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The epidemiology of theileriosis in Rwanda and implications for the control

Thomas BAZARUSANGA

Thesis for obtaining the degree of Doctor in Veterinary Sciences (PhD)
Laboratory of Parasitology
Department of Virology, Parasitology and Immunology
Faculty of Veterinary Medicine
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Promoters: Prof. Dr. Jozef Vercruysse
Dr. Dirk Geysen

This thesis is dedicated to the late Prof. Pasteur Kageruka

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List of abbreviations

%:	Percentage
°C:	Celsius temperature scale
AEZ:	Agro-ecological zones
DNA:	Deoxyribo-nucleic acid
CTL:	Cytotoxic T lymphocyte
ECF:	East Coast Fever
EDPRS:	Economic development and poverty reduction strategy
EIR:	Entomological inoculation rate
ELISA:	Enzyme linked immunosorbent assay
FAO:	Food and agriculture organisation
HL:	High land
IFAT:	Indirect fluorescent antibody test
INEAC:	Institut national pour l'étude agronomique du Congo
ISAR:	Institut des sciences agronomiques du Rwanda
ITM:	Infection and treatment method
MDG:	Millenium development goal
MHC:	Major histocompatibility complex
PIM:	Polymorphic immunodominant molecule
PCR:	Polymerase chain reaction
RFLP:	Restriction fragment length polymorphism
SELISA:	Slide enzyme linked immunosorbent assay
TBD:	Tick-borne disease
UK:	United Kingdom

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Chapter 1

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Introduction

Theileriosis is a disease caused by an Apicomplexan protozoan parasites belonging to the family of Theileriidae. The *Theileria* spp. infecting animals include *T. velifera, T. taurotragi, T. buffeli, T. mutans, T. annulata* and *T. parva. T. parva* transmitted mainly by *Rhipicephalus appendiculatus* tick and causing a disease of cattle (*Bos spp.)* and buffalo (*Syncerus caffer*) known as East Coast Fever (ECF) is the most pathogenic of all Theileriidae.

In susceptible cattle, ECF is characterised by enlargement of superficial lymph nodes, a sustainable fever and diverse clinical signs associated with invasion of non-lymphoid tissues with parasitized lymphoblasts. ECF causes high mortality with death occurring approximately three weeks after infection, mainly as a result of severe pulmonary oedema.

Recovered cattle from ECF become solidly immune against a homologous and a number of heterologous strains. Neutralising antibodies directed against sporozoites are induced in immune animals although they do not confer protection. The immune response is principally mediated by cytotoxic lymphocytes directed against the intralymphocytic-schizonts. Immune cattle remain carriers of the parasite and potential sources of infection for ticks.

Ticks become infected with *T. parva* when they feed on infected cattle. The occurrence of the disease is determined by the density of infected animals and the numbers of ticks infesting the host. The distribution of the vector is dependent on climatic conditions that regulate their survival. Depending on the relative suitability of climates for tick survival, epidemiological states ranging from epidemic to stable or unstable endemic are recorded over the entire range of *R. appendiculatus* distribution.

Available field studies document the epidemiology of ECF in eastern and southern Africa and variable degrees of endemicity to ECF are recorded among geographic regions and within a single locality. In central Africa, little is known about the endemic status of ECF and in Rwanda, there has been relatively no study aimed at specifically defining the field situation resulting from the complex interaction between the parasite, the cattle host and the tick vector in different regions. The state of endemicity prevailing in a given area determines the control approach to be applied.

The financial losses incurred, directly and indirectly, by ECF are extremely high. With more than 38% of the African total bovine population affected and an estimated mortality of 1.1 millions cattle per year, ECF remains probably the most important cattle disease in terms of economic losses and restriction of livestock development in affected countries of eastern, central and southern Africa.

ECF is controlled by different methods or a combination of methods: vector control, restriction of contact between ticks and cattle hosts, immunisation and treatment. Tick control by acaricide application is the common method used. However, the method is expensive and impractical to sustain. A break down in the control regimen results in disease and death of the animals. The most cost effective and sustainable control method is immunisation. Vaccination programmes using infection with live parasites and simultaneous treatment with a long acting tetracycline is being applied by an increasing number of countries although the method has been characterised by incomplete protection due to antigenic variants in field strains.

The objective of the present chapter is to review: (1) the available literature on the complex epidemiological relationship between the parasite, the host and the vector in different geographic areas endemic to ECF, (2) the different control measures in use to protect susceptible cattle against the disease and (3) the status of theileriosis in Rwanda and the control options applied to combat it.

1 The epidemiology of East Coast Fever

1.1 The parasite *Theileria parva*

Theileria parva (Theiler, 1904), the causative agent of ECF in cattle is an important tick-borne blood parasite of cattle and wild ruminants in eastern, central and southern Africa. Three subspecies are recognized in *T. parva*, namely *T. parva parva* causing classical ECF, *T. parva lawrencei* responsible for Corridor disease transmitted from buffalo to cattle and *T. parva bovis*, the causing agent of Zimbabwe theileriosis, a more benign form also known as "January disease" (Uilenberg et al., 1982). The distinction is principally based on clinical and epidemiological parameters but the phylogenic significance of this classification remains a matter of discussion (Allsopp et al., 1989). The trinomial classification has been abandoned as cross-immunity and DNA based characterisation data did not substantiate subspecies within *T. parva* complex.

It was recommended that the parasite stocks be described as either cattle or buffalo-derived parasites (Anon, 1989). Cattle derived parasites are readily transmissible between cattle, whereas buffalo-derived parasites are transmitted with low efficiency between cattle and occurs mainly where cattle are exposed to ticks that have fed on buffalo. Although cattle-derived and buffalo-derived parasites both produce acute fatal diseases in cattle, the infection differs in that the later invariably gives rise to lower levels of piroplasm parasitaemia than classical cattle-derived *T. parva* (Young et al., 1973). Major differences in clinical picture and mortalities have been reported among cattle-derived parasites (Lawrence, 1981) but whether the severity of the disease and outcome is dose-related or host-dependent resistance remain to be elucidated.

While the molecular biology-based methods confirmed the existence of *T. parva* as a single parasite species, it is known that within *T. parva*, stocks with distinct antigenic characteristics exist (Radley, 1978; Irvin, 1987; Nambota et al., 1997; Geysen et al., 1999). The variations in pathogenecity and incidence displayed in different geographic areas are compatible with the extreme antigenic diversity within *T. parva* species, and with variations in host and vector relationships. The existence of different immunogenic strains of *T. parva* that do not necessarily cross-protect poses a serious

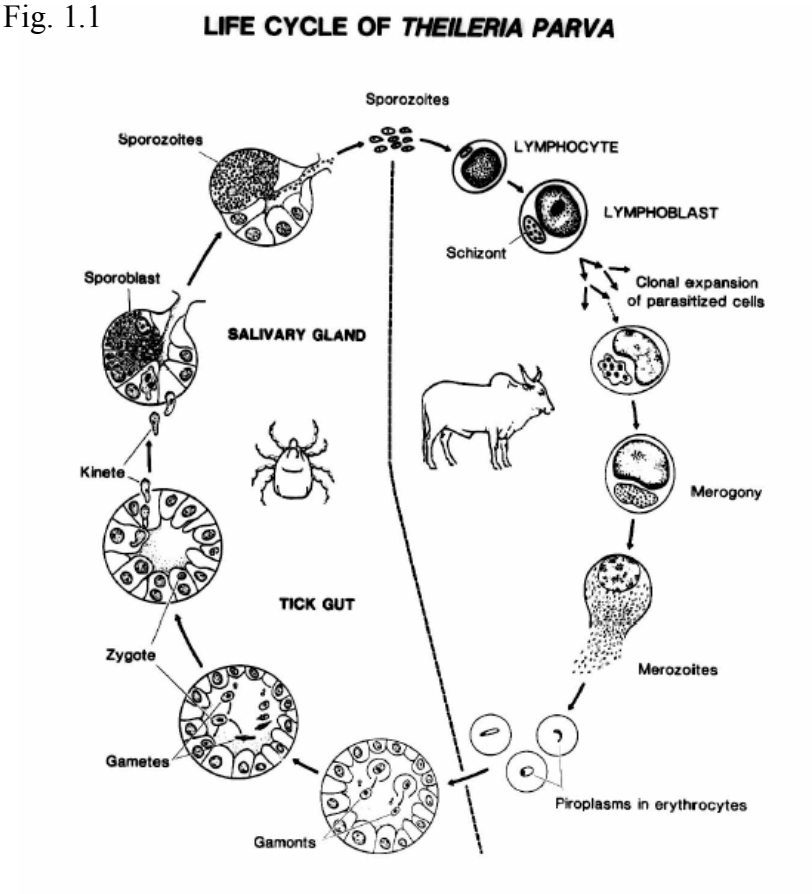
hindrance to the design of efficient control measures as they have to be carefully matched to prevailing diversity among *Theileria* strains in the field.

1.2 The life cycle of *Theileria parva*

T. parva alternates between cattle and ticks (Fig 1.1) and its life cycle involves the sequential invasion of two different cell types in cattle host and a sexual cycle in the vector. Ticks become infected with *T. parva* when they feed on cattle or buffalo carrying in their erythrocytes the piroplasm stage of the parasite. Piroplasme are released within the tick gut and differentiate into male and females gametes. Pairs of gametes fuse to produce diploid zygotes. These will invade cells in the gut wall and undergo a two-step meiotic division, resulting in large number of developmental stages which develop into motile kinetes (Gauer et al., 1995) and migrate to the salivary glands through the heamolymph. The kinete is now called a sporont. During tick feeding, sporoblasts are produced in a sporogony process of nuclear mitotic division. Maturation is then stimulated and haploid sporozoites are produced after three to four days feeding. These infective sporozoites are subsequently released into the saliva and injected into the mammalian host when ticks are taking their blood meal. Inoculated sporozoites invade bovine T and B lymphocytes by a complex receptor-mediated process and begin to develop. The parasite completes the first stage of its lifecycle in lymphocytes which consists in a logarithmic multiplication phase (schizogony) and the formation of the invasive forms (merogony) for the next stage. Resulting merozoites are liberated from the lymphocytes and invade the erythrocytes in which they are referred to as piroplasms. The erythrocytic stages are infective to ticks and are characterised by limited proliferation. This is in marked contrast to most of the other members of the *Piroplasmia* in which the main multiplication phase occurs in the erythrocytic phase.

The epidemiological implication of a sexual cycle in *Theileria* is the potentiality for recombination. This may well be the key to the complex field situation which prevails in certain regions endemic for *T. parva.* it has been suggested that genetic exchange is expected to occur between different *T. parva* genotypes when no physical obstacles (isolation by time or geographical distance or both) or biological obstacles (either cryptic biological speciation or clonality) exist in the field (Tibayrenc, 1998).

Fig. 1.1



1.3 The vector populations

The occurrence of ECF closely follows the geographic distribution of the main vector *R. appendiculatus*, a three host ixodid tick.

1.3.1 Vector range and seasonal dynamics

R. appendiculatus ticks have been reported in 17 countries of eastern, central and southern Africa. Within its range the abundance varies considerably, being governed by climatic conditions. In a particular region, the overall transmission of *T. parva* infection is mainly driven by the macro and micro climatic requirements of the tick and by the host availability (Norval et al., 1992). Under marginal conditions, adaptations such as diapausing behaviour of the tick might become a more important factor. In hot and drier areas of central and southern Africa, *R. zambeziensis* ticks replace *R. appendiculatus* (Lawrence et al., 1983) but its role in the epidemiology of

T. parva in dryer climate areas has not been yet defined. Limited populations of *R. duttoni* occur in some areas of southern Africa (Angola) where no case of ECF has been reported, implying a negligible role in the *T. parva* transmission.

In ECF endemic areas, the key determinant of the transmission and the epidemiology of *T. parva* infection are the distribution and the abundance of *R. appendiculatus.* Variation in *R. appendiculatus* occurrence influences greatly the transmission possibilities, creating different epidemiological states over the entire range of the *T. parva* distribution (Short and Norval., 1981; Billiouw et al., 1999). Three different phenologies are recorded over the range of *R. appendiculatus* distribution. The phenology is defined by whether there is one, two or many tick generations per year resulting in complex *T. parva* transmission patterns. The numbers of feeding stages of ticks on cattle control the total level of *T. parva* infection within the tick population. The transmission of *T. parva* is further complicated by the occurrence of diapausing behaviour in *R. appendiculatus* (Berkvens, 1990; Madder, 1999).

Non-diapausing populations occur in tropical eastern Africa (Norval et al., 1992) where transmission of *T. parva* parasite is continuous due to year round presence of all *R. appendiculatus* tick instars. In southern Africa, unfed adult ticks remain inactive (diapause) when unfavourable conditions prevail (Short and Norval, 1981, Rechav, 1982; Pegram and Banda, 1990, Berkvens et al., 1995) and the occurrence of only one generation a year coinciding with the rainy season (Norval et al., 1991) reduces the transmission period to four months. In the transition zone in central Africa, a rainy season and a dry season *T. parva* transmission peak might prevail and this needs to be determined.

Exceptional epidemiological states are observed at the fringes of the vector distribution areas when survival of the tick populations is not secured due to climatological and environmental conditions. Lessard et al. (1990) have reported the presence of antibodies to *T. parva* from fifteen countries in eastern and southern Africa, but eleven countries are commonly threatened by ECF (Mukhebi et al., 1992). A clear understanding of the effect of cyclical climatic changes with alternating prolonged dry and wet seasons may lead to a more comprehensive picture of the *T. parva* transmission.

1.3.2 Vector to host transmission of *T. parva*

Transmission of *T. parva* from tick vector to cattle host occurs during a blood meal of infected ticks. The successful transmission of *T. parva* infection is determined by survival rates of the parasite in ticks and by mechanisms of host resistance to feeding ticks. The extreme physiological adaptations of the tick for survival on and off host are regarded as a series of hazards for the survival of the *Theileria* parasite. Survival for *Theileria* is simpler in the cattle than in the tick (Walker, 1990).

At the engorgement stage of tick feeding, many millions of piroplasms are ingested even from cattle with low parasitaemia (Shaw and Young, 1994). The majority of piroplasms ingested by the tick do not survive the first hazards posed by digestive enzymatic activity and phagocytosis in the gut and appear to be rapidly destroyed (Walker, 1990). However, vast numbers of sporozoites are usually found in salivary glands of infected ticks.

The parasite is transmitted transstadially, meaning infection picked up in one instar will be transmitted by the next instar (Lawrence et al., 2004). *R. appendiculatus* is a three-host tick and each instar feeds on a separate host. Larvae or nymph that feed on infected bovine will transmit *T. parva* infection in their subsequent instars as nymphs or adults. The absence of transovarial transmission constitutes an additional important obstacle in the survival of the parasite. The only possibility is onwards transmission of the infection to the mammalian host.

When an infected tick is attached to the host, the tick-feeding lesion is a hostile environment for the parasite. Walker (1990) stated that in addition to the non-specific inflammatory reactions to the tick attachment site, there is also an immune response by the cattle to the proteins in the tick saliva. Individual animals develop resistance to *R. appendiculatus* as a result of repeated exposure (Lawrence et al., 1994) which may affect the transmission of *T. parva* by impairing the feeding of the ticks.

There is also evidence that various breeds of cattle exhibit differences in resistance to ticks (Norval et al., 1988; Rechav et al., 1990; Paling et al., 1991). Fivaz et al. (1989) and Leitch (1989) demonstrated that tick resistant cattle are capable of limiting the level of *T. parva* transmission compared to tick-susceptible cattle. Different breed types exist in the ECF-endemic regions but their relative level of resistance to tick infestation is to be established.

In endemic areas, transmission of *T. parva* is influenced by the numbers of ticks that successfully attach to the host. Approximately, only half of the total *R. appendiculatus* numbers applied on resistant cattle feed successfully (De Castro et al., 1985). In the field, the overall epidemiology of *T. parva* infection will be determined by the proportion of ticks that are infected.

The laborious work of tick dissection as the method of determining tick infection rates has limited the number of field ticks analysed as these generally show low abundance, often with only one infected acinus. Nymphal ticks fed as larva on carrier animals show lower abundance than adults because of small blood intake during previous instars (Sonenshine, 1990) and the subsequent *T. parva* infection prevalence is lower in nymphs (Marcotty et al., 2002). Consequently, the ability of nymphs to transmit theileriosis will be low compared to adult ticks (Short and Norval, 1981).

Differences in infection rates are also observed between the sexes in *R. appendiculatus*. Female ticks develop higher abundance levels than male ticks. Biological differences occur between populations of *R. appendiculatus* in different geographical areas. Depending on the origin, much higher infections of *T. parva* can develop in some ticks stocks than in others (Ochanda et al., 1998). The genetic basis of differences in magnitude of vector competence for geographically different *R. appendiculatus* requires further clarification.

Estimation of transmission intensities in various epidemiological situations is best quantified by the number of infective bites per animal per year and can be derived from data on tick infestation on cattle and the *T. parva* infection rate for a certain region. The infection rates in ticks is generally low (Moll et al., 1986) but vary greatly in various endemic areas (Walker et al., 1981; Kariuki et al., 1995, Ogden et al., 2003). The underlying mechanisms in variability of tick infection prevalence in different areas have not been fully elucidated.

Methods most commonly used to identify infection in *R. appendiculatus* involve detection of the infective sporozoite stages in stained tick salivary glands (Young and Leitch, 1982; Voigt et al., 1995). The staining method is cumbersome and lacks specificity especially in areas where *T. parva* and *T. taurotragi* co-exist (Norval et al., 1992). The PCR methods are highly specific and allow processing of a large numbers of samples. However, PCR methods are expensive and their wide-spread use in the field is limited. The development of more user-friendly PCR protocols would help carry-out extensive field surveys.

1.4 The Bovine host

1.4.1 Host sensitivity

The introduction of *T. parva* infection into a previously unexposed cattle population results in an epidemic situation with mortality up to 95% in all age categories of cattle. When the disease is established, the older animals that have survived primary infection become immune. In these situations, only calves and newly introduced stocks are at risk of primary infection and mortality is estimated high. The animals have come into contact with *T. parva* infection early in life and clinical disease is rare even among young animals. A balanced equilibrium between parasite, vector and host in which all co-exist with the virtual absence of clinical disease can exceptionally occur when *T. parva* transmission is continuous and occurs early in life. This situation termed as "endemic stability" may remain unstable under different epidemiological circumstances. An assessment of the level of endemic stability achieved becomes a prerequisite for the design of efficient control programmes.

Measures applied to prevent ticks infestations greatly influence the incidence of ECF as they determine the chances of host-vector interactions. Endemic stability depends on the degree of exposure of the host to *R. appendiculatus*. This in turn determines the age at first contact of the *T. parva* infection by the majority of the cattle populations. A better understanding of the degree of contact between infected ticks and cattle would help define areas of stable or unstable endemicity to *T. parva* infection.

In stable endemic regions, animals develop variable degrees of resistance against *T. parva* infection. The ability to acquire resistance to infection with *T. parva* is inherited and varies between breeds and types of cattle (Paling et al., 1991). Selecting resistant animals may therefore have a role in the control of the infection.

European taurine breeds are the most susceptible to *T. parva* infection than the African *Bos indicus* cattle (Norval et al., 1992). Moll et al. (1986) believes that taurine cattle would die if introduced in ECF-endemic areas. Cattle in endemic areas of *T. parva* are usually Zebu or Sanga breeds (Young et al., 1981). The cattle breed of Rwanda but also Burundi and parts of Uganda, Democratic Republic of Congo and Tanzania is a Sanga type called Ankole (Mason and Maule,1960). The Ankole breed

which resulted from a mixture of humped zebu (*B. indicus*) and humpless Hamitic Longhorn (*B. taurus*) cattle has a partial *Theileria* tolerance (Paling et al., 1991).
This tolerance is likely to be a result of centuries of natural selection within the Ankole cattle population, surviving in the ECF endemic areas of central Africa. However, Ankole cattle are characterised by poor production traits. Young (1981) suggested that cattle from ECF endemic areas may represent a useful genetic pool from which cattle of higher productivity, but *Theileria* tolerant, could be developed. Extensive breed improvement programmes are taking place in most of Africa. A number of pure taurine cattle population and various degrees of their crosses with Zebu or Sanga type have increased markedly over the past years. The incidence of *T. parva* infection and the economic losses due to ECF in improved stocks have not been yet evaluated.

1.4.2 Carrier state

Infective cattle may be clinically ill, recently recovered or persistent carriers. Carrier animals are defined as animals that have recovered from the primary infection and consequently maintain piroplasms in the circulating blood at levels high enough to infect ticks but not necessarily detectable by routine diagnostic techniques (Medley et al., 1992). A fundamental difference between primary and carrier *T. parva* infection is the degree of parasitaemia. This in turn affects the level of infection acquired by feeding ticks. The effectiveness with which infected animals transmit *T. parva* infection to ticks is not fully understood.
During primary infection, the parasitaemia in the host is high but short-lived. High infection rates in feeding ticks are usually produced (Norval et al., 1992). Carrier cattle generally carry very low piroplasm infections. Based on his mathematical model, Medley et al. (1993) have shown that in endemic areas, the low infectivity of carrier animals is outweighed by their great numbers. The study concluded that in susceptible cattle, the number of animals developing clinical disease will increase the rate of dissemination of *T. parva* infection but the contribution of carriers to the overall transmission will far be the most important.
However, it is not well established what proportion of recovered animals become carriers. It is known that there are strains that disappear rapidly from the body after recovery. The *T. parva* Muguga stock and the *T. parva* Pugu stock do not induce

carriers (Young et al., 1986; Dolan, 1999). The number of *T. parva* strains inducing a carrier state is not known but is expected to be high. Young et al. (1981) found the carrier state in adult cattle to approach 100% in an endemic area in Kenya.

It is further established that transmission of *T. parva* infection from carrier animals is sporadic (Dolan, 1986, 1999). The relapsing nature of parasitaemia which is well documented in the case of Babesia and *T. mutans* (Perry et al., 1985) has now been established in *T. parva* carrier states (Young et al., 1986, 1990). Geysen (2000) reported large differences in the frequency of detectable carrier states which did not appear to be correlated with time. The author argues that the parasite densities seem to fluctuate and periodically fall below the level of detection.

The mechanisms involved in the maintenance of the carrier state are not known, although more evidence points in favour of the survival of the schizont stages in sites of low immunological surveillance (Dolan et al., 1986). Its importance was reemphasized when *T. parva* parasites were isolated, using standard tissue culture techniques, from animals three and seven months after vaccination by the infection and treatment method (Kariuki et al., 1995). Although *T. parva* schizogony in red blood cells has been reported (Conrad, 1986; Fawcett, 1987), it seems unlikely that piroplasms maintain the carrier state.

1. 5 The epidemiological states of ECF

1.5.1 Criteria to assess the epidemiological states

The epidemiological state of theileriosis caused by cattle-derived *T. parva* and transmitted by *R. appendiculatus* is determined in a population of cattle as stable or unstable endemicity by using a combination of indicators: herd antibody prevalence, disease incidence, age group of cattle affected by the disease and case-fatality (Norval et al., 1992). Endemic stability for *T. parva* is defined as a state whereby the large majority of the cattle population is infected and immune by six months of age and little or no clinical disease occurs (Moll et al., 1984, 1986), which implies that any other situation should be qualified as unstable. This has caused considerable confusion and inconsistency as two completely different epidemiological situations are both called unstable.

Billiouw (2005) suggested that the epidemiological states of ECF is better described by the trend in the infection prevalence and the age at first contact and the case fatality ratio are the most discriminating criterion. The author argued that excluding situations in which the tick population fails to establish, the natural states in ECF epidemiology can be classified as epidemic or endemic states. While an ECF epidemic which defines a state of the disease in which high morbidity and mortality occur in cattle of all categories has the advantage of being easily measured, the endemic state in which the majority of the population is infected and immune and only calves are at risk is more complex and does not meet the strict criteria of endemic stability.

Young (1981) postulated that endemic stability of theileriosis is a combination of regulatory mechanisms involved in development of protective factors against *Theileria* infection in calves, the level of *Theileria* infection in the tick vector population and the size of the vector population feeding on cattle. This state can be found when the cattle possess a low innate susceptibility to *T. parva* infection (Morzaria et al., 1988) and *R. appendiculatus* is virtually continuous throughout the year (Yeoman, 1996). Norval et al. (1992) reserve the term epidemic for introduction of the infection into a previously disease-free area with a full susceptible cattle population.

Based on different equilibrium in the transmission dynamics and the frequency of the disease, Billiouw (2005) redefined new epidemiological states with regards to ECF as: (1) epidemic when the age at first contact is less than 2 years, case fatality is close to 100% and decreasing while the infection prevalence is increasing, ((2) first level endemic stability where age at first contact is less than 2 years, case fatality is about 50% and infection prevalence is stable, (3) second level endemic stability when age at first contact less than 2 years, case fatality is about 25% while infection prevalence is stable and (4) ultimate endemic stability where age at first contact is less than six months, case fatality is close to 0% and infection prevalence is stable.

In the author's classification of the epidemiological states with regards to ECF, the undemically unstable state used to describe a state in which only a proportion of the cattle population become infected and immune by six months of age and clinical disease is seen (Norval et al., 1992) is an unnatural state induced by restricted cattle-tick contact. This situation is artificially created when acaricides are administered at very high frequency but may also occur naturally at the fringes of the vector

distribution in case of unfavourable climatic conditions for tick survival such as unusual prolonged dry and wet seasons or El Nino events. This situation is considered epidemiologically unstable since there is still potential for increased transmission of infection.

1.5.2 Tools to measure the epidemiological states

Disease incidence and prevalence are the minimum data sets used to assess the epidemiological states of ECF. Adequate data on disease incidence are difficult to obtain and need prospective studies with close animal supervision (Norval et al., 1992). The common method used to detect a case definition of ECF under field conditions is based on a combination of clinical signs and the demonstration of schizonts/piroplasm in microscopic examinations.

However, reports from several countries were based on the presence of piroplasm alone which has confused the disease incidence data. It is for instance not possible to discriminate between piroplasms of *T. parva* from those of other *Theileria* spp. which frequently occur in mixed infections in the field (Norval et al., 1992). In addition, detection of piroplasm alone may merely indicate that an animal is a carrier. A long lasting carrier state following recovery gives rise to low numbers of erythrocytes infected with *Theileria* piroplasm (Neitz, 1957, Paling et al., 1991) and detection of piroplasms in stained-blood smears may be difficult and impractical for large-scale surveys.

In many parts of Africa, areas of stable or unstable epidemiological states have been based on prevalence results of serological diagnostic tests in extensive field surveys (Norval et al., 1992; Perry et al., 1996). The most widely used serological assay in Africa has been the indirect fluorescent antibody test (Burridge and Kimber, 1972, Goddeeris et al., 1982). Both the schizonts and piroplasm can be used as antigen to detect *T. parva* antibodies, although the schizonts antigen is preferred as it confers a longer duration of the serological response. However, the serological cross-reaction with other *Theileria* species and the high cost due to low through put of IFAT limits its use especially in regions where several *Theilerial spp*. coexist.

Other serological methods which have been developed include enzyme-linked immunosorbent assay (ELISA) and slide enzyme-linked immunosorbent assay (SELISA), a modification of the standard ELISA technique. The commonly used

ELISA assay is based on PIM-based antigen expressed as recombinant fusion protein with enzyme glutathione S-trasferase (GST) enzyme (Katendi et al., 1998). The PIM-based ELISA is highly sensitive and specific and is practical for use in the screening of large number of bovine sera for antibodies against *T. parva* in the epidemiological studies.

In comparison with the IFAT and conventional ELISA, the schizont antigen-based SELISA is as sensitive as IFAT and ELISA but has additional advantage of being simple to perform under field conditions. However, studies have shown that the sensitivity of serological assays varies with the presence and the intensity of *T. parva* transmission (Billiouw et al., 2005) and may not be indicated in highly seasonal *R. appendiculatus* occurrence regions of southern Africa.

An alternative to serological methods is direct detection of *T. parva* by PCR-based DNA amplifications. The PCR method has been used to detect the prevalence of *T. parva* in field ticks and cattle (Ogden et al., 2003) and is the method of choice to discriminate between *T. parva* and other *Theileria* spp. infecting cattle in the field (Geysen, 2000). Recently, the PCR method has come to play a major role in *T. annualata* prevalence surveys in the nature (Aktas et al., 2002, Dumanli et al., 2005). In *T. parva* infections, the high cost involved in PCR protocols has limited extensive prevalence studies in the field.

However, although a higher sensitivity of PCR method than conventional blood smear examination to detect *T. parva* has been reported (Ogden et al., 2003), the sensitivity of PCR has not yet been evaluated in different field conditions. Available reports describe that PCR method can only detect half of the *T. parva* carriers in experimentally infected animals (Geysen, 2000). In addition, Jarvi et al. (2002) have reported a significantly lower sensitivity of PCR compared to serology in detecting *Plasmodium* in chronically infected birds. It may be therefore useful to determine the sensitivity of PCR methods in detecting *T. parva* parasites present at low densities in carrier cattle under different epidemiological conditions in the nature.

2 Control methods and limitations

Clinical cases of ECF as in many other tick-borne diseases can be treated by chemotherapy, but the most commonly used methods are different forms of prevention.

2.1 Curative methods

Clinical cases of ECF can be treated by chemotherapy. The majority of developments in chemotherapy have occurred in the last twenty years (Norval et al., 1992). There are presently three effective drugs for treatment of ECF: Halofuginone lactate (Terit), Parvaquone (Clexon and Parvaxone) and Buparvaquone (Butalex).

Halofuginone is easy to administer but is ineffective when used in early infection. The recommended dose is 1.2 mg/Kg orally at 48 hour intervals. However the high toxicity (Norval et al., 1992) and its long waiting period for milk and meat consumption have limited its use in many countries. Subsequently, a very safe and effective Parvaquone compound was developed. This drug is effective against schizont and sporozoite stages of *T. parva* but requires 2 treatments at a dosage of 10mg/Kg at 48 hour intervals and do not require milk withdrawal after administration.

In many endemic ECF, the most commonly used antitheilerial drug is Buparvaquone, a parvaquone derivative. McHardy et al. (1985) have shown that Buparvaquone is 8 times more effective than Parvaquone in vivo against *T. parva* infection but Butalex is 5 times more expensive and had a relatively longer waiting period for meat consumption. In the field, a single treatment at a dosage of 2.5 mg/Kg is effective, but a second injection may be required at 48 hours interval, as some strains of *T. parva* are not fully controlled by one dose (McHardy, 1989). Buparvaquone is the most commonly used for treatment against ECF on smallholder farm in most of endemic areas

The availability of therapeutic means is a significant development in the control of ECF. However, there are two constraints to the widespread use of medication: the drugs are too expensive for most African farmers and rapid, accurate diagnosis and administration of the drug are required for effective therapy (Norval et al., 1992). In addition, the widespread use of chemotherapy has increased the number of carriers over the past years. Treated and recovered animals become carriers of infection

(Dolan, 1986) and parasites surviving treatment will be transmitted by recovered cattle (Dolan, 1999).

2.2 Vector control

The control of ECF is most commonly achieved through the control of the vector *R. appendiculatus* by means of acaricide application. The practice of cattle dipping has been widely accepted and understood by farming communities and veterinary authorities. In the field, scientists claim that acaricide use can prevent cattle from being infected provided the procedure is respected. However, traditional chemically-based control methods will not work in the long term as they are expensive (Pegram et al., 1996; de Castro, 1997), environmentally damaging (Mbogo et al., 1996) and require costly infrastructures and adequate knowledge of the application principle.

As an alternative to short-interval dipping with acaricide, various forms of farm-based management that restrict contact between vector ticks and cattle host have been increasingly adopted in most of the affected areas. However, studies have shown that even in zero-grazed small holder farms, cattle contract ECF mostly through cut and carried forage with infected ticks (Gitau et al., 1994). In much of the endemic areas, different management systems ranging from the most intensive use of acaricide, to systems where very little or even nothing needs to be done except to maintain the existing endemic stability prevail (Billiouw, 2005). The endemic stability is an apparent natural ecological balance between parasite and host.

Genetic adaptation of organisms to their environment, including diseases and pests, is the basis for the enzootic equilibrium. Moll et al. (1981) noted a rapid recovery of calves from *T. mutans* and *T. parva* infection in endemic areas, which could be related to increased immuno-responsiveness of the calves. This resistance has been selected over generations as a result of the constant infection pressure exerted on the cattle population in the endemic areas. The protective factor involved in such resistance to *Theileria* infection is not well understood. However, a possibility is that cattle in endemic area for theileriosis have been selected for low susceptibility to *Theileria* infection. However, Norval et al. (1992) have ascertained that the potential resistance of indigenous breeds is ignored and large numbers of cattle in several countries are still subjected to unnecessary intensive acaricide application. Young (1981) has reported that animals raised in endemic areas to ECF develop increased resistance to

tick infestation which reduces the feeding success of ticks, the weight of replete ticks and the transmission of *Theileria* parasites.The ideal strategy would be to make maximum use of acquired resistance to both ticks and the disease they cause, modified to suit each ecological region or epidemiological state.

2.3 Vaccination

Most of the animals at risk are maintained in extensive management systems and traditional tick control and chemotherapy alone hardly represent efficient methods for preventing damage caused by ECF. The limitations associated with these control methods and the shift away from intensive acaricide use in Africa have prompted a search for new, more effective and sustainable control strategies.

It was generally recognized that cattle in enzootic areas could become immune after surviving a *T. parva* infection. Neitz (1953) found that cattle exposed to tick infection and given oxytetracycline during the incubation period could survive and develop immunity. This knowledge was used to develop an immunisation method based on a tick stabilate inducing infection followed by concomitant treatment (Radley et al., 1975).

This method of vaccination commonly known as “Infection and Treatment Method (ITM)” has been tested in the field in several countries in eastern and southern Africa. Results demonstrated that it is very effective in a given area if properly administered (Paling and Geysen, 1981; Musisi et al., 1989; Mutugi et al., 1988). Nevertheless, the method was found to be insufficient for the ECF region-wide application.

The immunity conferred by one strain affords protection against only a limited number of different *T. parva* stocks (Cunningham et al., 1973; Irvin et al., 1983). Failure to protect fully has been attributed to the existence of variability in the parasite population. Determining live parasite immunisation approach must be based on the parasite immunological diversity found in an area (Geysen et al., 1999).

2.3.1 Basis of the immune responses

A number of research studies have focused on analysis of the immune responses to *Theileria* parasites with the aim of defining the nature and antigenic specificity of responses that confer protection in order to identify potential protective antigens. These have provided evidence that immunity to the parasite can operate at two levels:

blocking of the infection by antibody specific for sporozoite surface antigen and killing of parasitized leucocytes by cytotoxic T cells.

Animals in endemic areas respond to *T. parva* infection by mounting humoral responses that decline over months in the absence of challenge. The serum from immune cattle contains antibodies against all stages of the *T. parva* parasite (Burridge and Kimber, 1972). The most relevant antibody responses are those directed against sporozoite surface antigens (Musoke et al., 1982). Antibodies against sporozoites appear to recognise a wide range of *T. parva* isolates and are correlated with some protection (Musoke et al., 1984).

In vitro studies have showed that a highly conserved 67-KDa protein (p67) and to a lesser extent the polymorphic immunodominant molecule (PIM) were the major neutralising antigens (Toye et al., 1991; Nene et al., 1992). Immunisation of cattle with *Escherichia-coli* derived recombinant p67 gave approximately 70% protection of cattle against severe disease after laboratory challenge (Musoke et al., 1992). However, only a small proportion of immunised cattle were able to show complete neutralisation of infection while the rest showed schizont parasitosis of varying severity.

In addition, Musoke et al. (2005) reported that the p67 vaccine was able to reduce severe ECF in cattle by only 47% and 52% at the coast and central Kenya, respectively. The exact nature of sporozoite-neutralising response against *T. parva* under natural circumstances is still unkown, but high titres of neutralising antibodies induced by vaccination with recombinant p67 are thought to play an important role. However, high antibody titres are not found during natural infection. The sporozoite coat is only exposed for a short time to circulating antibodies while the invasion of a host cell occurs rapidly. In the field, the effect of natural boosting of vaccine induced neutralising antibodies and the proportion of severe reactions resulting from inadequate protection will be difficult to predict under different epidemiological conditions.

There is strong evidence that the long lasting protective immune mechanisms in *T. parva* are cell-mediated and targeted against the schizont-infected lymphocytes (Pearson et al., 1979; McKeever et al., 1994). Animals subjected to a single immunisation by infection and treatment produce little or no sporozoite-neutralising antibodies (Musoke et al., 1992, 2005). The principal effectors of the protective cellular immunity are CTLs directed against the schizont-infected lymphocyts (Emery

and Morrison, 1980; Eugui and Emery, 1981; Morrison et al., 1987; McKeever et al., 1999).

The immune cells responsible for protection through lysis of infected cells belong to CD8+ T-cell subpopulation and are MHC class-I (MHC-I) restricted and parasite specific (Goddeeris et al., 1986a; Goddeeris et al., 1990). The strain specificity of CTL response induced is consistent with the existence of immunological heterogeneity in populations of *T. parva* (McKeever, 2001), which is maintained through sexual recombination in the vector (Morzaria et al., 1992).

Other cellular mechanisms may also contribute to protection of immune cattle against challenge. Baldwin et al. (1987, 1992) isolated parasite-specific CD4+ T cell clone with cytotoxic activity but their role in the immune response had not been determined.

2.3.2 Immune characteristics of host and parasite

2.3.2.1 Host specific immune response

The clinical outcome of *T. parva* infection in cattle may vary with an identical dose of the same parasite stock. Anon (1989) classifies *T. parva* reactions as severe, moderate, mild and subclinical reactions. The reactions depend on the susceptibility of the cattle breed or type (Paling et al., 1991) and on the general condition of the animal and immunity, either active or passive (Perry et al., 1985).

Bos indicus cattle from ECF-endemic areas were found more resistant to *T. parva* infection than *B. indicus* and *B. taurus* cattle from ECF-free areas (Ndungu et al., 2005). In addition, studies have shown that the majority of calves born in enzootic ECF became immune by natural exposure within the first year of life without showing clinical signs (Barnett and Blocklesby, 1966). Barnett (1957) explained the innate resistance of calves from endemic area as the result of genetic selection for *Theileria* resistant animals.

It is believed that because of their innate ability to withstand challenge by virulent forms of *T. parva*, indigenous breeds of cattle may survive without tick control in eastern and central Africa (Dolan and Mc Hardy, 1977; Moll et al., 1986) while taurine breed would not survive if introduced in these areas (Moll et al., 1986). Ndungu et al. (2005) observed a difference in clinical development of ECF between *B. indicus* and *B. taurus* cattle. A variety of cattle breeds exist in Africa which show

differences in parasite susceptibility (Radley, 1978; Dolan and McHardy, 1977) but the differing degrees of resistance to *T. parva* infection among different cattle breeds needs to be elucidated.

Although the mechanism of resistance is not well known, the ability to mount effective protective immunity is likely to be dependent on more effective cell mediated response in indigenous cattle than other breed stocks (Pearson et al., 1982; Baldwin et al., 1986). The effector cells are genetically restricted and variation between animals in the CTL responses and protection is therefore a function of the host type.

2.3.2.2 Parasite strain specific immunity

The main epidemiological problem in the immunisation of *T. parva* has been the existence of immunologically different strains that do not cross-protect (Radley et al., 1975; Paling and Geysen, 1981; Irvin et al., 1983). Even a combination of three East African *T. parva* stocks (Muguga triple cocktail) did not induce always broad protection over the entire ECF zone (Radley et al., 1979). Twenty two stocks from widely separated areas as Zambia, Malawi, Uganda, and Kenya have been characterised on the basis of mAbs raised against three stocks (Muguga, Kiambu5 and Marikebuni) and all fall into one of these three groups described (Minami et al., 1983). It thus appears that there is limited heterogeneity in *T. parva* stocks in many areas of eastern Africa (Irvin et al., 1989; Mutugi et al., 1990a; 1990b).

The lack of cross-protection in some stocks is thought to be dependant on the specificity of the CTL. It is believed that some form of antigenic competition among strain specific epitopes drives the CTL-based immune response. The strain specificity of the immune response has been related to the different MHC-I haplotypes (Goddeeris et al., 1990). There is also evidence that the polymorphism in the T-cell receptor (TCR) repertoires of the host might play a role in the affinity differences towards different epitopes. Some animals might lack good affinity receptors for certain epitopes of *T. parva* strains (Morrison et al., 1995).

Immunisation induces strain specific responses in some animals and cross-reactive CTLs in others which correlate with protection. These differences are defined mainly by the genetic make-up of the parasite. In the field, the complexity of strain composition might vary, depending on the transmission intensity prevailing in

different epidemiological situations. Parasite immunisation for a certain area must be based on developing a study of the immunological diversity in that area. Two different approaches are being followed: A “local strain approach” using a broadly protective local stock of *T. parva* and a “cocktail approach”, using a combination of stocks to provide a wider coverage against variants in a wide enzootic area (Dolan, 1987).

Extensive vaccination campaigns have been conducted in Zambia, Tanzania and Uganda. Immunised animals remain persistent carriers of infection. Inherent to the carrier state is the risk of introduction of foreign *T. parva* strains in the case of the cocktail approach. Geysen et al. (1999) reported that in southern Zambia, parasites with a different genotype from that of local parasites, introduced as part of the immunisation campaign have become established and are causing disease. This has resulted in the local strain approach to vaccination against ECF in Zambia. In addition, genetic recombination between *T. parva* stocks may take place giving rise to antigenic variants and an unpredictable epidemiological outcome in the field.

2.3.2.3 Interactions between host and parasite

Survival of a *T. parva* infection can be viewed as a race between the parasite and the host immune system (Emery and Morrison, 1980), whereby the parasite tries to outnumber the host’s defence force. The parasite seems to manipulate its environment for fast multiplication while depressing effective immune responses. The interaction might be complex, involving not only transmission intensities, but also dominant and stage specific immune responses and making predictions hazardous (Cebula and LeClerc, 1997).

The outcome of the host-parasite interaction could account for the more variable results observed with certain *T. parva* stocks. It is also known that certain stocks of *T. parva* provide protection against a wider range of parasites than other immunising stocks. *T. parva* Marikebuni protects against challenge with the homologous and other heterologous parasites including *T. parva* Muguga, whereas the later does not always protect against heterologous challenge (Irvin et al., 1983).

It can be envisaged that the stock conferring wider protection contain a greater variety of genotypes. However, this seems to contradict with data from cross-immunity trials in which stocks derived from a relatively homogenous field situation, as later defined

by molecular characterisation by Bishop et al. (1994), gave broader protection than would be expected from their lack of genotype complexity.

In endemic areas, the complexity of the parasite stocks can be seen as a result of a strong strain restricted evolution in the CTL response induced by the parasite. This response would be beneficial for the parasite's survival and transmission under conditions where susceptible host species prevail. Evolution towards a dominant strain would restrict parasites with similar epitopes for establishment and maintenance of infection in the same host.

Cohabitation of parasites in the same host species would only be possible if the components did not show cross-reaction, meaning that they should express differences in the epitopes that are important in inducing protective responses. The preliminary evidence that the incidence of infections with mixed parasite genotypes increases with age (Oura et al., 2005) is consistent with frequent infection of already infected animals.

However, challenge with antigenically similar parasites might also result in establishment of infection in the same animal in some instances, since such challenge infections are not controlled by the immune response until 7-10 days after challenge. Studies have shown that following challenge of immune cattle with sporozoites, small numbers of parasitized cells are usually detected for a few days in the draining lymph node and the prepatent period to detection of parasites is similar to, or slightly longer than that in susceptible animals, indicating that the immunity does not prevent infection.

Furthermore, immunisation and challenge of calves with molecularly distinct but cross-protective parasite populations revealed that infection results in transmissible erythrocyte forms in spite of a protective immune response (Katzer et al., 2006). The ability of parasite to persist in immune cattle conveys an enhanced flexibility in fine-tuning a stable relationship between parasite and mammalian host.

2.3.3 Immunogenic diversity in *T. parva*

Maintenance of heterogeneity in *T. parva* parasite virulence would only be possible if there was a virtual lack of effective cross-immunity between strains (Gupta et al., 1994). Differences in the immune responses of the host are often influenced by genetic differences among parasite populations. There is evidence that

epidemiological differences might influence this diversity (Bishop et al., 1994). Antigenic diversity within and between parasite stocks from different geographic regions is a common feature although the extent seems to be limited (Irvin et al., 1983; Hoove et al., 1995). However, the mechanisms generating *T. parva* diversity in different field situations are not well understood.

In an attempt to characterise breakthrough strains through analysis of extensive cross-immunity data, Geysen. (2000) has classified *T. parva* stocks into four categories. There was no indication of correlation between the hierarchical ordering of the parasite stocks based on cross-immunity and monoclonal antibody (mAb) profiles with the geographic origin of the parasites. Shiels et al. (1995) found extensive polymorphism in *T. annulata* isolates with no geographic correlation between genotype and geographical location.

In addition, molecular characterisation data revealed similar profiles for *T. parva* Uganda and Mariakani (Kenya), suggesting that they could be related (Conrad et al., 1989; Chen et al., 1991). It has been suggested that there are only a limited number of largely different stocks in the field. Moreover, the solid immunity engendered by natural *T. parva* infections in different epidemiological environments presumes the presence of a few different strains in a certain area. A clear understanding of the *T. parva* heterogeneity which exists in a region would help identify possible breakthrough strains to optimise the immunization programmes against ECF.

However, the subsequent question is how to establish a correlation between the degree of relatedness amongst different stocks and the cross-protection data. Cross-immunity trials could provide a good idea of the immunogenic and antigenic diversity found in *T. parva*. However there is evidence that many cross-immunity studies were conducted with stocks that have been characterised as a mixture of different *T. parva* genotypes (Conrad et al., 1989, Goddeeris et al., 1990). Moreover, cross-immunity trials are cumbersome and expensive for use in field isolates. An *in vitro* screening method would be of interest in epidemiological studies of *T. parva* stocks from the field.

In recent years, considerable effort has been devoted in identifying markers that distinguish immunologically different strains of *T. parva.* A series of monoclonal antibodies raised against the schizonts can readily discern antigenic differences between parasite stocks from various locations (Minami et al., 1983). However, none

of the available monoclonal antibody-based methods correlate with cross-immunity and the nature of diversity remains undefined.

A PCR-RFLP based method using highly resolving markers provides currently the most useful technique for characterising *T. parva* isolates in the field. A number of polymorphic markers from multiple loci is now available. Among those, the PIM gives the high resolution, subdividing the field parasites into different groups. In addition, there is evidence that the polymorphic nature of PIM protein is strongly correlated with the antigenic diversity in ECF (Toye et al., 1995; Geysen et al., 2004). Analysed of the PIM-derived *T. parva* diversity in the field could help to establish possible relationships between genotype data with their immunologic properties.

3 Overview on East Coast Fever (ECF) in Rwanda

The importance of ECF (theileriosis) in Rwanda and its control measures must be viewed in the light of the fundamental agricultural changes which are taking place throughout the country. The increasing demand in arable land is putting considerable pressure on pasture, while the traditional cattle system, governed by security aspects and social status is loosing its significance. This process inevitably results in a shift of the extensive grazing practice to a more intensive way of livestock production using cattle of more productive genotypes. The upgrading of local breeds by crossbreeding with exotic cattle greatly increases the risk of major economic losses caused by theileriosis. Formulation of appropriately designed control measures is a key strategy for the success of this livestock improvement program.

3.1 Physical characteristics

Rwanda is a small land-locked central African country, lying within latitudes 1°-3° south and longitudes 29°-31° east. Situated immediately south of the Equator, it borders the Democratic Republic of Congo (DRC), Uganda, Tanzania and Burundi. The altitude ranges between 1000m and 3000m and is dominated by mountain ranges and high land plateaus of the great watershed between the Nile and the Congo river basins (Congo-Nile Crete).

The relatively diverse relief has divided Rwanda into four major agro ecological zones (AEZs). The different AEZs are described in Figure 1.2 and Table 1.1. The

medium continental zone (AEZ 2) dominated by a central populous plateau lies between 1500 and 1700 meters above sea level. In the west, the cold conditions of the medium zone became tempered by Lake Kivu which separates Rwanda from DRC (AEZ 3).

East of the plateau, the relatively low land region is located between 1000-1500m (AEZ4) while the mountainous volcanic region in the high land in the northern region reaches altitudes above 1900m (AEZ1). In this region, the Kalisimbi peak culminates at more than 4000 m. This part of the volcano-chain is the only area free from *R. appendiculatus* (FAO report, 1982). In a tick distribution study, Lessard et al. (1990) reported that *R. appendiculatus* was present over 95, 6% of the Rwanda territory.

Climatic conditions in Rwanda are favourable for year round tick development. The average annual temperature in Kigali is 21°C with only small variations between the rainy and the dry season (Fig 1.3). The country enjoys high rainfall from October to June followed by a short dry season (Fig. 1.4). The average monthly rainfall is 85 millimeters, which supports a wide range of crops and vegetation on whatever available patch of land available.

Table 1.1 Description of the four agro-ecological zones of Rwanda and farming systems

Agro-Ecological Zones (AEZ 1-4)	Altitude	Temperature (°C)	Annual rainfall (mm)	Period of rain in a year	Farming system
1. High land (AEZ 1)	>1950 m	14-17	>1400	Sept-July	Restricted
2. Central high plateau (AEZ 2)	1650-1950	18-20	1200-1300	Oct-June	Restricted
3. Western high plateau (AEZ 3)	1550-1900	19-21	1100-1250	Oct-June	Free-range
4. Eastern plateau (AEZ 4)	1000-1550	21-24	800-950	Nov-May	Free-range (South) Fenced (North)

Figure 1.2 Subdivision of the major agro ecological zones (AEZs) in Rwanda

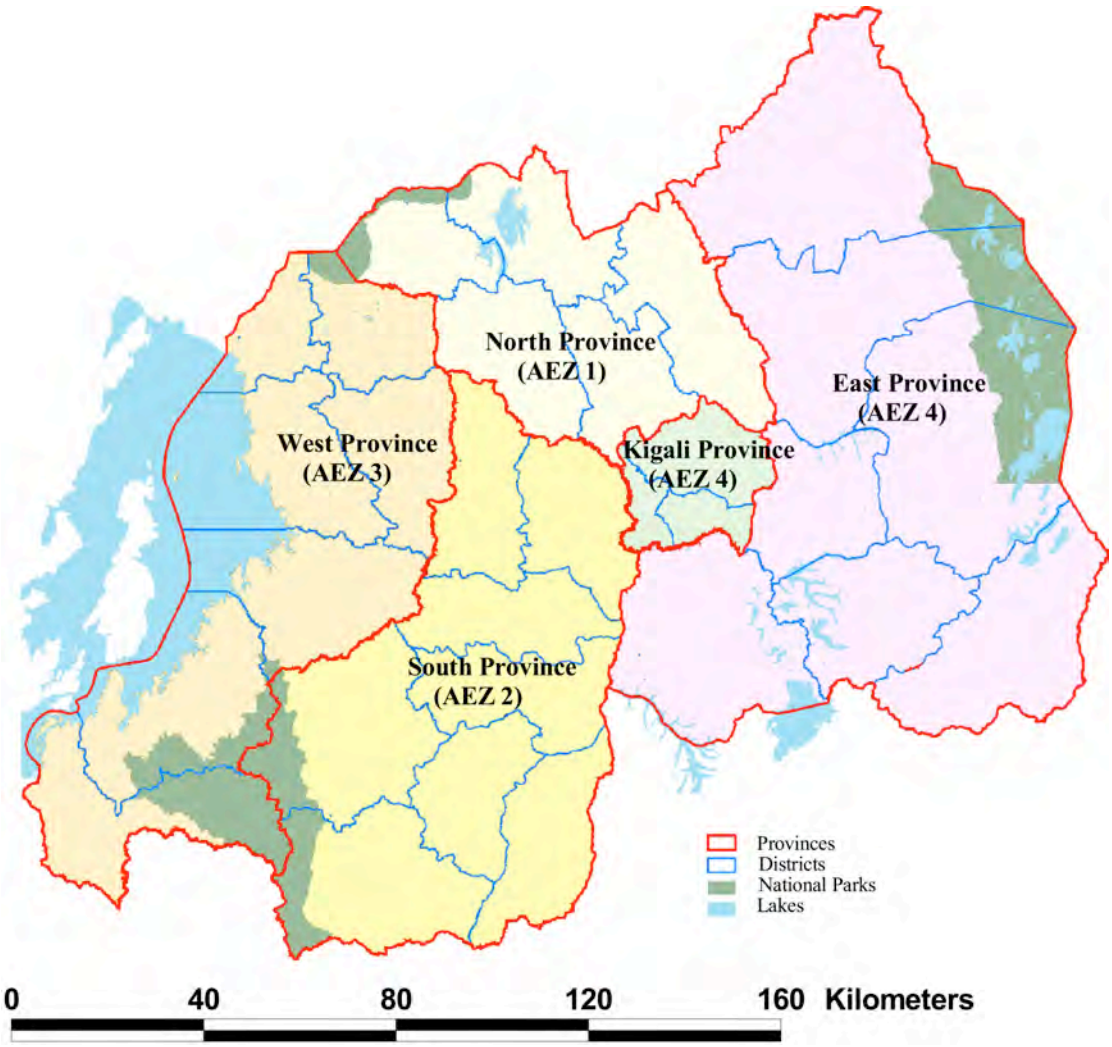


Fig. 1.3 Average temperature in the four AEZs.

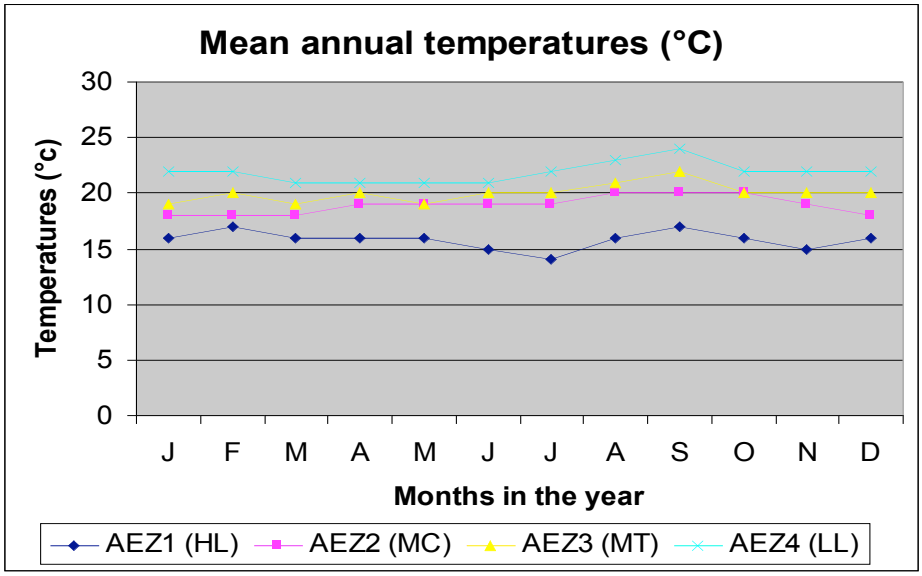
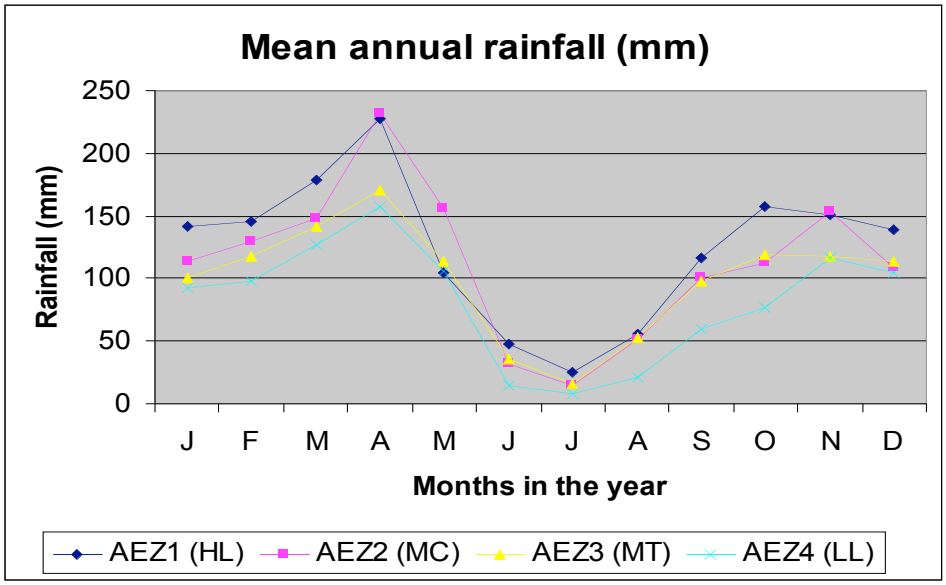


Fig. 1.4 Average rainfall in the four AEZs



3.2 Socio-economic characteristics

Rwanda is rebuilding its economy after the tragic genocide of 1994, followed by several waves of population movements. A recent socio-demographic survey put the mid-2002 population at 8.6 million people. The annual growth rate is estimated at 2.8%. More than 90% of the population lives in rural areas and with little more than 26000 Km^2 of national territory, Rwanda is among the most densely populated

country. Under such high pressure for land, the priority of the Rwandan government is dual: Ensuring the economic development while reducing poverty of the fast growing human population as outlined in the recently prepared EDPRS (Economic development and Poverty Reduction Strategy) paper.

The agriculture strategy emphasises major changes in land tenure, land utilisation and production systems, as well as relationship between agriculture and other sectors of the economy. Among measures supporting the overall priority, the livestock sub-sector was committed to play a crucial role as a prime means of improving the living conditions of the majority of the rural population. A specific program of providing "one cow to every poor family" was the centrepiece of the poverty reduction strategy in the rural sector.

3.3 Livestock sector

The 1990 decade has been a period of devastating and economic decline in Rwanda with severe repercussions on the livestock sector. Cattle are Rwanda's main livestock and it has been estimated that 90% of the national herd have been lost during the 1994 genocide. However, in the after war era, an influx of an estimated 600 000 heads of cattle from returning refugees put the new figures broadly at the pre-crisis level (Ministry of Agriculture report, 1998).

Traditionally, Rwanda is a country of breeders and cattle farming is a daily practice. Livestock, particularly cattle perform a wide variety of economic and social functions in households and national economies. They are a primary investment resource which generates food (milk and meat), cash income, employment and capital stock. They provide manure and draught power for crop production. They are a store of wealth which provides social status and cultural value.

In an effort to restore the rural economy, considerable effort was made to improve and expand the contribution of the livestock sector to the national agriculture output. Initially, the increased output from livestock should help the country meet the ever growing demand for protein, but eventually the government wishes to export any surplus of the primary products such as milk, beef and by-products.

However, the rapid population growth and subsequently the population density have greatly reduced pastures both in quantity and quality. Local breeds are of Sanga type, half-Zebu animals with small humps, called "Ankole". Their production under

common rural conditions is fairly poor. An increase in the number of cattle to meet the ever growing needs in cattle products is hardly possible. Therefore priority is given to upgrade their quality.

In this context, the intensification of livestock production seems to be the only answer and some considerable progress is being made along those lines. In an effort to increase local milk production, the national strategy is to introduce cross-breed cattle to the smallholder farming sector through distribution of heifers and extensive artificial insemination programs. The number of cross-breed has increased markedly in the past years due to a successful artificial insemination programme (Table 1.2).

However, whatever steps taken in this direction, they must include the control of animal diseases, mainly the tick-borne diseases of which ECF is the most important constraint in the development of livestock industry in Rwanda (FAO report, 1982).

Similar upgrading approaches have been attempted at the ISAR (Institut des Sciences Agronomiques du Rwanda) experimental stations. A milk selection program was started early 1970s and the results were encouraging.

Nevertheless, the diffusion of cross-breed animals has been very severely hampered by their high susceptibility to ECF. Two years after the program has started, the government decided to stop all diffusions of cross-breeds until an immunisation technique was elaborated. This problem was not confined to Rwanda alone, but all the neighbouring countries and even those beyond have been facing similar problems. The importance of tick borne-diseases has increased significantly as many affected countries seek to improve their cattle production by introducing exotic "*Bos taurus*" type of animals which are highly susceptible.

Table 1.2 Cattle breed in the four agro ecological zones (extracted from 2006 agricultural survey report, Ministry of Agriculture, Kigali, Rwanda)

Cattle population/AEZs	High land	Medium continental	Medium tempered	Low land	**Total**
Local breeds	174 102	255 164	145 606	460 513	**1 035 385**
Improved breeds	25 355	7 322	30 194	25 906	**88 777**
Total	**199 457**	**262 486**	**175 800**	**486 419**	**1 122 179**

3.4 The history of East Coast Fever

It is difficult to ascertain the distribution of *Theileriosis* in indigenous cattle at the turn of the century. The first description of the endemic form of *Theileriosis* in eastern and Central Africa was made by Bruce et al. (1910) in Uganda, who described a disease of calves known locally as "AMAKEBE" which has reportedly been recognised in that country for generations (Mettam and Carmichael, 1936).

In the same report, the authors quantified the mortality in indigenous calves as follows: "when young calves are nursed, the mortality may be as low as 10-25%. Amongst the agricultural tribes, however, especially under adverse climatic conditions, the mortality is much more severe and up to 40% may succumb annually to this disease alone". Similar clinical reports of an endemic disease in calves came from Rwanda (de Greef, 1919), Tanganyika (Tanzania) (Mettam and Carmichael, 1936) and the Belgian Congo (DRC) (Van Saceghem, 1925).

In Rwanda, this severe disease of calves was locally called "IKIBAGARIRA". Sunshine exposure during the early stage of the grazing time of the calves was incriminated as the cause of this devastating disease in calf population. This is traduced in the traditional husbandry practices by farmers, which consist of keeping calves indoors for almost one year.

3.5 Control methods and constraints

The current strategy of controlling tick and tick-borne diseases in Rwanda is aimed at reducing tick populations by the use of acaricides. This control method is difficult to pursue for a number of reasons. These include high cost due to high frequency of treatment, eventual development of tick resistance to acaricides and increased environmental pollution (Mbogo et al., 1996) and lack of appropriate infrastructures in small farming ECF regions. In recent years, therapeutic Buparvaquone (Butalex, Mallinkrodt veterinary, UK) drugs are available. Drug treatment is effective when started early, but it is extremely expensive and many farmers cannot afford to have their animal treated.

The only alternative strategy is that of integrated control, in which the continuous contact of livestock with vectors and parasites establishes a state of stable equilibrum (Lawrence, 1990). Tatchell (1992) refers to integrated control based on knowledge of

tick ecology, economic justification and endemic stability. The later has been defined as a stable situation with high prevalence of infection but no or little clinical disease in the target population. This situation is obtained when no or minimal tick control is applied and new born animals are infested early in life.

However, the situation may remain unstable in cases where a highly susceptible cattle population is introduced (Anon, 1989). Immunisation would allow a new approach to tick control based on economical considerations. Comparative studies of three tick control methods (intensive, strategic and no control) have shown that strategic tick control combined with immunisation against ECF produced the best economic results (Webb, 1996, Wanjohi et al., 2001). Furthermore, it appears that after 3 years of vaccination programs, animals originally not immunised against ECF withstood lethal challenge, suggesting that immunisation of cattle in the field may favour the establishment of endemic stability to ECF.

However, caution must be made when planning immunisation as a control strategy, since vaccination engenders a carrier state infective to ticks (Morzaria et al., 1987). It follows that vaccination using foreign *T. parva* stocks may introduce antigenic variants of *T. parva* into geographic areas where non-vaccinated cattle are enjoying endemic stability with indigenous strains. This may result in disease outbreaks caused by the antigenic variants. The foreign *vs* local strain approach in immunisation strategy remains a matter of controversy.

3.6 Development of immunisation programmes

Jizierski et al. (1959) described the successful immunisation of Rwandan cattle against ECF at the INEAC (Institut National pour l'Etude Agronomique du Congo Belge) using infection and treatment method based on the work of Neitz (1953). However, the lengthy drug administration (Aureomycin) meant that the method was far from practical. Using the same method, several other attempts were made by breeding stations to immunise selected breeding bulls in 1970's. A supernatant of ground ticks was inoculated into each animal, followed by four injections of whatever tetracycline was available at the dosage of 5 mg/Kg body weight.

Based on the previous work done at Muguga, an FAO project was implemented in early 1980s with the main objective of developing an immunisation control program. A stabilate-based vaccine using three local strains was developed and used especially

in modern farming. Despite the fact that the vaccine stocks have been lost during the 1994 war, it is likely that the vaccine would yield little success in immunising the current herd due to its diverse origin. Little is known about ECF in the after war era and no figures can be given on the exact distribution. Despite the lack of authentic data, ECF must be considered as the most important disease in Rwanda (Bazarusanga, 1999). Formulation of an efficient control policy has to be based not only on a sound knowledge of the disease epidemiology and the vector ecology but also on the nature of the farming system, the general economic situation and the socio-economic considerations prevailing in the country.

4 Conclusion

Since the discovery of *T. parva* in the very beginning of the last century, a number of studies have been carried out to understand the epidemiology of the disease it causes in order to design appropriate control strategies. However, interaction between the parasite, the cattle host and the vector ticks in different environments has led to more complex epidemiological relationships of this parasitic infection. While an apparent natural ecological balance refer to as "endemic stability" between parasite and host where little or no clinical cases occur in some areas, different situations of unstable endemicity prevails in some others, leading to high mortalities in susceptible populations (Norval et al., 1992; Billiouw et al., 2005). The nature of intervention needs therefore to match with the epidemiological situations that prevail in different geographic regions endemic to ECF.

In a particular region, the overall epidemiology of the disease will be driven mainly by the climatic requirements of the vector (Norval et al., 1992) and the ratios of contact between ticks and the cattle hosts. Data on the presence of *R. appendiculatus* are therefore essential for a reliable assessment of different epidemiological states. There is a pressing need for new extensive studies to define areas of differing *T. parva* transmission intensity.

In Rwanda, important changes in agriculture production strategies to meet the MDG (Millennium Development Goal) have put more emphasis on livestock production, particularly cattle breeding. The *"one cow to every poor household"* countrywide program is viewed as the back-bone of the Economic Development and Poverty Reduction Strategy (EDPRS) in medium term. The important cultural value

surrounding a cow in a daily living of a Rwandan farmer has been the major driving force for the successful implementation of this program.

More than 90% of the work force is involved in rural farming. Providing cattle to every rural family has been identified as the only measure to address the poverty problem. A number of pure exotic breed as well as cross breed cattle have been acquired through national budget and donor assistance. However, tick-borne diseases remain a serious obstacle and the sustainability of the program is subjected to efficient control of these pathogens, particularly theileriosis.

Climatic conditions are ideal for year round tick survival in most of Rwanda and ECF is endemic (Paling and Geysen, 1981; Bazarusanga, 1999). However, little is know about the abundance of *R. appendiculatus* and the disease they transmit in different farming communities throughout the major agro ecological regions. Previous studies have reported that *R. appendiculatus* is the most dominant species (Paling and Geysen, 1981). Given the rapid changes that are taking place with regards to land use and vegetation cover throughout the country, a better understanding of the variations in vector distribution and the subsequent infection dynamics would contribute to define adequately the range of existing epidemiological states, but most importantly to develop suitable control options to be implemented.

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Objectives of the thesis

General objective

The overall objective was to assess the epidemiological situation of East Coast Fever (ECF) prevailing in the major ecological regions of Rwanda. The obtained results would lead to recommend appropriate control strategies as supporting measures to the livestock production.

Specific objectives

Three specific objectives were set as a pre requisite to achieve the broadly defined objective with regard to ECF as follows:

1. To collect data on the prevailing tick populations and their relative ecological distribution
2. To estimate the intensity of *Theileria parva* transmission by *Rhipicephalus appendiculatus* field ticks in relation to existing farming systems
3. To assess the prevalence of *Theileria* infections in the country

Chapter 2

An update on the ecological distribution of Ixodid ticks infesting cattle in Rwanda: Countrywide cross-sectional survey in the wet and the dry season

Adapted from:

Bazarusanga, T., Geysen, D., Vercruysse, J., Madder, M., 2007. An update on the ecological distribution of Ixodid ticks infesting cattle in Rwanda: Countrywide cross-sectional survey in the wet and the dry season. Exp Appl Acarol, 43: 279-291.

2.1 Introduction

Tick infestations and tick-borne diseases (TBD) are considered as one of the most economic constraint to successful cattle industry in Rwanda. Direct effects of ticks on their hosts include tick toxicosis, metabolic disturbances, anaemia and tick worry which can result in production losses or deaths (O'Kelly and Seithert, 1969). In addition to damages caused on hides and skins, tick-bite wounds can become secondarily infected with bacteria (Lightfoot and Norval, 1981). Current breed improvement programmes in Rwanda are severely hampered by the protozoan tick-borne parasites, especially East Cost fever (ECF) (Bazarusanga, 1999). Although anaplasmosis, babesiosis and cowdriosis are also widespread in the country, they are of secondary importance (Bazarusanga, 1999).

Control of ticks and TBDs is chiefly focused on ECF and relies on the use of costly drug treatment and acaricide applications. More recently, the use of the most environmentally compatible and cost-effective immunisation method (Mukhebi et al., 1995; Pegram et al., 1996) has been proposed as an alternative for the control of ECF. A bilateral research project between the Belgian and the Rwandan governments to identify potential ECF vaccine strains has been initiated. However, though ECF can be controlled by means of immunisation technology, which combines the infection and treatment method (Radley, 1981) with strategic acaricide use, a breakdown in tick control may cause considerable losses from other tick-borne diseases.

Successful control strategy of ticks and the diseases they transmit depends on improving our understanding on the distribution and the dynamics of the tick vector in the field. There is no recent estimate on the geographical distribution of the tick species infesting cattle as well as their impact on livestock production in the country. The only available report describes the dominant distribution of three major Ixodid tick species (*Rhipicephalus appendiculatus, Amblyomma variegatum* and *Boophilus decoloratus)* (FAO, 1982) as a result of small tick collections from a region-wide limited survey.

As a result of recent molecular studies (Murrell et al., 2000, Beati and Kearans, 2001) the taxonomic status of the genus *Boophilus* has been reviewed and it has been proposed to place this genus in the genus *Rhipicephalus*. Considering that no final decision has been taken yet, this genus will still be called *Boophilus* throughout the study.

The present study was conducted as part of an extensive epidemiological investigation aimed at collecting baseline data for the development of an effective immunisation programme against ECF. This paper reports on the magnitude of the host' tick burdens, the between season dynamics and the geographical distribution of the variety of the tick species infesting cattle in all the four ecological regions of Rwanda.

2.2 Material and Methods

Ticks were collected during the dry period (August-September 2002) and the wet season (April-May 2003) surveys from a total of 204 (102 per season) randomly selected cattle in the four bio-climatic zones. In each zone, an average number of 6-10 sites were randomly chosen. Three herds were randomy selected in each site and ticks were collected on one animal in a herd. In total, a half body tick collection was performed on 36 animals in the high land, 54 in the medium continental, 54 in the medium tempered and 60 in the low land zones. All visible ticks were collected by the means of fine-forceps after the animals had been restrained on the pen floor. Because of their small size, a hand was thoroughly passed throughout the hair of the animal to collect the maximum of the immature stages. The ticks from each animal were preserved in separate labelled vials containing 70% ethanol, and subsequently counted and identified to genus and species level using a standard stereomicroscope and with the aid of an identification guide of Walker et al. (2003). Part of the specimens was brought to the Institute of Tropical Medicine of Antwerp for subsequent re-identification. Sex was determined in all adult ticks collected. Larvae and nymphs were classified in the same group as immature stages.

2.3 Results

The species and number of ticks collected in the different agro-ecological zones are summarised in Table 2.1 and 2.2. A total of 12814 ticks belonging to six species were collected on the same number of animals (102) during the dry season (6101) and the wet season (6713) surveys in the four ecological zones of Rwanda. Except the dominant position of *R. appendiculatus* tick species, which outnumbered (91.84%) all others in the total tick collection, *B. decoloratus* (6.07%) and *A. variegatum* (1.24%) were also commonly collected. The other rhipicephalids namely *R. compositus*

(0.31%) and *R. evertsi evertsi* (0.46%) together with *Ixodes cavipalpus* (0.08%) were incidental or sporadic infestations.

2.3.1 *Rhipicephalus appendiculatus*

The abundance of adult *R. appendiculatus* ticks was higher at lower altitudes ranging from 11 ticks per animal in AEZ 1 up to 72 ticks in AEZ 4. Ticks were active throughout the year although variation was observed between the different zones. The activity of immature ticks of this species was mainly during the wet season. Similarly to the adults, immatures were less abundant at higher altitudes (AEZ 1). The sex ratio (males:females) was (1:0.7).

2.3.2 *Boophilus decoloratus*

The number of adult *B. decoloratus* ticks collected was relatively low ranging from less than 1 tick per animal in AEZ 4 up to 9 ticks in AEZ 3. Both immature and adult ticks were most abundant at intermediate altitudes (AEZ 2 and 3). No seasonal pattern could be found for this species. The sex ratio (males:females) was (1:2.4). This one host tick had the highest adult: immature ratio (1:0.64).

2.3.3 *Amblyomma variegatum*

Adults of *A. variegatum* were predominant in the dry season and both adults and immatures quasi-absent in the cold high land zones (AEZ 1). The average number of adult *A. variegatum* ticks was less than 2 ticks per animal. Immature stages were predominant in the wet season. The sex ratio (males:females) was (1:0.5).

Table 2.1 Seasonal and ecological tick species distribution in Rwanda

AEZs	Season	Number of animals	Total tick number	Tick burden per animal	Tick species distribution as per sex and stages																	
					R. appendiculatus			*R. compositus*			*R. evertsi eversti*			*A. variegatum*			*B. decoloratus*			*I. cavipalpus*		
					M	F	I	M	F	I	M	F	I	M	F	I	M	F	I	M	F	I
1	Wet	18	156	8.67	93	49	6	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0
	Dry	18	318	17.67	154	68	9	0	0	0	0	0	0	5	1	0	9	61	10	0	1	0
2	Wet	27	2833	104.90	1260	871	491	0	0	0	21	11	0	2	2	9	14	68	84	0	0	0
	Dry	27	1430	52.96	690	593	100	0	0	0	0	0	0	13	3	8	3	13	2	2	3	0
3	Wet	27	1423	52.70	594	394	206	2	1	0	2	0	0	2	0	22	47	74	79	0	0	0
	Dry	27	2254	83.48	1141	703	37	9	5	0	0	0	0	28	19	10	62	113	126	0	1	0
4	Wet	30	2301	76.70	1249	811	179	3	8	0	5	9	1	11	5	9	4	6	1	0	0	0
	Dry	30	2099	77.74	1022	990	58	0	4	1	7	3	0	7	2	1	0	0	2	0	2	0
Tot		204	12814	62.81	6203	4479	1086	17	23	1	35	23	1	68	32	59	139	335	304	2	7	0

AEZ stands for agro ecological zones: 1= High lands, 2= Central high plateau, 3= Western high plateau, 4=Eastern plateau. **M**= adult males, **F**= adult females, **I**= immature stages.

2.3.4 Other rare species

The activity patterns of *R. compositus, R. evertsi evertsi* and *I. cavipalpus* are difficult to describe due to the low numbers of ticks collected for these species (table 2.2). *Ixodes cavipalpus* was however only collected in the dry season (only 9 ticks on 204 animals).

Of the 39 (19%) animals found uninfested, a large proportion (21) belonged to the high land zones. The maximum number of tick infestation (559) on a single animal was found in the low land region. The average tick burdens per animal were significantly lower in the high land (13 ticks) as compared to 68-78 ticks (table 2.2) in the other areas. All pre-imaginal stages (11%), which include larvae and nymphs, constituted 8% of the dry seasonal sample whereas in the wet season, they represented 3%.

Table 2.2 Ecological preferences of various tick species prevalent in Rwanda

AEZs	Tick species						Total
	R.appendiculatus	*R.compositus*	*R.evertsi evertsi*	*A.variegatum*	*B.decoloratus*	*I.cavipalpus*	
1	379 (79.96 %)	8 (1.68%)	0 (0%)	6 (1.26%)	80 (16.88%)	1(0.21%)	474
2	4005 (93.95%)	0 (0%)	32 (0.75%)	37 (0.86%)	184 (4.31%)	5 (0.11%)	4263
3	3075 (83.63%)	17 (0.46%)	2 (0.05%)	81 (2.20%)	501 (13.62%)	1 (0.02%)	3677
4	4309 (97.93%)	16 (0.36%)	25 (0.56%)	35 (0.79%)	13 (0.29%)	2 (0.04%)	4400
Total (%)	11768 (91.84%)	41 (0.31%)	59 (0.46%)	159 (1.24%)	778 (6.07%)	9 (0.08%)	12814 (100%)

2.4 Discussion

2.4.1 Tick species diversity

The results obtained are consistent with previous reports as far as the presence of important cattle ticks throughout the region (Paling and Geysen, 1981; Kaiser et al, 1988), but this is the first study so far performed in Rwanda on an ecological and seasonal basis. Three main species, *R. appendiculatus*, *B. decoloratus* and *A. variegatum* were commonly collected whereas the remaining group of three other species (*R. compositus, R. evertsi evertsi and I. cavipalpus*) represented incidentally or sporadic infestations. It is rare to find more than six tick species infesting their host in a given ecological zone (Punyua et al., 1991). No recent information on ticks is available in Rwanda and our knowledge is mainly based on unpublished data and personal communications. However, East Coast fever transmitted by the brown ear tick (*R. appendiculatus*) and *Anaplasma marginale* along with *Babesia bigemina* caused by *B. decoloratus* are described as the major tick-borne pathogens affecting cattle in Rwanda (FAO, 1982; Bazarusanga, 1999, Bazarusanga et al., 2007a). This clearly corroborates our results and confirms earlier reports on major tick occurrences in the country.

It is generally admitted that potential distributions of tick species are principally determined by the bio-climatic envelop (Cummings, 2002; Olwoch et al., 2003; Estrada-Penã, 2003). The three dominant tick species are therefore suggested to be in equilibrium with the current climate. However, Pearson and Dawson (2003) reported that while the bio-climatic approach could provide a useful first approximation, many other factors such as the dispersal ability by host and vegetation might have a potential impact in the mapping of various tick species. In the present study, it is hypothesised that with massive influx of cattle populations in Rwanda during the post war period, introduction of new tick species may have occurred. The smaller populations of *I. cavipalpus* together with the rhipicephalids other than *R. appendiculatus* may not only be an indication of the known poor host specificity in those species (Hlatshwayo et al., 2002), but also the unsuitability of the habitat for their development and survival. Hutchinson (1957) defined the fundamental ecological niche as comprising those environmental conditions within which a species can survive and grow. It is usually accepted that the climate can not only determine

the tolerance range of a species, but also involves selection against phenotypes that are poorly adapted to local conditions (Davis and Shaw, 2001). The small numbers recovered might be considered as accidental importations of species that did not find their ecological preference.

Rhipicephalus evertsi evertsi has been the most studied species and is more common in regions receiving between 400 and 1000 mm of rain fall (Macivor and Horak, 2003). Similarly, the climatic requirements for *I. cavipalpus* ranges from 900-1000 mm mean annual rainfall and three months continuous dry season in Uganda to 500-750 mm in Tanzania and Kenya (Matthysse and Colbo, 1987). High moisture values recorded in most regions of Rwanda may have been critical for the development and establishment of both species. Although their numbers were much lower, *R. evertsi evertsi* contributed to the tick worry problems by causing open wounds at their aggregation sites.

2.4.2 Abundance, phenology and relative distribution

In addition to the relatively important variety in tick species, the tick burden in the country was found very high with a negative gradient from the eastern plateau (AEZ 4) to the high land (AEZ 1) zones. The particularly higher population pressure on land in the AEZ 1, which leads to more intensification of the livestock production, coupled with unfavourable climatic conditions at an altitude of over 1950 m, undoubtedly resulted in low tick survival and infestations. The observation of lower tick infestations in high land correlates with earlier findings from Paling and Geysen (1981), where high tick burdens were recorded in the whole of Rwanda except at higher altitudes. Most regions of the country provide an optimal temperature for the development of all tick instars on a year round basis.

The superabundant pattern recorded in *R. appendiculatus* seems to dictate the entire picture of the tick population in Rwanda. This relatively dominant position in all the zones of the country reflects the ecological plasticity of this species, which is most frequently found between 1000 m and 2000 m altitude (Berggren, 1978). The presence of ticks throughout the year indicates that *R. appendiculatus* has more than one generation a year as is observed in more equatorial regions of its distribution where no synchronisation of the life cycle is needed (Madder et al., 2002).

Boophilus decoloratus, the second most important species also had a ubiquitous distribution with a preference for intermediate altitudes as previously recorded by Matthysse and Colbo (1987). The later authors describe the presence of *B. decoloratus* in Uganda at elevations from just over 600 meter to 2300 m. The lack of a seasonal pattern and the simultaneous occurrence of both adult and immature ticks indicate a continuous development and reproduction of this species as recorded in many other countries (Matthysse and Colbo, 1987; Berkvens et al., 1998).

In the present study, special care was taken in the identification of *Boophilus spp*. It is of interest to note the absence of *B. microplus*, whose trend to invade new areas previously colonised by *B. decoloratus* has been demonstrated by various authors (Theiler, 1962; MacLeod and Mwanaumo, 1978; Berkvens et al., 1998, Tønnesen et al, 2004). The spread of *B. microplus* has been recorded in neighbouring Tanzania (Lynen, personal communication) and it is believed that there are large geographic areas at risk of colonization by *B. microplus* in Africa (Estrada-Penã et al., 2006).

While *A. variegatum* is known to occur in semi-arid as well as humid regions (Yeoman and Walker, 1967), light infestations were found in this study and the species was virtually absent in highly humid zones. This is most likely related with the high altitude in this zone (AEZ 1). In neighboring Uganda, *A. variegatum* was also only found up to altitudes of 1800 m (Matthysse and Colbo, 1987) corresponding to the altitude range in AEZ 2.

Our results are in agreement with observations in Zimbabwe where *A. variegatum* previously occurred in drier lowveld areas surrounding the Zambezi valley (Norval , 1994) but later on established in western parts of the highveld plateau and started to encroach on the predominantly heartwater-free central and eastern highveld regions (Peter et al., 1998). *Amblyomma variegatum* showed similar climatic preference as *R. appendiculatus* and was almost absent at higher elevations. No seasonal differences in tick numbers were observed in contrast to populations in southern Africa where this species is characterized by a strict seasonal activity with a morphogenetic diapause in the engorged female ticks (Pegram et al., 1988).

2.4.3 Unbalanced ratios between mature and immature stages

In this survey, lower infestation of immature stages on cattle compared to adult stages was not unexpected, since host-specificity of the pre-imaginal stages is generally very low (Norval, 1982). Furthermore, the method used to collect immature stages was not the most efficient. More larvae and nymphs would probably have been collected had the cattle hair been combed. In previous studies, a poor correlation between larval and adult results has also been observed and was due to the differential efficiency in sampling methods (Randolph and Rogers, 1997; Zieger et al., 1998). The field data can only give relative estimates of the abundance of the different life-stages of the tick on their hosts.

To correct the under-counting of the immature stages, Randolph (1994, 1997) proposed a correction by multiplying all the actual counts of larvae by 100 and those of nymphs by 10 for three host ticks. However, the particular life cycle of various tick species should be a key variable in whatever step taken for adjustments in the different development stages. In the three most prevalent species, differences in adult:immature stage ratios were noted. The highest ratio was found in the one host *B. decoloratus* species while the three hosts' *R. appendiculatus* species had the lowest ratio probably due to the relatively higher likelihood in off-host dependent mortality in the two or three host tick species.

Moisture appears to be critical for the off-host developmental stages since mortality is highest where it is both too dry and too wet. Although ticks are found on their host on a year round basis in the equatorial regions, Randolph and Rogers (1997) stated a discernible seasonality especially in the immature ticks. Lower numbers in the immature stages found during the wet season in Rwanda are in line with earlier reports and may be explained by high-moisture dependant mortality during the rain period. However, it is believed that seasonal patterns in equatorial areas are not only determined by abiotic factors (Randolph, 1994), superimposed biotic factors such as the recent changes in the managerial system in Rwanda with most animals kept indoors may act negatively on the probability for the larvae and nymphs to successfully contact a host.

Despite the low number of immature stages, the present results are consistent with the general agreement in relation to the overlapping pattern in the activity of all tick instars earlier described in the region (McCulloch, 1978; Paling and Geysen, 1981;

Kaiser et al., 1982). This would mean an almost constant presence of transmitting instars of TBDs throughout the whole year.

It can be concluded that *R. appendiculatus* is the predominant tick species in Rwanda and close to the geographical centre of its distribution. As in many parts of eastern and central Africa, tick populations experience no prolonged dry season and all stages are present throughout the year in the whole country, except in the mountainous marginal regions.

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Chapter 3

Estimation of the *Theileria parva* entomological inoculation rate (EIR) and its confidence intervals in three different farming systems in Rwanda

Adapted from:

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3.1 Introduction

Data on prevalence, infestation burden and seasonal dynamics of ixodid ticks are important variables in the epidemiology and control of East Coast fever (ECF). In Rwanda, *R. appendiculatus* is the most abundant tick on cattle (Paling and Geysen, 1981, Chapter 2).

The numbers of *R. appendiculatus* ticks on the host is an indicator of the possible intensity of transmission of *T. parva* infection (Lessard et al., 1990). However, the overall *T. parva* transmission is mainly determined by the proportion of infected *R. appendiculatus* in this population that successfully attach to the host. Data obtained from field ticks show variation in prevalence of infection (Walker et al., 1981; Moll et al., 1986, Kariuki et al., 1995, Watt et al., 1997) as a function of variable vector/host contact conditions. Differences in husbandry practices influencing host-tick interactions result in different epidemiological situations of *T. parva* infection (Gitau et al., 2000; Rubaire-Akiiki et al., 2004).

In Rwanda, tick load infesting animals varies depending on the management method. Paling et al. (1991) reported a mean daily *R. appendiculatus* tick burden ranging from 20 to over 150 per animal. A number of farming systems from free-range to restricted grazing or fencing is practiced. Zero-grazing is currently being promoted to prevent contacts between ticks and cattle. However, the incidence of ECF remains high even in zero-grazed farming units, probably due to ticks present in the cut and carried forage (Gitau et al., 1994).

Estimation of the infection rates in vector populations is essential in the calculation of the intensity of transmission of vector-borne diseases and in evaluating the effect control programs may have on the transmission of these pathogens. Transmission intensity of *T. parva* at a given site is estimated by the entomological inoculation rates (EIR). This parameter gives the number of infective ticks attaching on an animal and can be determined as a product of the number of ticks feeding on cattle for a given period of time and the proportion of them that are *T. parva* infected (Geysen, 2000).

The prevalence of infection in host-seeking ticks is expected to be low (Moll et al., 1986; Ogden et al., 2003). Precise estimation of tick infection prevalence therefore requires the examination of large numbers of ticks. The routine method based on the detection of infective sporozoites of *T. parva* in stained individual tick salivary glands (Young and Leitch, 1982; Voigt et al., 1995) is cumbersome, particularly when large

numbers of ticks must be analysed. In addition, this method does not allow the discrimination between *T. parva* and *T. Taurotragi* which frequently co-exist in *R. appendiculatus* (Norval et al., 1992).

The PCR methods are as sensitive as the standard staining method (Chen et al., 1991; Watt et al., 1997) but have the advantage of being highly specific and subject to pooling of samples. A PCR-based pooling method has been successfully used to detect *Onchocerca volvulus* infection in pools of the *Simulium* vector population (Katholi et al., 1995). To estimate the infection prevalence from the proportion of positive pools, however, pooling must be random and the PCR assay must be able to detect a single infected *R. appendiculatus* in a pool containing large numbers of uninfected ticks (Katholi et al., 1995).

The objectives of this study was (i) to estimate the *T. parva* infection prevalence in *R. appendiculatus* ticks collected from the grass and (ii) to estimate the *T. parva* entomological inoculation rate (EIR) and its confidence intervals, from the estimated infection prevalence in ticks and the tick burden on cattle in three farming systems in Rwanda.

3.2 Materials and methods

3.2.1 Study environment and farming systems

The study was conducted in the eastern low (AEZ 4) and central high plateaus (AEZ 2) of Rwanda (Fig. 1.2) where the highest *R. appendiculatus* tick loads are observed on cattle (chapter 2). Most of the animals in the study areas are of Ankole type kept under different management systems. The eastern low plateau is subdivided in a non-project and a project zone. (i) The non-project zone is located in the southern part and a free-range farming system is the rule. Adult animals move from pasture to pasture over very long distances depending on water and feed availability whereas calves are kept indoors for almost their first year of life. Acaricides are infrequently applied (once every 2 to 3 months) and the tick infestation is high. (ii) A more structured husbandry system is found in the project zone in the northern part of the eastern plateau and all age categories (calves and adult cattle) are kept on permanently fenced farms. A weekly acaricide application is practiced. (iii) The densely populated central high plateau has a marked land shortage for livestock and agriculture activities. The majority of cattle owners are small holder farmers keeping a limited number of cattle.

Restricted grazing on small land pastures is the rule and tick control is practiced at variable frequencies.

3.2.2 Tick abundance

Data on tick numbers used in this study were derived from a cross-sectional tick survey conducted in the wet season in April-May 2003 as described by Bazarusanga et al. (2007a). Half-body tick counts were performed on 12, 15 and 18 randomly selected animals in the fenced, free-range and restricted grazing systems, respectively on the eastern low (AEZ 4) and central high (AEZ 2) plateaus (Fig 1.2).

3.2.3 Questing *R. appendiculatus* collections

In the wet season in April-May 2004, a total of 150, 700 and 750 adult questing ticks were collected from pasture through blanket dragging techniques as described by Short and Norval (1981) in the fenced, open and restricted grazing systems, respectively. To increase the chances of collecting a high number of questing ticks, visited sites in each farming system were selected in locations where higher numbers of ticks were found on cattle during the previous tick survey. After collection, the ticks were immediately put in 70% alcohol. Once at the laboratory, the ticks were thoroughly washed with sterile water to remove the alcohol, air-dried and kept at −20°C in sealed plastic bags containing silica gel.

3.2.4 Pooling of ticks and DNA extraction

Pooling aims at obtaining proportions of positive results around 50% because these proportions are estimated with more precision in statistical models (i.e. logistic regressions). With estimated infection prevalence in field ticks ranging between 2 and 3% (Paling et al., 1991; Moll et al., 1986; Swai et al., 2006), a pool size of 30 ticks would have a pool prevalence of 45 to 60%. However, increasing the pool size while the number of ticks remains constant reduces the number of pool results, which reduces the precision of the estimation. In addition, low observation numbers present a high risk of all positive or all negative observations, which cannot be analysed in a logistic regression. Last but not least, the dilution of individual DNA might violate the assumption that one infected tick makes the pool positive or, if the DNA is

concentrated, possible PCR inhibitory substances inherent to tick material would be concentrated too. The selection of pool size therefore depends on numerous considerations, including labour and test cost.

Parasite DNA was extracted from 5 pooled ticks using a modified Boom method (1990). Each tick was cut into two pieces using a sterile scalpel blade. Half-tick pieces of 5 whole ticks were pooled together in a 1.5 ml tube containing 250µl of lysis buffer (60mM Tris-HCl, pH 7.4, 60mM EDTA, 10% Tween 20, 1% Triton X-100, 1.6M Guanidine-HCl), 250µl of RODI (reverse osmosis de-ionised) water and 50µl of Proteinase K (Sigma, 20mg/l) and left for over-night incubation at 56°C in a shaking thermomixer. A volume of 4µl of Diatomaceous Earth (Sigma) was then added. The mixture was incubated for one hour at 37°C in the thermomixer, followed by short centrifugation for 20 seconds and the supernatant discarded.

The resultant pellet was washed with 900µl of 70% ethanol (v/v) at 4°C, centrifuged for 20 seconds and the supernatant discarded. The rinsing with ethanol was repeated and the pellet was washed with 900 µl acetone and dried for 20 seconds in a thermoblock at 50°C. To the dried pellet, 90 µl of TE buffer (10mM tris, 1mm EDTA, pH 8) was added and the mixture was incubated for 20 minutes at 60°C under shaking. The final product was subjected to short centrifugation for 40 sec before a 50µl volume of supernatant was extracted and transferred to a new tube.

3.2.5 PCR and RFLP analysis

Extracted DNA was used as a template in a semi-nested PCR amplification of the *Theileria* spp. Cox III mitochondrial locus. Pairs of primers CoxIII F/CoxIII R and CoxIII nR/CoxIII F (Fig. 3.1) were used in the first and second round of a semi-nested PCR, respectively. A 5µl volume of DNA was used as template in a final 25µl PCR reaction volume. Reaction mixtures in the first round contained 1µl of Y sub (Yellow Sub^{TM} GENEO BIO Products, Germany), 3.33µl of milli-Q water, 12.5µl of buffer (20 mM tris-HCL, pH 8.4, 100 mM KCl, 0.2% Triton X-100, 1.6 mM $MgCl_{2)}$, 2µl of dNTP's (Deoxyribo-nucleotide triphosphate), 0.4µl of each primer (25 umol/µl) and 0.37µl of *Taq* polymerase (Silverstar DNA polymerase, Eurogentec, Belgium). Each sample was overlaid with fine mineral oil and amplification took place in a heating block of a programmable thermocycle (PTC-100^{TM}, MJ Research) as follows: denaturation at 92°C for 30 sec, annealing temperature of 59°C for 45 sec and 1 min

elongation at 72°C. The amplification cycle was repeated 39 times. The second round of the semi-nested PCR reaction was carried out in a total volume of 25µl and 0.5µl of the first round was added to the reaction mixture comprising 24.5µl of master mix: 1µl of Y sub (Yellow Sub^{TM} GENEO BIO Products, Germany), 7.95µl of milli-Q water, 12.5µl of buffer (20 mM tris-HCL, pH 8.4, 100 mM KCl, 0.2% Triton X-100, 1.6 mM $MgCl_{2)}$, 2µl of dNTP's (Deoxyribo-nucleotide triphosphate), 0.4µl of each primer (25 umol/µl) and 0.25µl of *Taq* polymerase (Silverstar DNA polymerase, Eurogentec, Belgium). The amplification conditions were as described in the first round, although an annealing temperature of 56°C was used and the amplification cycle was repeated 24 times.

Standard detection with ethidium bromide staining was used after electrophoresis of the amplified samples together with a 100bp molecular weight marker. Positive samples were further analysed to discriminate *T. parva* and *T. taurotragi* infections. Restriction was carried out in a final volume of 15µl, consisting of 4µl DNA and 11µl of RFLP mix containing 0.3µl of restriction enzyme (Biolabs, New England), 9.2µl of milli-Q water and 1.5µl of buffer. The *RsaI* restriction enzyme was used and incubation was done overnight at 37°C. A 4µl volume of the restriction product of each sample was mixed with 2µl of loading buffer and placed on PAGE gel in an electrophoresis tank at 100 V for 2h 40 min. The gel was then incubated in sybr Green for 40 min before photography using UV light and green filter.

Fig. 3.1 *Theileria parva* Cox III gene showing the annealing sites (Red=CoxIII F; Blue=CoxIII R; and Green= CoxIII nF) and *RsaI* restriction sites (Bold).

Atgcgaaacagcgcacaatcatatttaaa**gtac**attaatataattaacatatttgaaactctatatctgtttttata**gtac**aggatt
agataccctggaatatattgattccacatataagaactttattataatgtatgtaaatcaatatttattatacggaactacacttaaa
tatttaagtgttggtgaatttttatgaacagtttaacaatatttataaatagtattagagagataatgacttctacaactatggtaat
gtacgctatatttggaatgtttatattttcagaaatattagtgttttcgacatttatatggggatattttcatttaagattatcgaatcc
aattttactagctgaattaaatgtagaagcctatcttcaaatctctgacgttttaaatactggaagtattttagtatcaataattcttc
atagagttcaagaaagtgccaactttgaaacagacttttttatggaacaattgctattaatagggtttatatttttatcgttacaaaa
tgatgaatattcattaattctatcttatgtgaacaattattggatgacattatatttttcattttaacaggacttcattcgttacatgtat
gtgccggaggcatttttt**gtac**taatacagtcatatttttatgaaggtgatggatctcaaagagatgaagaattcaatgctggagt
gtactggcattttgtagaaatgatttggatagctttaacaatgttgttatttctagcttaa

Fig. 3.2 Cox III positive sample (lane 10) as compared to Cox control amplicon in the first round (lane 13) and the second round (lane 15) of a semi-nested PCR. M is a 100bp marker.

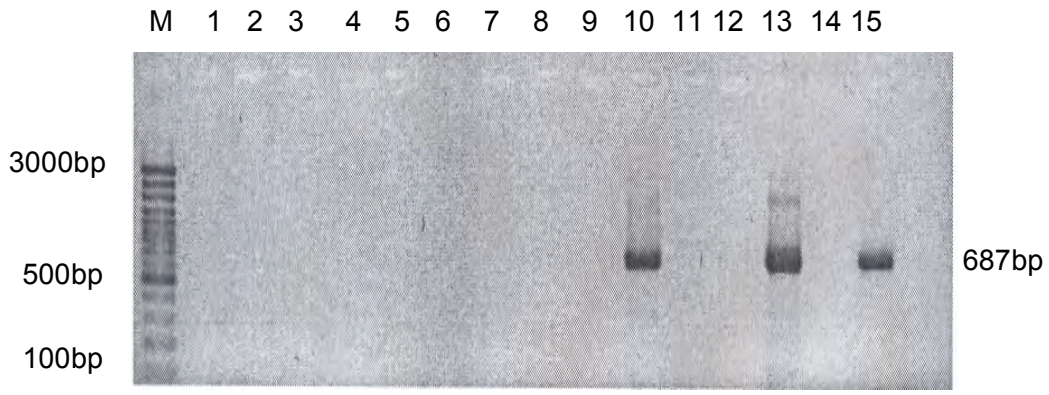
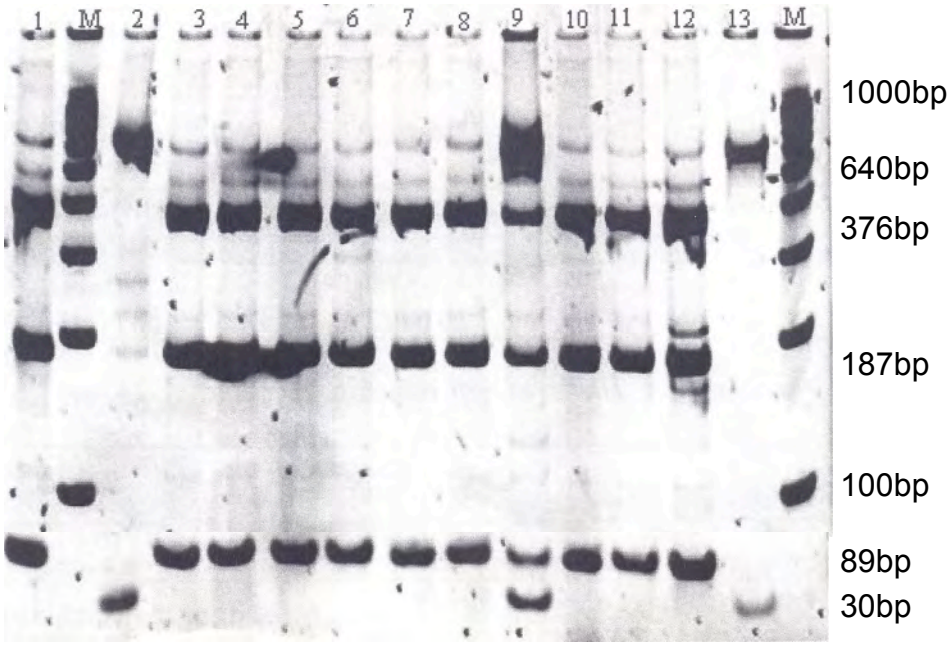


Fig 3.3 Species-specific RFLP profiles of Cox PCR positive amplicon (682bp long) on Sybr Green stained PAGE gel using *Rsa*I restriction enzyme. *T. parva* and *T. taurotragi* profiles were sized against 1000 base pair markers (M). *T. parva* positive profiles (lanes 1; 3-8 and 10-12) show 3 bands (low=89bp; medium=187bp and high= 376bp) whereas the two bands (low=30bp and high= 640bp) characteristic of *T. taurotragi* are found in lanes 2 and 13. Mixed *T. parva/T. taurotagi* infections (lane 9) are characterised by the 5 bands (3 bands for *T. parva* and 2 bands for *T. taurotragi)*.



3.2.6 Statistical analysis and EIR estimation

Statistical analysis was done in Stata 9 (StataCorp2006. Stata Statistical Software: Release 9.2. Texas: Stata Corporation). The pool prevalence was defined as the proportion of pools of ticks that were positive at *T. parva* PCR analysis. The pooled tick prevalence data was analysed using the farming systems as discrete explanatory variable in a logistic regression. Tick abundance was analysed using the same explanatory variable in a negative binomial regression. The linear estimators and the standard errors were estimated for each farming system in both regressions. They were used to define, for each farming system, two separate normal distributions from which paired but independent random values were sampled 100,000 times. The exponents of the values sampled from the distributions defined by the negative binomial regression estimators were used to build half-body tick burden distributions. Whole-body tick burden distributions were constructed by summing paired (but independent) values from half-body tick load distributions. Distributions of infection

prevalence in tick pools were obtained by transforming values sampled from the distributions defined by the logistic regression estimators. The probability formula below was used to transform the estimated pool infection prevalence in individual prevalence: $\mathbf{P_i = 1-(1-P_p)^{1/n}}$ **,** with Pi standing for individual prevalence, P_p for pool prevalence and n for number of ticks in pools.

This transformation relies on two assumptions: samples belonging to a pool are independent and one positive sample in the pool makes the pool positive. Paired (but independent) transformed tick burden and infection prevalence values were then multiplied to generate an EIR distribution. The EIR was expressed as the number of infected *R. appendiculatus* that attach to individual animal over an average tick feeding period (approximately one week). Finally, percentiles 2.5, 50 and 97.5 were estimated for the individual prevalence of infection in ticks, the tick burden and the EIR in each of the 3 farming systems.

3.3 Results

From the total 300 pools tested, 56 (18.67%) gave a positive *T. parva/T taurotragi* amplicon, showing a 682bp expected length (Fig.3.2). Higher number of positive pools was found in restricted (26%) and in fenced (23.33%) than the free-range (8.33%) farming systems. The RFLP method was further used to discriminate between *T. parva* and *T. taurotragi* DNAs. Table 3.1 shows the length of the expected *RsaI*-restriction fragments and Fig. 3.3 the species-specific profiles (*T. parva*: 3 bands at 376bp, 187bp and 89bp; *T. taurotragi*: 2 bands at 640bp and 30bp). *T. parva* was the predominant infection in the fenced and the restricted farming systems whereas *T. taurotragi* was the most prevalent infection in the free-range farming system (Table 3.2).

The pool prevalences and confidence intervals were calculated (Fig. 3.4B) and the total infection prevalence ranged between 1.72% and 5.18%. The free-range system had significantly lower *T. parva* infection in ticks than the fenced ($p=0.002$) and the restricted ($p=0.001$) farming systems but the *T. parva* infection prevalence did not differ between the fenced and the restricted farming systems (Fig. 3.4C).

The fenced farming system had significantly lower mean tick burden per animal than the free-range ($p<0.001$) and the restricted ($p<0.001$) whereas no significant difference was found between the free-range and the later farming system (Fig. 3.4A).

The EIR obtained in the three farming systems differed among them (Fig. 3.4D). The EIR was lower in the fenced farming system (1 infected tick/animal/week) than in the restricted (9 infected ticks/animal/week) but higher than in the free-range (1 infected tick/animal/ 2 weeks) farming systems.

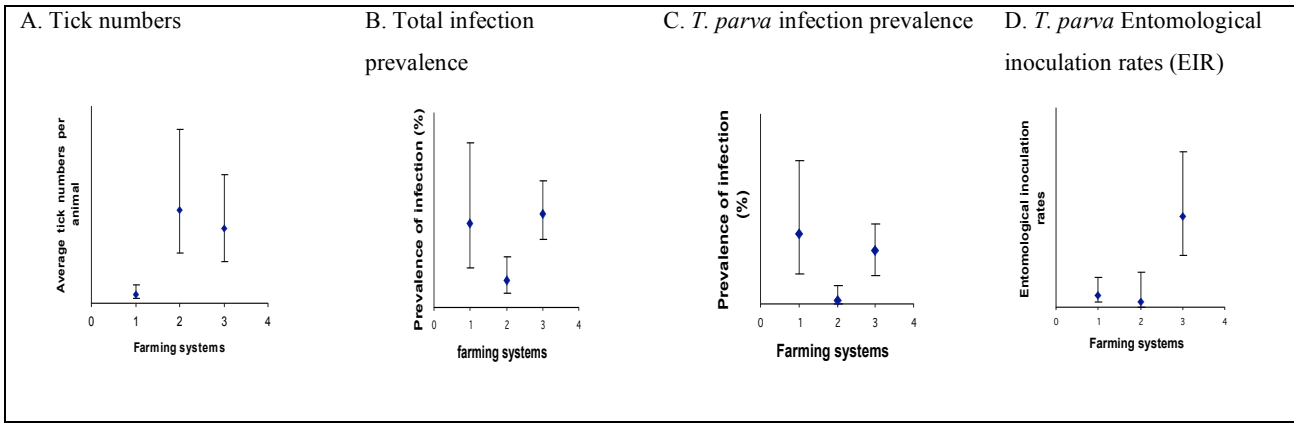
Table 3.1 *RsaI* restriction sites and the resulting fragment length

Number of *RsaI* restriction fragments	Fragment length (bp)	Restriction sites	
		From *RsaI*	To *RsaI*
1	376	264	640
2	187	77	264
3	47	30	77
4	42	640	…
5	30	…	30

Table 3.2 Total number of positive pools in the total tested per grazing system

Farming system	Total number of pools	Positive pools	*T. parva* (single infection)	*T. taurotragi* (Single infection)	*T. parva/T. taurotragi* (mixed infections)
Tethered	150	39 (26%)	27 (18%)	8 (5.33%)	4 (2.66%)
Free-range	120	10 (8.33%)	1 (0.83%)	9 (7.50%)	0
Fenced	30	7 (23.33%)	6 (20%)	1 (3.33%)	0
Total	300	56 (18.67%)	34 (11.33%)	18 (6%)	4 (1.33%)

Fig 3.4 Tick numbers, infection rates and entomological inoculation rates (EIR) in the three farming systems (1: Fenced; 2: Free-range; 3: Restricted)



1	28 (17-46)	5.18 (2.41-10.17)	4.37 (1.92-9.09)	1.20 (0.46-2.96)
2	292 (188-455)	1.72 (0.92-3.15)	0.17 (0.02-1.16)	0.49 (0.06-3.52)
3	233 (155-349)	5.84 (4.26-7.84)	3.89 (2.27-5.57)	9.06 (5.21-15.60)

3.4 Discussion

Suitable ecological conditions for tick development and survivals prevail in the plateaus of Rwanda and the year to year variation in tick numbers is not significant (Paling and Geysen, 1981; Paling et al., 1991; Chapter 2). The aim of this study was to estimate the EIR from tick numbers infesting animals and infection prevalence in field ticks. Previous reports on analysis of infection rates in field ticks were based on individual tick data, a time consuming and costly approach when large numbers need to be examined. The technique described here provides a simple way to estimate the prevalence of *Theileria* infection in pools of unfed, host-seeking *R. appendiculatus* ticks collected from the field. Based on the algorithm model developed by Katholi et al. (1995), the prevalence of *Theileria* infection in *R. appendiculatus* was estimated.

The prevalence of *T. parva / T. taurotragi* ranged between 1.7% and 5.2% in different farming systems. The infection rates in ticks was previously reported to vary from 0% and as high as 25% (Gitau et al., 2000). Our results are consistent with the *Theileria* infection rates detected by the salivary gland staining method in individual field *R. appendiculatus* earlier reported to be 1.8% in Rwanda (Paling et al., 1991) and

2.6% in Kenya (Moll et al., 1986). While there is no simple method for discriminating between *T. parva* and *T. taurotragi* infected salivary glands (Young et al., 1980; Voigt et al., 1995), the PCR-based methods could allow the differentiation between *Theileria* parasites co-infecting *R. appendiculatus*.

The *T. parva* infection prevalence detected by the pooling method was low and varied between 0.2% and 4.4 % in different farming systems but was in line with the 2.7% prevalence detected in individually PCR-tested *R. appendiculatus* collected from field sites in neighbouring Tanzania (Ogden et al., 2003; Swai et al., 2006). The infection level in the tick population is influenced by the presence of clinically diseased or carrier animals in the field (Norval et al., 1992). In study conducted in Tanzania, Swai et al. (2006) have shown that low prevalence prevails in endemic conditions where most of infected ticks acquire infection from carrier animals. It is also known that host-to-tick transmission from carrier cattle is low (Medley et al., 1993) and the *T. parva* carrier state is common in animals in Rwanda (Paling and Geysen, 1981; Bazarusanga, 1999).

In the present study, the proportion of *T. parva* infected ticks was much lower in the free-range than in the restricted and the fenced farming systems. The difference in tick infection rates between the various farming systems can be explained by the fact that in the free-range system, young animals are kept indoors in their first year of life to prevent contact with ticks. Under such management, the ticks feed only on low parasitaemic adult carriers. Our results are consistent with a 5-6 fold lower PCR-based detected parasitaemia previously reported in adult carriers than calves in the free-range farming system (Chapter 5).

Alternatively, the lower infection rates could be also a result of ticks feeding on hosts other than cattle in the free-range pastoral system. This could probably explain the high proportion (9/10) of *T. taurotragi* infected ticks found in the free-range management system. In this farming system, the pastoral system practiced will tend to cause variation in cattle host availability for feeding ticks. This will result in reduced transmission efficiency in that most parasites will die off in the infected ticks. The decline in *T. parva* infection levels in *R. appendiculatus* over time has been demonstrated by Ochanda et al. (2003).

In contrast, the system of fencing or confining animals will result in increasing contacts between ticks and cattle of all age categories, giving ticks many possibilities of feeding on high parasitaemic young animals (Purnell et al., 1974; Bazarusanga et al., 2008). This is in agreement with the reported positive correlation between infection rates and parasitaemia in cattle (Young et al., 1996). An interesting observation in the fenced farming system was the evidence that *T. parva* transmission remained significant although the cattle were dipped weekly. This finding is somewhat surprising, as one would predict that the level of transmission and challenge will be low. A similar high *T. parva* transmission has been reported in weekly dipped herds in Ouganda (Oura et al., 2007) suggesting that control of ECF in eastern Africa is hardly achievable by tick control alone.

When looking at the EIR calculations, the restricted farming system had much higher EIR than the free-range and the fenced farming systems. This is not surprising given the high tick counts and infection rates in field ticks found in the restricted managerial system. The higher challenge of *T. parva* infection detected in the restricted animals is likely to increase the risk of clinical ECF since the severity of the disease is dose-dependent (Barnett, 1957).

A similar situation would be observed in the fenced farming system but the reduced contact between animals and infected ticks through weekly acaricide application in this farming system lead to low levels of infection challenge and probably low clinical cases. In the free-range farming system, a significantly lower EIR was found although the highest tick numbers was collected on cattle. The majority of animals would receive low *T. parva* challenge leading to reduced number of diseased animals.

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Chapter 4

Epidemiological studies on theileriosis and the dynamics of *Theileria parva* infections in Rwanda

Adapted from:

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4.1 Introduction

Ecological and climatic variations induce changes in tick population dynamics that result in different epidemiological situations of theileriosis in the endemic regions (Fandamu *et al*., 2005). The epidemiology of the disease is further complicated by the presence of other *Theileria* spp. which are less pathogenic to cattle. However, only virulent strains of *T. mutans* have been described (Young *et al*., 1978), while *T. taurotragi* or *T. velifera* are benign infections.

Control of theileriosis in East Africa has proven to be difficult principally because of the lack of epidemiological information. Earlier investigations in many countries have been restricted to estimates of *T. parva* prevalence in limited geographic areas and none so far has addressed the question of the distribution of theilerial infection on a country wide basis. A gradient-based *T. parva* prevalence has been reported from a single district of Kenya (Gitau *et al*., 2000) and from the Eastern Province of Zambia (Billiouw, 2005).

These reports are based on serology results using the indirect fluorescent antibody test (IFAT) being the most commonly used detection method (Norval *et al*., 1992d). However, the performance of the IFA test in terms of sensitivity and specificity under field conditions is poorly documented. It is known that the test lacks specificity because of cross-reactions with *T. taurotragi*, which is of importance as the distribution of *T. taurotragi* overlaps with *T. parva* throughout much of eastern, central and southern Africa (Goddeeris *et al*., 1982; Norval *et al*., 1992d). Furthermore, IFAT lacks sensitivity in endemic regions when tick transmission is seasonal (Billiouw *et al*., 2005). The use of DNA based methods detects not only the presence of the parasite but can also be used to obtain prevalence data.

Animals can recover after a *T. parva* infection and become carriers for several years. These animals are immune to homologous infections. Specific identification of low *T. parva* parasite levels in naturally infected animals in the field is essential for epidemiological studies. The recently developed Polymerase Chain Reaction (PCR) techniques have a higher specificity and sensitivity than conventional diagnostic methods in determining theilerial infections in carrier animals (Bishop *et al*., 1992; d'Oliviera *et al*., 1995). The PCR methods have proven to be able to characterise and distinguish *T. parva* from multiple *Theileria spp*.-infections in field samples (Geysen *et al*., 1999, Ogden *et al*., 2003).

In Rwanda, little is known about the prevalence of theilerial infections although ECF is considered as the most important disease of cattle (Paling and Geysen, 1981). High *T. parva* prevalence rates have also been recorded in neighbouring Uganda (Rubaire Akiiki *et al*., 2004) and in Kenya (Gitau *et al*., 2000). The present study reports on results from three epidemiological surveys conducted both during dry and wet seasons and covering all four agro-ecological zones of Rwanda.

4.2 Materials and Methods

4.2.1 Experimental set up

Three surveys were organised: the first during the 1998 dry season, the second in dry season of 2002 and the third in the wet season of 2003. All the samples were tested for the presence of *T. parva* specific DNA. Full *Theileria* species determination was carried out on the samples from the 1998 survey only.

4.2.2 Herd structure and distribution

Cattle constitute the main livestock and are raised in all the four agro-ecological zones under different management systems. The AEZ 4 is the most important cattle area in Rwanda, holding 40% of the national herd (estimated at a total of 1,000,000 heads of cattle) whereas in the AEZ 3, a limited number (13%) is recorded just like in the highly populated part (AEZ 1) of the country (Fig. 1.2). Although indoor management strategy is the rule in Rwanda, the higher the number of cattle in the region, the less the indoor husbandry policy is successfully applied with the most communal grazing systems found in low land area and a gradual shift to a more developed zero grazing management systems in high land zones. Table 2.1 summarises the farming systems in use over the country.

Table 4.1 Farming system data according to the AEZs in Rwanda.

AEZ	Management system (%)			Breed type (%)			Age category (%)	
	Extensive	Semi-intensive	Intensive	Local	Cross	Pure	Heifer	Adult
1	73.1	17.8	9.1	62.5	27.5	10	26	74
2	47.9	40.7	11.4	68.3	21.2	10.5	24	76
3	55.8	39.8	4.4	89.2	10.5	0.3	18	82
4	44.1	47.6	8.3	67.8	23.2	9	17	83
Total	55.2	36.5	8.3	71.9	20.6	7.5	21.2	78.8

4.2.3 Animals and sampling

A total of 898 cattle were sampled during three country wide transversal surveys. For each survey, similar numbers of animals were randomly selected from each agro-ecological zone. In 1998, a total of 264 blood samples (half from calves under 7 months of age and half from adults) were collected. In the 2002 and 2003 surveys, a total of 317 animals of one year or older were sampled in each season. Blood samples were obtained by venopuncture into 10 ml venoject tubes containing EDTA. Tubes were kept on ice in a cool box and sent the same day to the National Veterinary Laboratory (NVL), Kigali. Upon arrival, blood drops were transferred to Whatman N°4 (Whatman, United Kingdom) filter papers the same day, air-dried overnight and stored until use at -20°C in plastic bags containing silicagel.

4.2.4 DNA extraction and PCR-RFLP assay

Parasite DNA was extracted from filter paper blots using a modified de Almeida *et al.* (1997) protocol as described by Geysen (2000). Blood-spot nested PCR methods for detection of *Theileria* infection were used. The p104 PCR (Iams et al., 1990) was applied to detect specific *T. parva* DNA in the samples whereas the 18S PCR/RFLP (Allsopp et al., 1993) was used for the identification of any *Theileria* species present in the samples. Table 4.3 shows pairs of primers used in the first and second rounds of the semi-nested p104 and 18S PCR and the amplification conditions in the PCR reaction. Briefly, PCR amplification was carried out in a total volume of 25 µl. Each

reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 20 pmol of each primer, 0.5 U *Taq* polymerase enzyme (Goldstar, Eurogentec) and 5 µl of a DNA solution as template. Each mixture was overlaid with 50µl of fine neutral mineral oil. In the semi-nested run, 0.5 µl of amplified product from the first run was added through the oil layer to the semi-nested mix at 84°C (Hot start principe), containing the same ingredients and concentration, except that 0.3 U *Taq* was used. The amplification programme was as follows: Step1: 94°C for 4 min; step 2: 94° C for 1 min; Step 3: annealing temperature of 58°C in the first run and, in the second runs of the semi-nested PCR, 60°C and 55°C for the p104 and the 18S loci, respectively, for 45 sec; Step 4: Extension temperature of 72° C for 1 min. Steps 2-4 were repeated 39 times in the first runs and 24 times in the second runs. Step 5 was a final extension phase at 72° C for 8 min and standard detection with ethidium bromide staining was used after electrophoresis of the amplified samples together with a 100bp molecular weight marker.

Theileria spp. differentiation was carried out using PCR-RFLP method (Geysen. 2000). The 18S DNA was digested overnight using *MspI* restriction enzyme at a temperature of 37°C. After electrophoresis, DNA digests were visualised on sybr green stained PAGE gels and the RFLP profiles of different *Theileria spp.* obtained were compared to species-specific profiles (Fig. 4.1).

4.2.5 Statistical analysis

Data were analysed using a logistic regression in Stata 8.0 (StataCorp, 2003) software. A first model was based on the 1998 data to evaluate the effects of age classes, agro-ecological zones and the interaction between the two on the prevalence of the parasite as detected using the PCR based p104 gene assay. The effects of the respective surveys, the agro-ecological zones and the interaction between the two were assessed in a second model, using the whole dataset. Coefficients with a P value less than 0.05 were considered significant.

4.3 Results

In the 1998 survey, 97 of the total 264 samples examined (36.7%) were found positive for *Theileria* spp., showing a fragment of approximately 1010 bp in the 18S pan species PCR (Fig. 4.1). These samples were considered positive for *Theileria* spp. Based on the different RFLP patterns, four *Theileria* spp. were identified: *T. parva* (62.9%), *T. mutans* (50.5), *T. taurotragi* (29.9%) and *T. velifera* (15.5 %). Table 4.2 shows that approximately half of the *Theileria* spp. positive samples were mixed infections [double (40.2%) and triple (9.3%)].

Table 4.2 Single, double and triple infections with *Theileria* spp. as identified by the 18S species-specific assay

Theileria spp.	Number	Percentage (%)
Single infections	50	51.5
T. parva	21	21.6
T. mutans	23	23.7
T. velifera	3	3.1
T. taurotragi	2	2.1
Double infections	39	40.3
T. parva/T. mutans	9	9.3
T. parva/T. taurotragi	20	20.6
T. parva/T. velifera	2	2.1
T. mutans/ T. velifera	8	8.3
Triple infections	9	9.2
T. parva/T. mutans/ T. taurotragi	7	7.1
T. parva/T. mutans/ T. velifera	2	2.1
Total	97	100

The prevalence of *T. parva,* determined by the p104 *T. parva* specific assay, was 66/264 (25.3%), 89/317 (28%) and 86/317 (27.1%) for the 1998, 2002 and 2003 surveys, respectively. There were no significant differences between the results of the three surveys showing the same prevalences during dry and wet seasons (2002 and 2003 surveys). Results from both 18S and p104 markers correlated for 94% of all *T. parva* positive samples.

Fig. 4.2 shows the prevalence of *T. parva* during the three surveys for the four agro-ecological zones. The regional distribution of *T. parva* infections showed a geographically declining trend with highest prevalence in the AEZ 1 and the lowest in the AEZ 4. The prevalence of *T. parva* was significantly higher in the AEZ 1 compared to other AEZs ($P<0.05$). The *T. parva* prevalence did not differ between the AEZ 2 and AEZ 3 ($P>0.05$) but both zones showed significantly higher prevalences compared to the AEZ 4 ($P>0.05$). Age related results were only available from the 1998 study. Here, higher *T. parva* infections were observed in calves compared to adult cattle in all agro-ecological zones, except the AEZ 1 (Fig. 4.3).

Table 4.3 Primer sequences of semi-nested PCR assays for the 18S and p104

Primers	Sequences	PCR round	Primerpair	Annealing Temperature (°C)
18SF4	CGTTTTTACATGGATAACCGTGCTAA	1	18SF4/18SR4	58
18SR4	TAAAAACTGACGACCTCCAATCTCTAGT	2	18SF4/18SnR	55
18SnR	GGCATTGTTTATGGTTAGGA			
p104F2	CCACCATCTCCTAAACCACCGTT	1	pP104F2/P104 5	58
p104 5	TAAGATGCCACTATTAATGACACCACAA	2	p104 5/ P104 2nF	60
p1042nF	AACCACCGTTTGATCCATCATTCA			

Fig.4.1 *Theileria* species-specific profiles resulting from RFLP-PCR analysis of 18S loci using *Msp*1 enzyme digestion. Primers 18SF/18SR were used in the first run and 18SF/18SNR in the second run. Field samples (lanes 2-6 and lanes 11-14) were sized against a 1000 base pair marker (anes 1 and 15) and assigned to *Theileria* spp. by comparing the RFLP patterns with the established species-specific profiles (lanes 7-10). Multiple infections were detected when the summed size of the resulting RFLP fragments exceeded the size of unrestricted PCR product.

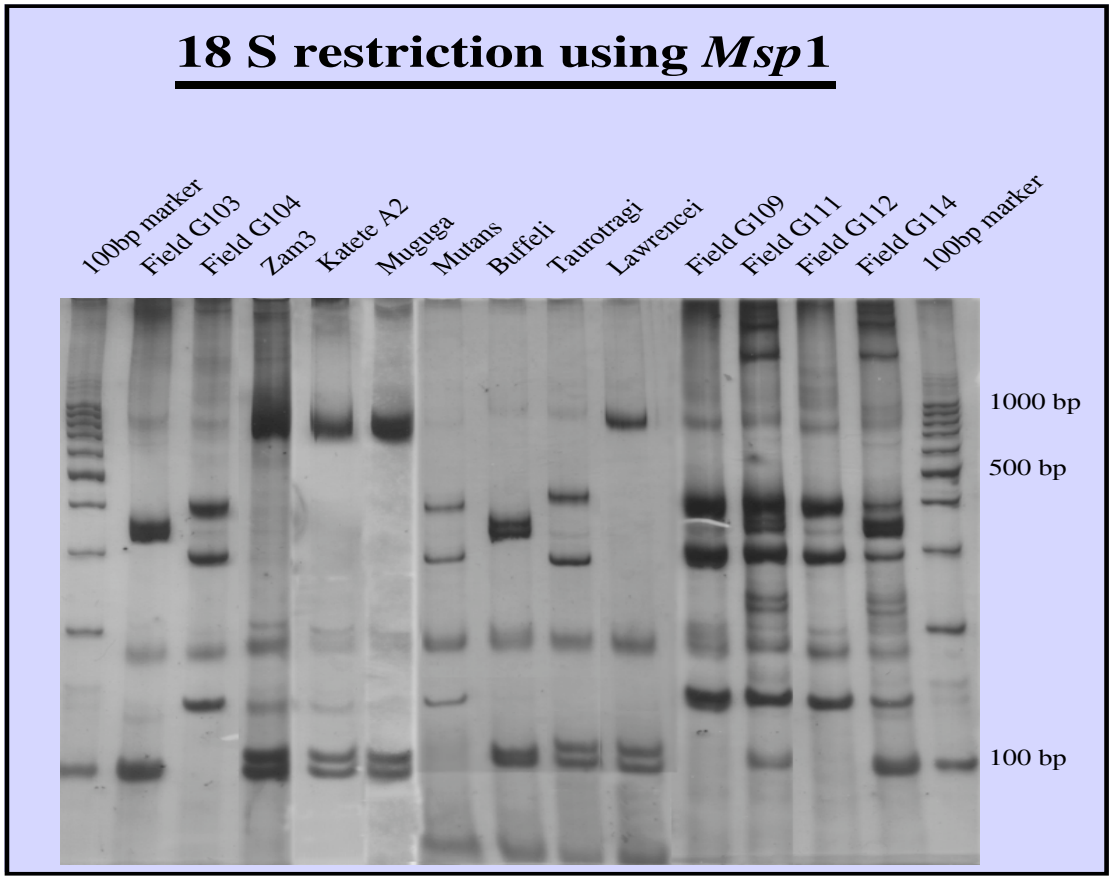


Fig.4.2. The prevalence of *T. parva* infection according to the 4 agro-ecological zones

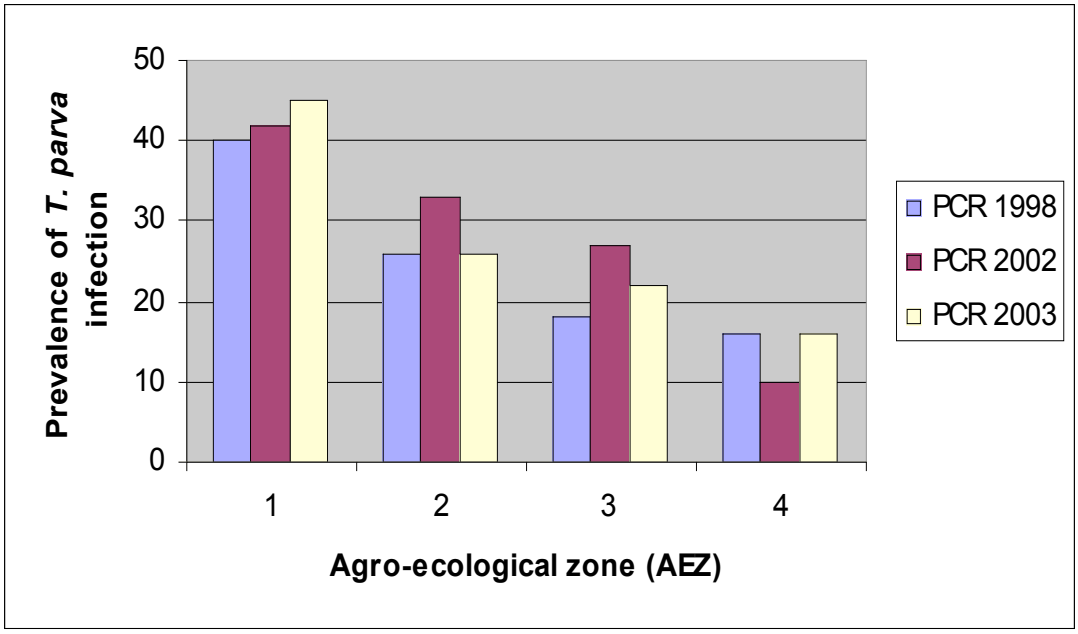
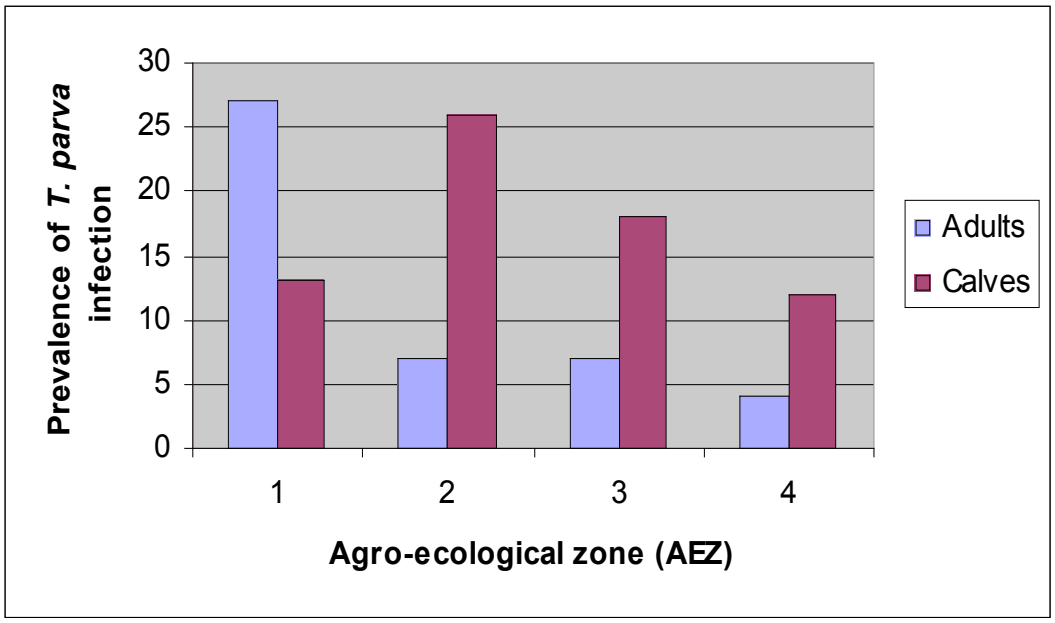


Fig.4.3 The prevalence of *T. parva* infection (1998 survey) according to age



4.4 Discussion

The results obtained by the 18S-PCR assay revealed the coexistence of four *Theileria* spp. (*T. parva, T. mutans, T. taurotragi and T. velifera)* reflecting the presence of different tick species as previously described in Rwanda (FAO, 1982). The presence of multiple *Theileria* spp. is a common feature in bovine samples from eastern Africa

(Norval *et al*., 1992a). In the present study, a high number of samples had mixed infections with *Theileria* spp. Despite this complexity, the pan species PCR method gave clear discrimination between the different *Theileria* parasites in field samples. *T. parva* and *T. mutans* were the most dominant infections, reflecting the relative importance in Rwanda of *R. appendiculatus* and *Ambyomma variegatum* tick populations, as described earlier by Paling and Geysen (1981). Similar results were obtained in a sero-survey conducted in neighbouring Tanzania (Swai, 2000), confirming that *T. parva* and *T. mutans* are the most important theilerial species in the region.

T. parva was the most prevalent species and there was a good correlation between prevalences obtained by the pan *Theileria* 18S and by the *T. parva* specific p104 assays. The high prevalence of *T. parva* infections is not surprising since the main vector, *R. appendiculatus* accounted for more than 80% in the total tick collections from Rwanda (FAO, 1982). This is to be expected as climatic conditions are very favourable for tick survival (Elb and Anastos., 1966; Newson, 1978) allowing intensive host-tick interactions and subsequent challenge of cattle with *T. parva*.

Studies of the dynamic of *T. parva* infections in the country indicate stable parasite prevalence throughout the year in all regions of the country. Despite the limitation of PCR as a detection method for carrier animals due to the fluctuating nature of parasitaemia in these animals (Geysen, 2000), the prevalence of *T. parva* infections was relatively high throughout the country.

Calves were significantly more infected with *T. parva* than adult cattle in almost all regions. This could be explained by higher parasitaemias in primary infections in young animals as compared to fluctuating parasitaemias in carrier adults. Similar higher PCR prevalences in calves have been reported in Mbarara district of neighbouring Uganda (Oura *et al*., 2005) or in ECF endemic regions of Kenya where almost half of local zebu calves were found infected by the age of 6-7 months (Moll *et al*., 1986). These high *T. parva* prevalences are an indication of the high transmission intensities in the region, giving infections early in life.

One exception was found in the AEZ 1 where the majority of calves were free of *T. parva* infection. It is thought that in this region, tick populations reflect a seasonal occurrence. Paling and Geysen (1981) reported on the presence of *R. appendiculatus* ticks in the whole of Rwanda except in regions of over 2000 m altitude where climatic conditions are marginal for tick survival. Systematic tick inspection of the animals

was done during the dry and wet season field surveys and ticks were found to be virtually absent during the rainy season in the AEZ 1. These observations suggest that *T. parva* transmission will be interrupted during the rainy season in these areas. Most of the calves in the high land would probably not have been in contact with infected ticks. In areas with less intensive *T. parva* transmission like Zambia, all calves were found to be free of *T. parva* infection up to the age of 6 months and only 50% were infected before the age of 15 months (Billiouw *et al*., 2002). Additional work on the dynamics of tick populations would help to clarify this particular situation of the AEZ 1 areas.

Although *T. parva* was present throughout the country, significant prevalence differences were found among geographic regions. These are in line with the findings of Deem *et al*. (1993) and reflect different levels of exposure to *T. parva* infection. The results of this study are in agreement with these seroprevalence studies where positive correlations were found between prevalence rates and the intensity of *T. parva* infection (Gitau *et al*., 2000; Maloo *et al*., 2001a). The declining trend in the prevalence of *T. parva* infections in higher zones having a relatively less suitable ecology to low land areas is an unexpected result.

The hypothesis of variable levels of vector competence amongst ticks from different ecological areas (Ochanda *et al*., 1998) is probably a likely explanation for the decrease in *T. parva* prevalence apart from the altitude influencing tick population dynamics. However, the tick population is not the only factor that determines the *T. parva* epidemiology in a region. The breed type, the tick control practices and the grazing system in the different agro-ecological zones will bring different levels of interaction between hosts and vectors. These have been reported to play a significant role in the epidemiology of *T. parva* infection in the east African region (Maloo *et al*., 2001b, Rubaire Akiiki., 2004) and this needs further investigation.

The present prospective study provides valuable epidemiological information on the endemic status of ECF in Rwanda, which is central to any future elaboration of strategic control measures. Investigations into the dynamics of tick populations and managerial practices are needed to characterise and understand the epidemiology of ECF in the different agro ecological zones of Rwanda.

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Chapter 5

The sensitivity of PCR and serology in different *Theileria parva* epidemiological situations in Rwanda

Adapted from:

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5.1 Introduction

Knowledge of the distribution of the vector and the disease is crucial in assessing the extent of the disease burden and in designing efficient control measures (Lessard et al., 1990). Seroprevalence reports based on indirect fluorescent antibody test (IFAT) have been used in many parts of Africa to determine areas of stable or unstable epidemiological states (Norval et al., 1992a; Perry, 1996). However, recent studies have shown that IFAT lacks sensitivity in ECF endemic regions when the tick transmission is seasonal (Billiouw et al., 2005). In addition, the method is not capable to detect early but patent infections (Burridge and Kimber, 1972).

DNA based detection methods have proven to be highly sensitive and specific in detecting early infections (Ogden et al., 2003) and variable levels of theilerial infections in carrier animals (Bishop et al., 1992; d'Oliviera et al., 1995). PCR techniques are now being applied in field surveys for the estimation of the prevalence of *Theileria annulata* (Aktas et al., 2002; Vatansever and Nalbantoglu, 2002; Dumanli et al., 2005) as well as *T. parva* infections under diverse epidemiological conditions (Ogden et al., 2003; Oura et al., 2005).

Most *T. parva* strains induce a carrier state for extensive periods of time in recovered animals (Dolan, 1986; Marcotty et al., 2002; Skilton et al., 2002). It is therefore expected that the proportion of carriers in the population is positively correlated with *R. appendiculatus* abundance. This was confirmed in various ECF endemic areas of Africa when serology was used as a diagnostic test (Gitau et al., 1997; Rubaire-Akiiki et al., 2004, Fandamu et al., 2005, Billiouw et al., 2005). However, we reported a negative correlation between *T. parva* prevalence estimated by PCR and tick abundance in the AEZ 4 in Rwanda (Chapter 2and 4).

It is hypothesised that this was due to a lack of sensitivity of the PCR in high transmission situations. While both, serology and PCR are reported to be specific (Skilton et al., 2002; Billiouw et al., 2005), the test sensitivities are mostly unknown in field conditions. Comparative evaluation of the performance of indirect and direct methods in situations with different *T. parva* transmission intensity becomes therefore of prime relevance in epidemiological studies.

The first objective of this study was to evaluate the sensitivity of serology and PCR in different ecological regions in Rwanda presenting variable degrees of infestation by *R. appendiculatus*. Apparent prevalences detected by individual serological (ELISA,

SELISA and IFAT) and parasitological (PCR) methods were compared on the same set of the samples using a Bayesian approach. This statistical method allows the estimation of the true infection prevalence and the various test sensitivities and specificities in the absence of a 100% sensitive and specific test (gold standard) (Lesaffre et al., 2007).

Bayesian models are iterative models that integrate data and prior information (expert's opinion) to make estimates. The prior information consists of the conditional probabilities underlying the relationship between, on one hand, the various test results and, on the other hand, the disease prevalence and the limits within which these parameters are allowed to vary. Similar multi-testing approaches were successfully utilised to estimate the true prevalence of porcine cysticercosis (Dorny et al., 2004) and calf giardiasis (Geurden et al., 2004).

The second objective of this study was to analyse, in an endemic field situation, the variation of PCR prevalence among IFAT positive animals in relation to their age and tick-control regimes. This was to confirm the low PCR sensitivity in adults living under high *T. parva* transmission intensity, compared to IFAT.

5.2 Materials and Methods

5.2.1 Study population and sampling protocol

Animals were recruited in two successive surveys (from September to November 2002 and from March to May 2003) in four agro ecological zones of Rwanda. A total of 635 local and cross-bred animals aged one year or more were randomly selected from 204 sites in 34 districts. The likelihood of re-sampling the same animals over the first (334 samples) and the second (301 samples) surveys was minimal as the sampling fraction was low in all sites. The samples of the two surveys were pooled for the statistical analysis. To further investigate the variations of PCR sensitivity amongst different cattle populations with variable levels of tick infestations, a second study was carried out on 194 samples (106 calves aged 4-6 months old and 88 adult cattle that had calved at least once) in AEZ 4. In this second study, a project (fenced) and a non-project (free-range) zones were defined according to acaricide regimes applied. In the project zone, animals were treated with acaricide on a weekly basis while in the non-project areas, a less regular acaricide use is observed.

Blood was collected by jugular venipuncture in 10 ml EDTA Venoject and Vacutainer tubes for PCR and serology testing, respectively. Sera were extracted from blood samples by centrifugation at 2500 rpm for 10 min. All serum samples were stored in 1 ml aliquots at -20° C until analysed. Sera from the first study (635 samples) were tested at IFAT, ELISA and SELISA whereas in the second study (194 samples), only the IFAT was applied. EDTA-treated blood drops were spotted onto Whatman filter paper (Whatman International, United Kingdom N° 4) and air-dried in the shade overnight. Filter papers were then stored in plastic bags filled with Silicagel and sent to the laboratory of the Institute of Tropical Medicine in Antwerpen for subsequent DNA extraction.

5.2.2 Serological tests

IFAT testing

Sera were screened for *T. parva* antibody schizonts using the indirect fluorescent antibody test (IFAT) as described by Burridge and Kimber (1972). An antibody titre of 1:160 or more was considered as positive.

ELISA testing

Antibodies to *T. parva* were detected in collected sera using a recombinant PIM-based ELISA as described by Katende et al. (1998). The ELISA results were read as optical densities (OD) obtained from the analysis of the samples in a Titertek Multiscan Mcc340 spectrophotometer. Controls were included in every test. The OD values were expressed as percent positivity (PP) relative to a reference strong-positive control serum (Wright et al., 1993). Any test serum with a PP value of 20 or above was considered positive (Katende et al., 1998).

SELISA testing

This assay was essentially a *T. parva* adaptation of the *Babesia bovis* antibody detection test developed by Kung'u & Goodger (1990). Teflon-coated slides were incubated in 95° ethanol for 1 hour. Whole-schizont-infected lymphoblast cells were used as antigen. The cells were diluted in plain phosphate buffer solution (PBS) complemented with 0.2% bovine serum albumin (BSA) and dispensed in 10 µl drops into each well of a Teflon-coated slide, left to dry for 60 min at 37°C and fixed in acetone for 10 min. Serum samples were diluted (1:640) in PBS buffer and 15 µl was

added to each well. Incubation was done at 37°C for 30 min in a moist chamber. Slides were then left for 1 hour in washing buffer (PBS) before the addition to each well of 15 µl of peroxidase conjugate diluted 1/200 in plain PBS (pH 7.2). The slides were incubated and washed as described before and the colour reaction was developed by the addition of 15 µl of DAB (3, 3'diaminobenzidine) substrate solution to each well, followed by a further incubation in the moist chamber for 20 min at ambient temperature. After a final washing and drying, slides were covered with mounting fluid (Mowiol 40-88) and cover-slips and examined under an ordinary light microscope. A positive and a negative reference serum were included on each antigen slide.

5.2.3 PCR–RFLP method

A *T. parva* specific p104 based-PCR assay was performed as previously described (Chapter 4) on all blood samples of the two studies. IFAT positive samples of the second study that tested negative for *T. parva* specific PCR were further analysed for the presence of *Theileria spp*. using the pan species 18S based PCR-RFLP method (Chapter 4).

5.2.4 Statistical analysis

The binary results of the four diagnostic tests were introduced separately for each agro-ecological zone in a Bayesian model (Annexes 5.1 and 5.2) to estimate the prevalence of *T. parva* infections and the sensitivity of the four diagnostic tests (Berkvens et al., 2006). The Bayesian model was run in Winbugs (http://www.mrc-bsu.cam.ac.uk/bugs). For Bayesian models to converge on a single solution, some limits must be imposed on conditional probabilities underlying the relationship between the disease prevalence and the test sensitivities and specificities. This is necessary to reduce de number of parameters to estimate to a lower value than the degrees of freedom of the datasets. All test sensitivities are unknown and it is the objective of this paper to evaluate them. Yet, their specificities were studied by different authors and were always reported to be close to 100%. IFAT specificity was estimated around 96% by Billiouw et al. (2002). The SELISA specificity can be assumed to be the same as that of IFAT since the two tests are based on the same

antigen-antibody reaction. The specificity of ELISA was reported by Katende et al. (1998) to be 94-98%. Finally, Geysen (2000) and Skilton et al. (2002) estimated that the PCR specificity was 100%. All test specificities were therefore set to one in our analyses. While it is acknowledged that unspecific cross-reactions in the serological tests may be possible and that the violation of the hypothesis might affect the output of the analysis, the assumption that all test specificities is 100 % is not very far from the truth, nor is it unreasonable, compared to limits applied on the test sensitivities.

In the Bayesian models, a total of 20,000 iterations were used in three chains, including a burn-in of 10,000. Convergence was ensured by examining plots of the variable values against iteration numbers for the 3 chains. The conformity of the model was evaluated using the criteria proposed by Berkvens et al. (2006).

In the second study, IFAT and PCR apparent prevalences and the conditional probability that a sample is positive at PCR while it is positive at IFAT were analysed using a logistic regression in Stata 9 (StataCorp 2006. Stata Statistical Software: Release 9.2. Texas: Stata Corporation). Discrete explanatory variables were the age class (calf or adult), the acaracide treatment (regular dipping or not) and the interaction between the two. Likelihood ratio tests were used to identify non-significant explanatory variables (P>0.05).

5.3 Results

5.3.1 Study 1

The apparent prevalences observed in the four agro-ecological zones (AEZs) using the four diagnostic assays are shown in Fig. 5.1. Their profiles are compared to the average tick load reported in the same agro-ecological zones in a parallel tick survey (Chapter 2). The Bayesian model provided estimations and 95% credibility intervals for the *T. parva* prevalence and the test sensitivities in each of the four agro-ecological zones. The data used in the four Bayesian models are presented in Table 5.1. The indicators of the Bayesian analyses are available in Annexes 3 and 4. They show a good fit of the data with the experts' constraints (Berkvens et al., 2006).

According to the Bayesian models, the prevalence showed little variations among agro-ecological zones and ranged between 83 and 85% (Fig. 5.2). Small variations were observed between sensitivities of the three serological tests, which ranged between 57 and 75% (Fig.5.2). IFAT and SELISA sensitivities were very similar in

the 4 zones. The sensitivity of the PCR test, however, was significantly lower in higher transmission areas (Fig. 5.2). As such, the PCR sensitivity was estimated to range from 66% in AEZ 1 to 24% in AEZ 2.

Table 5.1 Number of observations in each of the 16 classes defined by the combination of the 4 binomial test results in the 4 AEZs.

Tests				Agro-ecological zones (AEZs)				Total
IFAT	SELISA	ELISA	PCR	1	2	3	4	
−	−	−	−	24	34	40	29	127
−	−	−	+	8	12	6	4	30
−	−	+	−	3	7	11	11	32
−	−	+	+	12	17	9	4	42
−	+	−	−	4	3	7	12	26
−	+	−	+	0	0	0	0	0
−	+	+	−	1	3	6	7	17
−	+	+	+	9	4	8	3	24
+	−	−	−	1	4	4	6	15
+	−	−	+	3	1	4	0	8
+	−	+	−	3	3	9	1	16
+	−	+	+	3	4	4	0	11
+	+	−	−	6	11	14	21	52
+	+	−	+	1	2	0	2	5
+	+	+	−	9	34	39	40	122
+	+	+	+	33	26	34	15	108
Total				120	165	195	155	635

+: Positive; −: Negative.

5.3.2 Study 2

In the second study, the IFAT seroprevalence (Table 5.2) was relatively constant between the four groups. Neither age class of the animals nor tick control were significant as explanatory variables (likelihood ratio test: P=0.07). On the other hand, PCR prevalence (Table 5.2) was significantly lower in adults than in calves (P<0.001) and in irregularly dipped cattle compared to regularly dipped herds (P=0.025). The conditional probability that a sample had a positive PCR result while it had a positive IFAT was significantly lower in adults than in calves (P<0.001) while tick control had no significant effect (P=0.22). Of the 64 seropositive samples that tested negative at the p104 PCR, 1 failed to be further characterised due to the lack of material, 54 contained *Theileria spp.*-DNA and 9 were negative at the 18S PCR. The following *Theileria* species were identified: *T. taurotragi* in 1 sample, *T. mutans* in 13, *T. velifera* in 3 and mixed *T. mutans* and *T. velifera* infections in 37 samples.

Table 5.2 Prevalence of *T. parva* infection as estimated by IFAT and PCR in calves and adult cattle in the project and non-project zones

Prevalence	Project zone (regular dipping)		Non project zone (irregular dipping)	
(%)	Calves	Adults	Calves	Adults
IFAT	70 (42/60)	50 (15/30)	78 (36/46)	72 (42/58)
PCR	88 (53/60)	17 (5/30)	70 (32/46)	12 (7/58)
PCR|IFAT+	86 (36/42)	13 (2/15)	69 (25/36)	14 (6/42)

PCR|IFAT+: proportion of PCR positives among the IFAT positive samples

Fig. 5.1 Tick counts and apparent prevalence detected by IFAT, SELISA, ELISA and PCR in the 4 AEZs

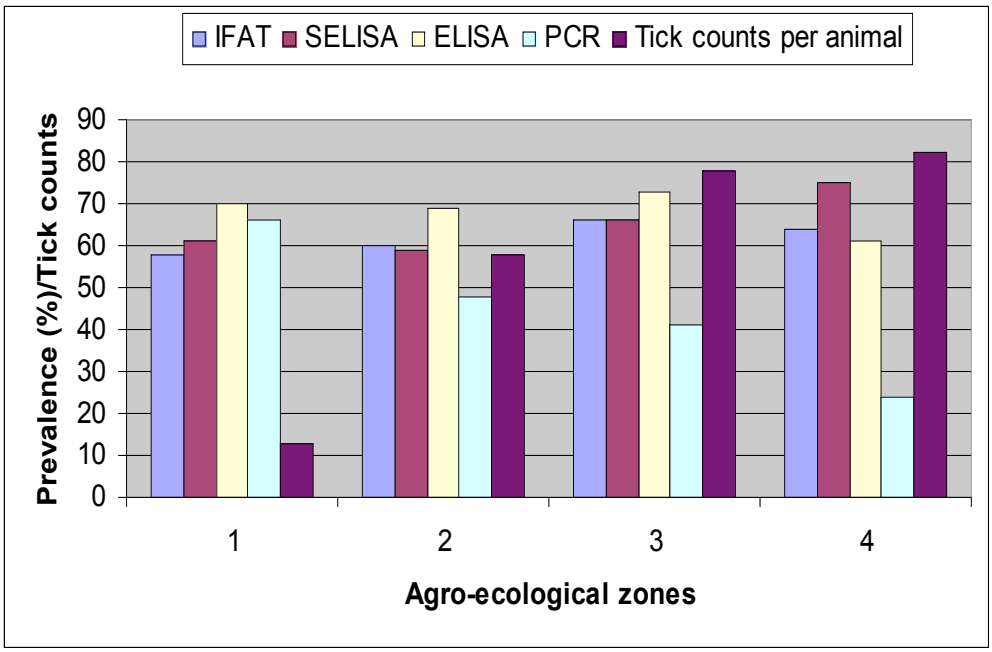
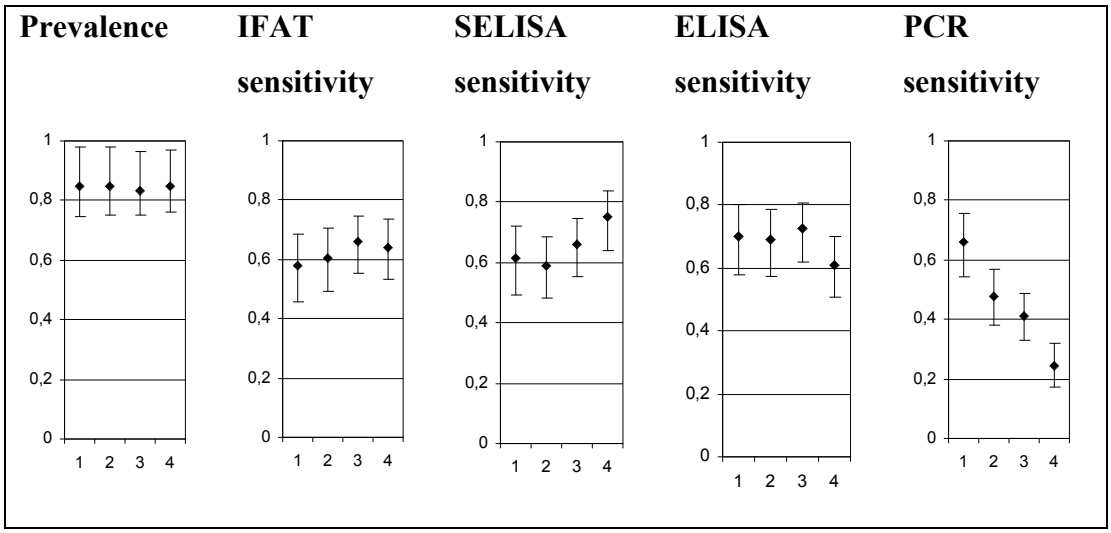


Fig. 5.2 Prevalence and relative test sensitivities estimated in the Bayesian model with their respective 95% credibility intervals in the 4 AEZs



1	**0.85 (0.74-0.98)**	**0.58 (0.46-0.68)**	**0.61 (0.49-0.72)**	**0.70 (0.58-0.80)**	**0.66 (0.54-0.76)**
2	**0.85 (0.75-0.98)**	**0.60 (0.49-0.70)**	**0.59 (0.48-0.69)**	**0.69 (0.57-0.78)**	**0.48 (0.38-0.57)**
3	**0.83 (0.75-0.96)**	**0.66 (0.55-0.75)**	**0.66 (0.55-0.75)**	**0.73 (0.62-0.81)**	**0.41 (0.33-0.49)**
4	**0. 85 (0.76-0.97)**	**0.64 (0.53-0.74)**	**0.75 (0.64-0.84)**	**0.61 (0.51-0.70)**	**0.24 (0.17-0.32)**

5.4 Discussion

Estimates of the prevalence of *T. parva* infection in endemic areas are usually based on apparent prevalence results of a single test. However, the true prevalence of the parasite in the absence of a gold standard test could be more accurately estimated by combining the results of several tests in a model (Enoe et al., 2000; Dorny et al., 2004; Berkvens et al., 2006; Lessaffre et al., 2007). In this study, a Bayesian approach using four diagnostic tests was applied to estimate the true prevalence of *T. parva* infection in four agro-ecological zones. Estimated prevalences ranged between 83 and 85 % in all zones, which are much higher than what was previously reported in Rwanda (Chapter 4).

The true prevalences estimated are also higher than apparent prevalence detected by individual tests. This is not surprising since all test specificities were set to 1, implying that any positive result generated by any of the four tests is considered as a true positive. The estimated prevalence is in line with the reported observation in ECF endemic areas in which close to 100% of the animals were carriers of *T. parva* (Young et al., 1981). In the present study, a good correlation between serology and PCR results was found in AEZ 1 but PCR results were significantly lower in the AEZ 4 where higher *R. appendiculatus* infestation levels occur (Paling and Geysen, 1981; Chapter 2). The lack of agreement between the diagnostic tests in high transmission conditions can either be explained by a lack of sensitivity of the PCR or by a lack of specificity of the serology.

The statistical inference heavily relies on the assumption that the specificity of diagnostic tests was 100%. It could be envisaged that the specificity of serology was overestimated if seropositive animals testing negative at PCR had, in fact, been infected but cleared the parasite before antibodies declined. A few stocks of *T. parva* have indeed been reported to disappear rapidly from the body after recovery (Dolan, 1986; Young et al., 1986; Bishop et al., 1992). The presence of such *T. parva* parasites in the AEZ 4 could explain the discrepancy between the PCR and the serological results. However, there was no evidence of such geographical differences among the *T. parva* genotypes in Rwanda (personal observations). Furthermore, the relative importance of the various cattle breeds was similar in the four ecological zones indicating no obvious differences in cattle susceptibility. Finally, unspecific serological results could be caused by cross-reactions.

Only cross-reactions with *Theileria taurotragi* are known to possibly affect the specificity of IFAT (Norval et al., 1992b) and SELISA while ELISA false positive results might be caused by the presence of glutathione S-transferase (GST) enzyme in the recombinant PIM antigen. Analogues of GST are present in *Schistosoma bovis* and *Fasciola hepatica* (Hiller et al., 1992) which occur within ECF endemic areas and the possibility of false-positive findings due to unspecific presence of GST-antibodies in the samples could not be excluded, although sera from cattle experimentally infected with *S. bovis* and *F. hepatica* tested negatively in the PIM-ELISA assay (Katendi et al., 1998). Since the serological tests are not all subject to the same type of cross-reaction, it is unlikely that their specificity was similarly affected in the four zones. In addition, the occurrence of *T. taurotragi* is low in the AEZ 4 (unpublished data), where the discrepancy between PCR and serology is the highest, and *T. taurotragi* was observed in only 1 out of 63 IFAT positive samples that tested negative at p104. Furthermore, the low serology specificity values that would be required to explain the disagreement with PCR results would seriously diverge from previous estimations (Billiouw et al., 2002, Katende et al., 1998).

In the second study, in the AEZ 4, animals were of the same breed and exposed to similar tick and *Theileria* spp. populations. Only the intensity and the duration of the exposure to *T. parva* were allowed to vary between young and adult cattle and between dipped and undipped herds. A good correlation was found between serology and PCR in calves but not in adults in which the PCR prevalence was much lower than the seroprevalence. The setup of this study excludes variations in cattle susceptibility, in *T. parva* genotype and in serological cross-reactions as reasons of the discrepancies since animals were homogenous and maintained in similar environmental conditions.

Evidence of lack of PCR sensitivity was previously reported in a study where only half of the *T. parva* carrier animals tested were positive at PCR (Geysen, 2000). Jarvi et al. (2002) also reported a lower sensitivity of a PCR-based method to detect *Plasmodium falciparum* in chronically infected birds, compared to antibody detection. In fact, PCR-based detection methods are less likely to detect low parasite densities because of a higher probability that no infected erythrocytes are present in the minute blood volume used in PCR reactions. In addition, the parasite densities in *T. parva* carriers seem to fluctuate with time and periodically fall below the detection level of PCR (Geysen, 2000).

The mechanisms involved in parasitaemia variations in *T. parva* carrier infections are not well understood. The survival of the parasite as a result of a dynamic equilibrium between the host immune defences and the evasion mechanisms of the parasite is the favoured hypothesis (Emery and Morrison, 1980; Young, 1980; Dolan, 1986). The higher PCR prevalence in young animals despite a lighter tick infestation and a shorter exposure is in agreement with observations in Uganda (Oura et al., 2005). This apparent contradiction might be explained by a lack of immunity and a higher parasitaemia related to primo infections. Although this hypothesis was not confirmed when blood samples were subjected to quantification at real-time PCR (personal observation), adult animals in high transmission intensity zones are likely to show solid immunity against the local circulating *T. parva* strains. Regular challenges probably boost the immune mechanisms resulting in controlled parasitaemia (without achieving sterile immunity) and in less frequent episodes of detectable levels of parasitaemia by PCR.

5.5 Conclusion

Though serology was reported to lack sensitivity in areas with seasonal *R. appendiculatus* occurrence (Billiouw et al., 2005), it appears to be the most sensitive available tool to detect chronic *T. parva* infections. The sensitivity of serological tests is relatively high and constant across tests and agro-ecological zones. Present PCR-based protocols lack sensitivity in detecting carrier *T. parva* infections in high transmission conditions. Increasing blood volume from which DNA is extracted could be considered to increase the sensitivity of the test. Alternatively, a repetition or a combination of different diagnostic tests with high specificity might provide a more accurate picture of the true prevalence of *T. parva* infection under various tick transmission intensities. Yet, such prevalence data might not allow the differentiation between endemic epidemiological states. Instead, the disagreement between serology and PCR in adult cattle may be, after validation of the method, a useful indicator of transmission intensity.

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5.7 Annexes

Annex 5.7.1 Bayesian model run in Winbugs

```
{
N <-sum(r[])
r[1:16]~dmulti(p[1:16], N)

p[1]<-theta[1]*(1-theta[2])*(1-theta[5])*(1-theta[11])*(1-theta[23])+(1-theta[1])*theta[3]*theta[6]*theta[12]*theta[24]
p[2]<-theta[1]*(1-theta[2])*(1-theta[5])*(1-theta[11])*theta[23]+(1-theta[1])*theta[3]*theta[6]*theta[12]*(1-theta[24])
p[3]<-theta[1]*(1-theta[2])*(1-theta[5])*theta[11]*(1-theta[22])+(1-theta[1])*theta[3]*theta[6]*(1-theta[12])*theta[25]
p[4]<-theta[1]*(1-theta[2])*(1-theta[5])*theta[11]*theta[22]+(1-theta[1])*theta[3]*theta[6]*(1-theta[12])*(1-theta[25])
p[5]<-theta[1]*(1-theta[2])*theta[5]*(1-theta[10])*(1-theta[21])+(1-theta[1])*theta[3]*(1-theta[6])*theta[13]*theta[26]
p[6]<-theta[1]*(1-theta[2])*theta[5]*(1-theta[10])*theta[21]+(1-theta[1])*theta[3]*(1-theta[6])*theta[13]*(1-theta[26])
p[7]<-theta[1]*(1-theta[2])*theta[5]*theta[10]*(1-theta[20])+(1-theta[1])*theta[3]*(1-theta[6])*(1-theta[13])*theta[27]
p[8]<-theta[1]*(1-theta[2])*theta[5]*theta[10]*theta[20]+(1-theta[1])*theta[3]*(1-theta[6])*(1-theta[13])*(1-theta[27])
p[9] <-theta[1]*theta[2]*(1-theta[4])*(1-theta[9])*(1-theta[19])+(1-theta[1])*(1-theta[3])*theta[7]*theta[14]*theta[28]
p[10]<-theta[1]*theta[2]*(1-theta[4])*(1-theta[9])*theta[19]+(1-theta[1])*(1-theta[3])*theta[7]*theta[14]*(1-theta[28])
p[11]<-theta[1]*theta[2]*(1-theta[4])*theta[9]*(1-theta[18])+(1-theta[1])*(1-theta[3])*theta[7]*(1-theta[14])*theta[29]
p[12]<-theta[1]*theta[2]*(1-theta[4])*theta[9]*theta[18]+(1-theta[1])*(1-theta[3])*theta[7]*(1-theta[14])*(1-theta[29])
p[13]<-theta[1]*theta[2]*theta[4]*(1-theta[8])*(1-theta[17])+(1-theta[1])*(1-theta[3])*(1-theta[7])*theta[15]*theta[30]
p[14]<-theta[1]*theta[2]*theta[4]*(1-theta[8])*theta[17]+(1-theta[1])*(1-theta[3])*(1-theta[7])*theta[15]*(1-theta[30])
p[15]<-theta[1]*theta[2]*theta[4]*theta[8]*(1-theta[16])+(1-theta[1])*(1-theta[3])*(1-theta[7])*(1-theta[15])*theta[31]
p[16]<-theta[1]*theta[2]*theta[4]*theta[8]*theta[16]+(1-theta[1])*(1-theta[3])*(1-theta[7])*(1-theta[15])*(1-theta[31])

theta[1] ~ dunif(0,1)     theta[9] ~ dunif(0,1)     theta[17] ~ dunif(0,1)     theta[25] ~ dunif(1,1)
theta[2] ~ dunif(0,1)     theta[10] ~ dunif(0,1)    theta[18] ~ dunif(0,1)     theta[26] ~ dunif(1,1)
theta[3] ~ dunif(1,1)     theta[11] ~ dunif(0,1)    theta[19] ~ dunif(0,1)     theta[27] ~ dunif(1,1)
theta[4] ~ dunif(0,1)     theta[12] ~ dunif(1,1)    theta[20] ~ dunif(0,1)     theta[28] ~ dunif(1,1)
theta[5] ~ dunif(0,1)     theta[13] ~ dunif(1,1)    theta[21] ~ dunif(0,1)     theta[29] ~ dunif(1,1)
theta[6] ~ dunif(1,1)     theta[14] ~ dunif(1,1)    theta[22] ~ dunif(0,1)     theta[30] ~ dunif(1,1)
theta[7] ~ dunif(1,1)     theta[15] ~ dunif(1,1)    theta[23] ~ dunif(0,1)     theta[31] ~ dunif(1,1)
theta[8] ~ dunif(0,1)     theta[16] ~ dunif(0,1)    theta[24] ~ dunif(1,1)

se[1] <- theta[2]
se[2] <- theta[4]*theta[2]+theta[5]*(1-theta[2])
se[3] <- theta[8]*theta[4]*theta[2]+theta[9]*(1-theta[4])*theta[2]+theta[10]*theta[5]*(1-theta[2])+theta[11]*(1-theta[5])*(1-
theta[2])
se[4] <- theta[2]*(theta[4]*(theta[8]*theta[16]+(1-theta[8])*theta[17])+(1-theta[4])*(theta[9]*theta[18]+(1-
theta[9])*theta[19]))+(1-theta[2])*(theta[5]*(theta[10]*theta[20]+(1-theta[10])*theta[21])+(1-theta[5])*(theta[11]*theta[22]+(1-
theta[11])*theta[23]))

r2[1:16] ~ dmulti( p[1:16], N)
for (i in 1:16)
{
        z1[i] <- equals(0,p[i])
        y1[i] <- max(z1[i],p[i])
        x1[i] <- max(r[i],1)
        d[i] <- r[i]*log(x1[i]/(y1[i]*N))

        z2[i] <- equals(0,p[i])
        y2[i] <- max(z2[i],p[i])
        x2[i] <- max(r2[i],1)
        d2[i] <- r2[i]*log(x2[i]/(y2[i]*N))
}
G0 <- 2 * sum(d[])
Gt <- 2 * sum(d2[])
bayesp <- step(G0 - Gt)
}
```

p[1:16] are the number of observations of each of the test result combinations (Table 1); theta[1:31] are the conditional probabilities (Annex 2) subjected to experts' constraints; se[1:4] are the sensitivity of the IFAT, SELISA, ELISA and PCR

Annex 5.7. 2 List and coding of the conditional probabilities

theta[1] = $\Pr(D^+)$

theta[2] = $\Pr(T_1^+ | D^+)$

theta[3] = $\Pr(T_1^- | D^-)$

theta[4] = $\Pr(T_2^+ | D^+ \cap T_1^+)$

theta[5] = $\Pr(T_2^+ | D^+ \cap T_1^-)$

theta[6] = $\Pr(T_2^- | D^- \cap T_1^-)$

theta[7] = $\Pr(T_2^- | D^- \cap T_1^+)$

theta[8] = $\Pr(T_3^+ | D^+ \cap T_1^+ \cap T_2^+)$

theta[9] = $\Pr(T_3^+ | D^+ \cap T_1^+ \cap T_2^-)$

theta[10] = $\Pr(T_3^+ | D^+ \cap T_1^- \cap T_2^+)$

theta[11] = $\Pr(T_3^+ | D^+ \cap T_1^- \cap T_2^-)$

theta[12] = $\Pr(T_3^- | D^- \cap T_1^- \cap T_2^-)$

theta[13] = $\Pr(T_3^- | D^- \cap T_1^- \cap T_2^+)$

theta[14] = $\Pr(T_3^- | D^- \cap T_1^+ \cap T_2^-)$

theta[15] = $\Pr(T_3^- | D^- \cap T_1^+ \cap T_2^+)$

theta[16] = $\Pr(T_4^+ | D^+ \cap T_1^+ \cap T_2^+ \cap T_3^+)$

theta[17] = $\Pr(T_4^+ | D^+ \cap T_1^+ \cap T_2^+ \cap T_3^-)$

theta[18] = $\Pr(T_4^+ | D^+ \cap T_1^+ \cap T_2^- \cap T_3^+)$

theta[19] = $\Pr(T_4^+ | D^+ \cap T_1^+ \cap T_2^- \cap T_3^-)$

theta[20] = $\Pr(T_4^+ | D^+ \cap T_1^- \cap T_2^+ \cap T_3^+)$

theta[21] = $\Pr(T_4^+ | D^+ \cap T_1^- \cap T_2^+ \cap T_3^-)$

theta[22] = $\Pr(T_4^+ | D^+ \cap T_1^- \cap T_2^- \cap T_3^+)$

theta[23] = $\Pr(T_4^+ | D^+ \cap T_1^- \cap T_2^- \cap T_3^-)$

theta[24] = $\Pr(T_4^- | D^- \cap T_1^- \cap T_2^- \cap T_3^-)$

theta[25] = $\Pr(T_4^- | D^- \cap T_1^- \cap T_2^- \cap T_3^+)$

theta[26] = $\Pr(T_4^- | D^- \cap T_1^- \cap T_2^+ \cap T_3^-)$

theta[27] = $\Pr(T_4^- | D^- \cap T_1^- \cap T_2^+ \cap T_3^+)$

theta[28] = $\Pr(T_4^- | D^- \cap T_1^+ \cap T_2^- \cap T_3^-)$

theta[29] = $\Pr(T_4^- | D^- \cap T_1^+ \cap T_2^- \cap T_3^+)$

theta[30] = $\Pr(T_4^- | D^- \cap T_1^+ \cap T_2^+ \cap T_3^-)$

theta[31] = $\Pr(T_4^- | D^- \cap T_1^+ \cap T_2^+ \cap T_3^+)$

T_1, T_2, T_3 and T_4 are the IFAT, SELISA, ELISA and PCR test respectively

Annex 5.7.3 DIC and pD values estimated using parent nodes and posterior means and obtained from the bayesian models ran for the four agro-ecological zones

	Model without constraints		**Model with experts' constraints**	
	Using parent nodes	**Using posterior means of the multinomial probabilities**	**Using parent nodes**	**Using posterior means of the multinomial probabilities**
High land				
DIC	**49.34**	**71.41**	**71.63**	**71.61**
pD	**-10.94**	**11.13**	**12.00**	**11.98**
Medium continental				
DIC	**52.90**	**76.19**	**76.29**	**76.34**
pD	**-11.43**	**11.86**	**12.45**	**12.50**
Medium tempered				
DIC	**49.02**	**80.36**	**78.95**	**79.17**
pD	**-19.48**	**11.87**	**12.06**	**12.27**
Low land				
DIC	**37.24**	**71.89**	**70.39**	**70.57**
pD	**-24.17**	**10.47**	**10.85**	**11.03**

DIC and pD values of the constrained models are similar whether their estimation is based on parent nodes or on posterior means of the multinomial probabilities, unlike the unconstrained model; in all models restricted by experts' opinion, pD values, which correspond to the number of parameters estimated in Bayesian models, are close to those estimated in the unconstrained models using posterior means.

Annex 5.7.4 Bayesp values obtained from the Bayesian models ran on the four datasets with different constraints applied on the parameters

	AEZ 1	AEZ 2	AEZ 3	AEZ 4
Model without constraints	0.47	0.46	0.60	0.62
Model with experts' constraints	0.44	0.44	0.54	0.55
Model constrained on estimates	$< 10^{-9}$	$3 * 10^{-5}$	0.001	0.006

Bayesp values indicates too severe constraints when diverting from 0.5; Bayesp value tend towards 0 when appropriate constraints are applied

Chapter 6

General Discussion

6.1 Introduction

Control of East Coast Fever (ECF) has been difficult to achieve in most of the endemic areas mainly due to complex factors affecting its epidemiology. In most of the affected countries, the transmission of the disease is extremely variable, involving factors affecting the parasite, the host and the vector in a widely differing ecological environment. While important epidemiological differences exist between eastern and southern Africa and within a single country, little is known about the epidemiology of ECF in central Africa, particularly in Rwanda.

The subject of this thesis was to gather and analyse the epidemiological data on ECF, and to optimise the use of the existing control methods. More specifically, the study aimed at collecting data on (1) the ecological distribution of the tick vector and its infection levels, (2) the factors affecting the vector-host transmission of *Theileria parva* infection and (3), and the dynamic of the infection prevalence in different ecological conditions.

In this chapter, the results will be discussed on a broader perspective with focus on methods to assess the epidemiology of ECF and optimal control strategies. A final conclusion and future prospects are presented in the last.

6.2 Prevalence estimates for epidemiological studies of *T. parva* infection under field conditions

In much of the endemic ECF regions, the status of the disease was based on seroprevalence results of a single diagnostic test. The sampling design of a single test survey implies that animals are classified as positive or negative. This is apparent prevalence which is not only affected by the characteristics of a diagnostic test applied (Greiner and Gardner, 2000; Basanez et al., 2004) but also by specific conditions such as intermittent and seasonal variation in tick occurrence in the case of *T. parva* infection (Billiouw et al., 2005).

To determine the true disease status of the animals, a multitesting approach offers a better estimate of the true prevalence of the disease (Dorny et al., 2004). In this study, the results of four diagnostic assays (ELISA, SELISA, IFAT and PCR) were used to assess the true prevalence of *T. parva* infection. Although some of the assays used in

the field surveys were previously evaluated for experimental or field studies, general scepticism is appropriate when extrapolating test parameters beyond the limit of the validation study. The lack of information on the test characteristics can seriously impact on the validity of the prevalence results estimated, as summarised in table 6.1. In the next two sections, discussion is made on the two types of bias for prevalence estimates.

Table 6.1 Estimates of the true and apparent prevalence of *T. parva* infection on the same set of the sample in four agro-ecological zones (AEZs) in Rwanda

Prevalence		Agro ecological zones (AEZs)			
		AEZ 1 (%)	AEZ 2 (%)	AEZ 3 (%)	AEZ 4 (%)
True (4 tests)		85	85	83	85
Apparent	IFAT	58	60	66	64
	SELISA	61	59	66	75
	ELISA	70	69	73	61
	PCR	66	48	41	24

6.2.1 Factors inherent to the diagnostic test

The epidemiological value of a laboratory test is commonly determined by its diagnostic sensitivity and specificity that reflects its clinical value. The diagnostic sensitivity and specificity are classically defined as intrinsic characteristics of the test (Martin et al., 1992; Thursfield, 1997) and are usually determined for specific test and a specific disease or infection. Test characteristics are usually estimated based on the "true disease status" of the animal, as determined by one or a combination of gold standard reference assays. A gold standard is an expert agreement rule that defines a reference for comparison and it is error free, by definition. Since even widely accepted gold standards are subjected to human or misclassification errors, it has been impossible to devise the perfect test. Consequently, all tests have non-perfect sensitivity and specificity.

In the light of these limitations, there are two basic ways to overcome these diagnosis misclassification errors. The first is directly from observed or experimental data, and the second is using expert opinion when data are scarce or missing. In field studies,

the latter alternative offers a better estimate of the true prevalence state. In this work, expert knowledge regarding sensitivity and specificity of the survey tests was included in a Bayesian model, since it has proved its value in circumventing the gold standard problem (Dorny et al., 2004).

The true prevalence estimated by a combination of four diagnostic assays was higher than apparent prevalence detected by any individual test. The high prevalence (>80%) of *T. parva* infection detected is consistent with previous observation that the proportion of carrier state in the cattle population is close to 100% in endemic areas of eastern Africa (Young et al., 1981). Our findings are therefore a clear indication that the results of single test surveys underestimate the prevalence and should be interpreted with caution. The use of more than one test on the same animal introduces extra variables required to cater for the interdependences of different tests. For example, an infected animal yielding a positive test result for a given test will not necessarily test positive in another test.

In the Bayesian model, inference on prevalence and test characteristics are based on test result data of at least 3 or 4 diagnostic assays, and on prior information, which comprises previously published estimates or expert opinion on model parameters (Suess et al., 2002; Branscum et al., 2005). A disadvantage of the Bayesian analysis is however the bias of the prior information, since the data at hand are established in the experimental conditions or on subjective expert opinion (Enoe et al., 2000).

The incorporation of prior information has been therefore the most controversial element in the Bayesian approach since prior knowledge does influence to a large extent posterior distributions. A sound model should include judgement on the importance of the prior information (Dunson, 2001; Basanez et al., 2004; Toft et al., 2005). Since there is no alternative to constrain the space on certain parameters, the use of the Bayesian approach provides a useful tool in setting steps from one state of knowledge over gaining information to a more advanced state of knowledge.

6.2.2 Extrinsic factors

For infectious diseases, serological tests are used frequently to determine the disease state of the animals. One of the major challenges in the interpretation of serological data is the need to adjust the result for misclassification errors. Although no formal relationship is reported to exist between sensitivity and prevalence, a reasonable

assumption for serological test is that the sensitivity varies with the stage of infection or with the immune status of the host.

In *T. parva* infection, the duration of antibody responses to infection may vary depending upon numerous factors. Burridge and Kimber (1972) have demonstrated that cattle are becoming seronegative two to four weeks after infection. After recovery, the development of carrier state may cause antibody levels to be maintained for longer period. However, antibody titres to schizont antigens may fall below the level of detection by serological method in the absence of rechallenge (Burridge and Kimber, 1973). This is particularly important in unimodal rainfall regions in southern Africa where the vector abundance and consequently *T. parva* challenge are highly seasonal (Berkvens et al., 1998). In contrast, the bimodal rainfall patterns in eastern Africa may assure a continuous *T. parva* challenge and a maximum efficacy of an IFAT survey. The choice of test is therefore important in Theileria infections where humoral responses are generally short-lived (Perry et al., 1985).

In recent years, molecular biology assays such as PCR have come to play a major role in parasitic diseases. The PCR method has proven to be more sensitive and specific in detecting *T. parva* infection than conventional assays under experimental conditions (Bishop et al., 1992). However, the high cost involved in the PCR analysis has limited its wide use in field surveys. Hence, when the survey tests are not locally (or regionally) validated, we stay ignorant about the diagnosis validity of the results.

We report in this study a comparison of the results from a field survey using different analysis based on serological (ELISA, SELISA and IFAT) and molecular diagnosis (PCR) for the estimates of the prevalence of *T. parva* in cattle. The reason why PCR was included in the design of the study was that no literature was available regarding the sensitivity of the assay under field conditions. The results showed a higher sensitivity of serological methods compared to PCR sensitivity in *T. parva* prevalence studies. Although it has been reported that some strains of *T. parva* do not induce a carrier state (Bishop et al., 1992; Oura et al., 2007), it is possible that seropositive but PCR negative animals may still be infected with the parasite but at a level below the threshold of PCR detection. In this context, the detection threshold of the assay used to measure the carrier status of the cattle would require further investigations and validation under field conditions.

The fluctuating sensitivity of serological methods previously reported in intermittent or seasonal tick occurrence areas in Eastern Zambia (Billiouw et al., 2005) was not

experienced in the case of Rwanda. The sensitivity of serological assays was similar in all seasons and in areas of high (>70 ticks per animal) and low (<15 ticks per animal) tick abundances. In the particular situation of Rwanda, they are two factors which could explain the high levels of *T. parva* antibody detectable by serological methods. First, the observed overlap in transmitting instars of *R. appendiculatus* assures a continuous transmission of *T. parva* infection from ticks to cattle. Second, the gradual shift from extensive to more restricted grazing systems allow for rapid and efficient transmission of infection between animals, since infection is picked up from carrier adult cattle as well as from calves undergoing their primary infection.

On the other hand, studies examining the sensitivity of PCR reveal a trend in decrease of PCR sensitivity when the tick numbers increased. There was a good agreement between the sensitivity of PCR and serological methods when tick numbers are low (<15 ticks per animal) but a significant decrease in PCR sensitivity was found as the tick numbers increased. Evidence for higher sensitivity of PCR method when tick challenge is low was also demonstrated in calves, carrying lower tick infestations than adult cattle.

In previous studies, PCR method was reported to be highly specific (Bishop et al., 1992), but its sensitivity has not been examined under variable field conditions. Available reports describe fluctuation of the sensitivity of PCR methods in detecting experimental *T. parva* carrier state (Skilton et al., 1998; Geysen, 2000). However, the negative association between the sensitivity of PCR and tick numbers found in this study has not been previously reported. The decrease in PCR sensitivity with increasing tick numbers implies that parasite densities in cattle are reduced below the detection level of PCR methods in high *T. parva* transmission conditions. The study concluded that although serological methods may lack sensitivity when *T. parva* transmission is intermittent, they remain the most sensitive tool to detect chronic *T. parva* infection in endemic areas of eastern and central Africa.

6.3 Epidemiology of ECF in Rwanda

6.3.1 The epidemiological significance of AEZs

Based on altitude, climatic parameters and agricultural activities, four agro ecological zones (AEZs) have been defined in Rwanda. The existence of particular climatic

conditions in a given area determines the relative abundances and the number of generations of developmental instars of ticks which in turn control the total level of transmission of tick-borne infections. Since the presence of *R. appendiculatus* is closely associated with the occurrence of *T. parva* infection, a question was put forward as to whether the different AEZs reflect different ecologies for *R. appendiculatus* distributions and hence, different *T. parva* infection challenge areas.
To address this question, a nation-wide tick distribution and a *T. parva* prevalence surveys were carried out in all AEZs. As a result of the tick survey, Rwanda became re-divided in two regions. Higher numbers (>70 ticks per animal) of ticks were found in all AEZs except in the AEZ 1 (< 15 ticks per animal) and *R. appendiculatus* accounted for more than 90% of the total tick loads. Among the reasons for this significant abundance of ticks include the geographical situation of the country and the suitability of the climate for tick development. The AEZ 1 was less ecologically suitable for tick survival than the rest of the country because of its higher elevation (1900 m) that leads to colder temperature in some periods of the year.
For the prevalence of *T. parva* infection, results indicated that no distinction could be made between the four AEZs. The prevalence of *T. parva* infection was estimated high (>80%) even in regions of low tick abundances. It became clear therefore from this survey that no correlation could be made between the geographic location of AEZs and the prevalence of *T. parva* infection. Our findings are however in contrast with the significant variation in prevalence of *T. parva* infections by AEZs earlier reported in many other countries of eastern Africa endemic to ECF (Gitau et al., 2000; Rubayire Akiiki et al., 2004). However, the survey results reported in these areas were mainly based on apparent prevalence data of a single diagnostic assay whereas the multitesting approach used to detect the true prevalence of *T. parva* infection in Rwanda improved significantly the prevalence estimates of infection in the field compared to results of each individual test. This implies that any reliable comparison could be hardly established between these results.

6.3.2 Methods to assess the epidemiological states of ECF

The epidemiological state of ECF in an area can be considered as either endemically stable or unstable. According to strict definition, endemic stability to ECF is a situation characterised by high prevalence of infection, low incidence and case-

fatality rate and low age-at-first contact of infection. In some instances, a combination of incidence and age-at-first contact provides necessary and sufficient information to allow accurate assessment of the epidemiological status of the disease (Billiouw et al., 2002), although the case fatality of infection is arguably the most complex and influential variable in the epidemiology of ECF. The major constraint is that adequate data set on disease incidence and hence case-fatality is difficult to obtain since it needs close supervision of monitored animals in longitudinal surveys. The organisation of these surveys is labour intensive and expensive.

The research carried out in this work was based entirely on cross-sectional surveys. Compared to longitudinal surveys, cross-sectional studies provide only a snapshot of event at a particular time. In this regards, transversal surveys are of limited value in the epidemiological studies of ECF, since the results of a single survey cannot reveal trends in disease incidence. Furthermore, estimation of the effect of risk factors on the dynamics of the disease is not possible in cross-sectional investigations. Indeed, these are less suitable to study the disease frequency and case fatality (Thursfield, 1997), important parameters in the assessment of the epidemiology of ECF.

6.3.3 Epidemiology and control

One of the aims of this study was to assess the difference in epidemiological pattern (level of challenge and degree of endemicity to ECF) in different AEZs and farming systems in Rwanda. Assessment of the epidemiological states prevailing in an area is cardinal when control strategies are discussed. In cross-sectional surveys, the infection prevalence in cattle and the inoculation rate of the disease are the two principal measurements required to assess the epidemiological state of ECF (Perry et al., 1985), although they do not provide answer to question that are relevant to what proportion of cattle suffer clinical disease or die because of the infection.

As described in previous section, the prevalence of *T. parva* infection in cattle was high in all AEZs. Disease prevalence reveals the proportion of animals that get infected in the cattle population, but a question remains on how many die of ECF from those infected. Studies attempting to correlate the prevalence and incidence of *T. parva* infection do not reveal a clear relationship (Gitau et al., 2000), although the lack of correlation could be masked by other factor such as differences in management systems (Gitau et al., 2000).

In the present study, the influence of managerial practices on the epidemiology of ECF was evaluated. Tick infection rates and available data on tick counts obtained from the three different farming systems (fenced, free-range and restricted) were combined to study the dynamic of transmission of *T. parva* infection. Infection challenge was significantly lower in the free-range (1 infected tick per animal every two weeks) and the fenced (1 infected tick per animal per week) than the restricted farming systems (9 infected ticks per animal per week). Since there is no variation in climatic suitability for *R. appendiculatus*, the difference in levels of *T. parva* challenge between farming systems will likely depend on different managerial systems applied. A similar variability in degrees of challenge of *T. parva* infections in function of differing managerial practices has been previously reported in high land Kenya (Gitau et al., 2000).

In the light of the importance of the managerial factors in the epidemiology of ECF in Rwanda, what are the implications for a rationalised control strategy?

Acaricide application is routinely applied at variable degrees to combat ECF in Rwanda. However, the presence of relatively high numbers of ticks in regularly dipped herds in the fenced farming system is a clear indication that strict tick eradication is hardly achievable. In view of the high endemicity to ECF and the ecological suitability for tick development and survival in the whole of Rwanda, sustainable control strategies have to be based on the constant interaction between infected ticks and cattle that led to varying levels of acquired immunity to ECF and other tick-borne diseases. In the particular situation of Rwanda where the level of infection challenge is a function of the farm-based managerial practices, any intervention for control of ECF will involve management-associated risk factors such as grazing practice and range (mixing carriers and primary affected animals) and the frequency of acaricidal application.

In the free-range farming system, calves up to one year old are kept in pen side or allowed to graze in home neighbourhood to prevent contact with ticks. The majority of ticks in this area likely become infected by feeding on carrier adult cattle. The subsequent *T. parva* challenge is low, reducing the case-fatality proportion. Under these conditions, only the calf population is at risk of primary infection and clinical disease. However, given the fact that young animals have lower tick numbers than adult cattle (Moll et al., 1986) and the dose of infection is low when derived from

carriers, the infection is likely to be less lethal. In addition, there is a protective or resistance effect in calve born from zebu cattle in endemic areas (Barnett, 1957; Barnett and Brocklesby, 1968; Kock et al., 1990) but this resistance decreased as calve grow up and was not owing to colostrum (Cunningham et al., 1989).

Barnett (1963) explained the innate resistance of calves from endemic areas as a result of genetic selection for *Theileria* resistant cattle which has been greater in eastern Africa where ECF is a serious disease for centuries. However, the author could not explain why this resistance would decrease in older calves. In this particular situation of the breakdown of the protective effect, timely administration of curative drugs to diseased animals will be the only realistic measure whereas the use of acaricides should be limited to the minimum required to prevent excessive tick loads in this area.

In contrast, the system of fencing or restricted farming allows for more interactions between ticks and cattle of all age classes. Ticks feed on adult carrier cattle as well as primary infected calves and higher level of infection is achieved in feeding ticks. However, reduced tick numbers through regular acaricide application leads to low infection challenge in the fenced farming system. These low levels of *T. parva* transmission would normally induce low incidence of ECF. In this respect, chemotherapy should be instigated in case of clinical severity. For effective treatment of acute infections of ECF, curative drug needs to be administrated early in the development of the disease. Cattle in the fenced farming system are kept under relatively good management conditions due to various farm-based project supports. Therefore, close monitoring of the occurring clinical case in the herd and its subsequent treatment is a relatively possible intervention for a common smallholder farmer of this managerial system.

On the other hand, tick control is applied at variable frequencies in the restricted farming system leading to high tick counts on animals. The high numbers of ticks infesting cattle coupled with their high infectivity would likely result in increased *T. parva* transmission levels and greater risks of clinical disease. Under these conditions, tick control measures could reasonably result in low levels of infection challenge and low incidence of the disease, a situation similar to what is described in the fenced farming system. Alternatively, a preventive vaccination before exposure of young animals to *T. parva* challenge should be recommended. The immunisation by means of infection and treatment method using Muguga Cocktail based stabilate is now being successfully used in neighbouring Tanzania and Uganda (Lynen et al.

unpublished communication; Oura et al., 2004) although it requires important logistics. However, this method seems to be an appropriate control strategy since the reduced herd size per smallholder and the restricted system would allow for adequate monitoring of immunised animals while minimising the logistics.

Specific epidemiological conditions are found in dairy production units that use highly productive but ECF susceptible taurine cattle. Such stall-fed animals are characterised by intensive acaricide application, very good management and the intensive input of labour and capital. Under these circumstances, the transmission of *T. parva* by *R. appendiculatus* is broken by regular and intensive use of acaricide that are effectively maintained at the correct concentration. Sporadic cases of clinical ECF are usually recognised early through good management, and animals are treated with antitheilerial compound. Because the cattle population is maintained susceptible through good tick control, high mortality will occur when the control effort breaks down and losses may occur in all age groups. In this situation where the entire herd remain susceptible to ECF, immunisation of the total cattle population is the best way to control the disease compared to tick control or chemotherapy (Muraguri et al., 1998; D'Haese et al., 1999; Penne et al., 1999).

6.4 Conclusion and future prospects

This study has assessed the epidemiological status of ECF in Rwanda through cross-sectional surveys. The outcome of this thesis has contributed to an improved understanding of the distribution of the tick populations in the major agro-ecological zones (AEZs) of the country. Most importantly, the analysis the results provided some insight in the dominant distribution of *R. appendiculatus* tick species and its repercussions on the transmission of *T. parva* infection.

The results of this study showed that although the *T. parva* infection pressure is generally high throughout the country, the intensity of infection is highly correlated with managerial practices (tick control practices, grazing practices) rather than classical ecological risk factors such as climatic suitability for tick development and the established positive correlation between the prevalence of infection in cattle and the vector abundances. However, though seroprevalence results did not reveal any difference between different AEZs, significantly lower tick counts were found in the high land zone. Moreover, while there is no indication of seasonal calving in Rwanda,

higher prevalence of infection was found in young animals (<1 year old) than adult cattle in all AEZs except in the high land zone, suggesting that a particular epidemiological situation may prevail in this part of the country. The study concluded that different endemic states for ECF may exist in Rwanda and these may results in different production losses.

However, we are aware that it is difficult to understand clearly the factors associated with acquisition of *T. parva* infections, the infection patterns and the outcome of these infections by a cross-sectional study. For accurate assessment of epidemiological states of ECF, data on ECF incidence and production loss are key epidemiological parameters and can only be obtained from longitudinal studies.

Our findings also stressed the usefulness of a multitesting approach over a single test use for accurate estimates of the true prevalence of *T. parva* infection. Furthemore, the study has provided evidence that the sensitivity should not be considered as value intrinsic to the diagnostic test (i.e constant and universally applicable) but as variable dependent on *T. parva* prevalence and intensity of infection transmission. Our study finally underscores the importance of structured incidence and case fatality studies in different farming systems to confirm our EIR-based endemic state predictions

6.5 References

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Summary/Samenvatting

Summary

Theileria parva that causes East Coast Fever (ECF) and transmitted by *Rhipicephalus appendiculatus* tick is one of the major limitations to the development of cattle industry in the eastern, central and southern Africa. Control of the disease has been hampered by the existence of different epidemiological states resulting from a number of intrinsic and extrinsic variables affecting the cattle host, the parasite and the vector ticks but most essentially by the dynamic interactions that establishes between them in different environments.

While it is widely recognised by many experts that ecological variations in *R. appendiculatus* occurrence leads to different epidemiological states of ECF between different regions and even within a single geographical region, there is a paucity of information on the status of *R. appendiculatus* distribution and the disease they transmit in the different ecological environments in Rwanda. The work described in this thesis aimed at gaining a better understanding of factors affecting the epidemiology of ECF in Rwanda, the ultimate goal being to come-up with suitable control approaches.

In the first chapter, a general introduction sets the problem of ECF in endemic areas on a broader sense and points out the direction of the present study. Current information on epidemiological factors governing the parasite-host-vector interactions and the shortcomings in the control methods are reviewed. Special attention is paid on identifying gaps in our knowledge with particular reference to the epidemiological situation of ECF in Rwanda. The overall scope of the study and specific research topics to be addressed in Rwanda are presented at the end of the chapter.

The second chapter describes the results of a tick survey carried out in the different ecological zones (AEZs) and over the rainy and the wet seasons. In this tick survey, six tick species were identified but *R. appendiculatus* was the most predominant tick species and accounted for more than 90% of the total tick loads. *Boophilus decoloratus* (6.07%) and *Amblyomma variegatum (1.24%)* were the second and third most important species respectively, whereas other ticks species [*R. eversti eversti (0.46%), R. compositus (0.31%)* and *Ixodes cavipalpus (0.08%*)] were rarely found. The season and the AEZ had no significant influence on tick abundances in most of

Rwanda except in the high land (AEZ 1) areas where significantly reduced tick numbers were recorded. The average tick numbers per animal was high in all except in the AEZ 1. Suitable climatic conditions for tick development prevail in most of Rwanda, resulting in year round overlap in different tick instars throughout the country. The epidemiological significance of the high numbers of ticks infesting cattle was further discussed in light of the development of livestock production system in Rwanda.

In chapter three, the prevalence of co-infecting *T. parva* and *T. taurotragi* infections in pooled *R. appendiculatus* field ticks were assessed using the species-specific RFLP-PCR method. The *Theileria* spp. infection rates varied between different farming systems and ranged from 1.7% in the free range system to 5.8 % in the restricted farming system. The prevalence of *T. taurotragi* infection detected in *R. appendiculatus* was in agreement with prevalence results found in cattle hosts.
T. parva was the most prevalent infection in *R. appendiculatus* but the proportions of infected ticks varied significantly between farming systems. Lower infection rate was found in the free-range where the majority of ticks feed on low parasitaemic carrier adult animals than in the fenced and the restricted farming systems characterised by frequent contacts between ticks and both calves and adult cattle. The intensity of *T. parva* transmission expressed as EIR was further measured through a model integrating data on infectivity in field ticks and the numbers of ticks infesting animals in different managerial systems. The results showed a significant effect of the farming system in the overall *T. parva* transmission. Similar infection rates were found between the fenced and the restricted farming systems but the EIR was 9 fold much higher in the later than in the former. Similarly, the restricted farming system had a much higher infection challenge than the free-range, although comparable tick numbers were collected in the two farming systems. The system of fencing and restricted favour higher infectivity of *R. appendiculatus* than the free-range farming systems but the tick control measures applied in the fenced compared to the restricted farming systems led to lower *T. parva* transmission intensity in the former.

The fourth chapter presents the prevalence results of *Theileiria* spp. infecting cattle in Rwanda as detected for the first time by PCR methods under field conditions. The PCR-RFLP method identified four *Teileria* spp. namely *T. parva, T. mutans, T.*

taurotragi and *T. velifera* but *T. parva* and *T. mutans* were found to be the most dominant species. These results were in accordance with the described tick phenology. The ecological and seasonal dynamic of *T. parva* infection was studied in more details in different regions. In all except the AEZ 1, calves are highly infected than adult cattle. Analysis of the overall *T. parva* prevalence results detected by PCR methods revealed a decreasing trend in infection prevalence as *R. appendiculatus* numbers increased. These results were unexpected and the sensitivity of PCR was questionable. It was therefore deemed necessary to compare the sensitivity of PCR with that of serological methods commonly used to detect *T. parva* infection under field conditions.

Chapter five presents an in-depth evaluation of the sensitivity of PCR and serological methods in different *T. parva* transmission areas (AEZs) in Rwanda. The Bayesian model that integrates both methods confirmed the high prevalence (83-85%) of *T. parva* infection throughout the country. However, agreement between the sensitivity of PCR and serological methods was only achieved in AEZ 1 where the lowest tick challenge was found (< 15 ticks per animals). However, while the sensitivity of serological assays remained constant throughout all the AEZs, the PCR sensitivity declined from lower (AEZ 1) to higher tick challenge areas in AEZ 4. The discrepancy between lower sensitivity of PCR than serology in high tick challenge AEZ 4 was further demonstrated between higher sensitivity of PCR in detecting *T. parva* infection in calves carrying low tick infestations than adult cattle and between dipped and undipped herds. In contrast, the sensitivity of the three serological methods used remains high and constant across tests and AEZs. We concluded that serological methods are the most suitable tool to detect carrier infections. However, a combination of PCR and serology would be a useful indicator of variable transmission intensities.

The final general discussion (Chapter 6) outlines the major findings of the thesis in a broader perspective. First, the methods to assess the epidemiological states of ECF are critically discussed. Then, the dynamic of *T. parva* transmission is analysed with regard to different AEZs and farming systems and possible control strategies are proposed with focus on the specific epidemiological situations found in the country.

Samenvatting

East Coast fever (ECF), veroorzaakt door *Theileria parva* en overgedragen door de teek *Rhipicephalus appendiculatus* is een van de belangrijkste hinderpalen in de ontwikkeling van de rundveehouderij in oost, central en zuidelijk Afrika. De controle van deze ziekte is bemoeilijkt door een geheel van intrinsieke en extrinsieke factoren die de gastheer, de parasite en de vector beinvloeden. De epidemiologie van deze ziekte zal verschillen naargelang de omgeving die voor dynamische verschillen in deze interacties zorgt.
Ecologische verschillen in de habitat van *R. appendiculatus* leiden tot verschillen in de epidemiologie van ECF in de verschillende regios en zelfs binnen eenzelfde geografisch gebied. Er is nog onvoldoende informatie over de verspreiding van de teek en de ziekte in de ecologisch verschillende gebieden van Rwanda. Deze thesis heeft tot doel de faktoren beter te begrijpen die de epidemiologie van ECF in Rwanda beinvloeden, met als uiteindelijk doel tot een betere aangepaste controle te komen.

Het eerste hoofdstuk plaatst het probleem van ECF in endemische gebieden in een breder perspectief en geeft de onderzoekslijn aan van de huidige studie. Een literatuur onderzoek brengt de huidige kennis samen over epidemiologische faktoren die bepalend zijn voor de interaktie tussen gastheer, de parasite en de vector en de tekortkomingen in de controle methodes. Speciale aandacht wordt besteed aan tekortkomingen in onze kennis en meer specifiek aan de epidemiologische ECF situatie in Rwanda. De algemene doelstellingen en de specifieke onderzoeksvragen in de Rwanda studie worden voorgesteld aan het einde van dit hoofdstuk.

Het tweede hoofdstuk beschrijft de resultaten van een teken veldstudie die in de verschillende agro-ecologische (AEZs) in het regen en het droog seizoen werd opgezet. In deze studie werden 6 verschillende teek soorten geidentificeerd, maar *R. appendiculatus* was de meest voorkomende teek met meer dan 90% van de totale teken aantallen. *Boophilus decoloratus* (6 %) en *Amblyomma variegatum* (1,24%) waren de tweede en derde meest voorkomende soorten, terwijl de andere teek soorten (*R. evertsi* (0,46%), *R. compositus* (0,30%) en *Ixodes cavipalpus* (0,08%)) sporadisch voorkwamen. Het seizoen, noch de AEZ had een significante invloed op de teken aantallen in Rwanda, behalve in het hoogland (AEZ1) waar duidelijk minder teken

werden gevonden. Overal was het gemiddelde aantal teken per dier hoog, behalve terug in het AEZ1. In het grootste deel van Rwanda zijn de klimatologische omstandigheden voor teken ontwikkeling gunstig waarbij de verschillende teken stadia over geheel het jaar over geheel het grondgebied voorkomen. De epidemiologische betekenis van de hoge aantallen teken is verder besproken in het kader van de ontwikkeling van de rundveehouderij in Rwanda.

In hoofdstuk drie wordt een methode besproken, gebruik makend van een soortspecifieke RFLP-PCR, om *T. parva* en *T. taurotragi* infecties in een staal van samengebrachte *R. appendiculatus* teken te bepalen. De Theileria spp. infectiegraden vertoonden verschillen tussen verschillende veeteelt systemen en varieerden tussen 1,7% in het vrije loop systeem en 5,8% in het vastgebonden systeem. De infectie graad van *T. taurotragi* in *R. appendiculatus* kwam overeen met de prevalentie resultaten van deze parasiet in runderen. *T. parva* was de meest voorkomende infectie in *R. appendiculatus*, maar de proportie van geinfecteerde teken was in belangrijke mate verschillend tussen de veeteelt systemen. Een lagere infectiegraad werd gevonden in het vrije loop systeem waarbij de meerderheid van de teken voeden op volwassen drager dieren met lage parasitaemie terwijl die hoger is bij dieren in weiden of in vastgebonden systemen omwille van het frekwente contact met teken. De intensiteit van *T. parva* transmissie, uitgedrukt als EIR werd nagegaan door de gegevens van de infectie graad in veldteken en aantal teken op de dieren in de verschillende landbouwsystemen in een model te vergelijken. Dit liet een signifikant effekt zien van de landbouwsystemen op de *T. parva* transmissie. Gelijkaardige infectie graden werden gevonden bij dieren in weiden of in vastgebonden systemen, maar de EIR was 9 maal groter in de weidedieren dan in de vastgebonden dieren. Bovendien hadden de gebonden dieren een veel hogere infektie druk dan deze in het vrije loopsysteem, niettegenstaande zelfde aantallen teken werden verzameld in beide systemen. Het weidesysteem en het vastbinden geven aanleiding tot een hogere *R. appendiculatus* infectiviteit dan het vrije loopsysteem, maar de methode van teken controle in het weidesysteem geeft een lagere *T. parva* transmissie dan in het gebonden systeem.

Het vierde hoofdstuk geeft voor het eerst PCR resultaten over het voorkomen in het veld van de verschillende Theileria's in Rwanda. De PCR-RFLP methode identificeerde 4 Theileria spp. namelijk *T. parva*, *T. mutans*, *T. taurotragi* en *T. velifera*, waarbij *T. parva* en *T. mutans* de meest voorkomende soorten waren. Deze resultaten waren in overeenstemming met de beschreven teken fenologie. De ecologische en seizoensdynamiek van *T. parva* infecties werd voor de verschillende regios in meer detail onderzocht. Buiten het AEZ1 gebied waren meer kalveren dan volwassen dieren in contact geweest met *T. parva*. Een analyse van de door PCR bepaalde *T. parva* prevalentie gaf een dalende trend weer naarmate de teken aantallen toenamen. Dit was een onverwacht resultaat welke de gevoeligheid van de PCR in vraag stelt. Daarom werd de gevoeligheid van de PCR methode vergeleken met die van routinematig gebruikte serologische testen.

Hoofdstuk vijf gaat dieper in op de gevoeligheid van PCR en serologie testen in de verschillende *T. parva* transmissie zones (AEZs). Het Bayesiaanse model dat de 2 testmethodes integreert bevestigt de hoge prevalentie (83-85%) van *T. parva* in Rwanda. Maar er werd enkel een overeenstemming gevonden tussen de gevoeligheid van PCR en serologie in AEZ1, waar de laagste teken aantallen werden gevonden (<20 teken/dier). Daarbij daalde de PCR gevoeligheid naarmate de teken antallen stegen (van AEZ1 naar AEZ4). Dit verschil in gevoeligheid voor PCR in de gebieden met hoge teken aantallen werd verder bevestigd door een hogere gevoeligheid van de PCR methode om *T. parva* infecties in kalveren te detecteren en in met acaricide behandelde dieren. Beide groepen dieren hebben minder teken. Daartegenover staat dat de gevoeligheid van de 3 serologische testen groot en constant blijft in de verschillende AEZs. Daaruit volgt de conclusie dat serologie de beste methode is om 'in contact' dieren op te sporen. Maar een combinatie van PCR en serologie zou een goede indicator kunnen zijn om verschillen in transmissie intensiteit aan te tonen.

De algemene discussie (Hoofdstuk 6) geeft de voornaamste bevindingen van de thesis weer in een breder kader. Ten eerste worden de methodes om de epidemiologie van ECF te onderzoeken kritisch bekeken. Dan volgt een analyse van de dynamiek van *T. parva* transmissie in de verschillende AEZs en veeteelt systemen, met de bespreking van mogelijke controle strategieen naargelang de verschillende specifieke epidemiologische situaties die voorkomen in Rwanda.

Curriculum vitae

CURRICULUM VITAE

Personal Identity

Name: Thomas Bazarusanga

Date and place of birth: 19th December 1966 in Burundi

Nationality: Rwandese

E-mail address: tbazar05@yahoo.fr; tomabazar@yahoo.fr

Education

1998-1999: MSc in Tropical Animal Health. Title: *Molecular characterisation of Theileria parva parasites in the field of Rwanda*
Institute of Tropical Medicine (ITM), Belgium

1997-1998: Diploma- Course in Tropical Animal Health and Production (CIPSAT)- ITM

1990-1995: DVM (Doctorat d'Etat en Sciences et Médecine Vétérinaires), Inter States School of Veterinary Sciences and Medicine (EISMV), University Cheick Anta Diop, Dakar, Sénégal

1987-1989: First level of Bachelor degree in Pharmacy, University of Burundi

1980-1987: Scientific Certificate, High School of Bujumbura, Burundi

Working experience

1995-1996: Assistant lecturer, Inter-states school of Veterinary Sciences and Medicine (EISMV), Dakar, Senegal

1996-2002: Livestock Programme Coordinator, Agriculture Research Institute (ISAR), Butare, Rwanda

2002-2008: Head of Parasitology Department, National Veterinary Laboratory, Kigali, Rwanda

Other activities

Regular visiting lecturer at the National University and at High School of Agriculture and Animal Production (ISAE), Rwanda

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