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Investigations into the carrier-state of *Theileria* sp. (buffalo) in cattle

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ABSTRACT

The *Theileria* are apicomplexan parasites transmitted by ticks to vertebrate hosts. Most *Theileria* species exhibit some form of host or vector specificity, since under endemic conditions only a limited number of tick species act as vectors and not all vertebrate hosts are able to maintain a persistent carrier state. Data for *Theileria* sp. (buffalo) suggest host specificity for African buffalo (*Syncerus caffer*). However, *T.* sp. (buffalo) infections in cattle co-grazing with African buffalo have been reported in Kenya and schizonts were cultured from these infected cattle, raising questions regarding host specificity. A Corridor disease outbreak in 2013 on a ranch in South Africa where cattle co-grazed with *Theileria parva* and *T.* sp. (buffalo) infected buffalo presented the opportunity to investigate the possible carrier-state of *T.* sp. (buffalo) in cattle using real-time PCR analysis. Almost all buffalo (n = 19, 95%) were infected with *T.* sp. (buffalo) and showed CP values (22-20) indicative of high parasitemia similar to that observed for buffalo in endemic areas. Conversely, only ~14-27% cattle (n = 69, 100, 96) were positive with CP values (31-40) suggesting low parasitemia and a carrier state epidemiology different from African buffalo. Long term monitoring of *T.* sp. (buffalo) positive cattle showed that most cattle lost their parasitemia or presented fluctuating parasitemia around the PCR assay detection limit. A single splenectomized animal showed a persistent carrier state. The general trends and epidemiology observed in cattle infected with *T.* sp. (buffalo) are similar to that seen for buffalo-adapted *T. parva*, for which a defined carrier state in cattle has not yet been proven. The study suggests that cattle may be infected by *T.* sp. (buffalo) but are not definitive hosts that play an important part in the epidemiology of this parasite.

1. Introduction

The *Theileria* are apicomplexan parasites transmitted by ticks to vertebrates that act as carriers and hosts (Bishop et al., 2004). Tick vector distribution, tick infectivity, persistence of the carrier state in the host species and host specificity determine parasite prevalence (Mans et al., 2015). This specificity may encompass restriction to a single species, a limited number of species or specific evolutionary lineages (Mans et al., 2015; Pienaar et al., 2018, 2020). The determinants of host specificity remain unclear, although parasite-host-tick co-evolution for piroplasms has been considered (Jalovecka et al., 2019). However, incidental records for *Theileria* in exotic hosts question the concept of host specificity and may have implications for hypotheses on clinical disease etiology, epidemiology, geographic distribution and speciation (Mans et al., 2015). In the current study we investigated the host specificity of *Theileria* sp. (buffalo).

It was posed that *Theileria* sp. (buffalo) show host specificity for African buffalo (*Syncerus caffer*) based on extensive screening of cattle in Uganda and South Africa in regions where this natural infection occurs (Oura et al., 2004, 2011a; Yusufmia et al., 2010; Mans et al., 2011, 2016; Pienaar et al., 2011a, 2014; Njiiri et al., 2015). However, recent studies aimed at investigating the host specificity of *Theileria parva* in buffalo, cattle and waterbuck in Kenya indicated a high prevalence of *T.* sp. (buffalo) in cattle (Githaka et al., 2014; Bishop et al., 2015), suggesting that *T.* sp. (buffalo) is not specific for African buffalo. However, the latter studies were restricted to a single farm (Marula) in Kenya (East Africa) where cattle and African buffalo share pastures. In South Africa, African buffalo and cattle are not allowed to share the same pastures, to limit the risk of Corridor disease (CD) outbreaks caused by transmission of *T. parva* from infected buffalo to cattle by the

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brown ear ticks, *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* (South African Animal Disease Act (Act 35 of 1984). Recently (2013), a CD outbreak did occur on a ranch (Bedrog) where cattle were allowed to graze on pastures frequented by African buffalo (Latif et al., 2019). This presented an opportunity to investigate the host specificity of *T.* sp. (buffalo) and to determine the potential carrier state of this species in cattle, since the buffalo resident on the ranch were infected with *T. parva* (Latif et al., 2019) and *T.* sp. (buffalo). The current study shows that while *T.* sp. (buffalo) can be detected in cattle, its epidemiology is closer to that of buffalo-adapted *T. parva*, where the duration of a definitive carrier state in cattle has yet to be defined.

2. Materials and methods

2.1. Ethics approvals

Animal experiments were approved by the Animal Ethics Committee of the Agricultural Research Council – Onderstepoort Veterinary Research (ARC-OVR) (AEC12.11; AEC11.13; AEC03.14).

2.2. Sample collection

Cattle were introduced onto a game ranch in 2013 (Bedrog, KwaZulu-Natal) within the tick vector endemic area that harbored buffalo. This resulted in an extensive CD outbreak among the cattle (Latif et al., 2019). After the CD outbreak was diagnosed, cattle were block treated with oxytetracycline, dipped and moved to a tick free pen for observation and sampling for a period of three months. Samples analyzed in the current study were collected from cattle and buffalo during the investigation of the CD outbreak on Bedrog ranch (Latif et al., 2019). This included 69 cattle sampled on 3 December 2013 shortly after the CD outbreak was reported, 100 cattle sampled on 14 January 2014 and 96 cattle sampled on 11 March 2014 during follow up visits, respectively. In addition, all 19 African buffalo from the ranch were sampled on 02 December 2013 (n = 9) and 21 January 2014 (n = 10). Buffalo from the Kruger National Park (KNP; n = 239) and the Hluhluwe-Imfolozi National Park (HIP; n = 94) as well as cattle (mixed Nguni breeds; n = 93) from diptanks adjacent to the parks previously screened for *Theileria taurotragi* (Pienaar et al., 2018), were also included in the analysis.

All buffalo in the study were immobilized with a combination of etorphine hydrochloride (M99®; Novartis, South Africa) and azaperone tranquillizer (StresniT; Bayer Pharmaceutical, South Africa). The dose was approximately 8 mg etorphine hydrochloride with 100 mg azaperone adjusted according to estimated body mass. The anesthesia was reversed by intravenous administration of diprenorphine hydrochloride (M5050; Novartis) and all the animals were observed until they were mobile, a process that generally took about 2–5 min.

2.3. DNA extraction and real-time hybridization assays for T. parva and T. sp. (buffalo)

DNA was extracted from 200 µl blood and eluted in 100 µl elution buffer using automated MagNa Pure technology (Roche Diagnostics, Mannheim, Germany). Each PCR reaction included 2.5 µl of DNA (~15–50 ng/µl). Real-time hybridization assays were used to test the samples for the presence of *T. parva* DNA using the LightCycler® 2.0 (Roche Diagnostics, Mannheim, Germany) and for *T.* sp. (buffalo) using LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) as previously described (Pienaar et al., 2011a, 2011b, 2014). Crossing-point (CP) values were calculated by the qualitative analysis mode of the LightCycler 4.0 software (Roche Diagnostics, Mannheim, Germany).

Briefly, for *T. parva* the Hybrid II assay was setup with 2 µL LightCycler-FastStart DNA Master PLUS HybProbe (Roche Diagnostics, Mannheim, Germany), 2 µL LightCycler® 480 Genotyping Master (Roche Diagnostics, Mannheim, Germany), 1U UDG (Roche

Diagnostics, Mannheim, Germany), 0·5 pmol TgF forward (TgF: 5′-GGTAATTCCAGCTCCAATAG-3′) and TpR reverse (TpR: 5′-AAAGTA AACATCCAGACAAAGCG-3′) primer, 0·1 pmol each of the *T. parva* specific anchor (Anchor: 5′-GGGTCTCTGCATGTGGCTTAT–FL) and probe (Probe: 5′-LCRed640-TCGGACGGAGTTCGCT—PH) pairs (final volume of 20 µl). Reaction conditions included an initial UDG activation step (40 °C, 10 min) and a pre-incubation step (95 °C, 10 min). An initial 10 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 10 s) and extension (72 °C, 15 s), followed by a touch-down procedure (60–56 °C, 15 cycles), followed by 20 cycles at 56 °C. These conditions were used on both Roche LightCycler® 2.0 and LightCycler® 480 systems (Roche Diagnostics, Mannheim, Germany) as described (Pienaar et al., 2011b). A cutoff of 37 °C was used.

For *T.* sp. (buffalo), primers utilized was a forward (TpF: CTGCAT CGCTGTGTCCCTT) and reverse (TgR: ACCAACAAAATAGAACCAAA GTC) primer set with specific anchor (TpA: GGGTCTCTGCATGTGGCT TAT–FL) and sensor (TsBuff: (LC640-TC*A*GACGGAGTT*T*A*C*T-PH; underlined bases indicate locked nucleic acids) probes. Assay conditions included 2 µL LightCycler-FastStart DNA Master PLUS HybProbe (Roche Diagnostics, Mannheim, Germany), 2 µL LightCycler® 480 Genotyping Master (Roche Diagnostics, Mannheim, Germany), 1U UDG (Roche Diagnostics, Mannheim, Germany), 0·5 pmol forward and reverse primer, 0·1 pmol each of the hybridization anchor and probe pairs (final volume of 20 µL). Reaction conditions included UDG activation (40 °C, 10 min) and a preincubation (95 °C, 10min), followed by 45 cycles of denaturation (95 °C, 10 s), annealing (58 °C, 10 s) and extension (72 °C, 15 s). Melting curves were obtained by ramping from 40 to 75 °C (5 data acquisitions per degree). Runs were performed using the LightCycler® 480 system and CP values calculated using the LightCycler Version 1.5.0 software as described (Pienaar et al., 2014). A cutoff of 37 °C was used.

2.4. Monitoring cattle under controlled conditions

Five cattle positive for *T. parva* (B24, B53, B120, B163, B164) and five cattle positive for *T.* sp. (buffalo) (B21, B79, B121, B123, B631) were moved from Bedrog to ARC-OVR for long term monitoring. Upon arrival B121 tested positive for *T. parva* and negative for *T.* sp. (buffalo) and was monitored as such. Cattle were kept under tick-free quarantine conditions and bled on a weekly basis for testing. Two animals positive for *T. parva* (B53, B120) and one animal positive for *T.* sp. (buffalo) (B21) were splenectomized on 5 August 2014 (8 months after CD outbreak) using standard procedures used for blood vaccine production at the institute by competent veterinarians.

2.5. Infection of a naive African buffalo and splenectomized Hereford with macroschizont-infected lymphoblastoid cell culture material

A *T.* sp. (buffalo) macroschizont-infected lymphoblastoid cell culture previously isolated from an African buffalo (Zweygarth et al., 2009), was used to infect a naïve African buffalo (Buffalo 114) as well as a splenectomized Hereford bovine by intravenous needle inoculation. The cell culture material was propagated as previously described in RPMI 1640 (Gibco 82400) containing 25 mM HEPES, 2 g/l sodium bicarbonate, and Glutamax^R, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.2 mM hypoxanthine, 100 IU/ml penicillin, and 100 µg/ml streptomycin and was grown at 37 °C (Zweygarth et al., 2009). Both animals were housed in the East Coast fever quarantine complex at ARC-OVR and were confirmed negative for *Babesia* and *Theileria* parasites using real-time PCR and blood smear analysis. The immobilized buffalo (section 2.2) received 50 ml of cell culture material (10 passages from frozen stock) intravenously containing a total of 8.28×10^7 infected cells (macroschizont- and microschizont-infected lymphoblastoid cells). The Hereford was injected intravenously with 30 ml of cell culture (14 passages from frozen stock) containing a total of 7.98×10^8 infected cells. Animals were monitored for any

Table 1
Summary of results for buffalo from Kruger National Park (KNP) and Hluhluwe-Imfolozi Park (HIP) and buffalo and cattle from Bedrog.

Locality	Species	Animals (n)	*T.* sp. (buffalo) Number (% + 95% CI)	*T. parva* Number (%: 95% CI)	Co-infections Number (%: 95% CI)
KNP	Buffalo	239	87 (36%: 32-43)	173 (72%: 66-78)	72 (30%: 25-36)
HIP	Buffalo	94	94 (100%: 96-100)	86 (91%: 84-96)	86 (91%: 84 - 96)
Bedrog (02/12/2013)	Buffalo	9	8 (89%: 56-98)	9 (100%: 70-100)	8 (89%: 56-98)
Bedrog (21/01/2014)	Buffalo	10	10 (100%: 72-100)	10 (100%: 72-100)	10 (100%: 72-100)
Buffalo (Total)	Buffalo	19	18 (95%: 75-99)	19 (100%: 83-100)	18 (95%: 75-99)
Bedrog (03/12/2013)	Cattle	69	10 (14%: 8-25)	32 (46%: 35-58)	5 (7%: 3-16)
Bedrog (14/01/2014)	Cattle	100	27 (27%: 19-36)	23 (23%: 16-32)	12 (12%: 7-20)
Bedrog (11/03/2014)	Cattle	96	21 (22%: 15-31)	23 (24%: 17-33)	7 (7%: 4-14)
Cattle (Total)	Cattle	265	58 (22%: 17-27)	78 (29%: 24-35)	24 (9%: 6-13)

adverse clinical signs, and the bovine for *Theileria* piroplasms using Giemsa stained blood smears. Both animals were bled on day 35 post infection to confirm successful transmission of *T.* sp. (buffalo) infection using real-time PCR as well as with Giemsa stained blood smears.

3. Results

3.1. PCR assay results for cattle and buffalo during a CD outbreak

Testing of 19 African buffalo from the ranch sampled on 02 December 2013 and 21 January 2014 indicated that 100% were positive for *T. parva* and 95% for *T.* sp. (buffalo), resulting in 95% co-infection (Table 1). In the case of *T. parva*, 46% of cattle were qPCR positive on the first sampling, which then decreased to 23% and 24% in consecutive months. For *T.* sp. (buffalo), 14% cattle tested positive during the first sampling and this value increased to 27% and 22% in consecutive months. This resulted in 9% co-infection for *T. parva* and *T.* sp. (buffalo).

Comparative data for buffalo sampled in National Parks indicate that 72% and 91% of buffalo tested positive for *T. parva* in the KNP and HIP, respectively (Table 1). Only 36% of buffalo tested positive for *T.* sp. (buffalo) in the KNP, resulting in low co-infection of 30% for *T. parva*. Conversely, 100% of buffalo from HIP were positive for *T.* sp. (buffalo), resulting in 91% co-infection with *T. parva*.

3.2. Comparison of *T. parva* and *T.* sp. (buffalo) CP values for various samples

In the case of *T. parva*, cattle from the CD outbreak showed an initial CP value range of 22–37 (average 31.8 ± 2.9), but this increased to 32-37 (average 34.7 ± 1.0) during subsequent samplings (Fig. 1). Buffalo from Bedrog showed CP value ranges from 20 to 30 (average 24.9 ± 2.2), which compared well with those from buffalo from the endemic KNP and HIP which showed CP value ranges from 20 to 35 (average: 26.8 ± 2.8) (Fig. 1).

For *T.* sp. (buffalo), CP values in cattle from Bedrog ranged from 31 to 33 (32.5 ± 0.77) during the first sampling shortly after the CD outbreak was identified, from 32 to 35 (33.7 ± 0.6) during the second sampling one month later and from 31 to 34 (32.8 ± 0.68) in the third month (Fig. 1). CP values in buffalo from Bedrog ranged from 22 to 30 (average 27.9 ± 2.0), comparing well with buffalo from endemic which ranged from 23 to 35 (average: 28.8 ± 2.4) (Fig. 1).

3.3. Monitoring *T. parva* and *T.* sp. (buffalo) positive cattle

Six cattle (B24, B53, B120, B121, B163, B164) positive for *T. parva* were moved from Bedrog to ARC-OVR for long term monitoring. One animal (B24) became negative shortly after arrival and did not test positive over a 24-month period. One animal (B164) with intact spleen that showed an increase in parasitemia died of natural causes ~ one year into the monitoring period. Two animals (B53 and B120) were

splenectomized a week after their CP values reached the PCR cutoff value (37 cycles) on 05 August 2014 (8 months after the outbreak). The animals showed intermitted CP values that fluctuated below the PCR cutoff value before steadily decreasing (increased parasitemia) ~6 months after splenectomy. For the non-splenectomized animals (B121 and B163) the CP values fluctuated around the detection limit (Fig. 2).

Four cattle (B21, B79, B123, B631) positive for *T.* sp. (buffalo) were moved from Bedrog to ARC-OVR for long term monitoring. All animals became PCR negative within 5 months, except for B79 for which CP values fluctuated around the cut-off for ~2 years of testing. B123 and B631 remained negative for the duration of the testing. One animal (B21) was splenectomized a week after its CP value reached the cutoff, after which it tested negative for ~6 months before it became PCR positive again (Fig. 2). This animal remained positive to date with a trend of detectable but fluctuating parasitemia (Fig. 2).

3.4. Infection of an African buffalo and a bovine using a macroschizont-infected lymphoblastoid cell culture of *T.* sp. (buffalo)

Neither the bovine nor the African buffalo showed adverse signs after infection using the cell culture material. On day 35 post infection both animals were monitored for the appearance of piroplasms using Giemsa stained smears and for the presence of *T.* sp. (buffalo) using real-time PCR. Buffalo 114 showed the presence of piroplasms and schizonts and tested positive for *T.* sp. (buffalo) using real-time PCR with a CP value of 33.2. The bovine did not show the presence of any schizonts or piroplasms and tested negative using real-time PCR.

4. Discussion

Theileria sp. (buffalo) remains an enigmatic parasite. It was first identified as a potential *T. parva* schizont cell culture isolate from African buffalo, that gave monoclonal antibody profiles different from that of other *T. parva* isolates (Conrad et al., 1987). Sequencing of the 18S rRNA hypervariable region indicated that its sequence differed from that of *T. parva* and it was tentatively suggested that it is a new species designated as *T.* sp. (buffalo) (Allsopp et al., 1993). Its status as unique species from *T. parva* has been established using 18S rRNA, S5 ribosomal, cytochrome oxidase I, polymorphic immunodominant protein (PIM), Tp6, Tp7 and Tp8 genes (Mans et al., 2011; Pienaar et al., 2014; Bishop et al., 2015). It was subsequently shown to extensively infect African buffalo from Uganda to South Africa (Allsopp et al., 1999; Chaisi et al., 2011; Mans et al., 2011; Oura et al., 2011a, 2011b; Pienaar et al., 2014).

While *T.* sp. (buffalo) has been considered to be host specific for African buffalo, recent reports suggest that it could also infect cattle (Githaka et al., 2014; Bishop et al., 2015). Even so, its epidemiology in cattle continues to present some conundrums. Screening of a large number of cattle from *R. appendiculatus* endemic regions in South Africa (regions known to be endemic for *T.* sp. (buffalo) in buffalo), did not detect *T.* sp. (buffalo) to date (Pienaar et al., 2011a; Mans et al., 2016).
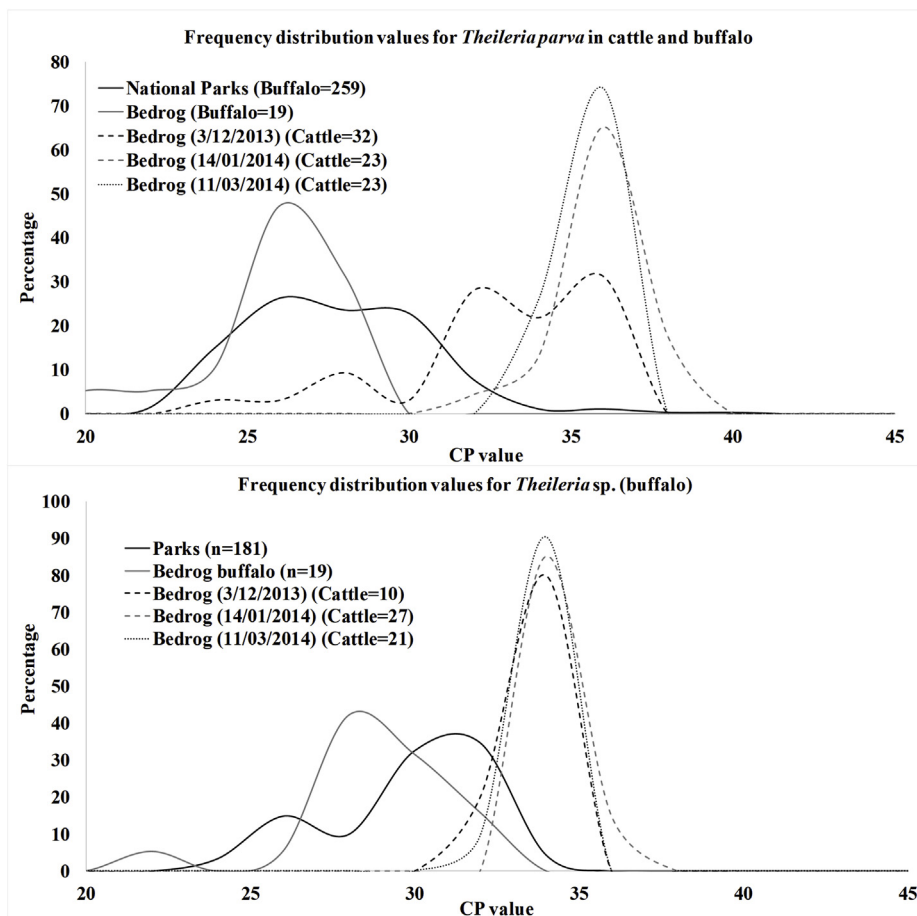
Fig. 1. Frequency distribution curves for *Theileria parva* and *Theileria* sp. (buffalo) positive animals. Indicated are frequency distribution plots of the CP values and the percentage observed in buffalo from National Parks, buffalo from the Bedrog ranch, and cattle from the Bedrog ranch sampled during the outbreak (3/12/2013) and monitored afterwards for *T. parva* (top panel) and *T.* sp. (buffalo) (bottom panel).

In Lake Mburo National Park (Uganda, East Africa), none of the cattle (n = 144) tested were positive for *T.* sp. (buffalo), even though all buffalo (n = 19; 100%) were positive (Oura et al., 2011a). In contrast, a recent study in Kenya (East Africa) where cattle and buffalo grazed on the same pastures (Marula farm), detected extremely high levels of infection for *T.* sp. (buffalo) in cattle (n = 63/86; 73%) (Githaka et al., 2014), suggesting a possible carrier state. Another study from the same farm identified 5/22 schizont cell lines (23%) from cattle to be *T.* sp. (buffalo) derived (Bishop et al., 2015). In another study from western Kenya (East Africa), a cohort of calves (n = 453) sampled across 20 random localities, showed infection for 406 animals at 51 weeks of monitoring, of all common *Theileria* species infecting cattle including *Theileria mutans*, *T. parva*, *T. taurotragi* and *Theileria velifera* (Njiiri et al., 2015). None was infected with *T.* sp. (buffalo) or *Theileria buffeli*. Another study that sampled cattle from two farms from central Kenya (East Africa), detected *T. parva* infection on both farms with prevalence ranging from 30 to 32% (Moumouni et al., 2015), which is comparable to previous epidemiological studies in Kenya (Odongo et al., 2010). In the previously refereed study, only one animal was positive for *T.* sp. (buffalo) at a prevalence of 0.6% (Moumouni et al., 2015). These observations in cattle contrast sharply with the prevalence of *T.* sp. (buffalo) in buffalo that generally range from 40 to 100% in endemic areas (Pienaar et al., 2014).

Cattle under carrier conditions (non-clinical disease progression) have been routinely tested since 2008 from regions in South Africa assumed to be endemic for *T.* sp. (buffalo) (Pienaar et al., 2014). Except for the Bedrog ranch, none of these cattle has tested positive for *T.* sp. (buffalo). A possible reason for this is the strict quarantine measures imposed in South Africa to keep buffalo and cattle segregated, in order to limit transmission of CD from buffalo to cattle. As such, opportunity for transmission of *T.* sp. (buffalo) from buffalo to cattle via its tick

vector would be limited and may explain the epidemiological distribution observed. Even so, significant CD outbreaks occur on a regular basis where cattle and buffalo come into contact within the CD endemic region of South Africa (Mbizeni et al., 2013), to assume that cattle would be exposed to *T.* sp. (buffalo). In this regard, CD outbreaks have been monitored for more than 15 years and no evidence exist that the disease has spread to areas outside the CD endemic area, even though the tick vector distribution is much wider (Pienaar et al., 2011a). This strongly argues against a natural carrier state in cattle for buffalo-adapted *T. parva* from South Africa, even though therapeutic carrier states may be induced with oxytetracycline (Latif et al., 2019). This contrasts to the very rapid spread of East Coast fever when the disease established itself within a 10-year period from 1902 to 1912 throughout its vector distribution in South Africa, during the East Coast fever epidemic (Neitz, 1957; Lawrence, 1986; Norval et al., 1992). The same argument may be applied to *T.* sp. (buffalo), i.e. it does not show an extensive distribution in cattle in South Africa even though cattle contact with its vector tick and the parasite may be expected on a regular basis. The epidemiology of *T.* sp. (buffalo) is therefore similar to that of buffalo-adapted *T. parva* in cattle, and would suggest that both parasites, even though they can infect cattle, have not adapted to a stable carrier state yet.

The Bedrog ranch presented an interesting opportunity to study possible transmission from buffalo to naïve cattle during its CD outbreak, since most buffalo on the ranch were infected with *T.* sp. (buffalo). In this case, 27% of cattle tested positive for *T.* sp. (buffalo) one month after the initial outbreak. Follow-up monitoring over a period of two months indicated that the prevalence dropped to 22% (CP values ranged from 31 to 35). Animals monitored for a longer period in the absence of infected tick vectors lost the infection completely, except for one animal splenectomized. A trend of lower CP values indicated that
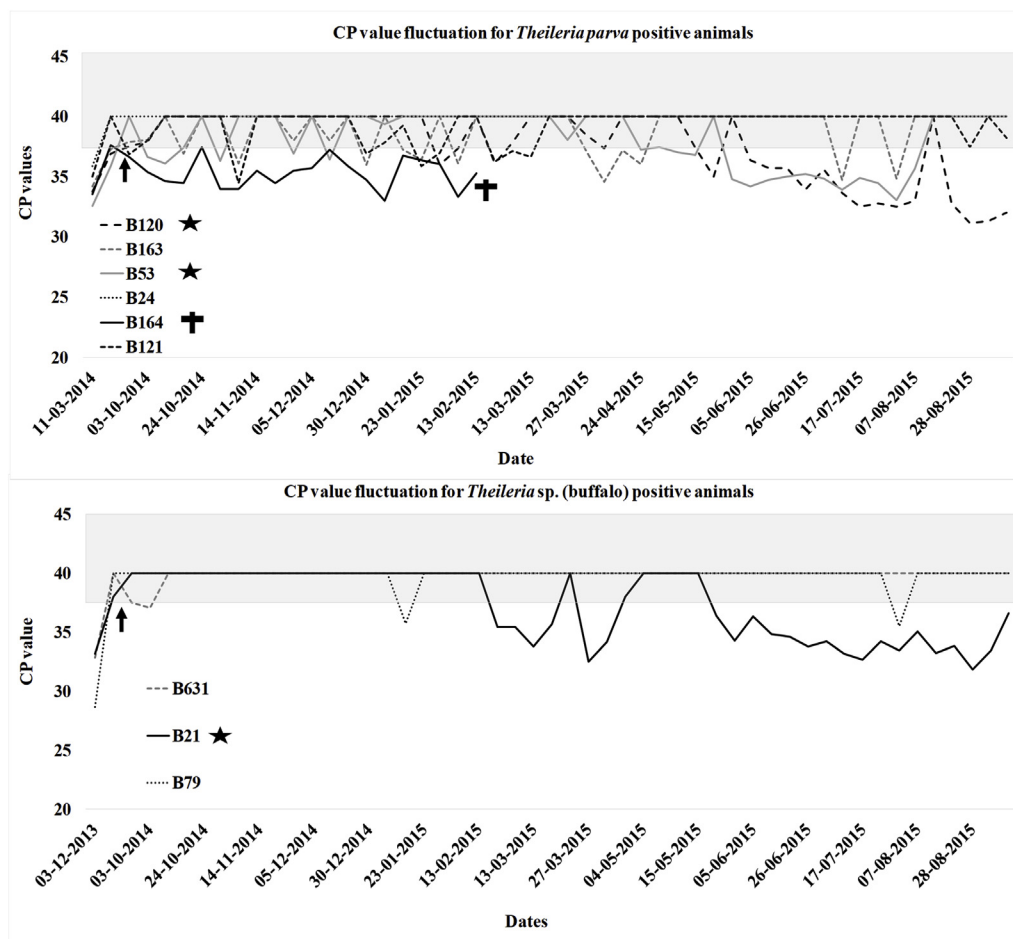
Fig. 2. Monitoring of *Theileria parva* and *Theileria* sp. (buffalo) positive animals over a period of 24 months. The gray shaded areas indicate CP values below the cutoff of the PCR assays. Stars indicate animals that were splenectomized and the arrow indicate the date of splenectomy. The cross indicates an animal that died of natural causes.

the parasitemia for *T.* sp. (buffalo) slowly increased for this positive animal. A similar trend was observed in a *T. parva* positive splenectomized animal. This suggests that buffalo-derived *T. parva* and *T.* sp. (buffalo) is cleared from intact animals (Mbizeni et al., 2013), and that the spleen and its related immunity play an important role in this process. The results also support the observations by Neitz (1958) that splenectomy can result in recurrence of parasitemia of buffalo-derived *T. parva*, most probably if performed while sufficient numbers of parasites are still circulating. In contrast, field derived cattle that previously tested positive for *T. parva* and subsequently became PCR negative (Mbizeni et al., 2013), were subsequently splenectomized but did not show *T. parva* recurrence (Latif, personal observation). According to some authors (Latif et al., 2019), the only difference between these latter cattle and the carriers from Bedrog ranch were oxytetracycline treatment.

A confounding factor in the current study is the fact that animals were block-treated with oxytetracycline during the peak of the CD outbreak (Latif et al., 2019). Oxytetracycline is used as part of the infection and treatment regime during East Coast fever vaccination (Young et al., 1990). It has also been shown that treatment with oxytetracycline during early stages of infection can prevent death due to *T. parva* (Brown et al., 1977). Furthermore, infection and treatment with oxytetracycline can lead to a carrier-state (Maritim et al., 1989). To date, the mechanism of tetracycline on apicomplexan parasite biology has not been resolved, although perturbation of mitochondrial protein synthesis and parasite differentiation has been implicated (Spooner, 1990; Shiels et al., 1997; Gaillard et al., 2015). It may therefore be

possible that non-natural carrier-states can be created using chemoprophylaxis and may explain in part the relatively long period that cattle were PCR positive for *T.* sp. (buffalo) in the current study (> 3 months) as well as the cattle positive for *T. parva*.

Overall, the data suggest that cattle may be infected by *T.* sp. (buffalo) and that infected schizonts may persist for some time (Bishop et al., 2015). However, transformation, maturation and merozoite production to produce a long term carrier state may not be viable. This may suggest that schizonts can persist for prolonged periods in the vertebrate host without progressing to the piroplasm stage, while animals remain PCR positive. *Theileria parva* schizonts could be cultured 210 days (7 months) after infection (Kariuki et al., 1995), making this a possibility for both *T.* sp. (buffalo) and buffalo-adapted *T. parva*. Alternative explanations may be that the strains between South Africa, Uganda and Kenya differ to such an extent to support different host-carrier behavior. *Theileria* sp. (buffalo) may then mimic the phenomenon observed between buffalo and cattle-derived *T. parva* in East and South Africa. In this case, the single annual lifecycle of *R. appendiculatus* in South Africa does not allow maintenance of a carrier state in cattle, because parasites are cleared in cattle before nymphal pickup can occur (Mbizeni et al., 2013). In East Africa, the concurrent presence of all life stages enables tick pickup from transient carriers and transmission to new cattle hosts. The tick vector for *T.* sp. (buffalo) would therefore also have a single annual life cycle in South Africa (even though the tick vector remains unknown), but would explain the absence of a carrier state in cattle in that geographic region.

Immunity to infection may partially explain the results observed. In

this case limited genetic diversity within geographic populations, linked with maternal immunity may limit subsequent homologous infection resulting in low prevalence. In the case of CD and cattle this may result in sterile immunity and loss of a parasitaemic carrier state, most probably because a stable carrier state is not well established in cattle. Results from the currently study suggest that for *T*. sp. (buffalo) a stable carrier state in cattle is also not supported. The prevalence data in the current study for Bedrog for *T*. sp. (buffalo) and *T. parva* in cattle (14-24% and 23-46%, respectively), and that obtained at Marula (73% and 76%, respectively), also differs significantly, suggesting that additional factors may play a role in disease transmission in these regions such as climate, tick infectivity or tick vector capacity. In the case of buffalo, high genetic diversity of *T*. sp. (buffalo) and *T. parva* will result in heterologous challenge and higher prevalence. This would, however, not explain the high incidence observed for *T. parva* and *T*. sp. (buffalo) in geographically restricted areas such as HIP (Pienaar et al., 2014). High incidences were also observed for other *Theileria* genotypes such as *T. mutans*-like 1, *T. mutans*-like 2 and *T. mutans*-like 3 (Mans et al., 2016). This would suggest that while immunity may play a role in prevalence, propensity for the natural carrier state may also have an impact.

The results from the current study have interesting implications for surveys of mammalian hosts for *Theileria* parasites using molecular methods and the potential epidemiological conclusions that may be derived from these studies. Detection of parasites in a vertebrate host may only imply recent transmission by the tick, or transient infection of host leucocytes, or establishment of chemotherapeutic-induced carrier states, especially where oxytetracycline treatment forms part of a regular treatment regime. It does not imply efficient leucocyte transformation, maturation to the piroplasm stage, or a long term carrier state in the host that would support infection of the tick vector under natural conditions (no prophylaxis). Useful conclusions regarding parasite epidemiology and host carrier status requires long term follow up over several seasons and sampling of sufficient host numbers in vector endemic regions. Incidental detection of *Theileria* in hosts not considered natural carriers should be contrasted to endemic stability expected for the *Theileria* genus in natural hosts and should be factored into epidemiological considerations regarding parasite transmission. An interesting case in point would be the infection of waterbuck (*Kobus defassa*) with *T. parva* under controlled conditions, while under conditions of co-grazing with infected buffalo no evidence was found for a carrier state (Stagg et al., 1983, 1994; Githaka et al., 2014).

5. Conclusions

While *T*. sp. (buffalo) can infect cattle, no evidence exists that a long-term carrier state occurs in cattle that would suggest that they are natural carrier hosts. This is similar to buffalo-adapted *T. parva* that causes CD in cattle. Confirmation of long-term carrier status is an important consideration in the epidemiology of theilerial diseases. While short-term incidental infections of non-optimal hosts may occur, these events may play a negligible part in the epidemiology of *Theileria* parasites, especially where the tick vectors exhibit annual life cycles.

Declaration of competing interest

The authors declare they have no conflict of interest regarding the work described in this manuscript.

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References

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.P.E., Cavalier-Smith, T., Bishop, R.P., Carrington, D.M., Sohanpal, B., Spooner, P., 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. Parasitology 107, 157–165.

Allsopp, M.T., Theron, J., Coetzee, M.L., Dunsterville, M.T., Allsopp, B.A., 1999. The occurrence of *Theileria* and *Cowdria* parasites in African buffalo (*Syncerus caffer*) and their associated *Amblyomma hebraeum* ticks. Onderstepoort J. Vet. Res. 66, 245–249.

Bishop, R., Musoke, A., Morzaria, S., Gardner, M., Nene, V., 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. Parasitology 129, S271–S283.

Bishop, R.P., Hemmink, J.D., Morrison, W.I., Weir, W., Toye, P.G., Sitt, T., Spooner, P.R., Musoke, A.J., Skilton, R.A., Odongo, D.O., 2015. The African buffalo parasite *Theileria* sp. (buffalo) can infect and immortalize cattle leukocytes and encodes divergent orthologues of *Theileria parva* antigen genes. Int. J. Parasitol. Parasites Wildl. 4, 333–342.

Brown, C.G., Radley, D.E., Burridge, M.J., Cunningham, M.P., 1977. The use of tetracyclines on the chemotherapy of experimental East Coast Fever (*Theileria parva* infection of cattle). Tropenmed. Parasitol. 28, 513–520.

Chaisi, M.E., Sibeko, K.P., Collins, N.E., Potgieter, F.T., Oosthuizen, M.C., 2011. Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa. Vet. Parasitol. 182, 150–162.

Conrad, P.A., Stagg, D.A., Grootenhuis, J.G., Irvin, A.D., Newson, J., Njamunggeh, R.E., Rossiter, P.B., Young, A.S., 1987. Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterization with anti-schizont monoclonal antibodies. Parasitology 94, 413–423.

Gaillard, T., Madamet, M., Pradines, B., 2015. Tetracyclines in malaria. Malar. J. 14, 445.

Githaka, N., Konnai, S., Bishop, R., Odongo, D., Lekolool, I., Kariuki, E., Gakuya, F., Kamau, L., Isezaki, M., Murata, S., Ohashi, K., 2014. Identification and sequence characterization of novel *Theileria* genotypes from the waterbuck (*Kobus defassa*) in a *Theileria parva*-endemic area in Kenya. Vet. Parasitol. 202, 180–193.

Jalovecka, M., Sojka, D., Ascencio, M., Schnittger, L., 2019. *Babesia* life cycle – when phylogeny meets biology. Trends Parasitol. 35, 356–368.

Kariuki, D.P., Young, A.S., Morzaria, S.P., Lesan, A.C., Mining, S.K., Omwoyo, P., Wafula, J.L., Molyneux, D.H., 1995. *Theileria parva* carrier state in naturally infected and artificially immunised cattle. Trop. Anim. Health Prod. 27, 15–25.

Latif, A.A., Troskie, P.C., Peba, S.B., Maboko, B.B., Pienaar, R., Mans, B.J., 2019. Corridor disease (buffalo-associated *Theileria parva*) outbreak in cattle introduced onto a game ranch and investigations into their carrier-state. Vet. Parasitol. Reg. Stud. Rep. 18, 100331.

Lawrence, J.A., 1986. East Coast Fever in Southern Africa 1901-1960. University of Zimbabwe, Harare (Monograph updated 2016).

Mans, B.J., Pienaar, R., Latif, A.A., Potgieter, F.T., 2011. Diversity in the 18S SSU rRNA V4 hyper-variable region of *Theileria* in bovines and African buffalo (*Syncerus caffer*) from southern Africa. Parasitology 138, 766–779.

Mans, B.J., Pienaar, R., Latif, A.A., 2015. A review of *Theileria* diagnostics and epidemiology. Int. J. Parasitol. Parasites Wildl. 4, 104–118.

Mans, B.J., Pienaar, R., Ratabane, J., Pule, B., Latif, A.A., 2016. Investigating the diversity of the 18S SSU rRNA hyper-variable region of *Theileria* in cattle and Cape buffalo (*Syncerus caffer*) from southern Africa using a next generation sequencing approach. Ticks Tick Borne Dis. 7, 869–879.

Maritim, A.C., Young, A.S., Lesan, A.C., Ndungu, S.G., Mutugi, J.J., Stagg, D.A., 1989. Theilerial parasites isolated from carrier cattle after immunization with *Theileria parva* by the infection and treatment method. Parasitology 99, 139–147.

Mbizeni, S., Potgieter, F.T., Troskie, C., Mans, B.J., Penzhorn, B.L., Latif, A.A., 2013. Field and laboratory studies on Corridor disease (*Theileria parva* infection) in cattle population at the livestock/game interface of uPhongolo-Mkuze area, South Africa. Ticks Tick Borne Dis. 4, 227–234.

Moumouni, A.P.F., Aboge, G.O., Terkawi, M.A., Masatani, T., Cao, S., Kamyingkird, K., Jirapattharasate, C., Zhou, M., Wang, G., Liu, M., Iguchi, A., Vudriko, P., Ybanez, A.P., Inokuma, H., Shirafuji-Umemiya, R., Suzuki, H., Xuan, X., 2015. Molecular detection and characterization of *Babesia bovis*, *Babesia bigemina*, *Theileria* species and *Anaplasma marginale* isolated from cattle in Kenya. Parasites Vectors 8, 496.

Neitz, W.O., 1957. Theileriosis, gonderiosis and cytauxzoonoses: a review. Onderstepoort J. Vet. Res. 27, 275–430.

Neitz, W.O., 1958. Can Corridor disease-recovered cattle serve as reservoirs of *Gonderia lawrencei*? Bull. Epizoot. Dis. Afr. 6, 151–154.

Njiiri, N.E., Bronsvoort, B.M., Collins, N.E., Steyn, H.C., Troskie, M., Vorster, I., Thumbi, S.M., Sibeko, K.P., Jennings, A., van Wyk, I.C., Mbole-Kariuki, M., Kiara, H., Poole, E.J., Hanotte, O., Coetzer, K., Oosthuizen, M.C., Woolhouse, M., Toye, P., 2015. The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. Vet. Parasitol. 210, 69–76.

Norval, R.A.I., Perry, B.D., Young, A.S., 1992. The Epidemiology of Theileriosis in Africa. Academic Press, London.

Odongo, D.O., Sunter, J.D., Kiara, H.K., Skilton, R.A., Bishop, R.P., 2010. A nested PCR assay exhibits enhanced sensitivity for detection of *Theileria parva* infections in bovine blood samples from carrier animals. Parasitol. Res. 106, 357–365.

Oura, C.A., Bishop, R.P., Wampande, E.M., Lubega, G.W., Tait, A., 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. Int. J. Parasitol. 34, 603–613.

Oura, C.A., Tait, A., Asiimwe, B., Lubega, G.W., Weir, W., 2011a. *Theileria parva* genetic diversity and haemoparasite prevalence in cattle and wildlife in and around Lake

Mburo National Park in Uganda. Parasitol. Res. 108, 1365–1374.

Oura, C.A.L., Tait, A., Asiimwe, B., Lubega, G.W., Weir, W., 2011b. Haemoparasite prevalence and *Theileria parva* strain diversity in Cape buffalo (*Syncerus caffer*) in Uganda. Vet. Parasitol. 175, 212–219.

Pienaar, R., Potgieter, F.T., Latif, A.A., Thekisoe, O.M.M., Mans, B.J., 2011a. Mixed *Theileria* infections in free-ranging buffalo herds: implications for diagnosing *Theileria parva* infections in Cape buffalo (*Syncerus caffer*). Parasitology 138, 884–895.

Pienaar, R., Potgieter, F.T., Latif, A.A., Thekisoe, O.M.M., Mans, B.J., 2011b. The HybridII assay: a sensitive and specific real-time hybridization assay for the diagnosis of *Theileria parva* infection in Cape buffalo (*Syncerus caffer*) and cattle. Parasitology 138, 884–895.

Pienaar, R., Potgieter, F.T., Latif, A.A., Thekisoe, O.M.M., Mans, B.J., 2014. Geographic distribution of *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) in Cape buffalo (*Syncerus caffer*) in southern Africa: implications for speciation. Parasitology 141, 411–424.

Pienaar, R., Latif, A.A., Mans, B.J., 2018. Investigations into the host specificity of *Theileria taurotragi*. Vet. Parasitol. 254, 30–35.

Pienaar, R., Josemans, A., Latif, A.A., Mans, B.J., 2020. The host-specificity of *Theileria* sp. (sable) and *Theileria* sp. (sable-like) in African Bovidae and detection of novel *Theileria* in antelope and giraffe. Parasitology. https://doi.org/10.1017/S003118201900132X.

Shiels, B., Aslam, N., McKellar, S., Smyth, A., Kinnaird, J., 1997. Modulation of protein synthesis relative to DNA synthesis alters the timing of differentiation in the protozoan parasite *Theileria annulata*. J. Cell Sci. 110, 1441–1451.

Spooner, P.R., 1990. Oxytetracycline inhibition of mitochondrial protein synthesis in bovine lymphocytes infected with *Theileria parva* or stimulated by mitogen. Parasitology 101, 387–393.

Stagg, D.A., Young, A.S., Leitch, B.L., Grootenhuis, J.G., Dolan, T.T., 1983. Infection of mammalian cells with *Theileria* species. Parasitology 86, 243–254.

Stagg, D.A., Bishop, R.P., Morzaria, S.P., Shaw, M.K., Wesonga, D., Orinda, G.O., Grootenhuis, J.G., Molyneux, D.H., Young, A.S., 1994. Characterization of *Theileria parva* which infects waterbuck (*Kobus defassa*). Parasitology 108, 543–554.

Young, A.S., Leitch, B.L., Dolan, T.T., Mbogo, S.K., Ndungu, S.G., Grootenhuis, J.G., De Castro, J.J., 1990. Evaluation of infection and treatment methods in immunization of improved cattle against theileriosis in an endemic area of Kenya. Vet. Parasitol. 35, 239–257.

Yusufmia, S.B., Collins, N.E., Nkuna, R., Troskie, M., Van den Bossche, P., Penzhorn, B.L., 2010. Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge of Hluhluwe-Imfolozi Park KwaZulu-Natal, South Africa. J. S. Afr. Vet. Assoc. 81, 45–49.

Zweygarth, E., Koekemoer, O., Josemans, A.I., Rambritch, N., Pienaar, R., Putterill, J., Latif, A., Potgieter, F.T., 2009. *Theileria*-infected cell line from an African buffalo (*Syncerus caffer*). Parasitol. Res. 105, 579–581.