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ASPECTS OF THE EPIDEMIOLOGY OF *THEILERIA PARVA* INFECTIONS IN CATTLE AND AFRICAN BUFFALO (*SYNCERUS CAFFER*) IN SOUTH AFRICA REVEALED BY TICK TRANSMISSION AND SUB-INOCULATION OF BLOOD

by

Wilhelm Heinrich Stoltsz

Submitted in fulfilment of the requirements for the degree

Magister Scientiae (Veterinary Science)

in the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Study Leader : Dr N E Collins
Co-Study Leader : Prof B L Penzhorn

2011

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DECLARATION

I declare that this dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

………………………………………….

Wilhelm Heinrich Stoltsz

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LIST OF ABBREVIATIONS

ARC-OVI	Agricultural Research Council – Onderstepoort Veterinary Institute
BoLA	bovine lymphocyte antigen
DNA	deoxyribonucleic acid
ECF	East Coast fever
EDTA	Ethylenediaminetetraacetic acid
FMD	foot and mouth disease
IFAT	indirect fluorescent antibody test
KNP	Kruger National Park
MAbs	monoclonal antibodies
MSI	macroschizont index
N.D.	not done
p.i.	post-infection
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RH	relative humidity

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THESIS SUMMARY

ASPECTS OF THE EPIDEMIOLOGY OF *THEILERIA PARVA* INFECTIONS IN CATTLE AND AFRICAN BUFFALO (*SYNCERUS CAFFER*) IN SOUTH AFRICA REVEALED BY TICK TRANSMISSION AND SUB-INOCULATION OF BLOOD

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Co-Promoter : Prof B L Penzhorn

Degree : MSc (Veterinary Science)
Department : Veterinary Tropical Diseases

The aim of this study was to investigate three key epidemiological aspects of *Theileria parva* infections in cattle and African buffalo (*Syncerus caffer*) in South Africa. The first of these was the possible behavioural change (i.e. transformation) of buffalo-derived *T. parva* (causing classical Corridor disease in cattle) to what might be considered cattle-derived *T. parva* (causing classical East Coast fever in cattle) after repeated tick-passage in cattle. For the first time a South African isolate of buffalo-derived *T. parva* was successfully transmitted using *Rhipicephalus zambeziensis* for eight passages in non-splenectomised cattle. This was achieved despite most animals developing fatal infections with extremely low piroplasm parasitaemias, and without chemotherapeutic intervention. This finding indicates that, contrary to earlier belief, Corridor disease is not a self-limiting disease in cattle, and given the opportunity, could well become established in a cattle population in the absence of buffalo. Despite repeated tick transmission in cattle of the South African buffalo isolate of *T. parva* used in this study, it did not exhibit the behavioural changes associated with “transformation” to typical cattle-derived *T. parva*.

Secondly, the potential role of the common waterbuck (*Kobus ellipsiprymnus*) in the selection of cattle-adapted subpopulations of parasites from buffalo-derived *T. parva* was investigated. Waterbuck captured in Kruger National Park (KNP) were screened by conventional and molecular diagnostic techniques for *Theileria* spp. infections. Laboratory-reared *R. zambeziensis* were fed on captive buffalo confirmed to be naturally infected with *T. parva*. The ensuing adult

ticks were fed on captive waterbuck and cattle. All the waterbuck were found to carry microscopically detectable *Theileria* sp. piroplasm infections, found by polymerase chain reaction (PCR) diagnosis to belong to a hitherto uncharacterised *Theileria* species. *R. zambeziensis* adults which fed as nymphs on the buffalo transmitted fatal *T. parva* infections to cattle. However, no transmission of *T. parva* to the waterbuck could be demonstrated clinically or by PCR diagnosis. Also, *R. zambeziensis* nymphs that were subsequently fed on the waterbuck failed to transmit *T. parva* to cattle in the ensuing adult stage, confirming the absence of *T. parva*-group infections in the waterbuck. The results suggest that buffalo in KNP probably do not carry *T. parva*-group parasites which are readily transmissible to common waterbuck and waterbuck are therefore unlikely to play an important role in the epidemiology of *T. parva*-group infections in cattle in South Africa.

Thirdly, to investigate the carrier state of buffalo-derived *T. parva* infections in cattle, blood from infected non-splenectomised and splenectomised carrier cattle was subinoculated to splenectomised cattle. *T. parva* infections were successfully transmitted by subinoculation of 1000 ml of blood at various intervals after infection to splenectomised recipient cattle. Donor animals comprised of recovered intact cattle, reacting intact cattle or splenectomised recovered cattle. Microscopically detectable piroplasm parasitaemias were detected in all recipients after inoculation. One splenectomised recipient developed a moderate clinical reaction, accompanied by a moderate schizont parasitosis, but recovered spontaneously, confirming persistence of schizonts in some *T. parva* carrier animals. By contrast, a *T. parva* piroplasm infection, persisting in a treated recovered splenectomised bovine, in the apparent absence of circulating schizonts, was serially (consecutively) passaged in splenectomised cattle. Seroconversion occurred in all recipient cattle. With the exception of the recipient which developed a clinical reaction and circulating schizonts, none of the recipients showed any clinical signs of *T. parva* infection. Upon homologous sporozoite challenge with *T. parva*, two out of three recipient animals with only microscopically detectable piroplasm parasitaemias developed fatal *T. parva* infections and one recovered after exhibiting severe clinical signs. These findings confirm the stage-specific immunity in *T. parva* and, contrary to popular belief, the possibility of long-term maintenance of piroplasm parasitaemias in the absence of schizonts in carrier cattle. The technique of subinoculating and establishing virulent *T. parva* carrier infections in splenectomised cattle also provides a method whereby buffalo-derived parasite stocks may be isolated and maintained for characterisation and the preparation of sporozoite stabilates for inclusion in *T. parva* vaccines.

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CHAPTER 1
LITERATURE REVIEW

Theileria species are protozoan parasites infecting wild and domestic animals in most tropical and subtropical regions of the world (Dolan, 1989). They have a schizogonous reproductive cycle, usually in lymphocytes of the vertebrate host, followed by a piroplasm stage in the erythrocytes. Transmission occurs via ticks of the family Ixodidae with clear evidence of a sexual cycle in the arthropod vector (Mehlhorn & Schein, 1984). They are classified along with another important genus infecting domestic animals, *Babesia*, as Apicomplexa: Acanoidasida: Piroplasmorida (Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblich, Lom, Lynn, Merinfield, Page, Poljansky, Sprague, Vaura & Wallace, 1980; Adl, Simpson, Farmer, Andersen, Anderson, Barta, Bowser, Brugerolle, Fensome, Fredericq, James, Karpov, Kugrens, Krug, Lane, Lewis, Lodge, Lynn, Mann, McCourt, Mendoza, Moestrup, Mozley-Standridge, Nerad, Shearer, Smirnov, Spiegel & Taylor, 2005).

The most important theilerial parasites affecting livestock in Africa are members of the *Theileria parva*-group. Despite considerable research effort being devoted to the study of these parasites over a period of several decades, they remain a major constraint to the development of the livestock industry, and consequently the economy, of many developing countries in Africa (Potgieter, Stoltsz, Blouin & Roos, 1988; Dolan, 1989; Mukhebi, Perry & Kruska, 1992). Even today *T. parva* infection remains a threat to millions of cattle in many countries of East, Central and southern Africa and is responsible for many cattle losses annually on the continent.

Mehlhorn and Schein (1984) extensively reviewed the life cycle of theilerial parasites. Three natural tick vectors, *Rhipicephalus appendiculatus*, *Rhipicephalus zambeziensis* and *Rhipicephalus duttoni*, are capable of transmitting *T. parva* (Norval, Perry & Young, 1992). When an infected tick feeds on a susceptible host, sporozoites are inoculated with the tick saliva into the host. The sporozoites attach to and rapidly enter lymphocytes where they develop into schizonts (macroschizonts) which cause host cell transformation and lymphoproliferation. A proportion of schizonts differentiate to microschizonts, giving rise to merozoites which enter erythrocytes and develop into piroplasms. Ticks transmit *T. parva* parasites only transstadially (i.e. from larva to nymph or from nymph to adult) and larval or nymphal ticks become infected when they ingest erythrocytes containing piroplasms. After a sexual cycle in the tick gut, a

mobile kinete is formed which enters the tick salivary gland. When the tick feeds again, after moulting to the next instar (nymph or adult), sporoblasts develop in the salivary gland, infective sporozoites are formed and a new cycle of development is initiated.

Members of the *T. parva*-group of parasites are morphologically and serologically indistinguishable (Burridge & Kimber, 1972; Burridge, Kimber & Young, 1973; Burridge, Young, Stagg, Kanhai & Kimber, 1974; Lawrence, 1979), but based on certain behavioural characteristics in the vertebrate host (and mainly for the sake of convenience) a trinomial nomenclature was adopted for the group (Uilenberg 1978; 1981; Lawrence, 1979): *Theileria parva parva* for parasites causing classical East Coast fever (ECF) and characterised by large numbers of schizonts and piroplasms; *Theileria parva lawrencei* for strains from the African buffalo (*Syncerus caffer*) causing Corridor disease or Buffalo disease in cattle and characterised by small numbers of schizonts and very few or no piroplasms; and *Theileria parva bovis* for strains with an intermediate character, producing Zimbabwe theileriosis or January disease and generally causing a milder disease than the other two. Buffalo act as reservoirs of *T. p. lawrencei*, but usually only mild or subclinical infection results in this host (Brocklesby & Barnett, 1966; Uilenberg, 1981; Grootenhuis, Leitch, Stagg, Dolan & Young, 1987).

These observed behavioural differences (pathogenicity and numbers of schizonts and piroplasms present in infected animals) of the respective causative organisms, as well as differences in the epidemiology of the respective disease syndromes caused by them, were once considered sufficiently characteristic to differentiate them as species and to warrant the inclusion of *T. p. lawrencei* and *T. p. bovis* in a separate genus, *Gonderia* (Neitz, 1957). In many parts of Africa, however, differentiation into three subspecies seems to be an over-simplification of the situation as it exists in nature. There appears rather to be a gradual range of parasites between the two extremes: the *lawrencei*-type with few schizonts and very few piroplasms on the one hand, and the *parva*-type with high parasitaemias and numerous schizonts on the other (Uilenberg, 1981).

Epidemiologically, the main distinguishing features of the different disease syndromes are that ECF is maintained in cattle and spreads rapidly in susceptible cattle populations; Corridor disease is transmitted mainly from buffalo to cattle and transmission between cattle can only be accomplished with some difficulty in the laboratory; and Zimbabwe theileriosis (which resembles Corridor disease more closely) is maintained in cattle in the absence of buffalo and

apparently only occurs in Zimbabwe (Neitz, 1957; Koch, Ocama, Munatswa, Byrom, Norval, Spooner, Conrad & Irvin, 1988).

ECF was introduced into southern Africa at the beginning of the twentieth century, causing morbidity and mortality rates in cattle approaching 100 % in infected areas (Theiler, 1904). Despite initial control attempts, the disease spread very rapidly and brought the economies of the countries in southern Africa to an almost complete halt, since farms and mines depended heavily on ox-drawn transport (Diesel, 1948). It was only after the implementation of a massive campaign aimed at vigorous tick control, stringent control over the movement of cattle (Diesel, 1948) and finally the introduction of a slaughter policy, all at a very high cost, that the disease was eventually eradicated in South Africa, 53 years after its introduction (Neitz, 1957).

Since the eradication of ECF during the 1950s, Corridor disease (of which the African buffalo is the major reservoir host) has become the most important theilerial infection posing a threat to the cattle farming industry in South Africa (Stoltsz, 1989). The fact that the African buffalo acts as a reservoir of foot and mouth disease (FMD) virus used to be the main reason for its confinement to well-fenced game reserves. However, restricting the movement of buffalo, together with the enforcement of quarantine measures and vigorous tick control in areas bordering on Corridor disease endemic areas, especially when outbreaks occur, has probably contributed largely to preventing the spread of the disease.

In the past, *R. appendiculatus* was considered to be the only vector of *T. parva* (Neitz, 1957; Barnett and Brocklesby, 1966, De Vos, 1982). *Rhipicephalus zambeziensis* was described by Walker, Norval & Corwin (1981) as a new tick species from eastern and southern Africa which closely resembled *R. appendiculatus*. It was subsequently demonstrated experimentally by Lawrence, Norval & Uilenberg (1983) and Norval, Fivaz, Lawrence & Brown (1985) that *R. zambeziensis* was a vector of buffalo-derived *T. parva.* In many parts of the Limpopo and Mpumalanga Provinces, *R. zambeziensis* and *R. appendiculatus* occur sympatrically (Norval, Walker & Colborne, 1982; Stoltsz & Blouin, 1990), including many game-farming areas and the Kruger National Park (KNP). It would also appear that *R. zambeziensis* is more efficient at transmitting buffalo-derived *T. parva* infections, which commonly produce very low piroplasm parasitaemias (Blouin & Stoltsz, 1989). *Rhipicephalus duttoni*, the third confirmed vector of buffalo-derived *T. parva*, does not occur in South Africa and is restricted mainly to parts of Angola (Da Graca & Serano, 1971).

Studies in Kenya (Barnett & Brocklesby, 1966; Young, Brown, Burridge, Cunningham, Kirimi & Irvin, 1973) have indicated that repeated tick-passage of buffalo-derived *T. parva* in cattle results in some profound behavioural changes of the parasite to the extent that it becomes indistinguishable from cattle-derived *T. parva* (the cause of ECF). On the basis of this behavioural transformation of *T. p. lawrencei,* together with the fact that all three *T. parva* "types" share common tick vectors (Neitz, 1957), that they cannot be distinguished morphologically or serologically (Lawrence, 1979), and that various degrees of cross-immunity exist between them (Neitz, 1957; Radley, Brown, Cunningham, Kimber, Musisi, Payne, Purnell, Stagg & Young, 1975b), the arguments for the synonymy of the three subspecies were considered to be manifold and conclusive. Uilenberg (1981) thus concluded that *T. p. parva*, *T. p. bovis* and *T. p. lawrencei* might well only be behavioural forms of the same parasite. In support of this, it was suggested that *T. p. parva* and *T. p. bovis* probably represented the adaptation, or selection of a subpopulation, of parasites of buffalo origin in cattle (Grootenhuis *et al*., 1987), and that *T. parva* should thus be regarded as the only valid species (Grootenhuis *et al*., 1987; Dolan, 1989). It was therefore suggested that the trinomial naming system should be discontinued and that *T. parva* parasites should be classified according to their host of origin (Perry and Young, 1993).

It could, therefore, be speculated that wherever Corridor disease[1] occurs, classical ECF[2] might well re-emerge spontaneously one day in areas from which it has long been absent (Uilenberg, 1981). Considering that ECF has been absent from South Africa for several decades, the susceptibility of the present cattle population, and the presence of both *R. zambeziensis* and *R. appendiculatus* in most of the cattle-farming areas of South Africa, a recrudescence of ECF, either through re-introduction of the parasite from neighbouring states such as Mozambique, or the transformation of buffalo-derived *T. parva*, could have serious consequences for the cattle-farming industry in this country.

A potentially useful tool in the control of Corridor disease (or *T. parva*-group infections in general) would be a chemotherapeutic drug that would sterilise the infection in carrier animals. Although several of these drugs have been developed which are very effective in treating clinical theileriosis in cattle (Dolan, 1986; Morgan & McHardy, 1986), none of these drugs, or combinations of drugs, has proven effective in sterilising infections in carrier cattle (Potgieter *et al*., 1988; Stoltsz, 1989). Thus, recovered cattle remain carriers of the parasite and could act as

[1] Caused by buffalo-derived *T. parva*
[2] Caused by cattle-derived *T. parva*

reservoirs of Corridor disease, although probably less efficiently than buffalo. Based on the reports of transformation of buffalo-derived *T. parva* after serial tick-passage in cattle, the use of these chemotherapeutic drugs to control Corridor disease outbreaks has been prohibited in South Africa (Potgieter *et al*., 1988).

A further consideration in applying such strict control measures has been the fact that effective immunisation of cattle against *T. parva*-group infections has been hampered by the inability to develop a safe vaccine with which cattle can reliably be immunised. A particular problem in immunisation against these diseases is that, contrary to earlier reports (Neitz, 1957; Barnett and Brocklesby, 1966), different strains of the parasite exist which do not cross-protect (Radley, Brown, Burridge, Cunningham, Musisi & Purnell, 1975a; Irvin, Dobbelaere, Mwamachi, Minami, Spooner & Ocama, 1983). Thus, cattle immunised with one particular parasite stock usually exhibit strong immunity against homologous (and occasionally heterologous) challenge, but are often fully susceptible to challenge with heterologous stocks (Young, Radley, Cunningham, Musisi, Payne & Purnell, 1977). Such breakthroughs in immunity occur more commonly if the heterologous stock is derived from buffalo (Radley *et al*., 1975b; Young *et al*., 1977; Young, Brown, Cunningham & Radley, 1978; Radley, Young, Grootenhuis, Cunningham, Dolan & Morzaria, 1979; Radley, 1981) and has been ascribed to the presence of a greater variety of immunogenically different strains of *T. parva* in this natural host than in cattle.

Disappointing results were achieved during immunisation of cattle with *in vitro*-cultured schizont-infected lymphoblastoid cells, due to severe restriction of the parasite-specific T-lymphocyte responses by the major histocompatibility complex (MHC) type of the host (Dolan, Teale, Stagg, Kemp, Cowan, Young, Groocock, Leitch, Spooner & Brown, 1984). Thus, immunisation of cattle can presently only be achieved by infection of cattle with infective sporozoites derived from ticks and subsequent chemotherapy to control the infection (Dolan, 1989; Young, Leitch, Dolan, Mbogo, Ndunga, Grootenhuis & De Castro, 1990). Due to the poor cross-immunity between different stocks of *T. parva*, a "cocktail" or combination of several stocks is often used to broaden the protection (Radley, 1981; Young *et al*., 1990). Disadvantages of the cocktail are, however, that it may not protect against all strains in the field, immunised cattle remain carriers and foreign strains of the parasite may be introduced into an area.

Much effort has been devoted to developing techniques which will differentiate between buffalo-derived and cattle-derived *T. parva* and to characterise different stocks according to their cross-immunising properties. Monoclonal antibodies (MAbs) have been raised against

schizont-infected cells and sporozoites. Those raised against sporozoites appear to detect common antigens in different stocks and are not suitable for strain differentiation (Dobbelaere, Spooner, Barry & Irvin, 1984; Musoke, Nantulya, Rurangirwa & Busscher, 1984). However, MAbs raised against the schizont stage detect antigen diversity amongst different stocks of *T. parva* (Pinder and Hewett, 1980; Minami, Spooner, Irvin, Ocama, Dobbelaere & Fujinaga, 1983; Conrad, Ole-Moiyoi, Baldwin, Dolan, O'Callaghan, Njamunggeh, Grootenhuis, Stagg, Leitch & Young, 1989) and appear to offer some scope for strain characterisation since, when different stocks of parasite are tested against a series of such MAbs, a profile of reactivity can be prepared for each stock.

Studies with buffalo-derived *T. parva* (Conrad, Stagg, Grootenhuis, Irvin, Newson, Njamunggeh, Rossiter & Young, 1987; Koch *et al*., 1988; Conrad *et al*., 1989) have revealed the presence of mixed parasite genotypes within an isolate (following cloning of parasite stocks *in vitro*), which agrees with the extent of antigenic diversity that is apparent *in vivo* (Young *et al*., 1978; Radley *et al*., 1979). A MAb has also been identified which appears to be specific for buffalo-derived *T. parva* stocks, since it reacts with most (but not all) buffalo-derived *T. parva* isolates, but not with any cattle-derived *T. parva* isolates tested (Newson, Naessens, Stagg & Black, 1986; Conrad *et al*., 1989).

Although different species of *Theileria* could be distinguished on the basis of isoenzyme polymorphisms (Musisi, Kilgour, Brown & Morzaria, 1981; Melrose, Brown, Morzaria, Ocama & Irvin, 1984; Allsopp, Gibson & Stagg, 1985), the differences detected in different stocks of *T. parva* were negligible and failed to differentiate between buffalo-derived and cattle-derived *T. parva* (Musisi *et al*., 1981).

Theileria parva-specific DNA probes, derived from a genomic library of a *T. parva* (Muguga) reference stock, were developed (Conrad, Iams, Brown, Sohanpal & ole-MoiYoi, 1987; Allsopp & Allsopp 1988). During subsequent studies (Allsopp, Carrington, Baylis, Sohal, Dolan & Iams, 1989), a probe homologous to one of these latter probes, but derived from a buffalo-derived *T. parva* stock, was also successfully used to probe for *T. parva*-specific sequences. Such diagnostic probes have the advantage that they provide information which relates specifically to the strain(s) of parasite present in the animals at the time of testing and that large numbers of samples can be tested rapidly. This is in contrast to the MAb profile test (Minami *et al*., 1983) which relies on the isolation and cultivation of the parasite *in vitro*, with possible concomitant modification of antigenicity, before the test can be applied.

In these studies (Conrad *et al*., 1987; Allsopp & Allsopp, 1988; Allsopp *et al*., 1989), restriction fragment length polymorphisms (RFLPs) were produced by hybridisation of the probes to restriction enzyme digested DNA of different stocks of *T. parva*. However, some isolates showed similar RFLP patterns (Conrad, *et al*., 1987; Allsopp & Allsopp, 1988), which allowed separation of different isolates into distinct groups.

Conrad *et al*. (1989) subsequently used two of their probes to characterise *T. parva* isolates from buffalo and cattle infected with buffalo-derived parasites. Both probes hybridised specifically to parasite DNA, but not to cattle or buffalo lymphoblastoid cell DNA, and the hybridisation patterns produced with infected buffalo cell DNA were similar to those seen when the parasites were obtained from cattle infected with the same parasite stock. These results indicated that the probes could be used to characterise theilerial parasites in buffalo and bovine cells and that the genotype of the parasite, as detected by these probes, was not affected by differences in the host cell. The RFLPs observed with hybridisation of the probes to restriction enzyme-digested DNA from sub-clones of a schizont-infected buffalo cell line showed that, in addition to the antigenic differences previously detected (Conrad *et al*., 1987), there were genotypic differences between parasites in buffalo isolates. Similarities in the hybridisation patterns of the sub-clones which were derived from the same cloned cell lines also supported previous observations that distinct theilerial populations could be separated by cloning cell lines *in vitro* by limiting dilution (Conrad *et al*., 1987). Some parasite clones obtained from buffalo also showed MAb profiles similar to some cattle-derived *T. parva* stocks. This would support previous observations that buffalo may also carry ECF-type parasites (Grootenhuis *et al*., 1987).

Results obtained by Allsopp & Allsopp (1988) similarly showed that the grouping of three *T. parva* stocks according to their RFLP patterns did not correspond with the previous grouping of these stocks according to their MAb profiles (Minami *et al*., 1983). It was concluded that neither grouping probably represented separation into strains, but that reagents of greater discrimination would put all three stocks into separate groups. The different RFLPs obtained for cloned and uncloned material from a cattle-derived *T. parva* isolate by these authors (Allsopp & Allsopp, 1988) demonstrated that mixed infections with cattle-derived *T. parva* also occur and that the cloning procedure had selected a subpopulation from the field isolate, as had been demonstrated for buffalo-derived *T. parva* isolates (Conrad *et al*., 1987; 1989).

Conrad *et al*. (1989) concluded that theilerial parasites derived from buffalo and cattle which underwent typical Corridor disease/buffalo-derived *T. parva* reactions, after being infected with

buffalo-derived parasites, did not show any specific phenotypic or genotypic characteristics that would distinguish them from cattle-derived *T. parva* parasites. They too felt that this may have been due to the presence of mixed parasite populations in the isolates or to the fact that the appropriate antigenic markers or DNA probes which would make this distinction possible *in vitro* had yet to be developed.

Allsopp *et al*. (1989) determined the nucleotide sequence of selected restriction enzyme fragments of three East African stocks of *T. parva* and found that there was much similarity between different fragments from different parasite isolates, with some completely conserved regions, as well as relatively variable regions. Improved discrimination between isolates of *T. parva* was obtained by hybridisation of two oligonucleotide probes to a selected variable region of the parasite DNA amplified by the polymerase chain reaction (PCR). The oligonucleotide probes did not hybridise to DNA of all parasite isolates, however, and no indication was found that the two probes were specific for either buffalo-derived or cattle-derived *T. parva*. Further evidence was found that the parasite isolates used contained more than one parasite genotype.

The successful eradication of ECF in South Africa is a unique achievement in Africa (Norval, *et al*., 1992). Neither chemotherapy nor vaccination appears to provide sufficient safeguards in the event of a recrudescence of ECF in this country. At present, outbreaks of Corridor disease occur only sporadically and control measures apparently have been sufficient to prevent the spread of the disease in cattle. The mere fact that buffalo-cattle contact does occur in this country poses a severe risk, however, with potentially catastrophic consequences if buffalo-derived *T. parva* transformed and became established in the cattle population in the absence of buffalo. Thus far, no such transformation has apparently occurred under natural conditions in South Africa, and limited attempts to transform a South African buffalo-derived *T. parva* isolate (Hluhluwe 3) in cattle in the laboratory have failed (De Vos, 1982). From studying the published data it would appear that transformation of buffalo-derived *T. parva* is rather the result of selective establishment of a subpopulation of *T. parva* parasites in cattle. The limited data available for South African *T. parva*-group parasites is not sufficient to draw any definite conclusions, but it may well be that indigenous buffalo populations harbour such ECF-type parasites. In addition, buffalo also regularly cross our national borders from neighbouring Zimbabwe and Mozambique and these animals could introduce potentially devastating parasite strains.

The need thus exists to develop diagnostic techniques which will identify *T. parva* carrier infections in cattle and buffalo, particularly where the latter are considered for relocation. Present serological techniques lack sensitivity to detect all carrier animals, particularly buffalo (Potgieter *et al*., 1988). Such techniques are also unsuitable for the confirmation of elimination of infections following possible chemosterilisation. Furthermore, serological tests do not distinguish the different types of *T. parva*, and various degrees of serological cross-reaction may occur with other *Theileria* spp., particularly *T. taurotragi* (De Vos, 1982; Stoltsz, 1989). In addition, these other *Theileria* spp. cannot be distinguished morphologically from the *T. parva*-group and occasionally even share the same tick vectors (Stoltsz, 1989).

Research in other parts of the world is concerned primarily with characterising strains according to their cross-immunising properties, rather than their biological characteristics. However, the epidemiological differences and differences in control strategies required for *T. parva* parasites responsible for Corridor disease and ECF, respectively, in South Africa, necessitates characterisation of *T. parva* isolates at a different level. Diagnostic techniques based on recombinant DNA technology hold considerable promise, but the *T. parva* group-specific probes and oligonucleotide probes developed thus far have been useful mostly as diagnostic tools to differentiate *T. parva* from other *Theileria* species in the laboratory (Allsopp, Baylis, Allsopp, Cavalier-Smith, Bishop, Carrington, Sohanpal & Spooner, 1993; Sibeko, Oosthuizen, Collins, Geysen, Rambritch, Latif, Groeneveld, Potgieter & Coetzer, 2008; Papli, Landt, Fleischer, Koekemoer, Mans, Pienaar, Josemans, Zweygarth, Potgieter & Latif, 2011). Although the specificity and sensitivity of these techniques to detect *T. parva* infections in carrier animals have markedly improved in recent years, they do not differentiate between buffalo-derived and cattle-derived parasites, although early evidence suggested that such differentiation may be possible (Baldwin, Malu, Kinuthia, Conrad & Grootenhuis, 1986; Conrad *et al*., 1989; Allsopp *et al*., 1989).

Several subsequent studies attempted to distinguish between buffalo-derived and cattle-derived *T. parva* isolates. Comparison of the entire rRNA transcription unit of various *T. parva* isolates revealed major sequence variation in the internal transcribed spacer (ITS) (Collins and Allsopp, 1999). Eleven characterisation probes derived from the ITSs of twelve *T. parva* isolates revealed greater heterogeneity amongst buffalo-derived than cattle-derived *T. parva* isolates, providing further evidence to suggest that cattle-derived *T. parva* represents the selection of a relatively homogeneous subpopulation of *T. parva* from a much greater, more diverse gene pool in buffalo. Although this panel of oligonucleotide probes allowed the differentiation of different genotypes

of *T. parva*, it failed to provide an absolute distinction between buffalo-derived and cattle-derived *T. parva* isolates. In addition, the detection of ITS sequence mosaics indicated that *T. parva* parasites undergo genetic recombination, suggesting that the buffalo-derived and cattle-derived *T. parva* parasite populations are not yet separate gene pools, and that therefore, no probe will distinguish between them unless it targets a sequence directly associated with the mechanisms responsible for the observed behavioural differences in cattle (Collins and Allsopp, 1999).

Sequence analysis of the p67 gene of *T. parva* in East Africa has revealed a 129 bp deletion in cattle-derived isolates which was not present in buffalo-derived parasites (Nene, Musoke, Gobright & Morzaria, 1996; Nene, Gobright, Bishop, Morzaria & Musoke, 1999). This, initially, led to an assumption that the deletion may be specific to cattle-derived isolates of *T. parva*. Subsequently, however, Collins (1997) found the same 129 bp deletion in the p67 gene of several buffalo-derived *T. parva* isolates from South Africa, at least one of which caused classical Corridor disease in cattle.

Subsequent characterization of p67 gene sequences from several *T. parva* isolates from cattle and buffalo from different geographical areas of South Africa (Sibeko, Geysen, Oosthuizen, Matthee, Troskie, Potgieter, Coetzer & Collins, 2010) revealed the presence of these two alleles, as well as two novel alleles, one of which had a different 174 bp deletion and the other not. Furthermore, variants of the original two alleles were detected in *T. parva* isolates from buffalo, with more variations present in large buffalo populations as opposed to smaller ones. These results suggest that p67 genetic profiles are more complex than previously thought, and thus cannot be used to differentiate *T. parva* isolates from cattle and buffalo.

Others have exploited the variable region of *T. parva* antigen genes, including the genes coding for the polymorphic immunodominant molecule (PIM) and p104, to generate restriction fragment length polymorphism (RFLP) profiles (using PIM-based and p104-based semi-nested PCR-RFLP assays) to differentiate between buffalo-derived and cattle-derived isolates from East Africa (Geysen, Bishop, Skilton, Dolan & Morzaria, 1999; Geysen, 2000; Bishop, Geysen, Spooner, Skilton, Nene, Dolan & Morzaria, 2001). Results indicated that profiles generated from buffalo-derived *T. parva* stocks are heterogeneous and more polymorphic than cattle-derived stocks, which are often homogeneous (Geysen *et al*., 1999; Geysen, Bazarusanga, Brandt & Dolan, 2004).

Characterization of South African isolates of *T. parva* from cattle and buffalo revealed, however, that p104 and PIM sequences from both host species were more diverse than previously thought (Sibeko, Collins, Oosthuizen, Troskie, Potgieter, Coetzer & Geysen, 2011). Variants of cattle-type alleles and buffalo-type alleles were detected in cattle isolates and "mixed" PIM sequences, exhibiting characteristics of both cattle-type and buffalo-type sequences, were found in both buffalo and cattle isolates. These "mixed" type PIM alleles were more prevalent in cattle than buffalo isolates. However, it is not clear to what extent these may indicate either historic or more recent recombination events of buffalo-derived and cattle-derived gene pools.

Micro- and mini-satellite PCR amplification of *T. parva*-specific sequences have confirmed previous observations of marked genetic diversity amongst *T. parva* stocks derived from both cattle and buffalo, and show considerable promise at elucidating the complex structure of *T. parva* populations (Oura, Odonga, Lubega, Spooner, Tait & Bishop, 2003; Oura, Asiimwe, Weir, Lubega & Tait, 2005). Micro- and mini-satellite markers developed for characterizing *T. parva* stocks enable detection of higher levels of polymorphism than PCR-RFLP methods which are based on individual loci. Most field isolates, particularly those from buffalo, contain complex mixtures comprising multiple *T. parva* genotypes, however, making it impossible to determine the genotypes of the parasites directly using microsatellite markers. Therefore, microsatellite markers have been used to characterize field isolates by defining multilocus genotypes (MLGs) for each sample based on the sizes of the predominant alleles at each locus (Oura *et al*., 2005; Oura, Tait, Asiimwe, Lubega & Weir, 2010), but the authors conceded that less prevalent genotypes would be overlooked using this approach. MLGs of *T. parva*, however, confirmed previous observations of marked genetic diversity in *T. parva* from calves in Uganda (Oura *et al*., 2005). Recently, Oura *et al*. (2010) compared MLGs from *T. parva* parasites from buffalo in Lake Mburo National Park in Uganda and from calves grazing inside and outside the park. Their results suggest that the *T. parva* gene pools in buffalo and cattle were distinct and there was no evidence to indicate transmission of buffalo-derived *T. parva* genotypes to the cattle population. They therefore suggest that separation of distinct gene pools may already be present in *T. parva*, perhaps warranting a re-examination of the original sub-speciation of cattle-maintained, ECF-inducing *T. p. parva* and buffalo-maintained, Corridor disease-inducing *T. p. lawrencei*. It should be noted, however, that their MLG analysis includes only the predominant genotypes; thus genotypes present at very low frequency may well have been overlooked.

It would seem, therefore, that despite extensive investigation and numerous efforts to identify specific markers that will consistently distinguish between *T. parva* genotypes and which can also be correlated directly with observed behavioural differences *in vivo*, the final chapter in the continuing saga that is the characterisation of *T. parva* has by no means been written.

RESEARCH HYPOTHESIS

African buffalo (*Syncerus caffer*) in Corridor disease endemic areas of South Africa are infected with *T. parva* populations containing parasite subpopulations which, upon transmission to cattle, may result in clinical disease indistinguishable from East Coast fever.

RESEARCH OBJECTIVES

1. To attempt repeated tick transmission (= serial passage) of a buffalo-derived *T. parva* isolate in non-splenectomised cattle, without chemotherapeutic intervention, in order to establish whether any behavioural change (as indicated by parasite morphology and level of parasitosis and parasitaemia) which corresponds to cattle-derived *T. parva,* occurs.

2. To determine if common waterbuck (*Kobus ellipsiprymnus*) may play a role in the *in vivo* selection of *T. parva* subpopulations (derived from buffalo) which exhibit behavioural characteristics of *T. parva* derived from cattle.

3. To investigate the nature of the carrier state of buffalo-derived *T. parva* in cattle and the transmissibility of such infections by subinoculation of blood in splenectomised cattle.

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CHAPTER 2

SERIAL TICK PASSAGE OF A SOUTH AFRICAN ISOLATE OF BUFFALO-DERIVED *THEILERIA PARVA* IN SPLENECTOMISED AND NON-SPLENECTOMISED CATTLE

INTRODUCTION

The most important theilerial parasites affecting livestock in Africa are members of the *Theileria parva*-group, which remains a major constraint to the development of the livestock industry, and consequently the economy, of many developing countries in Africa (Dolan, 1989). Members of the *Theileria parva*-group are morphologically and serologically indistinguishable (Burridge & Kimber, 1972; Burridge, Kimber & Young, 1973; Burridge, Young, Stagg, Kanhai & Kimber, 1974; Lawrence, 1979), but based on certain behavioural characteristics in the vertebrate host (and mainly for the sake of convenience) a trinomial nomenclature was adopted for the group (Uilenberg, 1978; 1981; Lawrence, 1979): *Theileria parva parva* for parasites causing classical East Coast fever (ECF) in cattle and characterised by large numbers of schizonts and piroplasms; *Theileria parva lawrencei* for strains from the African buffalo (*Syncerus caffer*) causing Corridor or Buffalo disease in cattle and characterised by small numbers of schizonts and very few or no piroplasms; and *Theileria parva bovis* for strains with an intermediate character, producing Zimbabwe theileriosis or January disease and generally causing a milder disease in cattle than the other two. In many parts of Africa, however, differentiation into three subspecies seems to be an over-simplification of the situation as it exists in nature. There appears rather to be a gradual range of parasites between the two extremes: the *lawrencei*-type with few schizonts and very few piroplasms on the one hand, and the *parva*-type with high parasitaemias and numerous schizonts on the other (Uilenberg, 1981).

Epidemiologically, the main distinguishing features of the different disease syndromes are: ECF is maintained in cattle and spreads rapidly in susceptible cattle populations; Corridor disease is transmitted mainly from buffalo to cattle and transmission between cattle can only be accomplished with some difficulty in the laboratory; and Zimbabwe theileriosis (which resembles Corridor disease more closely) is maintained in cattle in the absence of buffalo and apparently only occurs in Zimbabwe (Neitz, 1957; Koch, Ocama, Munatswa, Byrom, Norval, Spooner, Conrad & Irvin, 1988).

Studies in Kenya (Barnett & Brocklesby, 1966; Young, Brown, Burridge, Cunningham, Kirimi & Irvin, 1973) have indicated that repeated tick-passage of buffalo-derived *T. parva* in cattle results in some profound behavioural changes of the parasite, to the extent that it becomes indistinguishable from cattle-derived *T. parva* (the cause of ECF). These changes, characterised by the numbers of schizonts and piroplasms increasing from the low numbers associated with buffalo-derived parasites to the high numbers associated with cattle-derived parasites, usually occurred after four or five transmissions in experimental cattle. This adaptation of *T. parva* from buffalo to cattle has generally been referred to as transformation. On the basis of this behavioural transformation, together with the fact that all three parasite "subspecies" share common tick vectors (Neitz, 1957), that they cannot be distinguished morphologically or serologically (Lawrence, 1979), and that various degrees of cross-immunity exist between them (Neitz, 1957; Radley, Brown, Cunningham, Kimber, Musisi, Payne, Purnell, Stagg & Young, 1975), the arguments for the synonymy of the three subspecies were considered to be manifold and conclusive. Uilenberg (1981) thus concluded that *T. p. parva*, *T. p. bovis* and *T. p. lawrencei* might well only be behavioural forms of the same parasite. In support of this, more recently it was suggested that *T. p. parva* and *T. p. bovis* probably represented the adaptation, or selection of a subpopulation, of parasites of buffalo origin in cattle (Grootenhuis, Leitch, Stagg, Dolan & Young, 1987), and that *T. parva* should thus be regarded as the only valid species (Grootenhuis *et al*., 1987; Dolan, 1989).

Although cattle-derived *T. parva*, causing classical ECF in cattle, has been eradicated from South Africa (Anon., 1981), buffalo-derived *T. parva*, causing Corridor disease, is endemic in many buffalo populations within the distribution range of *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* in this country (De Vos, 1982; Potgieter, Stoltsz, Blouin & Roos, 1988; Stoltsz, 1989). Based on the observed behavioural transformation of buffalo-derived *T. parva*, it can be speculated that wherever buffalo-derived *T. parva* occurs, severe disease indistinguishable from ECF may well re-emerge spontaneously in areas from which it has long been absent (Uilenberg, 1981). Considering that ECF has been absent from South Africa for several decades, the susceptibility of the local cattle population, and the presence of both *R. zambeziensis* and *R. appendiculatus* in most of the cattle-farming areas of South Africa, the spread of Corridor disease or a recrudescence of ECF, either through re-introduction of the parasite from neighbouring countries, or the transformation of buffalo-derived *T. parva*, could have serious consequences for the cattle-farming industry in this country. This has been an important consideration for confining buffalo to well-fenced game farms and reserves and for implementing and maintaining strict control measures to restrict the movement of buffalo in

South Africa (Stoltsz, 1989), especially in the face of the lack of effective prophylactic drugs and safe and effective vaccines to control these diseases. Despite the availability of effective chemotherapeutic drugs for the treatment of clinical theileriosis, these have not been registered for use in South Africa in order to prevent the establishment of carrier states in cattle following treatment during outbreaks of Corridor disease (Potgieter, Roos & De Vos, 1985).

Laboratory investigations (Lawrence, Norval & Uilenberg, 1983; Blouin & Stoltsz, 1989[3]) have indicated that *R. zambeziensis* is a more efficient vector of buffalo-derived *T. parva* than *R. appendiculatus*. Neitz (1957) considered Corridor disease to be an end-stage disease in cattle with no tick transmission between cattle occurring under natural conditions. However, the carrier state of buffalo-derived *T. parva* in splenectomised cattle has been clearly demonstrated (Neitz, 1958; De Vos, 1982). It could perhaps be argued that many attempts to confirm persistent parasitaemias in suspected carrier cattle by tick transmission with *R. appendiculatus* may have failed due to *R. appendiculatus* being a less efficient vector.

The possibility of buffalo-derived *T. parva* transforming to classical ECF under natural conditions in South Africa has been a subject of much debate. Although sporadic outbreaks of Corridor disease have occurred over many decades in cattle which come into contact with infected buffalo within the distribution range of the vector ticks, no evidence exists to suggest that any such transformation has occurred in the field. It should be borne in mind, however, that because of the strict control measures employed for foot and mouth disease (FMD) and Corridor disease, contact between cattle and buffalo is very restricted in South Africa. In addition, stringent tick-control practices in Corridor disease-endemic areas have left very few opportunities for the transmission of *T. parva* from buffalo to cattle. Several attempts to demonstrate transformation of South African isolates of buffalo-derived *T. parva* in the laboratory in South Africa have also failed (De Vos, 1982), but these were conducted using splenectomised cattle and usually involved treating infected animals with chemotherapeutic drugs prior to attempted tick transmission by *R. appendiculatus*.

[3] See Addendum A

In the present study, repeated tick transmission by *R. zambeziensis* of a buffalo-derived isolate of *T. parva* in non-splenectomised cattle, without chemotherapeutic intervention, was investigated to determine if any behavioural change, as measured by parasite morphology and level of parasitosis and parasitaemia could be achieved.

MATERIALS AND METHODS

Parasite isolate

A buffalo-derived isolate of *T. parva* (Hluhluwe 3) that was obtained by feeding *R. appendiculatus* nymphs and adults collected from a buffalo in the Hluhluwe-iMfolozi Park on cattle (De Vos, 1982) was used throughout.

Experimental cattle

All cattle used from Passage 3 to Passage 8 were *Bos taurus* and *Bos indicus* crosses, non-splenectomised (intact), 24–48 months of age and reared under tick-free conditions at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI). They were confirmed by indirect fluorescent antibody test (IFAT) using a schizont antigen (Burridge and Kimber, 1972) to be negative for antibodies to *T. parva* prior to infection. The rectal temperatures of all animals were monitored on a daily basis and thin blood smears were prepared three times per week and examined for haemoparasites.

The incubation period was defined as the day on which a body temperature exceeding 39.5^{o}C was recorded post-infection. Following tick infestation of clinically reacting cattle, thin blood films were prepared every day. As soon as a temperature reaction of 39.5^{o}C or greater was recorded, lymph node biopsy smears were prepared from the parotid and superficial cervical (prescapular) lymph node. Blood films and lymph node biopsy smears were stained with Giemsa's stain and examined microscopically using a 10x objective lens and 100x oil immersion lens for the presence of schizonts and piroplasms.

Serial tick-passage of *T. parva* (Hluhluwe 3) in cattle

A schematic representation of the tick transmissions is provided in **Figure 1**.

The initial three passages were performed by De Vos (1982) prior to the commencement of the current study. The original natural infection in the buffalo from which infected ticks were collected represents Passage 0. The first transmission was to a splenectomised bovine (B9059-0 = Passage 1) which received only symptomatic treatment prior to *R. appendiculatus* nymphs engorging and subsequently transmitting *T. parva* to a splenectomised bovine (B9098-5 = Passage 2) by *R. appendiculatus* (De Vos, 1982). The latter animal was treated with halofuginone lactate[4] and, following recovery and demonstration of a persistent piroplasm carrier state, *R. appendiculatus* nymphs were fed on it for the preparation of a tick stabilate. This *T. parva*-infected tick stabilate was used to infect B9709-1 (= Passage 3), which was non-splenectomised and received no specific treatment (representing the first passage in non-splenectomised cattle in which no specific chemotherapeutic drug was used). *Rhipicephalus zambeziensis* was used for all subsequent tick transmissions (i.e. Passage 4 to Passage 8).

Ideally, no chemotherapeutic drugs were to be used for the treatment of clinical disease in animals as this could possibly apply unwanted selection to the infective parasite population. However, there was some concern that all reacting cattle during a particular passage attempt might die too acutely for ticks to pick up infection if no chemotherapeutic drugs were administered. To ensure continued tick-passage in intact cattle, it was thus decided to perform the passages in both treated and untreated cattle. Cattle in the treatment group (starting with B9659-8 of Passage 4 on the left side of the table) were to be treated with halofuginone lactate[4], a drug known to have activity against theilerial schizonts, but minimal effect on piroplasms (Njau, Mkonyi, Maleche, Kitaly & Maiseli, 1985), thus ensuring propagation and transmission of subsequent parasite populations with minimal effect on piroplasm parasitaemias.

During the initial transmissions (Passage 4 to Passage 5), animals in the untreated group were treated symptomatically with diuretics and anti-inflammatory drugs in an attempt to possibly extend the lives of the reacting animals sufficiently for ticks to pick up the infection, yet avoiding potential elimination of any sub-population of parasites through the use of specific anti-theilerial drugs. When it became clear that tick transmission could be accomplished reliably without the aid of specific chemotherapy (B9652-3 and B9873-5 from the untreated group, and B9656-4 and B9859-0 from the treatment group that did not receive specific chemotherapy and

[4] Terit, Hoechst AG

infected during Passages 4 and 5), no treatment was administered to any of the reacting cattle during subsequent passages (i.e. Passage 6 to Passage 8).

Subinoculation of blood in splenectomised cattle

On three occasions 1000 ml blood, collected in heparin as anticoagulant, was subinoculated from intact reacting cattle to splenectomised cattle (B9931-7 = passage 4, B9899-3 = passage 6 and B9954-2 = passage 7) to ensure that parasites may be recovered in the event of failure of subsequent tick transmission from the original reacting cattle from those passages.

Blood and lymph node smears

Thin blood smears and lymph node biopsy smears were prepared on glass microscope slides, air dried and fixed in methanol before staining with 10% Giemsa's stain for 40 minutes.

Macroschizont index (MSI)

MSI determinations were made in lymph node biopsy smears and in blood smears according to the method described by De Vos (1982): Fifty randomly selected schizonts were measured using an ocular micrometer and the number of nuclei in each was counted; the means of these parameters were determined. These were determined on the day of maximum observed parasitosis, *in extremis* or at post-mortem examination.

Ticks and tick feeding

The Killkenny strain of *R. zambeziensis* (Potgieter *et al*., 1988) or the Rietvlei cross strain of *R. appendiculatus* (De Vos & Roos, 1981), both of which had been maintained in the laboratory at the ARC-OVI, were used for all tick transmission attempts. Ticks were reared and fed essentially as described by Neitz, Boughton & Walters (1971). Laboratory-reared nymphs were applied in cotton ear bags or body bags to each animal. The ear bags were attached around the base of the ear and body bags to the skin of the back using a contact adhesive[5]. During each transmission attempt, 100–300 laboratory-reared *R. appendiculatus* or *R. zambeziensis* adults, which had fed as nymphs on reacting cattle, were applied in an ear bag to each ear of a bovine.

During tick passages, approximately 300 *R. zambeziensis* nymphs were applied in an ear bag on the first or second day of a clinical reaction confirmed to be due to *T. parva* infection (i.e. temperature reaction above 39.5°C and microscopic demonstration of schizonts following tick

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[5]Genkem, Bostik

transmission). Approximately 200 additional nymphal ticks were applied on alternate days to the same or different ear or body bags in order to establish a stacking of ticks over the period of acute clinical reaction and to ensure that some engorged nymphs could be collected even if an animal died acutely.

Engorged nymphs were allowed to moult in the laboratory at 26°C (± 1°C), relative humidity (RH) 75 % (± 5 %). Approximately 6–8 weeks after moulting, the ensuing adult ticks were applied in ear bags to cattle to attempt tick transmission of *T. parva*. In the majority of transmissions, only adult ticks which had completed their engorgement as nymphs during the period of maximum piroplasm parasitaemia (usually coinciding with the two days prior to the donor animal dying) were used.

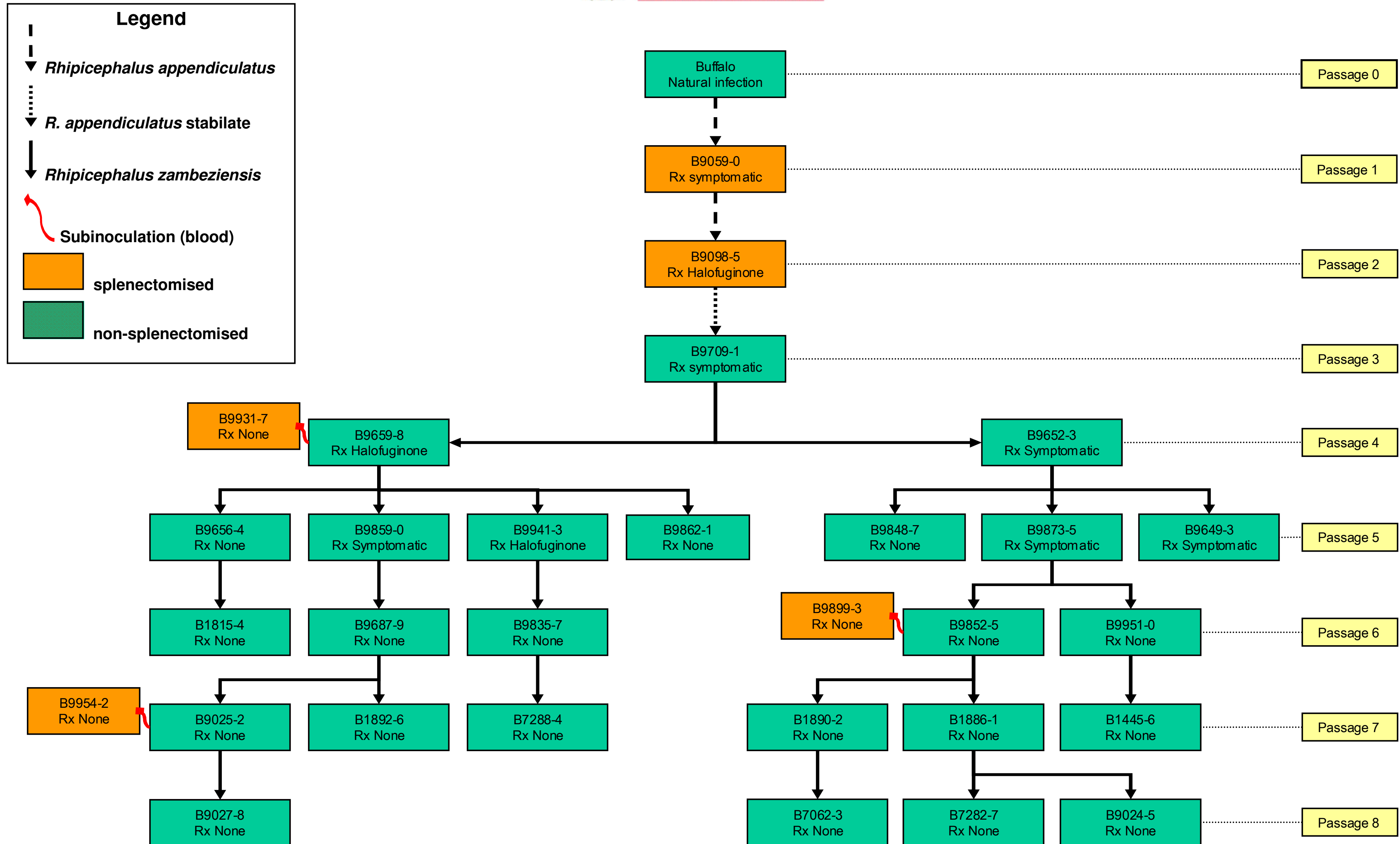

Figure 1 Diagrammatic representation of the serial passage of *T. parva* (Hluhluwe 3) in cattle.

RESULTS

Results of the serial tick passage of *T. parva* are presented in **Tables 1** and **2.**

Table 1 Summary of reactions of cattle infected in **Figure 1**

Passage No[a]	Animal No	Incubation Period (Days)	Time to Death (Days p.i.)	Mean size of macroschizonts (µm) (range)	Mean no of nuclei/ macroschizont (range)	Maximum MSI[b] (%)	Maximum piroplasm parasitaemia[c]
1	B9059-0	19	*	4.2 (2-12)	5.1 (2-24)	3.6	23/100F[c]
2	B9098-5	11	*	3.6 (3-7.5)	4 (1-18)	11.8	102/100 F
3	B9709-1	16	25	3.9 (1.5-11)	3.7 (1-22)	6.7	45/100 F
4	B9659-8	11	*	3.4 (2-8.5)	4 (1-15)	4	6/100 F
	B9652-3	11	20	3.8 (1.5-12)	4.6 (1-25)	7.2	19/100 F
5	B9859-0	12	22	4.1 (2-12)	5.2 (1-26)	6.3	72/100 F
	B9941-3	13	44**	3.2 (1.5-9)	4.8 (1-14)	2.3	15/100 F
	B9656-4	16	21	3.9 (2-11)	5 (1-28)	5.4	69/100 F
	B9862-1	12	18	3.6 (2-9.5)	4.2 (1-19)	9	11/100 F
	B9848-7	11	18	3.4 (1.5-11.5)	4.4 (1-21)	18.4	96/100 F
	B9873-5	12	21	3.9 (1.5-10)	3.8 (1-18)	8.9	24/100 F
	B9649-3	14	23	4.0 (1.5-12)	5.3 (1-26)	4.7	39/100 F
6	B9852-5	13	25	4.1 (2-12)	4.7 (1-22)	5.5	48/100 F
	B9951-0	13	23	3.9 (1.5-9.5)	4.4 (1-14)	8	123/100 F
	B9835-7	11	17	3.5 (1.5-8)	3.6 (1-12)	3.6	24/100 F
	B9687-9	15	24	4.2 (1.5-11)	4.4 (1-20)	12.2	146/100 F
	B1815-4	12	28**	3.7 (1.5-7.5)	3.9 (1-10)	2.8	30/100 F

Table 1 continued

Passage No[a]	Animal No	Incubation Period (Days)	Time to Death (Days p.i.)	Mean size of macroschizonts (µm) (range)	Mean no of nuclei/ macroschizont (range)	Maximum MSI[b] (%)	Maximum piroplasm parasitaemia[c]
7	B9025-2	16	39**	4.8 (2.5-12.5)	5.6 (1-32)	9.2	137/100 F
	B1892-6	12	27**	4.5 (2-11.5)	5.1 (1-22)	14.6	183/100 F
	B7288-4	15	38**	4.4 (1.5-10.5)	4.5 (1-18)	6.7	44/100 F
	B1890-2	20	53**	3.7 (1.5-7)	3.8 (1-10)	7.2	65/100 F
	B1886-1	12	19	3.9 (1.5-11)	4.6 (1-22)	8.6	87/100 F
	B1445-6	13	23	4.3 (2.5-12)	5.3 (1-32)	13.8	334/100 F
8	B9027-8	11	17	3.7 (1.5-8)	3.9 (1-10)	7.5	28/100 F
	B7062-3	12	20	4.1 (1.5-11.5)	4.6 (1-18)	7.4	71/100 F
	B7282-7	10	18	4.4 (2-10.5)	4.7 (1-16)	14.7	56/100 F
	B9024-5	13	21	3.9 (1.5-9.5)	4.5 (1-15)	8.2	166/100 F

[a] This refers to the number of tick passages in cattle
[b] MSI = macroschizont index
[c] Piroplasm parasitaemia is expressed as the number of piroplasms detected in 100 microscopic fields (100 F) at 1000x magnification, containing approximately 500 erythrocytes each
[*] = did not die
[**] = slaughtered (*in extremis*)

Table 2 Summary of means of selected parameters for tick-cattle passages of *T. parva* in intact cattle

Passage No	Mean Incubation Period (Days)	Mean size of macroschizonts (µm)	Mean number of nuclei per macroschizont	Mean Maximum MSI* (%)
3 n = 1	16	3.9	3.7	6.7
4 n = 2	11	3.6	4.3	5.6
5 n = 7	12.9	3.7	4.7	7.9
6 n = 5	12.8	3.9	4.2	6.4
7 n = 6	14.6	4.3	4.8	10
8 n =4	11.5	4.0	4.4	9.5

* MSI = macroschizont index

Intra-erythrocytic piroplasms were generally first detectable in thin blood smears in very low numbers approximately 15 to 18 days post-infection.

It would appear that in those animals in which the incubation period was somewhat extended, the animals tended to survive for longer, and in a number of those, the increased duration of survival was accompanied by higher macroshizont indices, an increased size of schizonts and higher piroplasm parasitaemias. However, these findings were not consistently observed in all infected animals.

In summarising the reactions of all animals infected during the experiment: The incubation period for tick-transmitted infections in non-splenectomised cattle ranged from 11 to 20 days (mean = 13.2 days), the mean size of macroschizonts ranged from 3.2 to 4.8 (mean = 3.9) and the mean number of nuclei per macroschizont from 3.6 to 5.6 (mean = 4.5). The maximum macroschizont indices ranged from 2.3 to 14.7 (mean = 7.2) and piroplasm parasitaemias from 6 to 334 (mean = 81) piroplasms per 100 microscope fields of 500 erythrocytes each. In none of the animals was a piroplasm parasitaemia of more than 1% detected, the highest parasitaemia of approximately 0.7 % being recorded in B1445-6 in Passage 7.

DISCUSSION

All previous attempts to transform buffalo-derived *T. parva* from South Africa in cattle failed to demonstrate any discernable behavioural change after as many as four consecutive tick passages in cattle (De Vos, 1982). It should be noted, however, that these transmissions were performed using splenectomised cattle which had recovered after chemotherapy. By contrast, in those experiments where transformation of buffalo-derived *T. parva* was reported (Barnett & Brocklesby, 1966; Young *et al.*, 1973), intact cattle were used and no specific chemotherapeutic drugs were administered to reacting animals. To approximate the conditions used by these authors more closely, non-splenectomised (as opposed to splenectomised) cattle were chosen for the transmission attempts from Passage 3 to Passage 8.

Unlike *R. appendiculatus*, *R. zambeziensis* could repeatedly transmit *T. parva* infections from lethally infected cattle with extremely low piroplasm parasitaemias, and without chemotherapeutic intervention, as had previously been noted by Potgieter *et al*. (1988). This can almost certainly be attributed to the fact that *R. zambeziensis* is more efficient at transmitting buffalo-derived *T. parva* infections than *R. appendiculatus* (Blouin & Stoltsz, 1989[6]).

The considerable variation observed in incubation periods, mean size of macroschizonts, mean number of nuclei per macroschizont and mean maximum macroschizont indices are likely due to variations in infection rates of the different tick batches used to transmit infections. No attempt was made to determine the salivary gland infection rates in the batches of infected ticks as it was expected that a low number of ticks would become infected during the acute stage of the clinical reaction. Studies have suggested that young piroplasms (i.e. those within 3 days of erythrocyte infection) are not infective or have low infectivity for ticks (Norval, Perry & Young, 1992).

The mean size of macroschizonts, mean number of nuclei per macroschizont, mean maximum macroschizont index and maximum piroplasm parasitaemias observed in this study are all consistent with those previously reported for buffalo-derived *T. parva* infections in cattle (Barnett & Brocklesby, 1966; De Vos, 1982; Dolan, 1989; Young *et al*., 1973). Perhaps the most significant of these parameters was the piroplasm parasitaemias. In none of the animals was a piroplasm parasitaemia of more than 1% detected, the highest parasitaemia of approximately 0.7% being recorded in B1445-6 in Passage 7. By contrast, Barnett & Brocklesby (1966) and

[6]Included as an addendum in this manuscript

Young *et al*. (1973) observed piroplasm parasitaemias to increase to 2.5% and 5%, respectively, after as few as four tick-passages in cattle.

Since *R. zambeziensis* generally develops higher salivary gland infection rates of buffalo-derived *T. parva* than *R. appendiculatus*, it has been suggested that infections transmitted by the former are more likely to be virulent (and therefore result in higher mortality) (Norval *et al.,* 1992) and thus could be expected to be self-limiting. Since buffalo-derived *T. parva* piroplasm parasitaemias are characteristically very low, however, especially in non-splenectomised cattle[7], it might be precisely this characteristic which would enable transmission to occur.

No behavioural change in the *T. parva* (Hluhluwe 3) isolate was observed *in vivo* after eight consecutive passages in intact cattle without chemotherapeutic intervention. Failure to reproduce the findings of Barnett and Brocklesby (1966) in the current investigation must cast some doubt on the validity of those earlier findings. At the time when the reports appeared, it was believed that cattle recovered from *T. parva* infection did not remain carriers. So, although piroplasms were observed in the experimental cattle used for the serial passages, these were assumed to be *T. mutans*. It is possible that some of these animals may have been carriers of *T. parva*. On the other hand, based on the observed host-parasite relationship of cattle-derived and buffalo-derived *T. parva* in cattle and buffalo respectively, it seems plausible that the former represents the selection and/or adaption of a subpopulation of the latter in cattle.

The observation that microschizonts are only very rarely observed in buffalo-derived parasite infections can probably be explained by the fact that macroschizont parasitoses are generally much lower than those of cattle-derived *T. parva*. In addition, the presence of only small numbers of dense nuclei (merozoites) in such microschizonts is likely the reason for the low piroplasm parasitaemias observed in buffalo-derived *T. parva* infection, even in splenectomised cattle.

It has been suggested that the selection of ECF-type parasites in cattle from buffalo-derived parasites is the result of the presence of non-diapausing populations of *R. appendiculatus* in East Africa (Norval *et al.*, 1992) and that the initial spread of ECF in southern Africa may well have been the result of a simultaneous introduction of such a non-diapausing population of *R. appendiculatus* when ECF was originally introduced. It could then be argued that the

[7] Piroplasm parasitaemia might only be microscopically detectable after splenectomy

successful eradication of ECF in cattle in South Africa can thus be largely attributed to the eradication and/or dying out of these tick populations. However, this does not explain why the disease persisted for several decades after its initial introduction. It would seem that the persistence of carrier infections, which at one time were thought not to exist or to occur rarely, may well be rather more common than previously thought. However, the absence of non-diapausing ticks may well be the reason why no selection of ECF-type parasites has occurred under natural conditions in South Africa. The high mortality, and strict control measures (including the prohibition of chemotherapy) implemented during buffalo-associated outbreaks limited exposure of cattle to buffalo-derived parasites and offered few opportunities for cattle-to-cattle transmission to occur. As demonstrated in this study, buffalo-derived *T. parva* infections can be maintained by tick transmission in cattle, provided nymphal ticks are present at the time of clinical disease, even in fatal infections. This situation is unlikely to occur in South Africa, however, where *R. appendiculatus* and *R. zambeziensis* exhibit strict seasonal patterns of abundance (Short & Norval, 1981)

The findings from this study indicate that, contrary to earlier belief, Corridor disease is not a self-limiting disease in cattle, and given the opportunity, could well become established in a cattle population in the absence of buffalo. This is particularly concerning in the light of the recent detection of sub-clinical infections of *T. parva* in cattle in South Africa (Thompson, Latif, Oosthuizen, Troskie & Penzhorn, 2008; Yusufmia, Collins, Nkuna, Troskie, Van den Bossche & Penzhorn, 2010), suggesting that the premises on which current Corridor disease control strategies are based may have to be reassessed.

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CHAPTER 3

ATTEMPTED INFECTION OF COMMON WATERBUCK (*KOBUS ELLIPSIPRYMNUS*) WITH BUFFALO-DERIVED *THEILERIA PARVA*

INTRODUCTION

It seems plausible that the African buffalo (*Syncerus caffer*) is the original host of *Theileria parva* (Young & Grootenhuis, 1985), a parasite exhibiting considerable genetic variation (Radley, Brown, Burridge, Cunningham, Musisi & Purnell, 1975; Young, Radley, Cunningham, Musisi, Payne & Purnell, 1977; Young, Brown, Cunningham & Radley, 1978; Radley, Young, Grootenhuis, Cunningham, Dolan & Morzaria, 1979; Conrad, Iams, Brown, Sohanpal & ole-MoiYoi, 1987; Allsopp & Allsopp, 1988; Bishop, Sohanpal, Allsopp, Spooner, Dolan & Morzaria, 1993; Collins & Allsopp, 1999; Oura, Odonga, Lubega, Spooner, Tait & Bishop, 2003; Oura, Asiimwe, Weir, Lubega & Tait, 2005). It is responsible for a number of disease syndromes in cattle in East, Central and southern Africa (Uilenberg, 1981), and places severe constraints on the livestock industries in many parts of the continent. Experimental evidence suggests that buffalo may harbour *T. parva*-group parasite populations of great genetic diversity, including parasites which cause East Coast fever (ECF) in cattle (= cattle-derived *T. parva* or East Coast fever-type) (Barnett & Brocklesby, 1966; Young, Brown, Burridge, Cunningham, Kirimi & Irvin, 1973; Uilenberg, 1981; Grootenhuis, Leitch, Stagg, Dolan & Young, 1987; Bishop, Spooner, Kanhai, Kiarie, Latif, Hove, Masaka & Dolan, 1994).

Farmers in East Africa have long believed that defassa waterbuck (*Kobus defassa*) are involved in the epidemiology of bovine theileriosis (Anon., 1981; Stagg, Bishop, Morzaria, Shaw, Wesonga, Orinda, Grootenhuis, Molyneux & Young, 1994). Apart from the fact that waterbuck are good hosts for *Rhipicephalus appendiculatus* (Young & Newson, 1973; Norval, Perry & Young, 1992), they often share their habitat with buffalo and cattle. It has been demonstrated that defassa waterbuck cells may be infected *in vitro* with *T. parva* sporozoites derived from buffalo, but not with those derived from cattle (Stagg, Young, Leitch, Grootenhuis & Dolan, 1983). These findings were confirmed when it was demonstrated that defassa waterbuck could be infected with buffalo-derived *T. parva* and may remain carriers of the infection for a considerable period (Stagg *et al.*, 1994). In addition, subsequent transmission to cattle of the

infection established in the defassa waterbuck showed that the population of parasites isolated from the cattle, after passage through the experimentally infected waterbuck, had a different genetic composition from the population used to infect the waterbuck. It would appear that the observed behavioural change, characterized by a higher schizont parasitosis and piroplasm parasitaemia in cattle, is the result of selection of a subpopulation of parasites of the ECF-type in the waterbuck, and is similar to that observed in the so-called transformation of buffalo-derived *T. parva* (Corridor disease-type) to typical cattle-derived *T. parva* after repeated passage in cattle (Barnett & Brocklesby, 1966).

Since ECF (cattle-derived *T. parva* infection) was eradicated from South Africa by the mid-1950s, the possible re-emergence of ECF in this country has been of considerable concern. Due to strict control measures and very limited contact between buffalo and cattle in Corridor disease-endemic areas, very few opportunities exist for the selection of ECF-causing subpopulations of *T. parva* in cattle. However, the finding that such transformation may occur during a single tick-passage of buffalo-derived *T. parva* in waterbuck indicates a much greater potential risk for the re-emergence of ECF in Corridor disease-endemic areas, especially in the light of the finding that buffalo-derived *T. parva* isolates from the Kruger National Park (KNP) contain p67 gene sequences characteristic of both cattle-derived and buffalo-derived *T. parva* (Collins, 1997). It therefore needs to be established whether the common waterbuck (*Kobus ellipsiprymnus*) is susceptible to infection with local *T. parva*-group isolates from buffalo, and whether this species of waterbuck could play a role in the rapid selection of ECF-type *T. parva* from buffalo-derived parasites in South Africa.

Common waterbuck in the KNP, a known *T. parva* endemic area, were thus screened for the presence of *T. parva* infection. Attempts were also made to transmit *T. parva* from these waterbuck after experimental infestation with *T. parva*-infected ticks. It has previously been established that *R. zambeziensis* is a better vector of buffalo-derived *T. parva* than *R. appendiculatus* (Blouin & Stoltsz, 1989) and transmission attempts using this tick as a vector would therefore maximise the possibility of transmitting buffalo-derived *T. parva* in these experiments. In order to minimise experimental manipulation and avoid artificially-induced selection of parasite subpopulations (for example by isolation in cattle and cryopreservation of sporozoite stabilates), only direct tick transmissions were attempted from one mammalian host to another.

MATERIALS AND METHODS

Study site

The KNP is the largest Corridor disease (buffalo-associated *T. parva*) endemic area of South Africa. Due to its size and geographic location (i.e. bordering on Zimbabwe and Mozambique where *T. parva* is endemic and also the route of original introduction of ECF into southern Africa in the latter part of 1901 (Sinclair, 1922)), *T. parva*-group parasites in the KNP are likely to be genetically more diverse and therefore more likely to contain heterogeneous subpopulations of ECF-type parasites. As large numbers of common waterbuck and *T. parva*-infected buffalo co-exist in the KNP, there may be a high probability of transmission of *Theileria* parasites between these two host species and, therefore, a greater possibility of detecting such infections, should they occur naturally.

Ticks and tick feeding

The Killkenny strain of *R. zambeziensis* (Potgieter, Stoltsz, Blouin & Roos, 1988) was used throughout. For transmission attempts, laboratory-reared *R. zambeziensis* nymphs were applied in a cotton ear bag to each ear of an animal (waterbuck, buffalo or bovine). The ear bags were attached around the base of the ear using a contact adhesive[8]. Engorged nymphs were allowed to moult in the laboratory at 26°C (± 1°C) and 75 % (± 5 %) RH. Approximately six weeks after moulting, the ensuing adult ticks were applied in ear bags to cattle or waterbuck to attempt tick transmission of *T. parva*.

Salivary gland infection rates of ticks fed on buffalo

Ticks were removed from the captive buffalo on Day 4 after infestation and their salivary glands dissected out and teased onto glass slides (Blouin & Stoltsz, 1989). The glands were fixed in Carnoy's fixative, stained with methyl green/pyronin (Irvin, Boarer, Dobbelaere, Mahan, Masake & Ocama, 1981) and examined with a light microscope for infective stages of *T. parva*.

Buffalo

Four African buffalo were captured in the KNP and transported to a tick-secure facility. Animals were immobilised using etorphine hydrochloride[9] and resuscitated with diprenorphine hydrochloride[10]. At the time of capture, thin blood films were prepared for microscopic

[8] Genkem
[9] M99, Novartis
[10] M50:50, Novartis

examination and blood was collected in vacutainer tubes with EDTA as anticoagulant for use in diagnosis of *T. parva* by hybridisation of polymerase chain reaction (PCR) products with species-specific oligonucleotide probes (Allsopp, Baylis, Allsopp, Cavalier-Smith, Bishop, Carrington, Sohanpal & Spooner, 1993).

Isolation of *T. parva* organisms from naturally infected buffalo

To obtain *T. parva*, *ca.* 1000 laboratory-reared *R. zambeziensis* nymphs were applied in ear bags to each of the four captive buffalo. After engorgement, replete nymphs were collected and allowed to moult in the laboratory. Batches of adult ticks thus obtained were thoroughly mixed and batches of *ca*. 300 were prepared for attempted transmission of *T. parva* to one of the experimental cattle and the four waterbuck.

Waterbuck

Seven common waterbuck, three adult females and four subadults (two males and two females), were captured in the central part of the KNP, an area known to harbour large numbers of both waterbuck and buffalo. Animals were immobilised using etorphine hydrochloride and resuscitated with diprenorphine hydrochloride At the time of capture thin blood films were prepared for microscopic examination and blood was collected in vacutainer tubes with EDTA as anticoagulant for PCR diagnosis. The three adult females were released after collection of the diagnostic samples.

The four subadult animals were transported to a tick-secure facility where they were held for *ca.* four months. They were allowed to acclimatise for four weeks before being immobilised again to attempt tick transmission of *T. parva*.

Attempted experimental infection of waterbuck with *T. parva*

Approximately 300 *R. zambeziensis* adults, which had fed as nymphs on four captive buffalo infected with *T. parva* were applied in ear bags to each animal. Six weeks after this tick feeding, 600 laboratory-reared *R. zambeziensis* nymphs were applied to each animal (*ca.* 300 per ear) in an attempt to infect the ticks with *T. parva* which the animals may have acquired naturally or during experimental tick feeding. Blood smears and blood in anticoagulant (EDTA) were collected from these animals on each occasion when ticks were applied.

Control cattle

Two cattle (*Bos taurus* and *Bos indicus* crosses), of 30–36 months of age and reared under tick-free conditions at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI), were used as control animals. They were confirmed to be negative for antibodies to *T. parva* by indirect fluorescent antibody test (IFAT) using a schizont antigen (Burridge & Kimber, 1972). One control animal was used to confirm the infectivity of the *T. parva*-infected adult *R. zambeziensis* which had fed as nymphs on buffalo in the KNP, and the second, to detect *T. parva* infection in adult *R. zambeziensis* which had fed as nymphs on the captive waterbuck, after they had been exposed to *T. parva*-infected ticks. The incubation period, time to death, macroschizont size, number of nuclei per macroschizont and maximum macroschizont index (MSI) were determined for reacting animals as described in Chapter 2.

PCR and oligonucleotide hybridisation

Blood samples collected in vacutainer tubes containing EDTA as anticoagulent were chilled on crushed ice immediately after collection, then placed in a horizontal position in a normal household freezer (-10°C ± 5°C) until processed for PCR analysis and hybridisation with five *Theileria* spp. oligonucleotide probes (Allsopp *et al.*, 1993) at the Molecular Biology Section, ARC-OVI.

RESULTS

Microscopic examination of thin blood films prepared from the seven waterbuck captured in the KNP revealed that all were carriers of *Theileria* sp. piroplasms. PCR diagnosis confirmed the presence of *Theileria* spp. infections, but oligonucleotide probes to five *Theileria* spp. (*T. parva*, *T. mutans*, *T. taurotragi*, *T. buffeli* and *Theileria* sp. (buffalo)) failed to hybridise to the PCR product, suggesting that the waterbuck were infected with a hitherto uncharacterised *Theileria* species.

Theileria sp. piroplasms were detected in thin blood films of the four captive buffalo and all were confirmed to be infected with *T. parva* by PCR diagnosis.

The *R. zambeziensis* adults which fed as nymphs on the captive buffalo transmitted fatal *T. parva* infection to one of the experimental (control) cattle (**Table 3**). Twenty of the adult ticks were removed from the bovine on Day 4 after infestation and the *T. parva* infection rate in their

salivary glands determined. Nineteen of twenty ticks were found to be infected, with an average of 27 infected acini per infected tick. Similar batches of *R. zambeziensis* adults fed on waterbuck apparently failed to transmit *T. parva* to the waterbuck, as PCR diagnosis failed to detect *T. parva* in the waterbuck six weeks after tick feeding. Similarly, a batch of approximately 250 pooled *R. zambeziensis* adults, which had fed as nymphs on the four waterbuck, failed to infect a susceptible bovine, as indicated by the absence of clinical disease and negative PCR diagnostic results.

Table 3 Course of experimental tick-transmitted infection of *T. parva* from buffalo to a bovine

Animal No	Incubation Period (Days)	Time to Death (Days p.i.)	Mean size of macroschizonts (µm) (range)	Mean no of nuclei per macroschizont (range)	Maximum MSI (%) (range)	Maximum piroplasm parasitaemia
B9049-9	13	20	4.2 (1.5-7.5)	5.1 (1-12)	3.6	11/100 F

MSI = macroshizont index
Days p.i. = days post infection
Piroplasm parasitaemia is expressed as the number of piroplasms detected in 100 microscopic fields (100 F) at 1000x magnification, containing approximately 500 erythrocytes each

DISCUSSION

Observations on defassa waterbuck in Kenya showed that these animals carry uncharacterized *Theileria* species (Stagg, 1992), and that they are extremely attractive hosts for *R. appendiculatus* ticks, the vectors of *T. parva* parasites in East Africa (Young and Newson, 1973). Defassa waterbuck are very resistant to *T. parva* infection by *R. appendiculatus* ticks which have been infected by feeding on buffalo, but these waterbuck can be infected if inoculated with large doses of infective *T. parva* sporozoites isolated from such ticks (Stagg *et al.*, 1994). It appears that only a subpopulation of *T. parva* is able to infect the defassa waterbuck, that this subpopulation has the characteristics of cattle-derived *T. parva* (ECF-type), and that these parasites can infect uninfected *R. appendiculatus* ticks which feed on the waterbuck and can subsequently be transmitted to susceptible cattle and cause ECF (Stagg *et al.*, 1983). These observations suggest that defassa waterbuck may be important intermediates in the passage of *T. parva* from buffalo to cattle in East Africa, and this encouraged us to investigate

whether a similar situation exists in the KNP, where common waterbuck and buffalo jointly inhabit large areas of the Park.

All the common waterbuck used in this work were found to be carrying a previously uncharacterized *Theileria* species, indicating that they were being fed on by infected ticks. Although it is unknown which ticks carry this *Theileria* species, the observation suggests that the waterbuck would also be acting as hosts for such vector ticks, which may or may not be the same as the vectors of *T. parva*. Despite this, none of the waterbuck was found to be carrying *T. parva* parasites at the time of their capture, including the three adult animals which had been exposed to tick infestation in a *T. parva*-endemic area for a longer period than the subadult animals. Batches of adult *R. zambeziensis* ticks, highly infected from buffalo with *T. parva* parasites and shown to be highly infective to cattle, were also fed on four of the waterbuck, and none of them became infected. This suggests that common waterbuck may not be susceptible to *T. parva* infection, but confirmation of this is needed by inoculating large doses of infective *T. parva* sporozooites isolated from ticks. It should be noted that this was the method by which defassa waterbuck were experimentally infected with *T. parva*, while these animals too are difficult to infect with *T. parva* by normal tick feeding (Stagg *et al*., 1994).

In summary, although our experiments are not definitive, they suggest that common waterbuck are unlikely to play an important role in the epidemiology of *T. parva* infections in cattle in South Africa. This is supported by the historical observation that ECF does not occur normally in South Africa although buffalo in the KNP have been shown to carry parasites with characteristics of cattle-derived *T. parva* (Collins, 1997; Sibeko, Geysen, Oosthuizen, Matthee, Troskie, Potgieter, Coetzer & Collins, 2010). One of the crucial differences between southern and eastern Africa in respect of the epidemiology of ECF could well be the absence of defassa waterbuck from the southern area.

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CHAPTER 4
SUBINOCULATION OF BLOOD FOR DETECTION OF *THEILERIA PARVA* CARRIER INFECTIONS IN CATTLE AND SUBSEQUENT DEMONSTRATION OF STAGE-SPECIFIC ANTIGENICITY IN RECIPIENT ANIMALS FOLLOWING AUTOLOGOUS CHALLENGE

INTRODUCTION

Transmission of *Theileria parva* by subinoculation of blood and organ suspensions in cattle was demonstrated by Theiler and Du Toit (1929) during early attempts to develop a vaccine to East Coast fever. Results were rather erratic and unpredictable and immunity seemed to be conferred only to those animals in which clinical disease was provoked following administration of large quantities of infective material. Similarly, mixed results were obtained during attempts to transmit buffalo-derived *T. parva* with lymph node material and blood from infected to susceptible cattle (Neitz, 1955; Neitz, 1957; Neitz, Canham & Kluge, 1955; Neitz, 1964).

Subsequent studies on immunity to *T. parva* found a major histocompatibility complex (MHC) barrier to immunisation with schizont-infected lymphoblasts (Dolan, Teale, Stagg, Kemp, Cowan, Young, Groocock, Leitch, Spooner & Brown, 1984). In MHC-matched animals, small numbers of schizont-infected lymphoblasts could be used to establish infection, whereas in unmatched animals, large quantities were required, providing an explanation for the erratic results previously reported.

Neitz (1962) subinoculated undisclosed volumes of blood from a *T. parva*-infected buffalo heifer to two splenectomised calves. Piroplasm parasitaemias were detected approximately four weeks after subinoculation and *T. parva* was subsequently transmitted by *Rhipicephalus appendiculatus* to susceptible cattle. During attempts by De Vos (1982) to isolate *T. parva* from buffalo by subinoculation of blood to cattle, intravenous inoculation of 50 ml and 100 ml of pooled blood (each sample obtained from two adult buffalo shot in the Hluhluwe Game Reserve) into two splenectomised cattle, respectively, resulted in the transmission of only *T. mutans* infections in both recipients. Similarly, two attempts to isolate uncharacterised *Theileria* spp. infections from buffalo (in a South African game reserve where buffalo-associated *T. parva* is not endemic) by

subinoculation of 500 ml of blood from each of two immobilised buffalo into two splenectomised cattle, respectively, resulted in rapidly rising *T. mutans* piroplasm parasitaemias in the recipient animals (Potgieter, Stoltsz, Blouin & Roos, 1988).

Although limited replication of *T. parva* piroplasms was found to occur *in vitro* (Conrad, Denham & Brown, 1986) it has been suggested that maintenance of *T. parva* carrier infections is dependent on the continued presence of schizonts and that replication of piroplasms probably does not significantly contribute to maintaining persistent infections (Jarrett, Crighton & Pirie, 1969; Burridge, 1974; Stagg, Brown, Crawford, Kanhau & Young, 1974; Norval, Perry & Young, 1992). Stage-specific antigenicity has been demonstrated in *Theileria annulata* (Uilenberg, Franssen & Perié, 1986), where maintenance of carrier infections in the absence of schizonts has been demonstrated to occur in infections with attenuated strains of this parasite.

During investigations to improve the diagnosis of low-level carrier infections in buffalo to confirm their *T. parva*-free status, to validate molecular diagnostic tests, or to confirm elimination of *T. parva* infections following attempted chemosterilisation (Stoltsz, 1985), subinoculation of blood from infected non-splenectomised and splenectomised carrier cattle to splenectomised cattle was investigated. Donor cattle comprised recovered intact cattle, reacting intact cattle or splenectomised recovered cattle

MATERIALS AND METHODS

Parasite isolate

The buffalo-derived Hluhluwe 3 isolate of *T. parva* (De Vos, 1982) was used throughout.

Experimental cattle

All cattle were *Bos taurus*/*Bos indicus* crosses, born and reared under tick-free conditions at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI) for the production of the commercial blood-based vaccines to bovine babesiosis and anaplasmosis. Prior to infestation with infected ticks or subinoculation of blood, they were confirmed to be negative for antibodies to *T. parva* by indirect fluorescent antibody test (IFAT), using *T. parva* piroplasm and schizont antigens (Burridge and Kimber, 1972).

Subinoculation of blood

One litre of blood was collected from the jugular vein of the *T. parva*-infected donor animal using a 1000 ml blood collection kit containing 10,000 IU heparin. Gentle agitation ensured thorough mixing of blood and anticoagulant and prevented sedimentation of the erythrocytes. The donor blood was administered slowly intravenously into the jugular vein of the recipient animal within one hour of collection.

Diagnostic tests and sampling

The rectal temperatures of all animals were monitored on a daily basis and thin blood smears were prepared three times per week and examined for haemoparasites. Following tick infestation, thin blood films were prepared every day. As soon as a temperature reaction of 39.5^{o}C or greater was recorded, lymph node biopsy smears were prepared from the parotid and superficial cervical (prescapular) lymph nodes. Blood films and lymph node biopsy smears were stained with Giemsa's stain and examined microscopically using a 10x objective lens and 100x oil immersion lens.

Duplicate samples of blood without anticoagulant were collected at weekly intervals for serological testing using the IFAT with either piroplasm (Burridge, 1971) or schizont antigen (Burridge & Kimber, 1972), incorporating some minor modifications (Gray & De Vos, 1981).

Ticks and tick feeding

The Killkenny strain of *Rhipicephalus zambeziensis* (Potgieter *et al*., 1988) or the Rietvlei cross strain of *R. appendiculatus* (De Vos & Roos, 1981), both of which had been maintained in the laboratory at the ARC-OVI, were used for all tick transmission attempts. For each transmission attempt, approximately 300 laboratory-reared *R. zambeziensis* nymphs were applied in an ear bag to each ear of a bovine. Engorged nymphs were allowed to moult in the laboratory at 26°C (± 1°C), 75 % (± 5 %) RH. Approximately six weeks after moulting, adult ticks were applied in ear bags to the same individual cattle on which the preceding nymphs had fed, in an attempt to demonstrate stage-specific immunity to *T. parva*.

Experiment 1 : Subinoculation from *T. parva*-recovered splenectomised carrier bovine

Splenectomised bovine B9098-5 (see **Figure 1, Chapter 2**) was infected during the second tick-passage of *T. parva* (Hluhluwe 3) using *R. appendiculatus* and had recovered following

treatment with halofuginone lactate[11]. Subinoculation of blood from B9098-5 to splenectomised bovine B9420-8 was performed on Day 1368 after tick infection. Subinoculation of blood from B9420-8 to B9621-9, and subsequent subinoculation from B9621-9 to B9595-7, both of which were splenectomised, was performed on Day 96 and Day 123, respectively, after infection with subinoculated blood (**Figure 2**).

Homologous challenge of *T. parva* piroplasm-infected carrier cattle

Approximately 300 laboratory-reared *R. zambeziensis* nymphs were fed in ear bags on B9420-8, B9621-9 and B9595-7, respectively. The ensuing adult ticks were then applied in ear bags to the same animal on which the preceding nymphs had fed.

Experiment 2 : Subinoculation from *T. parva*-recovered intact carrier cattle

Two non-splenectomised cattle (B9666-0 and B9665-2) were infected with *T. parva* (Hluhluwe 3) using a sporozoite suspension prepared from infected *R. appendiculatus* adults (Cunningham, Brown, Burridge & Purnell, 1973) (**Figure 3**). These two animals recovered from clinical *T. parva* infections following chemotherapy with parvaquone[12]. A volume of 1000 ml of blood collected in anticoagulent was subinoculated into B9408-5 and B9418-9, respectively, both of which were splenectomised cattle.

Experiment 3 : Subinoculation from *T. parva*-reacting intact cattle

Animals B9659-8, B9025-2 and B9852-5 (**Figure 4**) were non-splenectomised cattle from which 1000 ml of blood was subinoculated into three splenectomised cattle, B9931-7, B9954-2 and B9899-3, respectively, during the clinical phase of *T. parva* infections in the former (see Chapter 2). All three donor animals developed fatal infections and no chemotherapeutic drugs were administered prior to collection of blood.

[11] Terit, Hoechst AG
[12] Clexon, Coopers Animal Health Ltd, England

RESULTS

The results are summarised in **Figures 2, 3** and **4**.

Animal, B9098-5 (**Figure 2**) was a splenectomised carrier of buffalo-derived *T. parva* (Hhluhluwe 3 isolate). It developed severe clinical disease following infection using *R. appendiculatus* ticks, but recovered after treatment with halofuginone lactate and had a persistently low *Theileria* piroplasm parasitaemia for more than four years. One litre of blood in EDTA was subinoculated into B9420-8. Following the detection of small numbers of *Theileria* piroplasms in this animal, one litre of its blood was subinoculated into B9621-9. Similarly, after an initial period of several weeks in which no *Theileria* piroplasms were observed microscopically, small piroplasms were detected in very small numbers in the recipient animal and one litre of blood from this animal was subinoculated into B9595-7. The latter also developed a microscopically detectable piroplasm parasitaemia several weeks after inoculation.

Bovine B9666-0 (**Figure 3A**) developed a temperature reaction on Day 10 post-infection, which coincided with the first microscopic detection of *Theileria* schizonts in lymph node biopsy smears. This animal was treated with parvaquone at a dosage rate of 10 mg/kg intramuscularly on Day 11 and Day 13 post-infection. B9665-2 (**Figure 3B**) showed a biphasic temperature reaction and was treated at the same dosage rate on Days 11 and 13 as well as on Days 20 and 22 post-infection. In both animals *Theileria* sp. piroplasms were not microscopically detectable in thin blood smears for the duration of the experiment, but on Day 124 post-infection, a single *Theileria* sp. macroschizont was observed on a blood smear during routine microscopic examination of thin blood smears of B9666-0. Although no attempt was made to transmit *T. parva* from the two non-splenectomised cattle whilst no piroplasms were microscopically detectable in thin blood smears, no *Theileria*-infected acini were detected in batches of 35 and 42 adults of *R. appendiculatus* and *R. zambeziensis*, respectively, which had engorged as nymphs on B9666-0 (Blouin & Stoltsz, 1989).

On Day 260 post-infection, one litre of blood from B9666-0 was subinoculated into B9408-5 (**Figure 3A**). On Day 36 after receiving one litre of blood from B9666-0, the recipient animal developed a mild febrile reaction (ranging from 39.5^{o}C to 40.6^{o}C) which persisted for six days and coincided with the microscopic detection of a low-level *Theileria* sp. macroschizont parasitosis in peripheral blood smears and lymphnode biopsy smears. On Day 260 post-infection, one litre of blood from B9665-2 was subinoculated into B9418-5 (**Figure 3B**). This recipient

animal developed no febrile reaction and no schizonts were microscopically detectable, but small numbers of piroplasms were detected several weeks after inoculation.

Animals B9659-8, B9025-2 and B9852-5 (**Figure 4**) were non-splenectomised cattle from which 1000 ml of blood was subinoculated into three splenectomised cattle, B9931-7, B9954-2 and B9899-3, respectively, during the clinical phase *T. parva* infections during the serial tick-passage of *T. parva* (Hluhluwe 3) (see **Figure 1**, **Chapter 2**). All three of these donor animals developed fatal infections and no chemotherapeutic drugs were administered prior to collection of blood. Persistent piroplasm carrier infections were established in the splenectomised recipient cattle, but no attempts were made to further transmit these infections to recipient cattle.

In all three experiments, none of the recipient animals developed a febrile reaction after subinoculation of blood, with the exception of B9408-5. However, three splenectomised recipients (**Figure 2**) developed acute *T. parva* reactions following homologous challenge (Hluhluwe 3 isolate) using infected *R. appendiculatus*. Two of these (B9420-8 and B9595-7) died, whereas B9621-9 recovered after a severe clinical reaction.

The IFA test (using either piroplasm or schizont antigen) was used to test the recipient cattle for possible seroconversion after receiving the *T. parva*-infected blood. All recipient cattle seroconverted from negative to positive (i.e. titre of ≥ 1/160) when using piroplasm antigen between 14 and 21 days post-inoculation with *T. parva*-infected blood. With one exception (B9408-9), none of the recipients developed microscopically detectable schizont parasitoses, or developed a positive titre of ≥ 1/40 using schizont antigen, but all developed low-level piroplasm parasitaemias (less than 1/1000 erythrocytes). These infections nevertheless remained tick-transmissible (*R. zambeziensis*), causing fatal *T. parva* infections in susceptible cattle. Even after repeated needle passage in splenectomised cattle there was no appreciable reduction in virulence of the parasite.

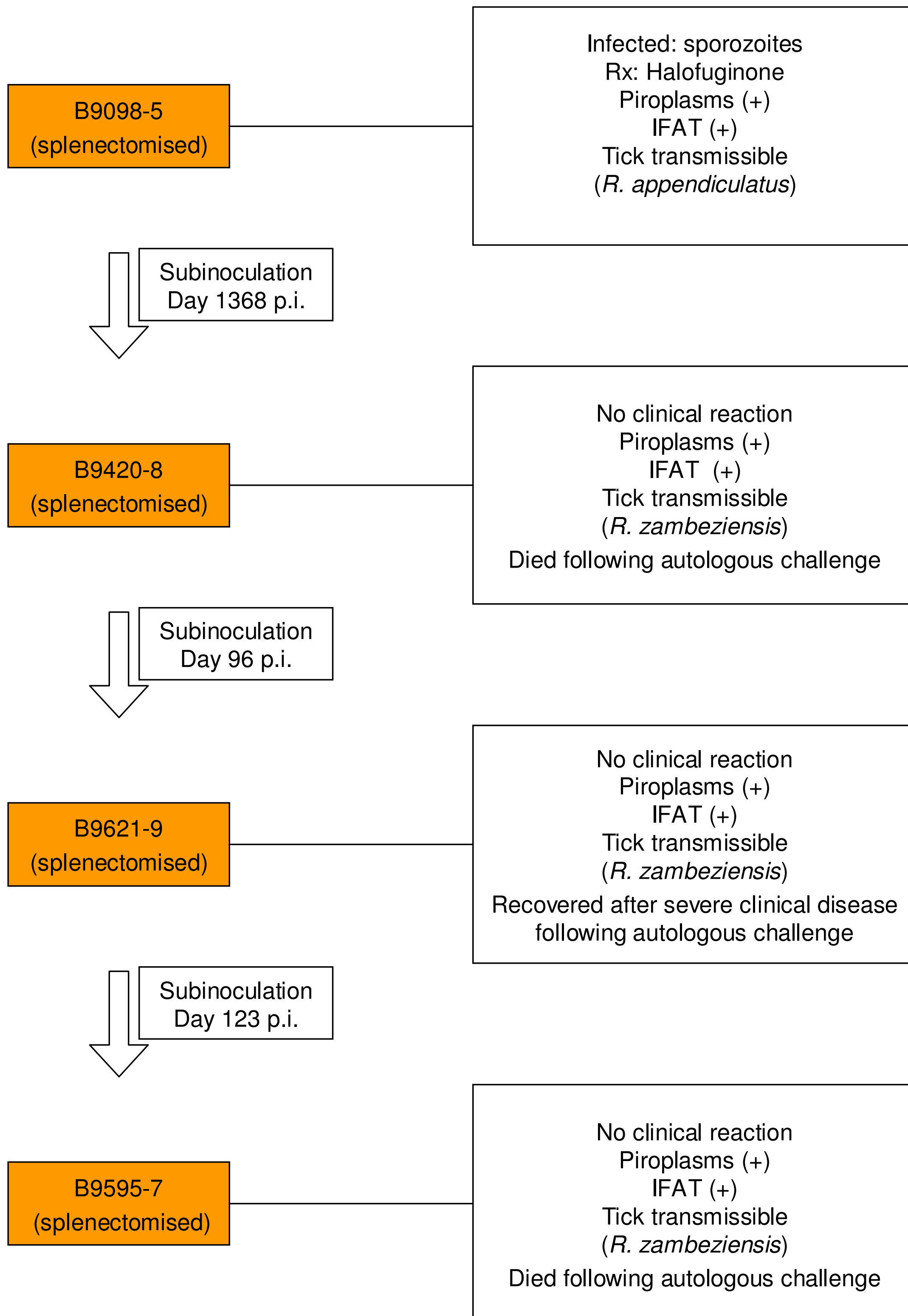

Figure 2 Schematic representation of the serial subinoculation of *Theileria parva* (Hluhluwe 3 isolate) in splenectomised cattle by intravenous inoculation of 1000 ml of blood (p.i. = post infection). IFAT (+) result indicates a titre of ≥ 1/160 using piroplasm antigen.

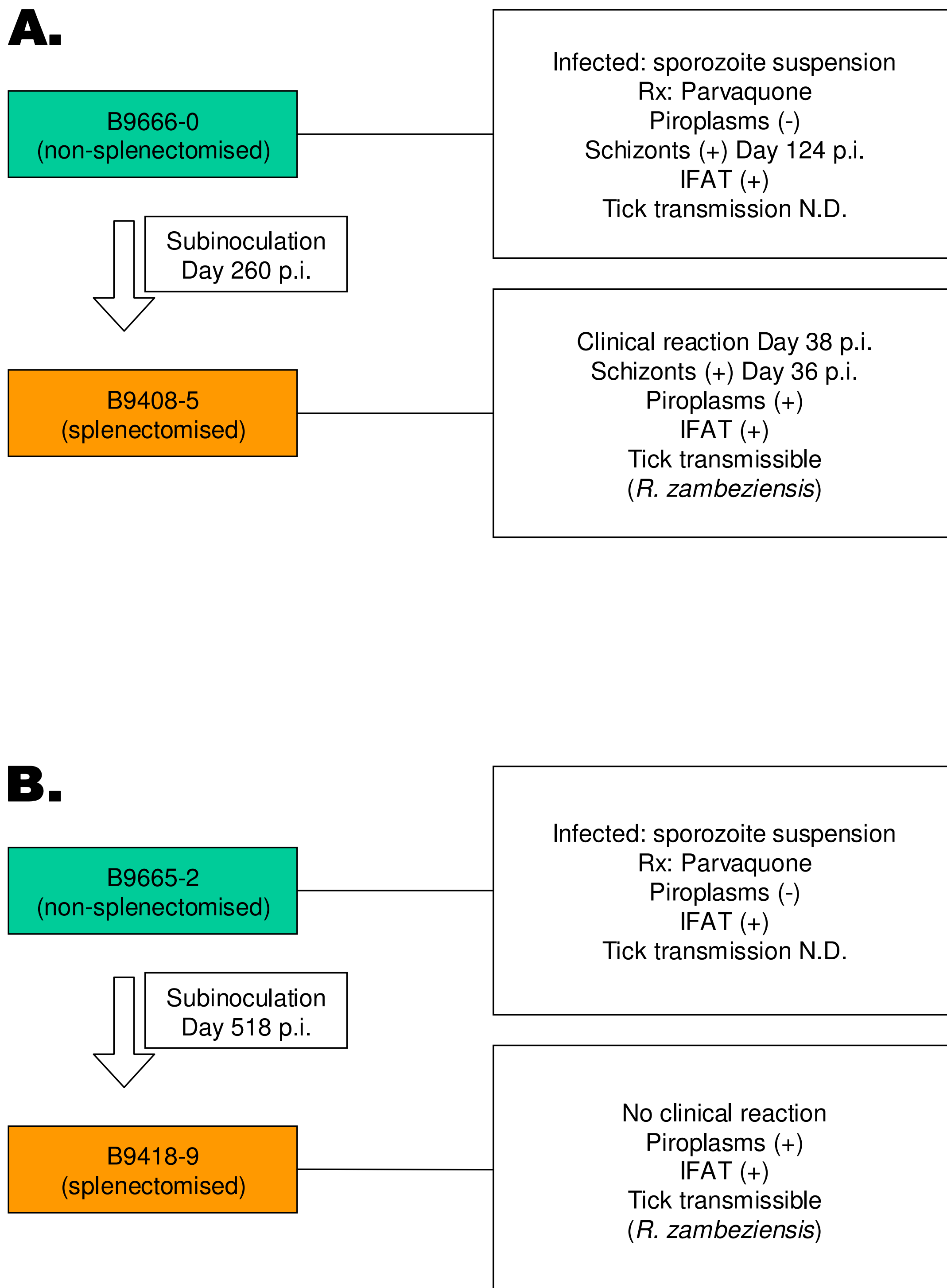

Figure 3 Schematic representation of the transmission of *Theileria parva* (Hluhluwe 3 isolate) from two non-splenectomised carrier cattle to two splenectomised cattle by intravenous inoculation of 1000 ml of blood (p.i. = post infection; N.D. = not done).

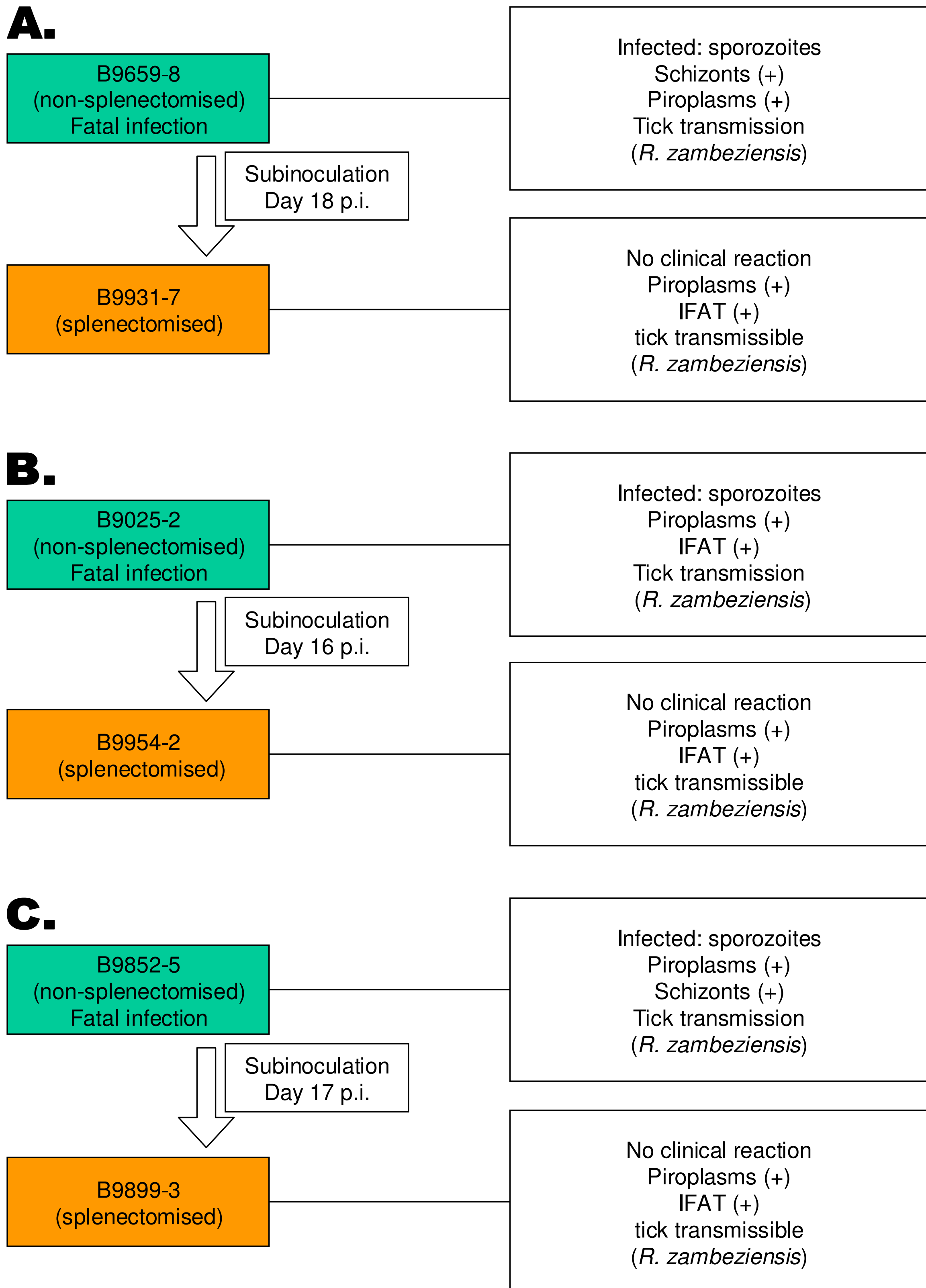

Figure 4 Schematic representation of the transmission of *Theileria parva* (Hluhluwe 3 isolate) to splenectomised cattle by intravenous inoculation of 1000 ml of blood taken from three lethally infected non-splenectomised cattle during the clinical phase of infection.

DISCUSSION

Theileria parva replicates mainly in lymphocytes and its pathogenic effects are associated with lymphocyte destruction; replication of merozoites (piroplasms) in erythrocytes is considered to be limited and heamolytic anaemia is therefore usually not a feature of clinical disease. In these experiments we were able to establish persistent piroplasm parasitaemias of buffalo-derived *T. parva* in splenectomised cattle by subinoculation of infected blood from various donor animals.

It would appear that in most recipient animals only piroplasm infections became established. These infections caused no detectable clinical disease and could apparently be maintained indefinitely in the recipient animals through replication of piroplams alone. Long-term maintenance of piroplasm parasitaemias in the absence of schizonts and lack of immunity to challenge with homologous sporozoites suggest that immunity in *T. parva* is also stage-specific, as has been demonstrated for *T. annulata* (Uilenberg *et al*., 1986).

Subinoculation of blood from *T. parva*-infected cattle or buffalo into splenectomised cattle may prove useful to identify carrier buffalo or cattle with very low parasitaemias, as well as provide a method for obtaining parasite isolates from such animals when isolation by tick transmission might not be possible.

Mixed theilerial infections harboured by buffalo make subinoculation of their blood into splenectomised cattle undesirable as a method for obtaining pure parasite isolates. This indirect method (i.e. subinoculation of blood) of identifying possible *T. parva* carriers may yet prove to be of some value, especially if the test animals have low parasitaemias and low specific antibody titres.

The spontaneous recovery of recipient animal B9408-5 may be explained by the extended incubation period of more than 36 days. It is assumed that B9408-5 received a sublethal dose of *T. parva*-infected lymphocytes during the blood transfusion. Histocompatibility probably played a role in that the donor and recipient could have had matched bovine lymphocyte antigen (BoLA) systems (BoLA types of the animals were not determined prior to subinoculation). These results confirm previous observations that schizonts may be persistently present for extended periods in *T. parva*-infected buffalo (Conrad, Stagg, Grootenhuis, Irvin, Newson,

Njamunggeh, Rossiter & Young, 1987) and *T. parva*-recovered cattle (Maritim, Young, Mutugi & Stagg, 1989).

The results obtained with subinoculation from B9098-5 suggest that *T. parva* piroplasms can multiply and maintain a carrier infection in the absence of schizonts. This was confirmed by the stage-specific immunity observed to *T. parva* in these recipient cattle, as the animals were fully susceptible to tick transmission of the *T. parva* parasites of which they were themselves the source of infection for the infected ticks used to challenge them. Buffalo-derived *T. parva* usually produces very low or undetectable piroplasm parasitaemias in cattle, which has generally precluded inclusion of such isolates in *T. parva* vaccines, which rely on large numbers of infected ticks being obtained from laboratory-infected donor cattle. Although the carrier state is well developed in buffalo and high salivary gland infection rates are usually obtained in ticks feeding on them, they may not make ideal laboratory animals for the production of sporozoite vaccines. After prolonged maintenance of such carrier infections in splenectomised cattle, characterisation of the parasites may be required to confirm that their antigenicity has not changed. Nevertheless, such carrier infections may yet prove useful where a particular parasite isolate/stock is required for repeated production of infected ticks, especially where such parasites may tend to produce only low piroplasm parasitaemias in cattle. Severe host immune responses to repeated tick infestation may preclude repeated infestations on hosts, but the effect of this can possibly be reduced by the administration of appropriate symptomatic (antihistamines/cortisones) or specific immunosuppressive chemotherapy to increase tick yield after repeated infestation. If antigenicity and immunogenicity of the original isolate are maintained after repeated subinoculation, however, subsequent source animals need only receive a blood inoculation to perpetuate the isolate in the laboratory.

Together with the high infection rates achieved in *R. zambeziensis*, establishment of persistent piroplasm parasitaemias in splenectomised cattle could make the inclusion of buffalo-derived parasite sporozoite stabilates, which until now have not been practicable to include in the ECF cocktail vaccines, much more feasible. Since it had long been realised that buffalo-derived parasites are genetically more diverse than those from cattle and often lead to vaccine breakdowns in the field (Radley, Young, Grootenhuis, Cunningham, Dolan & Morzaria, 1979; Radley, 1981; Young, Brown, Burridge, Cunningham, Kirimi & Irvin, 1973; Young, Brown, Cunningham & Radley, 1978), it was considered essential to include the Serengeti "transformed" isolate in the Muguga "cocktail" vaccine (Young *et al.*, 1973). Molecular characterisation of this isolate seems to suggest that it is antigenically very similar to *T. parva* (Muguga), however, and

therefore probably offers very little benefit in terms of broadening the antigenic diversity in the vaccine (Bishop, Geysen, Spooner, Skilton, Nene, Dolan & Morzaria, 2001).

The observed relative ease with which buffalo-derived *T. parva* piroplasm infections could be established and propagated in splenectomised cattle, even from acutely infected cattle, mostly in what seems to be the absence of a schizont parasitosis, thus provides a route by which such parasite isolates could conceivably be included in customised cocktail vaccines. Such vaccines could be produced for different geographic areas without the need to "transform" the parasite in order to have it produce higher parasitaemias for reliable sporozoite stabilate production. Rapid needle-passage may also select for a sub-population of parasites that may produce higher piroplasm parasitaemias, and/or reduce virulence of the parasite isolate, and granted such selection does not perceptibly reduce the immunogenicity of the isolate, could enhance the feasibility of including such isolates in future *T. parva* "cocktail vaccines".

It would have been desirable to characterise the parasite populations transmitted during each of the subinoculations in order to establish whether this may have resulted in the selection of any specific subpopulation of parasites from the original isolate and to what extent the genetic composition was retained during serial subinoculations. Unfortunately, no such methods were available at the time of experimentation and although samples were cryopreserved for possible future analysis, these were lost during a breakdown of the freezer facility.

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CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

"A single swallow does not a summer make"

"Absence of evidence is not evidence of absence"

Since the eradication from South Africa of *T. parva* infections which cause classical East Coast fever (ECF) in cattle, the re-introduction or recrudescence of the disease has always been of great concern to veterinary authorities in this country. These concerns were based on the knowledge that the vector ticks occurred widespread in most cattle-farming areas and that the local cattle population was totally naïve to infection with this parasite. The severity of the disease, the tremendous cost of the eradication scheme that lasted five decades (Diesel, 1948; Neitz, 1957) and the fact that no safe effective vaccines to protect cattle had been developed (Radley, 1981) only added to the concern. It was possible, however, to protect the local cattle population by strict veterinary surveillance along the national borders to prevent reintroduction by movement of infected cattle from neighbouring countries.

Although the presence of *T. parva* in some buffalo populations in the country was recognised shortly after the eradication of ECF (Neitz, 1957), the epidemiological differences between ECF and Corridor disease were considered to be significant enough to justify a somewhat different control policy with regard to the latter (Potgieter, Stoltsz, Blouin & Roos, 1988; Stoltsz, 1989). Control of Corridor disease was thus maintained by confining buffalo in endemic areas to well-fenced game parks, game farms and conservation areas, prohibiting the translocation of these animals, implementation of stringent dipping and quarantine procedures in the event of an outbreak, and allowing only disease-free buffalo from known *T. parva* and FMD-free areas to be translocated.

New concerns were raised following reports that buffalo-derived *T. parva* could become established in cattle in the absence of buffalo, giving rise to a disease indistinguishable from ECF (Barnett & Brocklesby, 1966; Young, Brown, Burridge, Cunningham, Kirimi & Irvin, 1973). Prior to these results being published, the general consensus in veterinary circles in South Africa was that buffalo-derived *T. parva*, although infective to cattle, resulted in such high mortality rates and such low piroplasm parasitaemias, that for all practical purposes it could be

deemed an end-stage disease in cattle. Also, the existing control measures appeared to be adequate, as no field evidence was found to suggest that cattle-to-cattle transmission of *T. parva* was occurring in South Africa, several decades after the eradication of ECF (De Vos, 1982; Potgieter *et al*., 1988; Stoltsz, 1989). Following the development of antitheilerial drugs, however, the potential risks associated with recovered cattle possibly becoming carriers of buffalo-derived *T. parva* was recognised and the use of such chemotherapeutic agents was prohibited in South Africa (Potgieter, Roos & De Vos, 1985; Potgieter *et al*., 1988).

The world-wide increase in ecotourism during the latter part of the twentieth century, and the particular lure of observing (and hunting) the "Big Five" dangerous game animal species of Africa (of which the African buffalo is one) created a massive demand for buffalo in many areas of South Africa where they no longer occurred naturally. Restrictions on the translocation of buffalo in the country remained in place, however, which obviously severely limited the available gene pool of buffalo suitable for stocking newly proclaimed wildlife conservation areas, game parks and privately owned game farms and also increased the value of buffalo to outrageous levels.

The development of accurate molecular diagnostic tools during the early 1990s (Allsopp, Carrington, Baylis, Sohal, Dolan & Iams, 1989; Allsopp, Baylis, Allsopp, Cavalier-Smith, Bishop, Carrington, Sohanpal & Spooner, 1993) created an opportunity to breed disease-free buffalo from infected parent stock and subsequently certify them free of *T. parva* infections. Several buffalo-breeding projects, in which certified disease-free buffalo could be raised for translocation and establishment of clean buffalo herds, were initiated by both government organisations and private entrepreneurs. Over the ensuing years, this has led to a significant increase in the number of disease-free buffalo available for translocation and, also, a proliferation in the number of properties within established *R. appendiculatus* and *R. zambeziensis*-endemic areas where buffalo are being kept.

As in the past, sporadic outbreaks of Corridor disease continued to be reported from Corridor disease endemic areas where infected buffalo and cattle came into contact within the distribution range of the known tick vectors (De Vos, 1982; Potgieter *et al*., 1988). However, a number of unexpected outbreaks of *T. parva* infections in cattle were also reported from areas where contact with infected buffalo could not unequivocally be confirmed (Thompson, Latif, Oosthuizen, Troskie & Penzhorn, 2008). In addition, *T. parva* infections were also increasingly being detected in buffalo herds in tick vector-endemic areas where only certified disease-free

buffalo were purportedly present (Stoltsz, 2001; Potgieter, 2005). These incidents have been attributed to illegal or clandestine introductions of infected buffalo, inadvertent introduction of infected ticks during game-capture operations and even failure of diagnostic tests to reliably detect carriers amongst certified disease-free animals that were subsequently introduced.

Recent attempts at characterising *T. parva* field isolates from buffalo and cattle in South Africa at a molecular level (Sibeko, Geysen, Oosthuizen, Matthee, Troskie, Potgieter, Coetzer & Collins, 2010; Sibeko, Collins, Oosthuizen, Troskie, Potgieter, Coetzer & Geysen, 2011) seem to suggest that parasites exhibiting characteristics of cattle-derived *T. parva* may be circulating in cattle and/or buffalo populations in this country. This raises renewed concerns, since the risk of ECF to the cattle population remains as high as ever before. In addition, it is inconceivable that the same drastic measures which were employed during the first half of the twentieth century to eradicate the disease in South Africa (Diesel, 1948) could ever be enforced in the current political dispensation in the country.

Following failed attempts to demonstrate transformation of buffalo-derived *T. parva* during limited tick-cattle passage in splenectomised cattle, and based on the lack of epidemiological evidence to suggest that buffalo-derived *T. parva* was able to establish itself in cattle in the absence of buffalo, De Vos (1982) concluded that the parasite associated with Corridor disease in South Africa was “more stable” than reported by others. These included Uilenberg (1981) who suggested that wherever buffalo-derived *T. parva* occurred, the parasite may spontaneously revert to a parasite indistinguishable from the cattle-derived *T. parva* which causes classical ECF.

The absence of any evidence of a behavioural change in the buffalo-derived *T. parva* (Hluhluwe 3 isolate) after repeated tick-cattle passage in non-splenectomised cattle for up to eight passages (this study) seems to support the notion that this isolate in particular (and, therefore, by implication all buffalo-derived *T. parva* isolates) may not contain suitable genetic subpopulations of *T. parva* parasites that could readily adapt to cattle, be maintained in cattle in the absence of buffalo, and produce the high schizont parasitoses and high piroplasm parasitaemias more characteristic of classical ECF. Given the evidence of extensive genetic variation and recombination amongst *T. parva* parasite subpopulations (Sibeko *et al*., 2010; 2011), and susceptibility of buffalo and cattle to both buffalo-derived and cattle-derived parasites (Norval, Perry & Young, 1992) an indeterminate number of isolates might have to be subjected to similar tick-cattle passage in order to possibly confirm the findings reported in East Africa by

Barnett & Brocklesby (1966) and Young *et al*. (1973). Even so, it could be argued that, unless a suitable subpopulation of parasites capable of adapting to cattle is present in the original isolate, no amount of manipulation in the laboratory will result in transformation. It would thus seem an insurmountable task to attempt definitive characterisation of *T. parva* isolates by tick transmission experiments in cattle in the laboratory.

Although extensive genetic diversity has recently been confirmed in buffalo-derived *T. parva* from South Africa (Sibeko *et al*., 2010; 2011), evidence was found to suggest that the diversity was more limited in *T. parva* populations from Hluhluwe-iMfolozi Park, than would normally be expected in large buffalo populations elsewhere. These molecular characterisation tools were unfortunately not available at the time when the *T. parva* (Hluhluwe 3 isolate) used in this study was obtained. Although parasite samples for possible future analysis were collected and cryopreserved during all tick-cattle passages of this isolate in cattle in the laboratory during this study, an unfortunate incident resulted in the loss of all preserved specimens. Consequently no molecular characterisation of this isolate at different stages of the tick-cattle passage was possible.

A significant finding in this investigation is that a virulent buffalo-derived *T. parva* isolate from South Africa (exhibiting characteristics of classical Corridor disease by producing low schizont parasitoses and very low piroplasm parasitaemias), causing high mortality and acute death in most infected cattle (thus labeling itself as self-limiting in cattle) and retaining these characteristics despite extensive manipulation through cattle-tick transmission in the laboratory, could nevertheless be repeatedly tick-transmitted amongst cattle. It therefore seems reasonable to assume that, given the opportunity, selection of subpopulations of parasite more suitable to cattle-to-cattle transmission may be inevitable, given the myriad of parasite genotypes found in buffalo in the field.

It begs the question though, why selection of cattle-adapted *T. parva* from buffalo-derived parasites was so readily achieved in East Africa and not in South Africa. The validity of the findings in East Africa might be questioned, based on the prevailing assumption at the time that cattle recovered from ECF did not develop persistent carrier infections (Norval *et al*., 1992). Consequently, the presence of *T. parva* carrier cattle amongst the experimental animals used during the tick-cattle passages of buffalo-derived parasites may have resulted in the mere selection of the cattle-derived parasites already present in one or more of the experimental cattle. Molecular characterisation of the Serengeti-transformed isolate of *T. parva* (originally isolated

from a buffalo) revealing its similarity to the *T. parva* Muguga isolate from cattle (Bishop, Geysen, Spooner, Skilton, Nene, Dolan & Morzaria, 2001) seems to support the suggestion that cattle-derived *T. parva* parasites were inadvertently introduced during the experimental procedures. However, all evidence still points to the fact that cattle-derived *T. parva* originated in buffalo. Based on the genetic diversity amongst cattle-derived *T. parva* isolates from different geographic locations in Africa, adaptation of buffalo-derived parasites in the cattle host probably occurred on more than one occasion under natural conditions. Granted, in the ecological and epidemiological situation as it exists in East Africa, where infected buffalo and cattle have co-existed for centuries in an environment conducive to rapid transmission amongst hosts (given the non-seasonality of *R. appendiculatus* nearer the equator) the opportunities for selection in cattle of genetically different subpopulations of parasites abound. Thus, the strict seasonality of the vector ticks in South Africa (Short & Norval, 1981) has likely been instrumental in limiting the spread of buffalo-derived *T. parva* in cattle, and also the possible selection of parasites that are better adapted to cattle-to-cattle transmission. This is due to the fact that in order for cattle-to-cattle transmission to occur in South Africa, carrier animals need to be present at the time of nymphal tick activity. The vector ticks complete only a single life cycle per year, and when adult stages of the tick are prevalent to transmit the disease (during mid to late summer), no nymphal ticks (which are most prevalent during late winter and spring) are present to pick up infection from reacting cattle to produce infected ticks during the ensuing adult stage.

The high mortality in cattle and extremely low piroplasm parasitaemias generally associated with buffalo-derived *T. parva* infections in recovered cattle in South Africa might have created a false sense of security that this is a self-limiting disease in cattle. However, persistent carrier infections as observed in both splenectomised and non-splenectomised cattle in the present (see Chapter 4) and previous studies (Neitz, 1957; Potgieter *et al*., 1988) suggest that recovered cattle may serve as a source of infection to ticks and subsequently cattle as well. In the absence of chemotherapy, very few cattle spontaneously recover after infection with buffalo-derived *T. parva*. On more than one occasion, however, sub-lethal infections have been transmitted to cattle in the laboratory (Stoltsz, 1993; Potgieter, 2005). Allowing chemotherapy of reacting cattle during outbreaks of Corridor disease would greatly increase the number of recovered carrier cattle. It seems inevitable then that under such conditions, sooner or later a subpopulation of parasites that might be readily tick-transmissible amongst cattle could become established in some animals. Therefore, the continued ban on the use of chemotherapy during outbreaks of Corridor disease seems particularly justified under field conditions in South Africa. These chemotherapeutic drugs are freely available in many countries of Eastern, Central and southern

Africa, however, and their illegal importation and use during Corridor disease outbreaks, perhaps even to obscure the presence of infected buffalo from veterinary authorities, could have serious long-term detrimental economic repercussions. Therefore, the continued ban on the registration of these products in South Africa and diligent policing to prevent their illegal importation and use should be maintained at all costs.

The proliferation of buffalo, which may harbour hitherto uncharacterised *T. parva* genotypes, in many cattle-farming areas of South Africa, could also dramatically increase the opportunities for buffalo-to-cattle transmission to occur. If, in addition, some genotypes are less pathogenic or more likely to produce persistent carrier infections in cattle, the risk of such infections spreading amongst cattle and/or reverting to virulence upon selective transmission, becomes even greater.

It has been suggested that the successful eradication of ECF in South Africa may have been due largely to the disappearance or eradication of non-diapausing *R. appendiculatus* which were introduced from East Africa at the time of introduction of the disease (Norval *et al*., 1992). Certainly the strictly seasonal occurrence of the different life cycle stages of *R. appendiculatus* and *R. zambeziensis* in South Africa (and/or the absence of non-diapausing strains of these ticks) practically negates the transmission of virulent *T. parva* amongst cattle. Outbreaks of ECF were reported from areas where the disease had apparently been absent for periods lasting two years or more, which led to the implementation of a slaughter-out policy to eliminate potential carrier cattle (Diesel, 1948). Had the outbreaks been due mainly to the presence of non-diapausing *R. appendiculatus*, these could hardly have accounted for the persistence of the disease in the cattle population for more than five decades, whilst faced with the stringent dipping policy and movement control that was practiced to eliminate the disease. Laboratory findings using *T. parva* (Muguga) and *T. parva* (Schoonspruit) isolates, where recovered cattle seemed to develop a sterile immunity (in the absence of a carrier state), led to the erroneous conclusion that cattle-derived *T. parva* did not result in persistent carrier infections in recovered animals (Norval *et al*., 1992). It is my opinion that the ultimate eradication of ECF in South Africa was due mainly to the adoption of the slaughter-out policy (which eliminated carrier cattle) that ultimately resulted in the eradication of ECF and not the eradication of a population of non-diapausing *R. appendiculatus* that may have been introduced with the original introduction of infected cattle from East Africa.

In the present study it proved possible to establish persistent piroplasm parasitaemias of buffalo-derived *T. parva* in splenectomised cattle by subinoculation of infected blood from various donor animals. For the first time, long-term maintenance of *T. parva* piroplasm parasitaemias in the absence of schizonts in splenectomised cattle was also demonstrated, and lack of immunity to challenge with homologous sporozoites suggests that immunity in *T. parva* is also stage-specific as has been demonstrated for *T. annulata* (Uilenberg, Franssen & Perié, 1986).

Subinoculation of blood from *T. parva*-infected cattle or buffalo into splenectomised cattle may prove useful to identify carrier buffalo or cattle with very low parasitaemias, as well as provide a method for obtaining parasite isolates from such animals when isolation by tick transmission might not be possible. Together with the high infection rates achieved in *R. zambeziensis* (Blouin & Stoltsz, 1989), establishment of persistent piroplasm parasitaemias in splenectomised cattle could make the inclusion of buffalo-derived parasite sporozoite stabilates, which until now have not been practicable to include in the ECF cocktail vaccines, much more feasible. In addition, the use of *R. zambeziensis* as vector, which consistently resulted in high salivary gland infection rates even with low piroplasm parasitaemias, may provide a practical means by which buffalo-derived *T. parva* isolates of greater genetic diversity, which should provide broader immunity to a variety of genetically diverse isolates in the field, could be obtained in sufficiently large quantities for inclusion in *T. parva* vaccines.

The current investigation on the possible role of waterbuck in the selection of *T. parva* subpopulations better adapted to cattle involved only a small number of animals and, most likely, only a limited number of buffalo-derived *T. parva* genotypes present in the country. At capture, none of the waterbuck was found to be carrying *T. parva* parasites, including the adult animals which had been exposed to tick infestation in a *T. parva*-endemic area for a longer period than the sub-adult animals. Batches of adult *R. zambeziensis* ticks, highly infected from buffalo with *T. parva* parasites and which were shown to be highly infective to cattle, were also fed on four waterbuck, none of which became infected. This suggests that common waterbuck may not be susceptible to *T. parva* infection, but confirmation of this would require inoculation of large doses of infective *T. parva* sporozoites of numerous isolates in large numbers of captive animals. It should be noted that experimental infection of defassa waterbuck in East Africa with *T. parva* was not achieved by normal tick feeding, but by inoculation of a large dose of infective sporozoites of a particularly virulent isolate of *T. parva*; numerous previous attempts with smaller doses and less virulent isolates had failed (Grootenhuis, 1995; Stagg, Bishop, Morzaria, Shaw, Wesonga, Orinda, Grootenhuis, Molyneux & Young, 1994). This seems to suggest that

T. parva infection in waterbuck is not a frequent occurrence and is unlikely to occur naturally under field conditions. It is also worth noting that the reports which incriminated waterbuck in possible outbreaks of Corridor disease in cattle, and which subsequently prompted their investigation, were based on anecdotal accounts rather than any empirical evidence. In summary, it can be stated that although the results of the experiments reported here are not definitive, they do suggest that common waterbuck are unlikely to play an important role in the epidemiology of *T. parva* infections in cattle in South Africa. Although it could be speculated that this may in part be attributed to genetic differences between defassa and common waterbuck, or the genetic composition of the *T. parva* parasites and dosage used, it could also be argued that, given the right combination of circumstances, a select subpopulation of buffalo-derived *T. parva* could be established in waterbuck. Therefore, where waterbuck and buffalo co-exist in *T. parva* endemic areas, transmission between these hosts, however rare this may be, could still, at least potentially, influence genetic diversity and subsequent epidemiology of *T. parva* infections in the area.

In conclusion, it seems imperative that studies on the population genetic structure of *T. parva* in South Africa should continue, to seek reliable molecular markers which correlate with *in vivo* behaviour of parasite subpopulations and to fully assess their potential threat to the cattle population in this country. Foremost amongst these should be a concerted effort to establish whether subpopulations of *T. parva* are present in the indigenous buffalo population which, upon cattle-tick passage, may result in the selection of virulent parasites which are readily transmissible amongst cattle, in the absence of buffalo. In addition, the possible existence of parasites of low pathogenicity that are being maintained in cattle in the absence of buffalo should be investigated, and if found to be present, the likelihood of these to revert to virulence, either by selective transmission of a subpopulation of parasites, or by genetic recombination with other cattle-derived *T. parva* or even buffalo-derived parasite subpopulations, should be assessed.

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ADDENDUM A
COMPARATIVE INFECTION RATES OF *THEILERIA PARVA LAWRENCEI* IN SALIVARY GLANDS OF *RHIPICEPHALUS APPENDICULATUS* AND *RHIPICEPHALUS ZAMBEZIENSIS*

ABSTRACT

BLOUIN, E.F. & STOLTSZ, W.H., 1989. Comparative infection rates of *Theileria parva lawrencei* in salivary glands of *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*. *Onderstepoort Journal of Veterinary Research*, 56, 211-213 (1989).

Three cattle, which had been experimentally infected with *Theileria parva lawrencei* and maintained as carriers of the infection, were each infested simultaneously with clean nymphal *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* in ear bags on separate ears. After moulting, the ensuing adult ticks were fed on rabbits for four days and their salivary glands were examined for infective stages of the parasite. Microscopic examination revealed significantly higher infection rates in the salivary glands of *R. zambeziensis* than in *R. appendiculatus* which may indicate an increased vector efficiency of *R. zambeziensis* for *T. p. lawrencei*.

INTRODUCTION

Corridor disease, caused by *Theileria parva lawrencei*, is transmitted from buffalo (*Syncerus caffer*) to cattle by *Rhipicephalus appendiculatus* (Neitz, 1955). In the Republic of South Africa (RSA) it is a controlled disease and is endemic to the Kruger National Park (KNP) and some of the Zululand game reserves (Potgieter, Stoltsz, Blouin & Roos, 1988). Sporadic outbreaks occur on farms bordering on, or in close proximity to, these endemic areas and have thus far always been associated with the presence of buffalo (Bigalke, De Vos & Barrowman, 1976).

De Vos (1981) indicated that *R. appendiculatus* was still considered to be the only known tick species capable of transmitting Corridor disease. *Rhipicephalus zambeziensis* was described by Walker, Norval & Corwin (1981) as a new tick species from eastern and southern Africa, including the RSA, which closely resembled *R. appendiculatus*. *R. zambeziensis* is generally found in hotter, drier areas than *R. appendiculatus* and the two species have often been confused in the past (Norval, Walker & Colborne, 1982). In the RSA, the distribution of *R. zambeziensis* has not been extensively surveyed but it has reportedly occurred on game species in the Kruger National Park (Horak, Potgieter, Walker, De Vos & Boomker, 1983). It is likely that this species is more widely distributed than present data indicate.

Lawrence, Norval & Uilenberg (1983) demonstrated that *R. zambeziensis* was an experimental vector of *T. p. lawrencei* and that infection rates in salivary glands were high. They also suggested that in certain parts of Zimbabwe *R. zambeziensis* may be the only vector of *T. p. lawrencei*. In a similar trial, a South African strain of *T. p. lawrencei* has been successfully used in the transmission of *T. p. lawrencei* between cattle (Potgieter *et al*., 1988) and infection rates in ticks were also found to be high. Since infection rates in *R. appendiculatus* fed on cattle are generally low (Young & Purnell, 1973), a comparison of the infection rates in the two tick species was conducted, using three *T. p. lawrencei* carrier cattle. This report represents the results of those trials.

MATERIALS AND METHODS

Three cattle (*Bos taurus* and *Bos indicus* crosses), reared under tick-free conditions at the Veterinary Research Institute, were infected with the Hluhluwe 3 isolate (De Vos, 1982) of *T. p. lawrencei.* Details of the infection of the individual animals are given in **Table 1**.

Ticks were reared and fed according to the methods of Neitz, Boughton & Walters (1971). Each animal was infested with 300 nymphal *R. appendiculatus* (Rietvlei cross) (De Vos & Roos, 1981) on one ear and 300 *R. zambeziensis* (Killkenny) (Potgieter *et al*., 1988) on the other ear. Engorged nymphs were collected and allowed to moult in an acaridarium at 25°C and 25 % relative humidity.

At approximately six weeks post-moulting, 150 of the ensuing adults of each species from each feeding were fed on rabbits. At four days post-infestation the ticks were removed. Each tick was placed into a drop of RPMI 1640 MEDIUM[13] and its dorsal exoskeleton was cut away. The salivary glands of individual ticks were dissected out and teased onto glass slides. The glands were fixed in Carnoy's fixative, stained with methyl green/pyronin (Irvin, Boarer, Dobbelaere, Mahan, Masake & Ocama, 1981) and examined with a light microscope for infective stages of *T. p. lawrencei*.

RESULTS

Despite the relatively small numbers of ticks recovered and examined (refer to **Table 2**), it was clear that significantly higher infection rates were obtained with *R. zambeziensis* ticks which fed as nymphs on Animals 9512-9 and 9418-5 (**Table 2**). The mean number of infected acini per infected tick was also higher in *R. zambeziensis* than in *R. appendiculatus*. No infective stages could be found in any of the ticks fed as nymphs on Animal 9666-0. This result was not surprising, since piroplasms could not be demonstrated microscopically in blood smears of the animal examined during this period. Subinoculation of blood from Animal 9666-0 to a splenectomized ox did, however, result in a patent parasitaemia in the latter animal, thus confirming the carrier status.

[13]Highveld Biological (Pty) Ltd, P.O. Box 488, Kelvin, 2054, RSA

Table 1 History of the infection of experimental cattle with *Theileria parva lawrencei* prior to tick feeding

Animal No.	Method of infection	Chemotherapy	Outcome
9512-9 (S)	Transmission with adult *Rhipicephalus appendiculatus*	Parvaquone[1] 10 mg/kg IM on days 11, 13, 26 and 30 p.i. Buparvaquone 2.5 mg/kg IM at 24 months p.i.* (5 treatments at days 0, 2, 4, 7 and 10)	Patent piroplasm parasitaemia 130 days p.t. 1/1000 rbc** Temporary disappearance of patent parasitaemia. Piroplasms reappeared 150 days p.t. < 1/10,000 rbc
9660-0	Transmission with adult *Rhipicephalus appendiculatus*	Parvaquone[1] 10 mg/kg IM on days 11 & 13 p.i.	Schizonts intermittently observed up to 124 days p.i. No patent piroplasm parasitaemia***
9418-5 (S)	Subinoculation of blood from a carrier ox****	No treatment required	Piroplasm parasitaemia 1/3000 rbc**

p.i. = post-infection
S = splenectomized
rbc = red blood cells
p.t. = post-treatment
I.M. = intramuscularly

* Attempt to sterilize piroplasm infection
** Parasitaemia during trial
*** Carrier status confirmed after subinoculation of blood to a splenectomized ox
**** Details given in Potgieter *et al.*, 1988

[1] Clexon, Coopers Animal Health Ltd, England
[2] BW 720C, Coopers Animal Health ltd England

Table 2 Comparative infection rates of *Theileria parva lawrencei* in the salivary glands of *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* fed on carrier cattle.

Tick species	Animal No.	Adult ticks fed on rabbits		Mean No. of infected acini per infected tick
		No. infected/ No. examined	% infection	
R. appendiculatus	9512-9	0/35	-	-
	9512-9*	0/25	-	-
	9418-5	4/72	5	4
	9666-0	0/60	-	-
R. zambeziensis	9512-9	31/62	50	18.7
	9512-9*	2/28	7	3
	9418-5	40/43	93	28
	9666-0	0/57	-	-

DISCUSSION

Chemotherapeutic treatment of cattle involved in Corridor disease outbreaks is not recommended, as this may result in the creation of carrier animals (Potgieter, Roos & De Vos, 1985). When such methods are used to establish experimental *T. p. lawrencei* infections in cattle, piroplasm parasitaemias are usually very low. Infection rates in *R. appendiculatus* fed on these carriers are also low. The initial results presented here, suggest that *R. zambeziensis* may be more efficient in picking up low level infections of at least certain isolates of *T. p. lawrencei* than *R. appendiculatus*. Higher yields of tick-derived parasite material for serological and immunological studies have thus been obtained by feeding *R. zambeziensis* on experimental carrier animals, but only when using animals with microscopically detectable parasitaemias.

The role that *R. zambeziensis* plays in the epidemiology of Corridor disease in the RSA can only be evaluated after further information on its distribution and abundance is obtained. It has been noted in Zimbabwe that, in the absence of tick control, *R. zambeziensis* appears to maintain lower levels of abundance than *R. appendiculatus* (Norval *et al*., 1982). The primary concern is that a more efficient vector might enhance the transmission of *T. p. lawrencei* between cattle and increase the possibility of the parasite changing its behaviour to that of *T. p. parva*, as reported by Barnett & Brocklesby (1966) and Young & Purnell (1973). This phenomenon has thus far not been observed in the RSA in the field or laboratory observations despite the fact that *R. zambeziensis* has probably existed in endemic areas for many years.

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