Occurrence of *Theileria parva* infection in cattle on a farm in the Ladysmith district, KwaZulu-Natal, South Africa

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ABSTRACT

Theileria parva causes widespread morbidity and mortality in cattle in endemic regions. An outbreak of theileriosis occurred on a farm near Ladysmith in KwaZulu-Natal, South Africa, which is not a declared Corridor disease-infected area. A survey of Red Brangus cattle from all age groups and areas of the farm was performed. Transmission of the parasite from infected animals on the farm to susceptible animals by tick transmission and tick-stabilate injection, was attempted. The survey indicated high numbers of animals with antibody titres to *T. parva* but only 6 infected animals, based on real-time PCR and RLB analysis. The transmission experiments failed to transmit the parasite. The study shows the difficulty in elucidating a source of infection and determining the dynamics of new infections in a herd where multiple possible sources are present and treatment with tetracyclines has taken place.

Key words: real-time PCR, RLB, transmission experiments, South Africa, *Theileria parva.*

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INTRODUCTION

Theileria parva is responsible for causing East Coast fever (ECF), Corridor disease and January disease, which may result in widespread losses in susceptible cattle. Transmission is by *Rhipicephalus appendiculatus, Rhipicephalus zambeziensis* and *Rhipicephalus duttoni,* all 3-host ixodid tick species. East Coast Fever is transmitted between infected cattle and susceptible cattle and Corridor disease between asymptomatic, carrier African buffalo (*Syncerus caffer*) and susceptible cattle. January disease is usually considered a milder form of the disease[8] and transmission occurs between infected cattle and susceptible cattle.

The greatest threat of *T. parva* infection in South Africa comes from infected buffalo coming into contact with cattle. *Theileria parva* is endemic in the declared Corridor disease-infected districts in the northeastern parts of South Africa. The Kruger National Park, Hluhluwe-iMfolozi Park and adjoining private nature reserves fall within this area. Corridor disease outbreaks occur in areas bordering on or near where infected buffalo herds graze. Buffalo are natural reservoirs of *T. parva* and if the vector tick species are present, cattle may also become infected. Corridor disease has been regarded as self-limiting in cattle because they usually die before the parasite develops to the piroplasm stage, which is infective to ticks[14]. Experimental evidence suggests that some bovines survive the disease, however, and may serve as reservoirs of infection[7].

Cattle that survive *T. parva* infection are considered to be permanent carriers of the parasite. Furthermore, carriers may arise from being immunised[2,5,6,9] or after being treated with anti-theilerial drugs[4]. In this way one can foresee that it is possible that new strains may be introduced into new areas or that a carrier state may develop which could interfere with control programmes already in place.

Between 2002 and 2004, a stud-cattle breeder in the Ladysmith district, KwaZulu-Natal, South Africa, lost a total of 42 cattle to a febrile disease, initially suspected to be babesiosis. The presence of *T. parva* was confirmed by the Onderstepoort Veterinary Institute, Agricultural Research Council (ARC-OVI). The Ladysmith district is outside the designated *T. parva*-infected area of the country. During the outbreaks in 2003 and 2004 the entire herd was treated with tetracyclines and regularly and intensively dipped against ticks. The number of deaths was reduced with this strategy.

The objectives of the study were (i) to determine the prevalence of *Theileria* species and other tick-borne infections in the herd, and (ii) to transmit *T. parva* from infected cattle on the farm to susceptible cattle as well as to replicate the disease process and pathology seen on the farm.

MATERIALS AND METHODS

Study site

The farm is located in the KwaZulu-Natal Midlands about 30 km from Ladysmith. A section of the farm borders on the Spioenkop Nature Reserve and antelope are occasionally seen on all areas of the farm.

Study herd

The herd consisted of *c.* 2000 Red Brangus cattle. They were kept extensively, except for young bulls that were kept on natural grazing in smaller paddocks and fed concentrates before the yearly sales. The animals were grouped in herds according to age and gender and moved between sections of the farm as the owner saw fit for pasture management.

Specimen collection

During January 2005, 170 Red Brangus cattle were sampled. The number of cattle from each sex and age group were chosen in proportion to their representation in the overall population on the farm. With the exception of calves, cattle in the smaller herds were chosen randomly. Calves were chosen according to which cow had been selected in order to determine whether there was any relationship between dam and calf in disease transmission. Blood in ethylenediamine tetraacetic acid (EDTA) (5 mℓ) and serum (5 mℓ) tubes were collected by venipuncture of the caudal vein or jugular vein. The blood in the serum tubes was allowed to coagulate and the serum was decanted into storage tubes. Both sets of specimens were stored at *c.* 4 °C.

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DNA extraction

DNA was extracted from 200 $\mu\ell$ EDTA blood using the blood and body fluid spin protocol and reagents described in the QIAamp® DNA Mini Kit (QIAGEN, Germany). DNA extracts were eluted in 100 $\mu\ell$ buffer AE and stored at –20 °C.

Theileria parva*-specific real-time PCR*

The recently described *T. parva*-specific real-time PCR assay was performed by making use of the Roche LightCycler V2.0 (Roche Diagnostics, Mannheim, Germany)[17]. The primers and probes used were obtained from TIB Molbiol (Berlin, Germany). A positive (*T. parva*-carrier buffalo) and negative (PCR grade water) control were included in each run.

Reverse Line Blot

The RLB, PCR and hybridisation protocols, reagents, equipment and commercial TBD-RLB membrane used were those recommended and produced by Isogen Life Sciences (Ijsselstein, the Netherlands). *Theileria* and *Babesia* genus-specific primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G) and biotin labeled RLB-R2 (5′-Biotin-CTA AGA ATT TCA CCT CTG ACA GT) were used to amplify a ~400 bp region of the V4 variable region of the 18S rRNA gene[12,13]. The RLB membrane contained *Theileria* and *Babesia* genus- and species-specific probes.

IFAT

Serum specimens were screened for the presence of antibodies against *T. parva.* The IFAT method used was the standard IFAT used at the ARC-OVI for diagnostic testing (Laboratory Manual Serology Volume II. Onderstepoort Veterinary Institute, Protozoology Division). Dilutions of 1:40, 1:80 and 1:160 were used. The 1:80 dilution is considered the standard cut-off titre at the ARC-OVI for positive reactions. It was decided to employ the 1:40 comparison, since this would increase the specificity of the test[10] and possibly aid in ruling out positives due to *T. taurotragi* cross-reactions[11,15]. Samples that show strong positive fluorescence at 1:40, but substantially weaker fluorescence at 1:80 or 1:160 are more likely to be *T. taurotragi* cross-reactions than those samples that react at all 3 dilutions, since cross-reactions are more likely to occur at low titres[15], thus increasing the specificity of the test. *Theileria mutans* was not detected on RLB at any stage, therefore was not considered in causing cross-reactions during IFAT assays.

Isolation of T. parva *from infected cattle*

Three young bulls (S24, 316 and 415) that had tested positive on the ARC-OVI DNA-Probe PCR assay during 2004 were selected. Clean, laboratory-bred *R. appendiculatus* nymphs were fed on the selected *T. parva*-infected bovines on the farm. After washing the bovines' ears with soap on 3 consecutive days, cotton ear-bags were attached. *Rhipicephalus appendiculatus* nymphs (n = 600) were placed in each ear-bag and allowed to feed for 4–6 days. On days 4, 5 and 6, the engorged ticks that had dropped off were collected out of the ear-bags. The nymphs collected were transported back to the ARC-OVI where they moulted and hardened over a period of 8 weeks. They were kept in an acaridarium, under standard conditions.

Prevalence of infection in the ticks, and preparation of a tick stabilate

A sample number of moulted adult ticks from each bovine were fed on rabbits at 60 ticks per rabbit for 3 days. This allowed for maturation of the *T. parva* parasites. The ticks were collected off the rabbits and used to determine *T. parva* infection prevalence in the tick's salivary glands and to prepare a stabilate of ticks from S24. The infection prevalence in the ticks' salivary glands was determined by dissecting out the glands, staining them with Feulgens stain and examining under a light microscope according to standard ARC-OVI protocol. A further small number of the dissected salivary glands were pooled and used for extraction of DNA and detection of *T. parva* and *T. taurotragi* using real-time PCR. A tick stabilate (10 ticks/mℓ of stabilate) was prepared from the remaining prefed ticks from bovine S24 according to the standard ARC-OVI protocol.

Tick feeding on susceptible bovines

The remainder of the adult ticks were divided into 2 groups and fed on intact, susceptible bovines 8182 (ticks from 316 and 415) and 8183 (ticks from S24). These bovines were housed in the East Coast Fever Stables at the ARC-OVI under strict tick control and in accordance with ARC-OVI ECF stable procedures.

The same method as described above for feeding the nymphs was used for feeding the infected adult ticks, but only 150 adults were applied to each ear. These bovines (8182 and 8183) were observed for clinical signs and their body temperatures monitored daily. A blood smear and an EDTA blood sample were taken daily up until day 35. Blood samples were taken by jugular venipuncture using Vacutainer© apparatus. Lymph node aspirates and smears were made when the lymph nodes became enlarged. Blood and lymph node smears were stained with Giemsa and examined for schizonts and piroplasms in lymphocytes and erythrocytes, respectively.

Attempted transmission of T. parva *in a tick stabilate*

Stabilate produced from ticks fed on bovine S24 was injected into a splenectomised, susceptible bovine (9378/8). A volume of 1.7 mℓ of the stabilate was injected subcutaneously over the parotid lymph nodes. The same observations and samples were taken as mentioned above.

RESULTS

Prevalence of piroplasms infections on the farm

Blood specimens were collected from 170 animals; the results are shown in Table 1. With the exception of 2 adult cows (3.3 %), none of the cattle were positive for *T. parva* on RLB analysis, while 5 adult cows (8.2 %) and 1 calf (1.7 %) were positive with real-time PCR analysis.

Two 1-year-old heifers (9.0 %), 3 adult cows (4.9 %) and 10 calves (17.2 %) gave clear positive results for *T. parva* antibodies on IFAT. If inconclusive results (titre 1:40) are added, the number of positive animals increases to: 1 adult bull (25 %), 3 1-year-old heifers (13.6 %), 5 2½-year-old heifers (27.8 %), 7 adult cows (11.5 %) and 18 calves (31.0 %).

None of the calves of cows that tested positive for *T. parva* on RLB or real-time PCR tested positive on either test. Two of these calves had positive and 1 had inconclusive results on the IFAT. One calf tested positive for *T. parva* on real-time PCR, whereas its dam did not test positive on the real-time PCR, RLB or IFAT.

On RLB analysis, none of the 4 adult bulls were positive for *T. taurotragi,* while 4 1-year-old bulls (57.1 %), 6 1-year-old heifers (27.3 %), 9 2½-year-old heifers (50 %), 36 adult cows (59 %) and 30 calves (51.7 %) were positive for *T. taurotragi.*

Positive reactions to *B. bigemina* on RLB were found in 1 2½-year-old heifer (5.6 %), 1 adult cow (1.6 %) and 4 calves (6.0 %). Positive reactions to *B. bovis* on RLB were found in 1 1-year-old bull (14.3 %), 2 2½-year-old heifers (11.1 %) and 6 adult cows (9.8 %).

Prevalence of infected salivary gland acini in adult ticks

Rhipicephalus appendiculatus adults that had fed as nymphs on bovine 316 on the farm had a mean of 18.08 infected salivary gland acini/infected tick; 92 % of the dissected ticks were found to be infected with a *Theileria* species. All *R. appendiculatus* adults that had fed as nymphs on bovine 415 had infected acini in the salivary glands (mean: 14.5 infected acini/in-

Table 1: **Occurrence of tick-borne piroplasm infections in Red Brangus cattle on a farm in the Ladysmith district, KwaZulu-Natal, South Africa, where an outbreak of theileriosis occurred.**

Group tested	Positive for *T. parva* (Real-time PCR)	Positive for *T. parva* (RLB)	Positive for *T. parva* (IFAT)*	Positive for *T. taurotragi* (RLB)	Positive for *B. bigemina* (RLB)	Positive for *B. bovis* (RLB)
One-year-old bulls (*n* = 7)	–	–	–	4	–	1
Adult bulls (*n* = 4)	–	–	(1)	–	–	–
One-year-old heifers (*n* = 22)	–	–	2 (1)	6	–	–
2½-year-old heifers (*n* = 18)	–	–	(5)	9	1	2
Adult cows (*n* = 61)	5	2	3 (4)	36	1	6
Calves (*n* = 58)	1	–	10 (8)	30	4	–

*Positive at titre ≥ 1:80; inconclusive results (titre 1:40) shown in brackets.

fected tick). Real-time PCR analysis of infected acini failed to reveal the presence of *T. parva* in both groups of ticks, but did show the presence of *T. taurotragi.*

The *R. appendiculatus* adults that had fed as nymphs on bovine S24 on the farm had a mean of 8.8 infected acini/infected tick; 57 % of the dissected ticks were infected. Real-time PCR analysis of infected acini was positive for both *T. taurotragi* and *T. parva.*

Attempted transmission of *Theileria* spp. to susceptible bovines

Adult ticks that had fed as nymphs on infected bovines 316 and 415 on the farm were fed on bovine 8182. The recipient did not develop any clinical signs associated with *T. parva* infection, and its body temperature remained within the normal range. On blood smears, schizonts were noted from day 11 tot day 28, and piroplasms were seen from day 15 onward. *Theileria taurotragi* was detected by RLB analysis.

Adult ticks that had fed as nymphs on infected bovine S24 on the farm were fed on bovine 8183. The recipient did not develop any clinical signs associated with *T. parva* infection and its body temperature remained within the normal range. Schizonts were seen on blood smears from day 11 to day 19, and piroplasms were seen from day 17 onward. *Theileria taurotragi* was detected by RLB.

Attempted transmission of *T. parva* in a tick stabilate to susceptible cattle

The tick stabilate was prepared from the adults ticks that had fed as nymphs on bovine S24. As mentioned previously, a proportion of the ticks from this batch from S24 were found to be positive for *T. parva* and *T. taurotragi* on real-time PCR of the salivary glands. The recipient (bovine 9378/8) did not show any clinical signs associated with *T. parva* infection and its body temperature remained within the normal range. No schizonts were seen on blood smears, but piroplasms were seen on day 35. *Theileria taurotragi* only was detected by RLB analysis.

DISCUSSION

Five of the 6 *T. parva*-positive cattle detected during the survey were adult cows. It is unlikely that the single positive calf had been infected *in utero,* since its dam tested negative on RLB, real-time PCR and IFAT. This indicates that this calf must have become infected through tick-feeding during the summer season of 2004/2005. Furthermore, none of the 5 infected cows had calves that tested positive for *T. parva* on RLB or real-time PCR. On IFAT, though, 2 of these calves were positive, and 1 had an inconclusive result (titre 1:40) for *T. parva* antibodies.

Positive and inconclusive IFAT results in calves may have been as a result of maternally derived antibodies since antibody prevalence in calves is considered an indicator of the immune status of the cow population[1]. If one were to agree with this suggestion, the IFAT results from these calves would indicate that the prevalence in the cow population is even higher than the cows' IFAT results illustrate.

Subsequent to the 2003/2004 outbreak, there were no further losses due to *T. parva* infection. The animals that tested positive on DNA probe PCR (ARC-OVI previous tests) were slaughtered and an intensive dipping regimen has been maintained on the farm. With the high numbers of animals testing positive on IFAT (19 with inconclusive and 15 positive – a total of 20 %), this could suggest that new infections are still occurring. It is also possible that many of these IFAT positives are as a result of cross-reactions with *T. taurotragi*[15], although 15 of the 34 animals that tested positive or had an inconclusive result on IFAT were negative on RLB for *T. taurotragi.* Furthermore, seroconversion after a booster challenge is 'immediate and short-lived', especially in situations where there is a vast difference in the challenge in different seasons[1]. This may very well be the case in infected animals on this farm and would also explain why the number of infected animals detected on real-time PCR and RLB was so low – parasitaemias may be extremely low resulting in few positives for parasite detection and confusing IFAT titres.

Since the entire herd had been treated with tetracyclines in the 2003/2004 summer season, an unknown number of animals that may have been incubating the disease were in effect vaccinated against *T. parva.* This ultimately complicated the investigation of the epidemiology of the disease in this herd: had there been no treatment with tetracyclines, one would have expected more deaths and fewer carrier animals.

Positive results for known cattle protozoal parasites, *T. taurotragi, B. bovis* and *B. bigemina,* provide insight as to the infectious challenges that the herd faces. These infections may also play a role in the susceptibility of the cattle to *T. parva* challenge on the farm. That is, bovines on the farm may have reduced immunity due to other infections that may make them more vulnerable to developing a *T. parva* infection. There does not appear to be a particular reason for the differences in prevalence of the various parasites in the different groups.

Transmission of *T. parva* from 3 known infected bovines on the farm, to susceptible cattle by *R. appendiculatus* live ticks and stabilate, could not be demonstrated. The salivary glands of some of the ticks that had fed on 1 of the bovines were found to be positive for *T. parva* and *T. taurotragi* on real-time PCR. This indicated that at least a small number of the ticks were infected with *T. parva.*

A reason for the *T. parva* transmission not having been successful may be that the infected cattle on the farm had very low parasitaemias during the tick feeding, resulting in very few of the ticks becoming infected with *T. parva.* Although tick salivary glands were shown, on dissection, to have extremely high numbers of infected acini, the pooled specimen from the batch of ticks fed on bovine S24 only gave a weak positive signal for *T. parva* on real-time PCR. It is clear from the transmissions and subsequent assays carried out on the susceptible cattle that the ticks had engorged well on the infected bovines, since *T. taurotragi* parasites

were transmitted. This occurrence would exclude the possibility of poor tick feeding on infected or susceptible bovines being the cause for failure of transmission of *T. parva.* Furthermore, the *T. taurotragi* parasitaemia in the bovines on the farm may have been higher than that of *T. parva.* This may have led to competition between the parasites, resulting in a *T. taurotragi* infection being successful in out-competing *T. parva.* This would also explain why there were schizonts seen on the blood smears of the susceptible animals, but the parasite DNA in their blood samples was below detection levels for *T. parva* on real-time PCR and RLB. The schizonts seen were most likely those of *T. taurotragi* and not *T. parva.*

With the development of the eco-tourism industry, many farmers are converting cattle ranches to game farms or parks as it is believed it may be a more profitable venture. By so doing, the interface between wildlife and livestock is increasing. These circumstances are conducive to diseases being spread from wildlife to livestock and *vice versa.* Furthermore, huge profits can be made by unscrupulous operators who are tempted to circumvent regulations and move buffalo from prohibited areas[3]. Under such circumstances, the risk of infected buffalo being introduced to areas outside the declared Corridor disease areas is high. Should outbreaks of *T. parva* occur in many of these areas simultaneously, they would become extremely difficult to contain and may result in the rapid and widespread establishment of the parasite in cattle and wildlife populations throughout South Africa.

As evidenced by the survey of cattle on the farm, there are several factors that may interact in the epidemiology of *T. parva.* It was originally thought that buffalo could not possibly be involved in the occurrence of the disease[16]. With infected buffalo being the major *T. parva* threat in South Africa, however, it would be naive to exclude this source of infection without weighing up the possibility of infected buffalo or ticks from infected buffalo areas being considered. It should be borne in mind that buffaloes from Spioenkop, Ithala and Weenen Nature Reserves sold at auction and taken to the Wasbank area of KwaZulu-Natal caused an outbreak of Corridor disease in cattle on that farm. As these buffaloes had not been tested individually for the presence of *T. parva,* it is possible that buffaloes from Spioenkop may have been infected with *T. parva,* although the Ezemvelo-KZN Wildlife had considered them to be 'Corridor disease-free'. It is also important to note that tenants renting a portion of the Ladysmith farm were involved in game-capture and translocation operations, which offers a further explanation how *T. parva*-infected ticks could have come onto the farm. A lone buffalo bull had also been shot in the area within the previous 10 years.

When studying the RLB and real-time PCR results of the survey, it is clear that the prevalence of *T. parva* appears to be low. By contrast, IFAT results indicate that exposure to *T. parva* may have been high. Extremely low parasitaemias may be the cause for few animals testing positive on RLB and real-time PCR, while high numbers tested positive on IFAT. When cattle are infected with a buffalo-associated *T. parva* strain, they typically show an extremely low parasitaemia[14].

When evaluating the tick-transmission experiments, it is also important to note that, although some of the adult ticks (fed on S24) were found to be *T. parva*-positive on real-time PCR, they failed to transmit the infection. The most likely reason for this failure is that the *T. parva* parasitaemias of the infected cattle on which laboratory-reared nymphs were fed on the farm, were probably very low. Very low schizont and piroplasm parasitaemias in cattle are characteristic in both Corridor disease and January disease[7,8]. In Zambia it is suspected that buffalo-associated *T. parva* may have transformed to cattle-adapted *T. parva*[14]. Thus, it is a possible indication that the source of the infection in the herd may have originally been associated with buffalo but has now adapted to the cattle host.

Theileriosis is a controlled disease in South Africa, and historically, government, veterinary services and farmers went to great lengths and expense to eradicate ECF. South Africa is considered free of *T. parva,* except in designated Corridor disease-infected areas including and bordering the Kruger National Park and Hluhluwe-iMfolozi Park. Therefore the national herd is essentially naive and completely susceptible to *T. parva.* It is imperative that outbreaks, as occurred on this farm, be controlled and contained, regardless of the source of infection. In this project, it has been demonstrated that elucidating sources of infection, confirming modes of transmission and determining prevalence of infection in infected herds, can be difficult at best. As is seen in other African countries, theileriosis, once endemic, remains a difficult and expensive disease to manage.

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