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## CHAPTER 2

### Literature Review

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The genus *Theileria* is a group of obligate, intracellular, tick-transmitted, apicomplexan parasites that infect wild and domestic ruminants throughout the world (Allsopp et al., 1993). Cattle and buffalo in Africa are usually co-infected with pathogenic, mildly pathogenic and non-pathogenic *Theileria* spp. These species are transmitted by different tick vectors and their geographical distribution depends on the distribution of their tick vectors.

#### 2.1 Classification of *Theileria* spp.

*Theileria* parasites belong to the super-group Chromalveolata; phylum Apicomplexa (Adl et al., 2005). Apicomplexan parasites are single celled eukaryotes with an apical complex in some of the life-cycle stages. Members of the family Theileridae (*Theileria* and *Babesia*) have schizont stages in lymphocytes. In *Theileria*, the piroplasm stages in erythrocytes lack a pigment (Irvin, 1987).

Classification: (Adl et al., 2005)

Supergroup: Chromalveolata

Superphylum: Alveolata

Phylum: Apicomplexa

Class: Aconoidasida

Order: Piroplasmida

Family: Theileriidae

Genus: *Theileria*

## 2.2 Life cycle of *Theileria* in cattle and tick vector

The life cycle of *Theileria parva* is a typical apicomplexan life cycle (Figure 2.1) with an alternation of sexual and asexual stages that are found in the mammalian and tick host. Sporozoites are inoculated into the bovine host by the tick during a blood-meal. These enter lymphocytes and develop into schizonts. The lymphocytes are transformed and immortalized by the parasite. Schizonts stimulate the host cells to divide, and as cell divides, the schizont also divides, resulting in infection of the daughter cells. This synchronization of the division of host cells and schizonts results in the invasion of various host tissues by infected cells, causing a severe and sometimes fatal disease (Kaba et al., 2005). Some of the schizonts develop into merozoites, which are then released into the bloodstream where they invade erythrocytes and transform into piroplasms which are the stages that are infective to the tick.

Inside the gut of the tick, the piroplasms differentiate into male (micro-) and female (macro-) gametes which then fuse to form a zygote. The zygote then enters gut epithelial cells and develops into a kinete. Kinetes then emerge from the epithelial cells and migrate to the salivary glands of the tick where they transform into sporoblasts, each of which produces thousands of sporozoites. The cycle is then continued by inoculation of the sporozoites into the mammalian host by the tick.

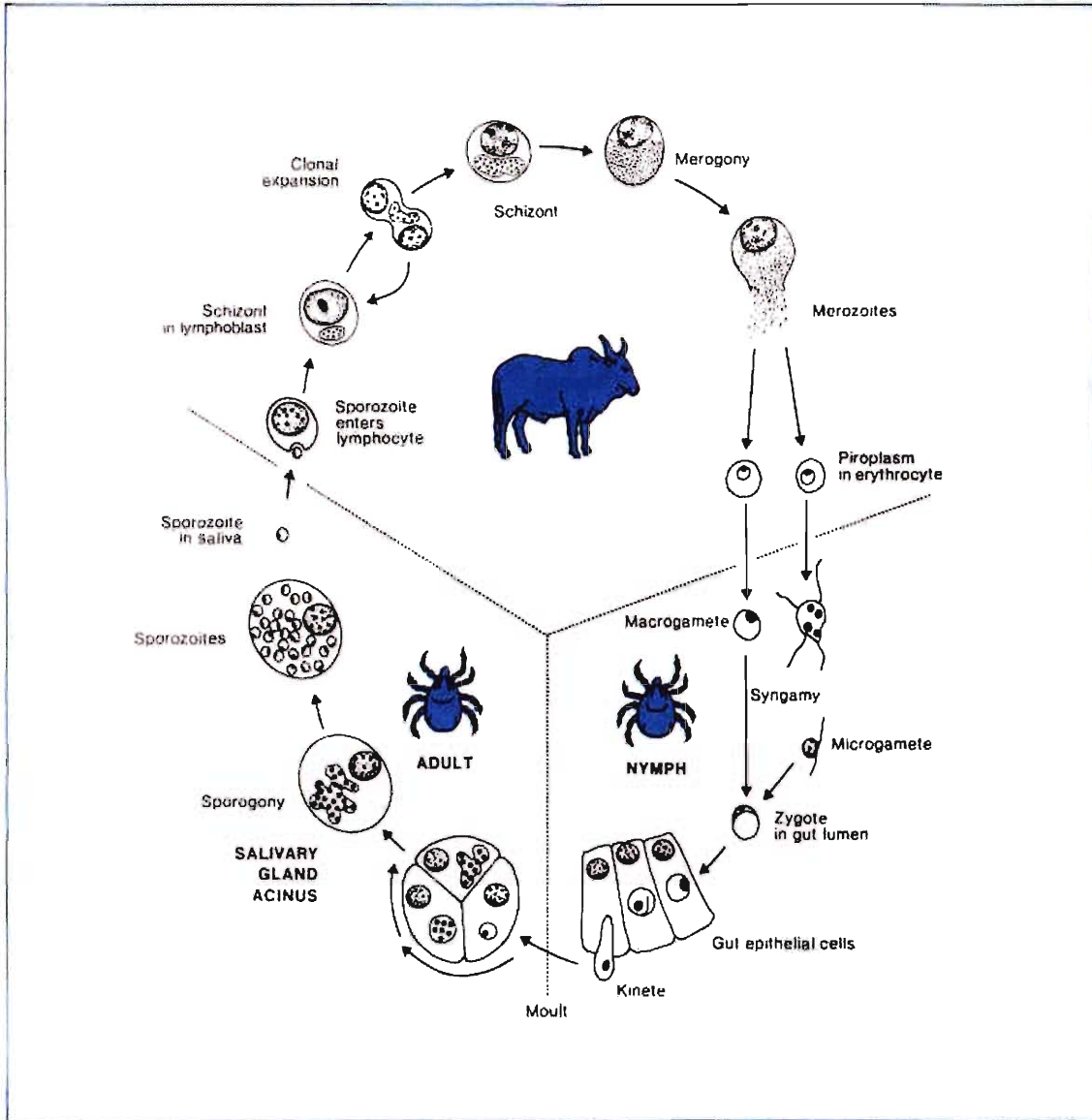


Figure 2.1: The life-cycle of *T. parva* (ILRAD, 1990)

### 2.3 *Theileria* species of buffalo and cattle in Africa

The most pathogenic (malignant) species of cattle are *T. parva* and *Theileria annulata*. These *Theileria* parasites are of major economic importance to the cattle industry due to high mortality and morbidity, cost of control and treatment, as well as loss in production by infected animals (Allsopp et al., 1993; ILRAD, 1990; OIE 2000; McKeever, 2001; Schnittger et al., 2002).

*Theileria parva* infects cattle and buffalo in eastern, central and southern Africa and is the causative agent of East Coast fever (ECF), January disease and Corridor disease in cattle (ILRAD, 1990; Gubbels et al., 1999). *Theileria parva* is transmitted transstadially by the three-host ticks, *Rhipicephalus appendiculatus*, *Rhipicephalus zambeziensis* and *Rhipicephalus duttoni* (Young et al., 1978b; Lawrence et al., 1983; Norval et al., 1992). The African buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* and infections in buffalo are usually asymptomatic, but are acute and usually fatal in cattle (Lawrence et al., 1994c). The African buffalo is an indigenous bovine of sub-Saharan Africa and has lived in harmony with *T. parva* and its vector *R. appendiculatus* long before cattle were introduced into the region (Grootenhuis, 1988). Other reservoir hosts of *T. parva* are water buffalo (*Bubalus bubalis*) and waterbuck (*Kobus defassa*) (Stagg et al., 1994; CFSPH, 2009).

*Theileria annulata* infects cattle, yak, water buffalo and camels in northern Africa, southern Europe, the Middle East and Central Asia where it causes tropical theileriosis (Sergent et al., 1935)

Mildly pathogenic and benign species of *Theileria* that infect cattle and buffalo in Africa are *Theileria mutans*, *Theileria velifera*, *Theileria buffeli*, *Theileria taurotragi* and *Theileria* sp. (buffalo) (Allsopp et al., 1993; Gubbels et al., 1999; Oura et al., 2004). *Theileria* parasites usually occur as mixed infections in infected animals (Georges et al., 2001) and although the benign and mildly pathogenic forms do not have any significant economic importance, they can interfere with the diagnosis of the pathogenic forms and therefore confuse their epidemiology (Lawrence et al., 1994a).

## 2.4 *Theileria parva* (Theiler, 1904)

*Theileria parva* is transmitted by the three-host ixodid ticks, *R. appendiculatus*, *R. zambeziensis* and *R. duttoni* (Jongejan et al., 1980; Lawrence et al., 1983). It occurs in 11 countries in Africa, extending from southern Sudan to northern KwaZulu-Natal in South Africa (Figure 2.2). The African buffalo (*Syncerus caffer*) is the natural host of this parasite and infected buffalo usually remain long-term, asymptomatic carriers (ILRAD, 1990; Uilenberg, 1999). The parasite is the causative agent of ECF, January disease and Corridor disease in eastern, central and southern Africa (Collins et al., 2002). These disease syndromes differ in their clinical symptoms, pathogenicity, epidemiology and host (cattle or buffalo) (Allsopp et al., 1993).

Due to these differences, *T. parva* was initially classified into three sub-species, namely *T. parva parva*, *T. parva bovis* and *T. parva lawrencei*. However, this classification was abandoned due to lack of molecular evidence as these sub-species are genetically similar (Norval et al., 1992; Allsopp et al., 1993). It has since been recommended that the different isolates should rather be classified as cattle- or buffalo-associated depending on the original host (Anon, 1989).

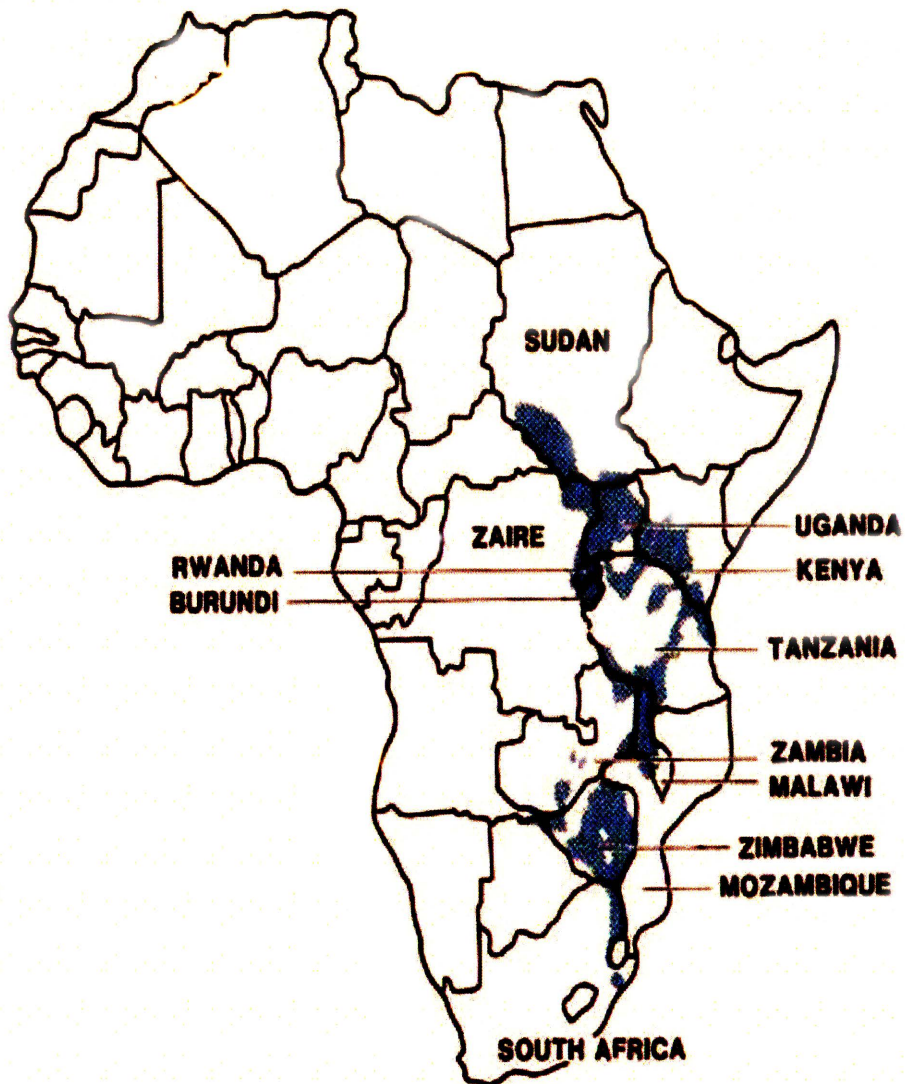


Figure 2.2: The distribution of *T. parva* in Africa (ILRAD, 1990)

### 2.4.1 East Coast fever (ECF)

ECF is a fatal disease of cattle caused by cattle-associated *T. parva* (previously known as *T. parva parva*) (Lawrence et al., 1994b). It is a major constraint to livestock production in Africa. Total annual losses due to ECF on the continent are estimated at around one million cattle and \$168 million loss in revenue (Mukhebi et al., 1992; Norval et al., 1992). Twenty-four million cattle are at risk of infection (Norval et al., 1992). The pathogenicity of *T. parva* is mainly due to transformation and proliferation of the host T-lymphocytes that is induced by the parasite during schizogony, resulting in lymphocytolysis, and usually death of the animal (Ebel et al., 1997; Nene et al., 2000). The clinical signs of ECF include fever, anorexia, decreased milk productions and nasal discharge (CFSPH, 2009). If untreated, death occurs within three to four weeks of infection (ILRAD, 1990). Naïve animals develop an acute infection, and if they survive, are able to mount an immune response that results in a carrier state with low levels of parasitaemia and disease (Beck et al., 2009). These animals usually become asymptomatic carriers and are therefore responsible for most of the transmission.

#### 2.4.1.1. The epidemiology of ECF in southern Africa

The disease was introduced into southern Africa in early 1900 by importation of cattle from the East coast of Africa after the rinderpest epidemic (Norval et al., 1992; Uilenberg, 1999). It was first reported in Zimbabwe in 1902 where it was introduced through a consignment of cattle brought from East Africa through Mozambique. The disease later spread to southern Zimbabwe and then southwards to Swaziland and neighbouring south-eastern Transvaal and Natal (now Mpumalanga and KwaZulu-Natal) provinces of South Africa until it reached the Cape of Good Hope (Cape Town) in 1910 (Norval et al., 1992).

An estimated 5.5 million cattle died in South Africa due to ECF (Stoltz, 1989). Due to its devastating effects in cattle, massive control strategies were implemented. These included clearing of infected pastures by removal of healthy cattle, intensive dipping and surveillance programmes, and mass slaughtering of infected cattle. This led to the total eradication of the disease from South Africa by 1955 (Stoltz, 1989; Lawrence et al., 1994b; Uilenberg 1999). Although the disease has been totally eradicated, cattle in South Africa are still at risk of infection because the tick vector is still present in some parts of the country (Stoltz, 1989; ILRAD, 1990).

## 2.4.2 January disease

January disease (also known as Zimbabwean theileriosis or malignant Rhodesian theileriosis) is a milder form of cattle-associated theileriosis that emerged in Zimbabwe after the eradication of classical ECF (Uilenberg, 1999). The symptoms of January disease are similar to but milder than those of ECF, and the two diseases can only be distinguished by seasonality. January disease is highly seasonal (occurs between December and March), with high mortalities in January which coincide with the availability of the tick host (Uilenberg, 1999). The causative agent was previously known as *T. parva bovis*. There is no evidence of the occurrence of January disease in South Africa (Stoltz, 1989).

## 2.4.3 Corridor disease

Corridor disease is the buffalo-associated form of the disease which still persists in most southern African countries, including South Africa (the causative agent was previously known as *T. parva lawrencei*) (Lawrence, 1992). The parasite does not cause disease in the African buffalo reservoir host, but it can be transmitted from buffalo to cattle by infected ticks. It is thought that this form of the parasite is not transmitted between cattle, as infected cattle usually die before piroplasms appear or are too few to infect new ticks (Uilenberg, 1999). The clinical symptoms are similar to those of ECF, except that death usually occurs within a short time after the onset of the first symptoms (Lawrence et al., 1994c). It is therefore regarded as a self-limiting disease in cattle (Norval et al., 1992). However, cattle infected by buffalo-derived *T. parva* can recover from infection after treatment by chemotherapy and become carriers of the parasite which are capable of infecting susceptible cattle (Potgieter et al., 1988).

### 2.4.3.1 The epidemiology of Corridor disease in South Africa

Corridor disease has become the most important form of theileriosis in South Africa after the eradication of ECF as it poses a threat to the cattle farming industry in this country (Stoltz, 1989). The disease was first recognized in the 'corridor' between the then Hluhluwe and Umfolozi (now Hluhluwe-iMfolozi) game reserves in the KwaZulu-Natal province of South Africa (Neitz et al., 1955; Lawrence et al., 1994a) and is currently endemic in the Kruger National Park (KNP) and in the Hluhluwe-iMfolozi game park, as well as in adjacent farms where cattle and buffalo are in close contact (Collins et al, 2002; Mashishi, 2002).

Measures used to control Corridor disease in South Africa include the prevention of contact between cattle and buffalo, regular dipping and spraying of all cattle in disease-infected areas and



testing of buffalo for theileriosis (and other controlled diseases) before translocation (South African Animal Disease Act 35 of 1984). However, despite all these strict control measures, sporadic outbreaks of the disease still occur in the country. In 1994, an outbreak of Corridor disease occurred in Warmbaths, Limpopo province, as a result of an illegal translocation of buffalo from an endemic area near the Kruger National Park (Collins et al., 2002). More recently, Thompson et al. (2008) reported an outbreak of theileriosis on a farm near Ladysmith in the KwaZulu-Natal province which is outside the declared Corridor disease endemic area. Infected buffalo from a neighbouring farm were suspected as the source of infection to cattle.

The phenomenon of ‘transformation’ of buffalo-associated *T. parva* to cattle-associated *T. parva* after serial tick passages in cattle has been described in East Africa by Barnett and Brocklesby (1966) and Young and Purnell (1973). However, experiments aimed at determining whether transformation can occur in South African *T. parva* isolates were unsuccessful (Potgieter et al., 1988; Norval et al., 1991).

#### **2.4.4 Control of *T. parva***

##### *2.4.4.1. Chemical control of ticks*

Tick control is one of the most important and effective ways of controlling theileriosis, and it was largely through tick control by use of acaricides that ECF was totally eradicated in southern African (Norval, 1989, Norval et al., 1992). Acaricides are usually applied by dipping or spraying. Other methods that have been used include the slow release of the chemicals from impregnated ear-tags and the pour-on method which is applied to the back of the animal and then spread over the entire body (Norval, 1989). Acaricides that have been used include the arsenical forms, organochlorines, organophosphates, carbamates, amides and synthetic pyrenoids (Norval, 1989). Although highly effective, chemical control is not sustainable due to the high cost of acaricides, development of resistance by ticks, cross-resistance between some of the acaricides, and the fact that acaricides are not environmentally friendly (Norval, 1989; Minjauw et al., 1998). Due to these disadvantages, other control measures had to be devised.

##### *2.4.4.2 Immunization of cattle*

The history of immunization of cattle against theileriosis in South Africa has been reviewed by Uilenberg (1999). Initial immunization trials involved using blood from sick or recovered cattle and later with material from the spleen and lymph nodes of infected animals. None of these methods were effective, as many animals died from fatal ECF or various bacterial infections. Subsequent

experiments resulted in the use of the 'infection and treatment' method that is currently used in the immunization of cattle against theileriosis in some African countries (Bishop et al., 2001).

The technique involves inoculation of the animal with an infective dose of sporozoites and simultaneous treatment with an antibiotic (e.g. oxytetracycline) to decrease the severity of infection (Cunningham et al., 1974; Brown et al., 1977). Although this provides life-long immunity to cattle, the technique has major limitations as it does not provide cross-immunity against all field strains; vaccination provides immunity to homologous strains of the parasite while animals remain susceptible to infection by heterologous strains (Uilenberg, 1999). This problem has been partially overcome by using 'cocktails' such as the Muguga cocktail which is composed of three *T. parva* stocks (Bishop et al., 2001). Other limitations include the fact that immunized animals remain carriers and therefore become reservoirs of infection for the tick; the infective dose is potentially fatal and preservation of the live parasites requires liquid nitrogen and cold storage, facilities that are usually lacking in developing countries where the disease is endemic (Figueroa and Buening, 1995; Uilenberg, 1999; Bishop et al., 2001; McKeever, 2001).

#### 2.4.4.3 Subunit vaccines

The problems mentioned above have resulted in the need for the development of other vaccination alternatives and research is focused on the identification of parasite proteins/genes, which could be used as subunit vaccines. Monoclonal antibodies against *T. parva* and *T. annulata* have been generated and are able to neutralize entry of sporozoites into host cells. The antibodies detect surface antigens such as SPAG-1 and TaSP (for *T. annulata*) and p67, p104, p150 and PIM genes (for *T. parva*) (Schnittger et al., 2002). Some of these have been used in animal experiments as candidate vaccine against theileriosis (Schnittger et al., 2002; Kaba et al., 2005; Musoke et al., 2005; Akoolo et al., 2008; Janssens, 2009).

#### 2.4.4.4 Chemotherapy

Tetracyclines were the first compounds to be used in the control of theileriosis. Their efficacy was limited though, as they have a suppressive effect only in the early stages of *Theileria* infection. Further research led to the discovery of the naphthoquinone compound, menotone, which demonstrated theileriacidal activity but could not be further developed, as it was too expensive to synthesize. Subsequently, more effective derivatives of this compound were developed. Hydroxynaphthoquinone compounds such as parvaquone and buparvaquone have all been effectively used in the treatment of *T. parva* infections, particularly against ECF (McHardy et al.,

1983). However, in South Africa, the treatment of Corridor disease has been discontinued due to the carrier state that these drugs induce in cattle (Potgieter et al., 1988).

#### **2.4.5 Diagnosis of *T. parva***

Diagnosis of *T. parva* is a crucial step in the control and treatment of the disease. Early detection of the parasite in the mammalian host also allows for proper treatment before clinical symptoms appear. The techniques used should therefore be specific and sensitive to low infections.

##### *2.4.5.1 Conventional parasitological techniques*

Conventional parasitological techniques include the microscopic examination of Giemsa-stained blood smears for the presence of piroplasms and examination of lymph node biopsies for schizonts in clinically suspected animals (Ogden et al., 2003; Oura et al., 2004). Although inexpensive and easy to perform, these techniques lack sensitivity and specificity as the piroplasms of *T. parva* cannot be differentiated from those of the non-pathogenic forms which commonly co-exist with *T. parva* in infected animals (Almeria et al., 2001).

##### *2.4.5.2 Serological techniques*

Serological assays detect serum antibodies to parasite schizont or sporozoite antigens (Katende et al., 1998; Billiouw et al., 2005). The most widely used serological method for the detection of *T. parva* infections is the indirect fluorescent antibody (IFA) test (Collins et al., 2002). However, the test has several disadvantages which include lack of specificity and sensitivity, cross reactivity, difficulty in standardization, subjectivity in interpretation of the results, inability to detect carrier animals, and diagnosed infections might not necessarily be active infections as the animal remains seropositive long after the infection has been cleared (Allsopp et al., 1993; Katende et al., 1998; Billiouw et al., 2005). Enzyme-linked immunosorbent assay (ELISA) has also been used for the detection of *T. parva* (Muraguri et al., 1999) antibodies and was found to be more sensitive than the IFA (Katende et al., 1998).

##### *2.4.5.3 Molecular biology techniques*

Advances in molecular biology have led to the development of more sensitive and specific diagnostic tests based on the detection and discrimination of parasite nucleic acid (DNA or RNA) sequences, and have decreased the subjectivity that usually occurs in interpreting results (Zarlenga and Higgins, 2001). *Theileria* parasites usually occur at low levels in infected animals, and molecular biology techniques are able to detect these low levels of infections. The identification of

carrier animals is important for the assessment of infection risk, since these animals serve as reservoirs of disease (Dolan, 1989). Inter- and intraspecific detection and characterization of parasites has become possible using molecular biology techniques (Monis et al., 2005).

### *I. Conventional Polymerase Chain Reaction (PCR)*

A widely used technique is the Polymerase Chain Reaction (PCR). The conventional PCR involves the use of a thermostable DNA polymerase enzyme to amplify target DNA, and a pair of oligonucleotides (primers) that are complimentary to the two strands of the target DNA (Mullis, 1990). Post PCR analysis involves electrophoresis of the PCR-product using an agarose gel and visualization (using a suitable stain) under UV light. The agarose gel separates DNA molecules according to their size and therefore the size of the separated molecules can be determined by comparison to DNA molecules of known length.

PCR is more specific and sensitive than conventional parasitological and serological techniques and has been widely used in epidemiological studies of bovine theileriosis (Katzner et al., 1998; Almeria et al., 2001; Ogden et al., 2003). However, despite all these advantages, cross-contamination and false-positives are often encountered (Zarlenga and Higgins, 2001).

### *II. PCR-based hybridization assays*

Polymerase chain reaction, coupled with hybridization, has increased the sensitivity and specificity of molecular diagnosis (Collins et al., 2002). DNA probes can be developed from variable regions of the gene target and used for simultaneous detection of related parasites from hosts and vectors (Figuroa and Buening, 1995). Primers corresponding to conserved sequences of a gene can be designed for PCR amplification and a variable region of the gene used to develop species-specific oligonucleotide probes. These species-specific probes can then be used in epidemiological studies and diagnosis of the parasites in mixed infections in hosts and vectors. Allsopp et al. (1993) used PCR amplification and species-specific probes to differentiate between the variable regions of the 18S rRNA gene of six *Theileria* species. This technique led to the identification of a novel *Theileria* sp. in buffalo which was designated *Theileria* sp. (buffalo).

Gubbels et al. (1999) developed a reverse line blot (RLB) hybridization method that combines both PCR and hybridization, and can simultaneously detect and differentiate different *Theileria* and *Babesia* species in infected hosts and vectors. PCR products are hybridized on a membrane on which species-specific probes are covalently linked. The products are visualized with chemiluminescence after a series of washes of the membrane with specific buffers. This assay has

since been used extensively in epidemiological surveys of theileriosis and babesiosis, and in many cases has resulted in the identification of novel *Theileria* and *Babesia* genotypes (Georges et al., 2001; Almeria et al., 2001; Oura et al., 2004; Nijhof et al., 2003; Salih et al., 2007; Matjila et al., 2008; Oosthuizen et al., 2008, 2009; Bhoora et al., 2009; Bosman et al., 2010; Chaisi et al., 2011).

The RLB assay is cost-effective as the membrane can be used several times if stored properly, it is reproducible and cross-reactions do not usually occur between the different species (Gubbels et al., 1999). However, the preparation of the membrane, hybridization step and the several post-hybridization washes involved make the assay laborious and time-consuming.

### III. PCR-based RFLPs

Several PCR-based restriction fragment length polymorphism (RFLP) assays have been used in the identification and characterization of *T. parva* isolates based on their unique polymorphic profiles after restriction digestion. PCR-RFLP assays based on the 18S rRNA, p104, polymorphic immunodominant molecule (PIM), p150 and cox III genes have been used to either differentiate between different *T. parva* stocks or between different *Theileria* spp. in mixed infections (Geysen et al., 1999; Bishop et al., 2001; Bazarusanga et al., 2007; Janssens, 2009, Sibeko et al., 2010). Although such assays are easy to perform, they require well equipped laboratories, and restriction enzyme digestion requires an overnight incubation which makes RFLP assays laborious and time-consuming (Sibeko et al., 2010).

### IV. PCR-based LAMP assays

Loop-mediated isothermal amplification (LAMP) is a simple, rapid and highly sensitive method for the amplification of DNA under isothermal conditions (Notomi et al., 2000; Nagamine et al., 2002). The test requires the use of four specific primers and DNA polymerase and is easy to perform as it requires the use of a regular laboratory heat block or water bath for the reaction (Notomi et al., 2000). Several LAMP assays have recently been developed for the detection of different *Theileria* and *Babesia* spp. Liu et al. (2008) developed a LAMP assay for diagnosis of ovine theileriosis in China and Thekisoie et al. (2010) developed two LAMP assays that target the PIM and p150 genes of *T. parva* in cattle and buffalo. The LAMP assays by He et al. (2009) and Wang et al. (2010b) target the 18S rRNA and 33-kDa major piroplasm surface protein (p33) genes of *Babesia orientalis* and *Theileria sergenti* respectively.

#### V. Quantitative real-time PCR (qPCR) assays

Real-time PCR has greatly improved molecular detection and differential diagnosis of closely related organisms. The assay simultaneously amplifies, detects and quantitatively analyzes target DNA sequences in real-time (Zarlenga and Higgins 2001; Monis et al., 2005). Post-PCR analysis is therefore not required and this reduces the risk of contamination, loss of the PCR product, and allows for rapid attainment of results (Bell and Ranford-Cartwright, 2002). Contamination is also minimized and the cycling instrument can be automated for large-scale processing of samples, thereby reducing both the time and labour required for analysis.

Real-time PCR using fluorescence resonance energy transfer (FRET) technology has been used for the detection and differentiation of *Theileria* and *Babesia* species in mixed infections by melting curve analysis. FRET involves the use of sequence-specific oligonucleotide (hybridization) probes that are labelled with fluorescence dyes (Reuter et al., 2005). Hybridization probes provide a simple way of analysing sequence variations using a single reaction and one set of probes as both amplification and hybridization occur in the same reaction (Caplin et al., 1999). Sibeko et al. (2008) developed a quantitative real-time PCR (qPCR) assay for the detection of *T. parva* in cattle and buffalo based on the 18S rRNA gene. This assay is currently used as the official test for the detection of *T. parva* in these animals in South Africa. In addition to the identification of *T. parva*, the assay can simultaneously detect *T. taurotragi* and *T. annulata* when the *Theileria* and *Babesia* genus-specific primer set is used. Recently, Papli et al. (2011) developed another qPCR assay for the detection of *T. parva* in cattle and buffalo in South Africa. The latter also targets the parasite 18S rRNA gene but is based on TaqMan probe chemistry. Comparison of the two *T. parva* qPCR assays indicated a good correlation in their ability to detect the parasite in infected animals (Papli et al., 2011). More recently, Pienaar et al. (2011) developed another qPCR, designated the Hybrid II assay, for the specific diagnosis of *T. parva*. The assay uses a single primer set to amplify both *Theileria* sp. (buffalo) and *T. parva*, and two distinct melting peaks are obtained for these species.

Other 18S rRNA gene qPCR assays for *Theileria* and *Babesia* spp. were developed by Criado-Fornelio et al. (2009) and Wang et al. (2010a). The former is used for simultaneous identification of *Babesia bovis*, *B. divergens*, *B. major* or *B. bigemina*, *Theileria annae* and an unidentified *Theileria* sp. in bovines, and the latter differentiates between *B. gibsoni*, *B. canis canis*/*B. canis vogeli* and *B. canis rossi* in canines. A nested qPCR assay based on the cytochrome oxidase subunit III (cox III) gene was described by Janssens (2009) for simultaneous detection and differentiation of *T. parva* and five co-infecting *Theileria* spp., in cattle.

## 2.5 Benign and mildly pathogenic *Theileria* species of cattle and buffalo in South Africa

Several benign and mildly-pathogenic *Theileria* species frequently co-exist with *T. parva* in infected animals. They are usually carried asymptotically, but under conditions of stress, malnutrition and immune-deficiency, some can also cause disease, loss of production and may increase the severity of theileriosis in infected animals (Noval et al., 1992; CFSPH, 2009). Although schizogony still occurs in the benign species, host cell transformation does not occur (non-lymphoproliferative) in this case and the pathology is mainly due to multiplication of piroplasms in the host red blood cells, resulting in anaemia, a condition that rarely occurs with *T. parva* infections (Nene et al., 2000). These parasites are transmitted by different tick species and therefore their geographic distribution coincides with the distribution of their tick vectors. *Theileria* spp. can be differentiated from each other based on their serological, morphological, epidemiological and molecular characteristics.

### 2.5.1 *Theileria mutans* (Theiler, 1906)

*Theileria mutans* is a parasite of buffalo, it is infective to cattle and can cause latent infections in sheep (Paling et al 1981; Allsopp et al 1993). It is transmitted by *Amblyomma* ticks (Uilenberg et al., 1976, 1982; Paling et al., 1981). Previously, *T. mutans* was implicated in all benign bovine *Theileria* infections worldwide (Gill, 2004). However, transmission, serology and phylogenetic studies have indicated that it is an African species and is different from benign *Theileria* species that were isolated from cattle in other parts of the world, namely, *T. orientalis* and *T. buffeli* (Uilenberg et al., 1977; Chae et al., 1999; Gill, 2004). Although generally considered as a benign species in buffalo, some strains of *T. mutans* have been associated with severe disease in cattle (Young et al., 1978a; b; Paling et al., 1981).

### 2.5.2 *Theileria* sp. (strain MSD)

This species was first identified from a naturally infected bovine at the Merck, Sharp & Dome (MSD) experimental centre at Hartebeespoort, Pretoria, South Africa (Chae et al., 1999). It was initially suspected to be a variant of *T. velifera*, but sequence and phylogenetic analyses based on 18S rRNA gene sequences indicated that it is most closely related to *T. mutans* (Chae et al., 1999; Martins et al., 2010; Chaisi et al., 2011; Mans et al., 2011). Although no attempts have been made to clarify the identity of *Theileria* sp. (strain MSD) after its first description by Chae et al. (1999), the identification of similar sequences in buffalo and cattle indicates that this genotype is circulating

in some buffalo and cattle populations in southern Africa (Martins et al., 2010; Mans et al., 2011).

### **2.5.3 *Theileria* sp. (buffalo)**

Conrad et al. (1987) reported on the presence of antigenically distinct *Theileria* parasites from the African buffalo in Kenya. The unknown parasite was thought to be buffalo-derived *T. parva* as it occurred several times among stocks that were isolated from buffalo and were characterized using monoclonal antibodies (Conrad et al., 1987). Subsequent sequencing of the 18S rRNA gene of the unknown parasite by Allsopp et al. (1993) indicated that it is a new species, as the 18S rRNA gene sequence was different from both cattle- and buffalo-derived *T. parva*. The new species was designated as *Theileria* sp. (buffalo). To date *Theileria* sp. (buffalo) has only ever been identified in buffalo, and is genetically closely related to *T. parva* and other pathogenic *Theileria* spp. (Chaisi et al., 2011; Mans et al., 2011). Recently, Zweygarth et al. (2009) established a macroschizont-infected lymphoblastoid cell line from an African buffalo infected with *Theileria* sp. (buffalo), suggesting that it is able to transform lymphocytes. However, it does not appear to infect cattle and its vector is unknown.

### **2.5.4 *Theileria buffeli/sergenti/orientalis* (Neveu-Lemaire, 1912; Yakimov and Dekhterven, 1930; Yakimov and Sudachenkov, 1931)**

*Theileria buffeli/sergenti/orientalis* is a group of closely related benign parasites of cattle and buffalo with a cosmopolitan distribution. They infect cattle and buffalo in Africa, Australia, Asia, Europe and the United States of America (USA) (Chae et al., 1998; Chansiri et al., 1999; Cossio-Bayugar et al., 2002; Aktas et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Gimenez et al., 2009; Chaisi et al., 2011; Mans et al., 2011). Ticks of *Haemaphysalis* spp. act as vectors in Australia, Asia and Europe, but the vectors in Africa and the USA are still unknown (M'ghirbi et al., 2008).

*Theileria sergenti* and *T. orientalis* were first described from eastern Siberia in the early 1930s by Yakimov and Dekhterev, and Yakimov and Soudatschenkov, respectively, while *T. buffeli* was first described from the Asian water buffalo (*Bubalus bubalis*) in 1908 by Schein (reviewed by Fujisaki et al., 1994). Their classification is confusing and it is still unclear if they represent the same species or different species. Biological differences such as the occurrence of macroschizonts and piroplasm morphology have been observed among isolates of the *T. buffeli/sergenti/orientalis* complex (Uilenberg et al., 1985). Uilenberg (2011) indicated that although the term "*T. sergenti*" has traditionally been used for this species, *T. sergenti* actually refers to a sheep parasite and it was incorrectly termed as a parasite of cattle and buffalo.



Due to all this confusion, Uilenberg et al. (1985) suggested that the benign species (*T. buffeli*/*T. orientalis*) should be classified as *T. orientalis*. The term *T. buffeli* is preferred over *T. orientalis* on the basis of molecular and biological data, as well as the fact that all characterized isolates are infective for buffalo (Steward et al., 1996; Gubbels et al., 2002; Gill, 2004). Moreover, the name *T. orientalis* is misleading as it implies that the parasite occurs only in the Far East, whereas it is known to occur all over the world. Gubbels et al. (2002) proposed that these organisms should be referred to as *T. buffeli* until more biological data becomes available for further classification, and the names *T. orientalis* and *T. sergenti* should only refer to isolates that have been previously described under these names.

#### **2.5.5 *Theileria taurotragi* (Martin and Brocklesby, 1960)**

*Theileria taurotragi* is a parasite of eland (*Taurotragus oryx*) and was first described from these animals in Kenya by Martin and Brocklesby (1960). However, fatal infections by this parasite in these animals have been never reported. It also infects cattle, sheep and goats (Uilenberg et al., 1982; Stagg et al., 1983). Like *T. parva*, it is transmitted by *R. appendiculatus* and *R. zambeziensis* (Uilenberg et al., 1982; Lawrence et al., 1983). It has been isolated from cattle together with *T. parva*, *T. annulata*, *T. mutans*, *T. velifera* and *T. buffeli*, from different parts of eastern and southern Africa (De Vos and Roos, 1981a; Oura et al., 2004; Bazarusanga et al., 2007; Salih et al., 2007; Sibeko et al., 2008). Infection in cattle is characterized by a transient fever and small numbers of microschizonts and piroplasms (De Vos and Roos, 1981b). In South Africa *T. taurotragi* infection has been associated with bovine cerebral theileriosis and Tzaneen disease (De Vos and Roos, 1981b; Stoltsz 1989). There are no reports of the occurrence of *T. taurotragi* from the African buffalo.

#### **2.5.6 *Theileria velifera* (Uilenberg, 1964)**

*Theileria velifera* was first described from cattle by Uilenberg (1964). It is a mild pathogen of the African buffalo and cattle (Noval et al., 1992; Oura et al., 2005) and is transmitted by ticks of the genus *Amblyomma* (Norval et al., 1992).

## 2.6 Molecular characterization and phylogeny of *Theileria* spp.

Initial studies on the characterization of *T. parva* and *T. annulata* involved the use of isoenzyme electrophoresis and monoclonal antibodies (Minami et al., 1983, Shiels et al., 1986) and RFLP analysis (Bishop et al., 1993; Geysen et al., 1999). These studies indicated that many isolates contained more than one genotype, and that different isolates have distinct phenotypic and genotypic profiles.

Molecular characterization using DNA and protein sequences has surpassed antigenic and phenotypic characterization in phylogenetic studies. DNA is suitable for studying phylogenetic relationships between organisms as it is passed down ancestral lineages and therefore reliably reflects ancestry.

The most commonly used marker in the characterization of *Theileria* spp. is the small subunit ribosomal RNA (18S rRNA) gene. This gene is highly conserved between all organisms but has variable regions which differ between species. Primers can therefore be designed in the conserved areas, and these will amplify a part of the gene from all related species, and species-specific probes can be designed from the variable regions in order to differentiate between the different species. This concept has been utilized in *Theileria* research for the development of several different *Theileria* species-specific diagnostic assays (Allsopp et al., 1993; Gubbels et al., 1999; Sibeko et al., 2008; Bhoora et al., 2009; Criado-Fornelio et al., 2009; Wang et al., 2010b; Papli et al., 2010), in the identification of new species and species variants (Nijhof et al., 2003; 2005; Altay et al., 2007; Oosthuizen et al., 2008; 2009; Bosman et al., 2010; Mans et al., 2011) and in determining phylogenetic relationships between species (Chae et al., 1998; Gubbels et al., 2002).

Other genetic markers that have been used in the characterization and phylogeny of *Theileria* spp. include the large subunit rRNA (28S rRNA), 5.8S rRNA and S5 genes (Bishop et al., 1995; 2000; Mans et al., 2011), internal transcribed spacers (ITS) (Collins and Allsopp, 1999; Bosman et al., 2010; Kamau et al., 2011), polymorphic immunodominant molecule (PIM) and p150 genes (Geysen et al., 2004; Sibeko et al., 2011); p67 gene (Musoke et al., 2005; Sibeko et al., 2010), major piroplasm surface protein (MPSP) genes (Kawazu et al., 1999; Gubbels et al., 2002), and the cytochrome c oxidase gene (Kairo et al., 1994; Hikosaka et al., 2010).

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