigemina* and *Ba. bovis* samples at the same time and from the same animals, the present study was able to directly compare certain aspects of the population biology of the two parasites. This is the first time that the role of genetic exchange has been systematically analysed in both *Babesia* species. This study has shown that *Ba. bovis* occurs in Central and Eastern Zambia as a single randomly mating population in which genetic exchange occurs frequently. Conversely, the *Ba. bigemina* population is probably sub-structured into five sub-populations. The results presented in Chapter Three show that the prevalence of *Ba. bovis,* in the wet season is almost twice that of *Ba. bigemina*, while in the dry season it is almost 25 times greater. This can be taken as indirect evidence of a higher transmission rate of *Ba. bovis* compared to *Ba. bigemina* in Eastern and Central Zambia and it would appear that the transmission rate of *Ba. bigemina* is insufficient to suppress the linkage disequilibrium brought about by the introduction of infected cattle and/or ticks from the other parts of the country through cattle movements. Combined with the possible preferential transmission of certain genotypes by the different vectors of *Ba. bigemina,* as postulated above, this might contribute to the observed linkage disequilibrium.

CHAPTER FIVE

General discussion

5.1 Development of PCR assays

Tick-borne diseases continue to be a hindrance to the development of the livestock industry in many countries. Sensitive, specific and cost-effective diagnostic methods are an important component in the study and control of these diseases. For epidemiological surveys and clinical diagnostic purposes, the polymerase chain reaction offers an ideal combination of sensitivity and specificity while being relatively high throughput. In Chapter Two of this thesis, several uniplex PCR assays that can be used to diagnose TBDs of cattle are described, which are based on amplification of the 18S and 16S rRNA, β-tubulin and cytochrome b genes. Attempts were also made to develop multiplex PCR assays for *T. parva, T. taurotragi, Ba. bovis, Ba. bigemina, Anaplasma* spp and *E. ruminantium* using the 18S and 16S rRNA genes; *T. parva, Ba. bigemina* and *Ba. bovis* using the β-tubulin gene and a duplex PCR assay for *Anaplasma* spp and *E. ruminantium* using the 16S rRNA gene. Among the uniplex assays, the cytochrome b and 18S showed higher sensitivity and specificity than the β-tubulin PCR assay. Because of a lack of material representing blood of known low parasitaemia, it was not possible to determine whether the 18S rRNA or cytochrome b gene was the more sensitive. However, previous studies have shown that the cytochrome b gene is more sensitive that the 18S rRNA gene (Salem *et al.* 1999). In addition, Bilgic *et al.* (unpublished results) has found that the *T. annulata* cytochrome b RLB assay was a thousand times more sensitive than the 18S rRNA RLB. As discussed in Section 2.4, this is likely to be due to the cytochrome b gene being present at a higher copy number per parasite genome (Brayton *et al.* 2005; Salem *et al.* 1999). Therefore, it can be predicted that the cytochrome b gene would have proved to be more sensitive than the 18S rRNA gene.

The high costs associated with the use of PCR continue to hinder its application in poorly resourced laboratories. The development and optimisation of multiplex PCR assays where several diseases can be diagnosed simultaneously offers an opportunity to reduce costs and increase the use of the technology for clinical diagnosis and epidemiological studies in most countries. However, the two multiplex PCR assays described in Chapter Two were not without problems. The 18S and 16S rRNA gene assay failed to amplify *E. ruminantium* and *Ba. bigemina*. For the other species, it generated non-specific bands which made

determination of the bonafide amplicons more difficult. Conversely, the β-tubulin gene PCR assay worked relatively well when used on laboratory-derived parasite DNA preparations. However, when it was used on field samples collected on FTA cards, multiple non-specific bands were observed after agarose gel electrophoresis of the PCR product, probably due to amplification of host DNA. Consequently this assay is unsuitable for use in field studies. Beyond the scope of this study, it is still possible that other genes, such as cytochrome b may be used to develop a sensitive and specific multiplex PCR assay for tick-borne pathogens. However, if such an assay were developed, issues such as PCR selection are likely to result in differing sensitivities in the amplification of the various templates. Therefore, considerable effort would be required to optimise such an assay, assessing template-to-product ratios at various concentrations of DNA for each parasite.

5.2 Epidemiology of TBDs in Zambia

The 18S and 16S rRNA uniplex semi-nested PCR assays described in Chapter Two were used for the epidemiological study of tick-borne parasites in Zambia. The decision to use this assay rather than the cytochrome b gene PCR assay was based on convenience of use, as it did not offer any advantages in terms of sensitivity or specificity. For each sample, the primary PCR assays were carried out using a pair of universal primers for the *Theileria* / *Babesia* genera and another pair of external primers was used for *Anaplasma* / *Ehrlichia*. Following this, individual species-specific assays were carried out by secondary semi-nested PCR assays specific for each parasite. This approach to some extent saved a lot of time and resources, and confirms why diagnostic tests that detect a combination of species are desirable.

PCR analysis of the blood samples on FTA cards from Central and Eastern Zambia showed that TBD pathogens of cattle are prevalent in all the three provinces sampled, with variations in prevalence among the provinces and between the two seasons. By conducting the study over both the dry and wet season in Central and Eastern Zambia, the effect of seasonality on TBD prevalence could be assessed. In general, the prevalence of the pathogens was higher in the wet season than in the dry season, and this was in agreement with the observed increase in tick burden on cattle. It was established that the farmers are aware of the increased risk of disease in the wet season; all the farms sampled treated their animals with acaricides in this season while in the dry season a proportion of them did not. However, in general, tick control was found to be inadequate and ticks were observed even on regularly treated cattle. This was particularly evident for traditional farmers in Eastern Province where a number of cattle were heavily infested with ticks. As demonstrated by

this and previous studies in Zambia (Minjauw *et al.* 1997; Pegram *et al.* 1989), in addition to transmitting diseases, heavy infestation with ticks can cause a significant reduction in cattle productivity. Therefore, programmes to educate traditional farmers are required in order to explain the need for adequate tick control and to highlight the consequences of inadequate tick control policies.

The results of this study demonstrate that a number of factors are responsible for the occurrence of TBD infections in cattle, some of which are common to all diseases. Cattle infected by one parasite are significantly more likely to harbour many other tick-borne pathogens. For example, it was found in this study that cattle which were PCR positive for *T. parva* were more likely to be positive for *Anaplasma* spp, *T. taurotragi*, *Ba. bovis* and *T. mutans*. This indicates that integrated disease control strategies (Eisler *et al.* 2003; Ghosh *et al.* 2007), targetting both the pathogen and the tick vector and are aimed at mitigating the impact of all ticks and the pathogens they transmit should be employed, rather than targetting each pathogen individually. For such an approach to be effective, several gaps in our basic knowledge of the biology of ticks and the pathogens they transmit must be addressed. These include (1) inoculum size and virulence of parasite species/strain; (2) heterogeneities in vector competence of transmission; (3) the force of infection (the rate at which susceptible animals acquire infection); and (4) immunity and carrier status (Eisler *et al.* 2003). Once ascertained, this basic knowledge should be combined with existing vector, epidemiological and economic models to provide a comprehensive theoretical frame-work for investigating and evaluating the impact of the various integrated disease and vector control programmes.

From the results in this study, only the prevalences of *T. mutans* and *Anaplasma* were sufficiently high to suggest the existence of endemic stability. While offering the opportunity to detect animals with active infections, PCR does not provide information on herd immunity. Tick-borne pathogen densities in carrier animals appear to fluctuate over time and periodically fall below the levels detectable by PCR (Geysen 2000; Herrero *et al.* 1998). Thus PCR-based approaches using blood samples collected on FTA filters may be less likely to provide evidence for low parasite densities than serological methods. This is because of the probability that no infected erythrocytes are present in the minute volume of blood on the portion of the FTA card that is excised and used to provide template DNA material. It is therefore likely that the PCR assays used in this study have underestimated the prevalence of these pathogens. Combining PCR with serological tests, which are able to detect antibodies in animals in which the parasitaemia is below the detection limit of

PCR and in those that have cleared the infection would improve the accuracy of the estimated prevalence. For example, Bazarusanga *et al*. (2008) found that the prevalence of *T. parva* was much higher when calculated using a combination of serological and PCR assays than on single diagnostic tests. In addition to providing an accurate estimate of pathogen prevalence, serological tests may provide useful information concerning herd immunity to these diseases, although this will depend on the nature of the protective immune response to each pathogen and the role of antibody in protective immunity.

The results presented in Chapter Three showed that carrier cattle with a higher number of infecting pathogens also had significantly lower PCV than those with no or few co-infecting organisms. This indicates that mixed infections of tick-borne pathogens are associated with loss in productivity of cattle, even in the carrier state. This observation is very important because most of the disease control measures currently recommended for control of TBDs favour the development of enzootic stability, i.e. a carrier status where the parasite, the vector and the host are in a state of equilibrium and clinical disease is virtually absent. This, in part, is because past efforts aimed at eradicating vector-borne diseases in many parts of the World (including sub-Saharan Africa) were largely unsuccessful (Eisler *et al.* 2003; Pegram *et al.* 2000). This begs the question, how does one encourage establishing endemic stability for multiple TBD pathogens while at the same time minimising the loss in productivity associated with such infections in carrier cattle? Previous studies of various disease control options for ECF in Zambia have shown that the combination of strategic tick control and immunisation resulted in the highest level of productivity, and at the same time reduced the potential risk from other TBDs (Minjauw *et al.* 1997). It would, therefore, be desirable to compare productivity losses that result from control strategies that encourage establishment of carrier status to all tick-borne diseases against other more targetted control options, to determine the methods that would give the best economic returns for farmers. Such comparisons should be done for both traditional farming systems, where most of the animals are local breeds, and commercial systems which largely rear exotic breeds. This comparison would be important, as the level and nature of endemic stability may be different. For example, introduction of exotic breeds of cattle in some areas of Tunisia resulted in changes in the state of endemic stability to *T. annulata* (Gharbi *et al.* 2006). Furthermore, cost-benefit analysis, carried out in the latter study, found that vaccination would be more effective in reducing losses from the sub-clinical forms of *T. annulata* infections. This assumed that vaccination would reduce the financial impact of infection by reducing the number of clinical cases and also by minimising the anaemia that occurs in sub-clinical infection (Gharbi *et al.* 2006). It is

therefore possible that vaccinating cattle against TBDs will reduce productivity losses associated with the sub-clinical disease, while at the same time encouraging the establishment of endemic stability. In this case, tick control would only be performed strategically in order to minimise losses (for example, reduced weight gains, tick worry) and not to prevent disease transmission, allowing boosting of protective immunity. At present this would be the best control strategy in the absence of methods that can effectively eradicate TBD parasites or their tick vectors. However, for such an approach to work efficiently, multiple parasite species vaccines may be required, although this is a complex issue, as the interaction between hosts and these parasite species has evolved over the millennia.

5.3 Population genetics

5.3.1 Development and application of the genotyping system

In the work presented in Chapter Four of this thesis, panels of mini- and micro-satellite markers for genotyping *Ba. bovis* and *Ba. bigemina* isolates were developed. The availability of the genome sequences of *Ba. bigemina* and *Ba. bovis* were important factors in the development of these markers. Without these genome sequences, the identification of these markers would have been a very difficult task. The number of repeat regions identified in the *Ba. bovis* genome was far higher than that identified in the *Ba. bigemina* genome using identical search parameters. While this difference may be a reflection of sequence variation between the genomes, it may in part be explained by the fact that the *Ba. bovis* genome was fully assembled, whereas the *Ba. bigemina* genome was represented by a collection of unassembled contigs. The presence of low complexity repeat regions is known to cause difficulties in genome assembly and often repeats are found at the extremities of unassembled contigs. Consequently, a proportion of the repeats within the *Ba. bigemina* genome may not be represented in the contig database. Future screening of a fully assembled *Ba. bigemina* genome will resolve this issue. By using stringent criteria to evaluate the suitability of each candidate marker locus, a large number were discarded. Only loci that were polymorphic, did not give multiple bands, gave few null alleles and were species-specific were used to genotype field isolates.

The use of the capillary electrophoresis allowed the resolution of PCR products to 1 bp. This not only allowed the exact size of alleles to be determined, but where multiple alleles were present they could be easily discriminated and enumerated. A problem encountered with the high degree of resolution offered by this method was that most alleles were not

separated by the exact motif length, but by a gradation of PCR product sizes, i.e., in some regions of the allelic spectrum there was no clear-cut demarcation between PCR products of differing sizes. Therefore it was necessary to create 'bins' for allele scoring and this was based on the distribution of PCR product sizes. In problematic regions, bins were generally 0.8 bp in range, with the size difference between successive 'bins' being as little as 0.5 bp. The highly polymorphic nature of many of the markers coupled with this high-resolution sizing method resulted in a plethora of alleles being identified at many loci. In laboratories where capillary electrophoresis is unavailable, the markers developed in this study may be of more limited use. An alternative method using electrophoresis would be required, such as the high resolution gel-based system used for *T. parva* genotyping in Uganda (Oura *et al.* 2005). To develop markers suitable for sizing using lower resolution technology, the initial screening process could be modified to select loci where either large and/or consistent intervals are identified between alleles, i.e. micro-satellite markers evolving in a step-wise manner. However, from this and previous studies (Oura *et al.* 2005; Weir 2006) it is evident that such loci are rare and it could take considerable time and effort to identify an adequate number of markers.

The quality of data generated by any population genetic study is limited by the choice of the genetic marker system used in the study. Over the years, a number of genotyping methods have been used (MacLeod *et al.* 2001), and while there is no such a thing as a 'perfect' marker system, each methodology has its own advantages and disadvantages. Isoenyme electrophoresis (Ben Miled *et al.* 1994; Schweizer *et al.* 1988) and monoclonal antibody profiles (Ben Miled *et al.* 1994; Minami *et al.* 1983; Shiels *et al.* 1986) gave way to methods using restriction fragment length polymorphism (RFLP) (Paindavoine *et al.* 1989) and random amplification of polymorphic DNA (RAPD) (Stevens & Tibayrenc 1995) because these approaches gave direct access to genotypic information rather than requiring inference from phenotypic data. However, these methods still require large amounts of parasite material that may only be obtained by amplification of original material in rodents or by culture. This may result in selection and loss of some genotypes. Another problem with these methods is that if a high proportion of isolates are composed of a mixture of genotypes, as was the case in this study, it becomes impossible to determine genotype frequencies, and assays may identify mixed infections as new genotypes, confounding the interpretation of the results. This problem maybe overcome by cloning each isolate, but this is time consuming and costly. More recently, new genotyping methods such as micro- and mini-satellite (Biteau *et al.* 2000) and single nucleotide polymorphism (SNP) (Coates *et al.* 2009) have gained in popularity. Microsatellites and

SNPs have been found to provide similar estimates of population genetic parameters in *Diabrotica virgifera virgifera* (Coates *et al.* 2009). However, with SNPs there is still the problem of identifying individual alleles in samples with mixed genotypes and are, as such, subject to the shortcomings of the methods described above. Micro- and mini-satellite analysis in combination with capillary electrophoresis as applied in this study permits easy identification of samples with a mixture of genotypes. The method is easy to perform and does not require amplification of isolates in rodents or cultures.

FTA filters offer a convenient method for storing parasite material as no cold chain is required when collecting samples in the field (Rajendram *et al.* 2006). However, for parasites with very low parasitaemia, the sensitivity of the PCR assay is compromised as only a very small amount of blood (125 µl) is spotted on the card. With low parasitaemia samples, it is possible that a very limited amount of parasite DNA is present on the part of the card that is excised to provide a template for PCR amplification. To overcome the problem in this study, lysates were prepared from larger volumes of blood and used to provide template DNA. However, the use of lysates is not without drawbacks as the amount of PCR inhibitors in such preparations is often high and may reduce the sensitivity of the PCR assay. In this study, this problem was overcome by diluting the lysate. An alternative approach would be to isolate DNA from the lysates using standard extraction protocols, although this may result in the loss of a proportion of parasite DNA.

The findings from Chapter Four indicate a large amount of diversity within *Ba. bovis* and *Ba. bigemina* populations, not only within a locality but also within a single host. This is in agreement with experiments by Timms *et al.* (1990) who found that the Australian Ka vaccine isolate was composed of a heterologous mixture of parasites clones. As every isolate analysed in this study comprised multiple genotypes, ticks feeding on these cattle are highly likely to become co-infected and this provides an opportunity for genotypes to recombine in the tick gut (Gough *et al.* 1998; Mackenstedt *et al.* 1995) and generate new random genotypes. Thus, the underlying conditions promoting genetic exchange are present, i.e. co-infection of ticks with multiple genotypes at the same blood meal where a single genotype does not completely dominate.

5.3.2 Population structure

This study determined that in Zambia, *Ba. bovis* occurred as a single, randomly mating population while in Turkey it occurred as two discrete populations. Although each of the Turkish populations was found to be approaching linkage equilibrium (LE), the sample

size was too small for definitive conclusions to be drawn. However, the calculated I^S_A for each Turkish population was too low for the populations to be considered clonal. The high level of genetic diversity and the absence of duplicate genotypes in the *Ba. bovis* populations across the two countries suggest limited self-fertilisation. It can therefore be concluded that in general *Ba. bovis* has a panmictic population structure. In a previous study of *T. annulata* in the same geographical region, a degree of sub-structuring according to district of origin was observed in populations from Turkey, which resulted in linkage disequilibrium when the populations were combined (Weir 2006). The results of the Turkish *T. annulata* study indicate that there was a degree of inter-mixing of *T. annulata* populations, through cattle movements between the districts, however this was insufficient to dissolve LD. In the current study, there is complete sub-structuring of the two *Ba. bovis* populations, suggesting a complete absence of inter-mixing. Studies of *Cryptosporidium parvum* populations in Turkey also found clear sub-structuring according to farms in some parts of Turkey (Tanriverdi *et al.* 2006). *Ba. bovis* is maintained in a purely cattle / tick transmission system with no paratenic host or *ex vivo* component to the life-cycle. Gene flow between areas, in this case, must be the result of movement of either the host or the vector. Therefore, it is likely that while cattle movement is occurring between certain areas of the country, it is completely absent between other areas, resulting in genetic isolation of particular parasite populations.

The results of the population genetic analysis of *Ba. bovis* (in Zambia and Turkey) indicate a very high level of diversity with each MLG being unique. This level of diversity could have significant implications for the development of a sub-unit vaccine for this parasite species. Research on developing a vaccine initially focused on the major immunodominant antigens expressed on the surface of the infected red cells (Allred *et al.* 1994; Leroith *et al.* 2005). However, recent research coupled with the publication of the genome sequence (Brayton *et al.* 2007), has shown that these antigens (VESA 1) are encoded by a multigene family which undergoes antigenic variation (Al Khedery & Allred 2006). The mechanism is probably similar to that defined for the *var* genes in *P. falciparum* (Scherf *et al.* 1998). These findings have lead to the view that such antigens are unlikely to be useful for a vaccine due to the high levels of antigenic variation and work on candidate vaccine antigens has focused on a series of less dominant antigens, such as RAP-1 and Hsp20 (Brown *et al.* 2006). There are limited data on the levels of polymorphism in these antigens but the data presented in this thesis imply that many will have the potential to be polymorphic particularly if they are under immune selection. Furthermore, the finding that the Turkish population is sub-structured suggests that such antigens will evolve

independently in different populations. The material generated in this study would be very useful for assessing the diversity of vaccine candidate antigens as well as testing whether they are subject to diversifying selection.

In contrast to *B .bovis*, linkage disequilibrium was observed in the *Ba. bigemina* populations from Eastern and Central Zambia regardless of whether they were analysed as a single population or separately. Based on the analysis of the whole population using the STRUCTURE programme, it was shown that the *Ba. bigemina* population was genetically sub-structured into five distinct sub-populations and this would explain the LD observed when the isolates were treated as a single population. The sample sizes of the sub-groups were too small to determine whether any of them comprised a single randomly mating population (panmixia). However, given the low I^S_A, the high genetic diversity and the absence of duplicate genotypes, the underlying population structure of this parasite is very unlikely to be clonal or epidemic. A clonal population is genetically homogenous, with identical genotypes deriving either from a parental genotype by clonal propagation or from parasite populations where there is strong inbreeding and self-fertilisation, i.e. the existing sexual reproduction (out-crossing) is insufficient to break the clonal pattern (Tibayrenc & Ayala 2002). Similar to many other apicomplexan parasites, an obligate sexual phase is known to occur in *Ba. bigemina* (Mackenstedt *et al.* 1995). However, in *P. falciparum*, the population genetic structure has been shown to be clonal in regions of low transmission and panmictic in regions of high transmission (Anderson *et al.* 2000). This means that the existence of a sexual phase by itself is not enough to ensure panmixia, as a minimum rate of transmission must be met for a panmictic population structure to occur. In a panmictic population, all individuals should be potential mating partners and this requires that there be no restrictions, either genetic or spatial upon mating. The high level of genetic diversity and the high number of genotypes in individual samples indicates that sexual recombination of *Ba. bigemina* genotypes does occur in the ticks. However, as discussed in Section 4.4.2.2, the low rate of transmission (evidenced from the low prevalence reported in Chapter Three) may be responsible for the departure of the *Ba. bigemina* population structure from LE in Eastern and Central Zambia. Nevertheless, this study demonstrates that the population structure of *Ba. bigemina* is close to panmixia. To definitely establish the population structure of this parasite, a new study, with a larger sample size must be undertaken.

5.4 Areas of future study

The epidemiological and population genetic data and their analysis presented in this thesis have raised a number of issues which may form the basis for further studies on tick-borne diseases.

- It was discovered that *Ba. bigemina* is almost entirely absent from the cattle population during the dry season. In future, it would be worthwhile conducting experiments to determine the force of transmission of this parasite from different species of tick vectors in the dry and wet seasons in order to determine the factors that are responsible for the variations in transmission between the seasons. It would also be possible to determine the relative competency of each of the vectors in transmitting this disease under varying conditions.

- The study showed that the *Ba. bigemina* population in Zambia is genetically sub-structured causing the population genetic structure of the parasite to deviate from LE. To investigate the basis for this sub-structuring and to conclusively determine whether random mating does occur in this parasite, a large number of samples from cattle hosts and the different vectors of *Ba. bigemina* in different regions of the country should be collected and analysed using the markers developed in this study. It would also be useful to investigate whether micro-satellite genotyping can shed light on the variation of the antigenic and virulence properties of the parasite; i.e is the selection for antigenic diversity able to overcome a low level of genetic exchange.

- The samples collected in this study provide a significant resource for also studying the population genetics of *T. parva* and the molecular epidemiology of the rickettsial pathogens. With *T. parva*, the results of this and a previous study (Fandamu *et al.* 2005) have shown there is no association between an animal's vaccination status and whether it is positive for ECF. In addition to this, Geysen *et al.* (1999) suggested that clonal expansion of components of the Muguga vaccine cocktail was responsible for the outbreaks of ECF in some parts of Southern Zambia. Geysen *et al*. used the PCR-RFLP method, whose limitations were mentioned above, in the characterisation of the strains and found that components of the Muguga cocktail that persisted in the Zambian population were the Muguga and the Serengeti transformed strains. This was in contrast to the results from a Ugandan study conducted by Oura *et al*. (2004), who used micro- and mini-

satellites and found that the Kiambu 5 was the component of the vaccine that persisted in cattle. Consequently, a number of questions have been raised about the conclusions of the Geysen *et al*. study (Geysen 2008; McKeever 2007) and it would be interesting to test the conclusions of this study, while at the same time characterising the carrier status induced by the local Zambian strains used to vaccinate cattle against ECF, by applying the *T. parva* markers developed by Oura *et al.* (2003).

- Another area where the markers developed in this study will be useful is in the characterisation of vaccine breakthroughs in areas where live vaccines are used to vaccinate cattle against babesiosis. A number of molecular methods have been used in an attempt to identify and genetically compare breakthrough strains from vaccine strains (Berens *et al.* 2005; Dalrymple 1990; Lew *et al.* 1997b; Wilkowsky *et al.* 2008). However, the resolution of these markers has been very low and in most cases they have failed to distinguish isolates from different areas. Recently, Wilkowsky *et al*. (2009) described a set of mini-satellite markers which can be used to genotype *Ba. bovis* isolates. However, these markers were all in coding regions, some of which are known to have antigenic properties (Hines *et al.* 1995a; Tebele *et al.* 2000) and are therefore unlikely to be neutral as they may be under selective pressure. The neutral markers described in this study would be more appropriate for the discrimination of vaccine breakthrough genotypes.

In summary, this study has shown that molecular tools are useful in the study of the epidemiology and basic biology of TBDs parasite populations in the field. Further research is needed on these important parasites of livestock, so that cost effective disease control strategies, based on sound knowledge of their epidemiology and host response to infection can be designed and implemented.

Appendix 6.1 MLGs used in the population genetic analysis of *Ba. bovis*

Samples representing 120 isolates derived from Turkey and Zambia were used for formal population genetic. A multi-locus genotype was generated for each sample, representing the predominant allele detected at each of the eight locus. MLGs for those samples that did not amplify from at least one locus are not included. Allele sizes are indicated in base pairs (bp).

CHAPTER 6

Appendices

Appendix 6.1 MLGs used in the population genetic analysis of *Ba. bovis*

Sample id	Region	Country	Markers							
			Bbv5	Bbv19	Bbv20	Bbv25	Bbv26	Bbv50	Bbv52	Bbv58
DALI001	Aydin	Turkey	208	225	223	155	155	155	176	199
DALI002	Ayidin	Turkey	208	155	209	155	209	155	176	211
DALI003	Ayidin	Turkey	177	209	155	156	178	208	155	211
DALI004	Aydin	Turkey	177	155	209	156	208	155	176	199
DALI005	Ayidin	Turkey	176	155	155	156	209	155	176	211
DALI006	Aydin	Turkey	175	209	209	155	155	148	211	211
DALI007	Aydin	Turkey	176	155	223	156	155	116	176	199
DALI008	Aydin	Turkey	176	209	230	156	214	148	176	199
DALI009	Aydin	Turkey	176	209	155	155	209	171	155	211
HAO001	Aydin	Turkey	204	270	231	231	210	173	246	209
HAO002	Aydin	Turkey	204	273	231	232	213	161	241	208
HAO003	Aydin	Turkey	205	273	232	232	214	153	244	211
HAO004	Aydin	Turkey	205	273	231	283	213	164	244	208
HAO005	Aydin	Turkey	173	273	235	226	213	174	248	208
HAO006	Aydin	Turkey	199	273	235	228	213	155	248	208
HAO007	Aydin	Turkey	202	274	230	231	213	173	242	199
HAO008	Aydin	Turkey	205	273	235	211	214	155	248	214
HAO009	Aydin	Turkey	204	273	231	217	214	174	246	199
HAO010	Aydin	Turkey	205	272	231	230	213	174	242	199
HAO011	Aydin	Turkey	205	273	231	230	214	153	248	199
HAO012	Aydin	Turkey	204	273	231	228	214	174	250	199
HAO013	Aydin	Turkey	205	273	231	215	213	159	251	199
YAZ001	Aydin	Turkey	204	273	231	209	213	174	251	208
YAZ002	Aydin	Turkey	208	273	232	232	213	174	253	214
YAZ003	Aydin	Turkey	207	274	232	209	213	171	250	208
YAZ004	Aydin	Turkey	204	273	231	390	213	174	242	199
YAZ005	Aydin	Turkey	108	273	231	217	213	174	248	199
TIR001	Izmir	Turkey	175	155	220	156	213	144	155	211
TIR002	Izmir	Turkey	211	155	209	155	155	174	176	211
TIR003	Izmir	Turkey	239	261	222	155	209	155	176	211
TIR004	Izmir	Turkey	208	209	223	309	209	155	208	199
TIR005	Izmir	Turkey	176	209	155	155	368	133	176	211
TIR006	Izmir	Turkey	175	264	155	156	209	104	176	211
TIR007	Izmir	Turkey	175	261	155	208	178	171	176	211
TIR008	Izmir	Turkey	176	261	209	209	178	155	176	199
TIR009	Izmir	Turkey	176	209	155	156	209	148	208	211
TIR010	Izmir	Turkey	176	209	155	156	208	135	208	211
UNK001	Unknown	Turkey	205	276	232	215	227	177	244	155
UNK002	Unknown	Turkey	206	273	231	217	213	174	244	199
UNK003	Unknown	Turkey	205	273	232	217	213	171	244	199
LUS001	Lusaka	Zambia	208	262	262	151	230	143	245	211
LUS002	Lusaka	Zambia	208	260	223	225	236	144	245	199

Appendix 6.1 continues

Sample id	Region	Country	Markers							
			Bbv5	Bbv19	Bbv20	Bbv25	Bbv26	Bbv50	Bbv52	Bbv58
LUS003	Lusaka	Zambia	208	260	223	281	304	143	247	211
LUS004	Lusaka	Zambia	208	261	222	281	236	144	245	199
LUS005	Lusaka	Zambia	208	261	222	281	238	144	247	211
LUS006	Lusaka	Zambia	208	261	222	281	185	174	245	199
LUS007	Lusaka	Zambia	207	260	222	281	213	174	245	199
LUS008	Lusaka	Zambia	209	260	228	312	359	144	244	211
LUS009	Lusaka	Zambia	208	261	222	370	515	144	244	154
LUS010	Lusaka	Zambia	208	261	210	309	213	146	266	211
LUS011	Lusaka	Zambia	208	272	228	312	213	143	245	202
LUS012	Lusaka	Zambia	208	261	215	309	106	161	247	208
LUS013	Lusaka	Zambia	208	272	144	309	371	162	245	199
LUS014	Lusaka	Zambia	208	272	228	283	313	170	267	205
LUS015	Lusaka	Zambia	208	261	269	312	216	172	233	208
LUS016	Lusaka	Zambia	208	272	233	155	213	180	247	202
CEN001	Central	Zambia	209	272	229	312	213	144	244	211
CEN002	Central	Zambia	207	267	229	276	213	144	266	211
EAS001	Eastern	Zambia	225	261	227	298	213	164	245	199
EAS002	Eastern	Zambia	207	261	229	171	196	170	244	199
EAS003	Eastern	Zambia	208	272	229	298	213	164	232	199
EAS004	Eastern	Zambia	207	272	229	232	217	172	242	208
EAS005	Eastern	Zambia	207	261	228	298	213	176	242	199
EAS006	Eastern	Zambia	207	261	229	211	298	174	233	211
EAS007	Eastern	Zambia	208	261	231	238	124	170	247	199
EAS008	Eastern	Zambia	208	272	228	211	124	144	249	208
EAS009	Eastern	Zambia	209	261	233	289	180	144	244	199
EAS010	Eastern	Zambia	207	262	231	212	113	144	211	199
EAS011	Eastern	Zambia	208	272	228	327	213	171	240	208
EAS012	Eastern	Zambia	208	261	228	272	213	162	247	205
EAS013	Eastern	Zambia	208	261	169	254	162	178	246	202
EAS014	Eastern	Zambia	172	272	223	228	213	171	247	211
EAS015	Eastern	Zambia	207	272	232	231	213	236	247	202
EAS016	Eastern	Zambia	209	262	225	284	213	170	243	205
EAS017	Eastern	Zambia	208	272	226	149	213	210	245	199
EAS018	Eastern	Zambia	207	261	224	372	262	162	248	199
EAS019	Eastern	Zambia	208	272	223	283	154	170	242	199
EAS020	Eastern	Zambia	208	261	262	205	154	143	248	205
EAS021	Eastern	Zambia	208	275	219	149	180	176	245	199
EAS022	Eastern	Zambia	208	277	228	209	244	143	267	211
EAS023	Eastern	Zambia	181	272	228	211	213	170	233	208
EAS024	Eastern	Zambia	181	265	228	138	324	172	215	199
EAS025	Eastern	Zambia	209	260	229	411	214	168	249	211
EAS026	Eastern	Zambia	261	272	227	231	213	170	247	199
EAS027	Eastern	Zambia	181	270	224	207	274	171	244	205
EAS028	Eastern	Zambia	181	272	229	426	178	143	244	205
EAS029	Eastern	Zambia	207	261	229	257	213	170	244	199
EAS030	Eastern	Zambia	206	266	219	211	213	170	266	199
EAS031	Eastern	Zambia	201	272	229	284	154	143	247	199
EAS032	Eastern	Zambia	207	226	230	312	214	168	266	211
EAS033	Eastern	Zambia	208	261	231	154	124	144	244	199

Appendix 6.1 continues

Sample id	Region	Country	Markers							
			Bbv5	Bbv19	Bbv20	Bbv25	Bbv26	Bbv50	Bbv52	Bbv58
EAS034	Eastern	Zambia	209	272	228	219	123	162	252	199
EAS035	Eastern	Zambia	210	223	227	144	178	143	247	199
EAS036	Eastern	Zambia	230	263	229	219	180	143	248	211
EAS037	Eastern	Zambia	209	261	228	155	162	142	266	211
EAS038	Eastern	Zambia	209	272	229	209	257	170	266	199
EAS039	Eastern	Zambia	207	272	228	155	213	143	266	199
EAS040	Eastern	Zambia	207	261	228	219	312	143	248	205
EAS041	Eastern	Zambia	261	272	228	211	213	170	247	211
EAS042	Eastern	Zambia	210	272	228	284	213	166	247	199
EAS043	Eastern	Zambia	208	261	225	281	297	144	244	199
EAS044	Eastern	Zambia	181	261	226	211	208	143	247	199
EAS045	Eastern	Zambia	209	261	227	211	312	143	244	199
EAS046	Eastern	Zambia	208	272	224	209	210	170	247	211
EAS047	Eastern	Zambia	207	145	228	284	213	143	246	199
EAS048	Eastern	Zambia	208	113	230	160	213	170	244	199
EAS049	Eastern	Zambia	209	237	228	114	213	139	244	199
EAS050	Eastern	Zambia	208	261	229	181	247	166	247	199
EAS051	Eastern	Zambia	209	272	419	211	124	143	247	202
EAS052	Eastern	Zambia	207	273	156	183	213	143	247	199
EAS053	Eastern	Zambia	209	223	232	211	124	143	247	199
EAS054	Eastern	Zambia	209	261	230	209	178	143	247	199
EAS055	Eastern	Zambia	208	393	262	209	124	210	264	199
EAS056	Eastern	Zambia	208	261	228	492	124	143	247	211
EAS057	Eastern	Zambia	209	261	102	154	178	144	244	199
EAS058	Eastern	Zambia	406	261	227	154	162	143	247	199
EAS059	Eastern	Zambia	208	228	232	284	244	251	247	199
EAS060	Eastern	Zambia	177	262	281	167	178	143	461	211
EAS061	Eastern	Zambia	125	261	233	177	213	158	236	199
EAS062	Eastern	Zambia	213	272	227	177	213	210	244	205

Appendix 6.2 MLGs used in population genetic analysis of *Ba. bigemina*

Samples representing 52 isolates derived from Zambia were used for formal population genetic. A multi-locus genotype was generated for each sample, representing the predominant allele detected at each of the eight locus. MLGs for those samples that did not amplify from at least one locus are not included. Allele sizes are indicated in base pairs (bp).

Appendix 6.2 MLGs used in population genetic analysis of *Ba. bigemina*

Sample id	Province	Markers								
		Bbg2	Bbg7	Bbg9	Bbg13	Bbg14	Bbg18	Bbg23	Bbg34	Bbg42
Lus01	Lusaka	280	254	278	156	267	173	309	274	347
Lus02	Lusaka	273	255	279	160	264	173	318	248	332
Lus03	Lusaka	280	255	276	156	268	173	309	274	347
Lus04	Lusaka	281	252	273	157	271	173	299	251	290
Lus05	Lusaka	273	258	291	156	271	173	299	248	290
Lus06	Lusaka	280	255	279	158	268	173	309	274	210
Lus07	Lusaka	282	272	276	132	265	173	318	216	358
Lus08	Lusaka	281	272	277	156	265	173	318	216	358
Lus09	Lusaka	281	272	276	159	265	173	318	216	357
Lus10	Lusaka	281	272	276	195	208	174	318	216	358
Lus11	Lusaka	273	272	275	194	265	173	318	216	292
Lus12	Lusaka	281	252	276	156	267	173	309	274	347
Lus13	Lusaka	273	255	279	156	270	173	318	262	332
Lus14	Lusaka	272	252	276	155	271	173	155	220	290
Lus15	Lusaka	279	155	276	195	267	173	155	219	287
Lus16	Lusaka	288	258	279	160	267	173	309	271	358
Lus17	Lusaka	284	156	281	155	264	175	155	271	351
Lus18	Lusaka	276	258	279	160	242	173	309	223	379
Lus19	Lusaka	273	156	277	160	195	173	155	226	346
Lus20	Lusaka	280	280	276	195	195	178	155	261	333
Lus21	Lusaka	280	255	279	157	271	173	309	219	328
Lus22	Lusaka	273	258	271	194	270	173	318	251	347
Lus23	Lusaka	273	258	268	194	268	173	318	248	384
Eas01	Eastern	273	256	271	155	266	174	309	260	357
Eas02	Eastern	274	156	276	194	266	173	309	260	252
Eas03	Eastern	281	242	265	195	273	173	309	257	316
Eas04	Eastern	276	155	271	157	263	173	309	257	347
Eas05	Eastern	281	155	275	194	176	174	155	219	356
Eas06	Eastern	281	271	276	195	264	173	318	216	358
Eas07	Eastern	281	155	277	194	265	173	155	216	359
Eas08	Eastern	274	156	265	155	305	173	309	260	379
Eas09	Eastern	298	155	268	195	195	174	155	274	347
Eas10	Eastern	282	280	262	195	195	173	155	218	355
Eas11	Eastern	281	272	276	194	265	174	318	216	358
Eas12	Eastern	282	155	158	195	178	171	275	219	351
Eas13	Eastern	281	272	276	194	265	173	318	216	358
Eas14	Eastern	282	155	291	157	175	173	155	274	360
Eas15	Eastern	281	242	262	157	265	173	309	216	274
Eas16	Eastern	282	155	262	155	268	174	155	219	274
Eas17	Eastern	282	155	277	156	155	174	318	216	358
Eas18	Eastern	274	242	262	195	276	173	318	258	186
Eas19	Eastern	281	214	262	157	266	173	309	260	274
Eas20	Eastern	281	263	262	195	276	175	309	260	274
Eas21	Eastern	282	159	154	195	177	178	155	262	356
Eas22	Eastern	272	160	291	306	178	177	155	258	359
Eas23	Eastern	213	160	292	194	177	175	155	220	177
Eas24	Eastern	155	252	262	195	177	173	318	219	274
Eas25	Eastern	281	260	262	195	267	170	309	256	340
Eas26	Eastern	281	156	262	157	177	173	318	260	316

Appendix 6.2 continues

Sample id	Province	Markers								
		Bbg2	Bbg7	Bbg9	Bbg13	Bbg14	Bbg18	Bbg23	Bbg34	Bbg42
Eas27	Eastern	281	255	262	195	301	173	309	260	316
Eas28	Eastern	282	257	265	195	268	173	309	219	154
Eas29	Eastern	274	260	262	195	276	173	309	262	364

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