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Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa

Mamohale E. Chaisi[a,b], Kgomotso P. Sibeko[a], Nicola E. Collins[a], Fred T. Potgieter[c], Marinda C. Oosthuizen[a,*]

[a] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa
[b] Department of Biology, National University of Lesotho, Roma 180, Lesotho
[c] Agricultural Research Council-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110, South Africa

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ABSTRACT

Theileria parva is the causative agent of Corridor disease in cattle in South Africa. The African buffalo (*Syncerus caffer*) is the reservoir host, and, as these animals are important for ecotourism in South Africa, it is compulsory to test and certify them disease free prior to translocation. A *T. parva*-specific real-time polymerase chain reaction (PCR) test based on the small subunit ribosomal RNA (18S rRNA) gene is one of the tests used for the diagnosis of the parasite in buffalo and cattle in South Africa. However, because of the high similarity between the 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo), the latter is also amplified by the real-time PCR primers, although it is not detected by the *T. parva*-specific hybridization probes. Preliminary sequencing studies have revealed a small number of sequence differences within the 18S rRNA gene in both species but the extent of this sequence variation is unknown. The aim of the current study was to sequence the 18S rRNA genes of *T. parva* and *Theileria* sp. (buffalo), and to determine whether all identified genotypes can be correctly detected by the real-time PCR assay. The reverse line blot (RLB) hybridization assay was used to identify *T. parva* and *Theileria* sp. (buffalo) positive samples from buffalo blood samples originating from the Kruger National Park, Hluhluwe-iMfolozi Park, the Greater Limpopo Transfrontier Park, and a private game ranch in the Hoedspruit area. *T. parva* and *Theileria* sp. (buffalo) were identified in 42% and 28%, respectively, of 252 samples, mainly as mixed infections. The full-length 18S rRNA gene of selected samples was amplified, cloned and sequenced. From a total of 20 sequences obtained, 10 grouped with previously published *T. parva* sequences from GenBank while 10 sequences grouped with a previously published *Theileria* sp. (buffalo) sequence. All these formed a monophyletic group with known pathogenic *Theileria* species. Our phylogenetic analyses confirm the distinction between *Theileria* sp. (buffalo) and *T. parva* and indicate the existence of a single group of *T. parva* and two *Theileria* sp. (buffalo) 18S rRNA gene variants in the African buffalo. Despite the observed variation in the full-length parasite 18S rRNA gene sequences, the area in the V4 hypervariable region where the RLB and real-time PCR hybridization probes were developed was relatively conserved. The *T. parva* specific real-time PCR assay was able to successfully detect all *T. parva* variants and, although amplicons were obtained from *Theileria* sp. (buffalo) DNA, none of the *Theileria* sp. (buffalo) 18S rRNA sequence variants were detected by the *T. parva*-specific hybridization probes.

* Corresponding author. Tel.: +27 12 529 8390; fax: +27 12 5298312.
E-mail address: marinda.oosthuizen@up.ac.za (M.C. Oosthuizen).

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1. Introduction

The haemoprotozoan parasite, *Theileria parva*, transmitted mainly by the ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*, is the causative agent of Corridor disease in cattle in South Africa, and East Coast fever (ECF) in eastern and central Africa (Norval et al., 1991; Uilenberg, 1999). ECF was introduced into South Africa in the early 1900s and eventually eradicated in the 1950s, but a different form of theileriosis, Corridor disease, persists (Lawrence et al., 1994). The African buffalo (*Syncerus caffer*) is the reservoir host; infections are asymptomatic in buffalo but potentially fatal in cattle. It is thought that Corridor disease is transmitted mainly from buffalo to cattle but not between cattle, as infected bovines usually die before piroplasms appear or piroplasms are too few to infect new ticks (Lawrence et al., 1994; Uilenberg, 1999). Cattle that survive an acute ECF infection are able to mount an immune response that results in an asymptomatic carrier state and therefore become sources of infection for tick vectors (Potgieter et al., 1988; Norval et al., 1992; Altay et al., 2008; Beck et al., 2009). The strict control measures that were put in place to control theileriosis in South Africa after the eradication of ECF were designed to prevent the creation of carrier animals and the subsequent spread of the disease in the cattle population.

In South Africa, *T. parva* is endemic in buffalo in the Kruger National Park (KNP) and the Hluhluwe-iMfolozi Park, and Corridor disease occurs in neighbouring farms and game parks where cattle and buffalo are in close contact in the presence of vector ticks (Collins, 1997; Mashishi, 2002). In addition to *T. parva*, the African buffalo is the natural host of the relatively benign *Theileria mutans* and the apathogenic *Theileria velifera*, both of which are transmitted by *Amblyomma hebraeum* (Norval et al., 1992). *Theileria buffeli* and the hitherto uncharacterized *Theileria* sp. (buffalo) have also been identified in some buffalo populations in South Africa (Stoltz, 1996; Zweygarth et al., 2009; Mans et al., 2011) and the local tick vectors of these parasites are unknown. *Theileria* sp. (buffalo) was first reported in 1993 from a buffalo in Kenya (Allsopp et al., 1993), and very little is known about this parasite.

Buffalo play an important role in the epidemiology of several other livestock diseases in South Africa, including foot-and-mouth disease, bovine brucellosis and bovine tuberculosis. Infected buffalo are isolated by approved fences in national and provincial game parks as well as in a limited number of buffalo breeding projects, under veterinary supervision. Farming of buffalo and cattle on the same farm is not allowed. In South Africa, buffalo are important for eco-tourism and this has led to an increasing demand for buffalo (Collins et al., 2002). There are several breeding projects, regulated by the veterinary authorities, that breed "disease-free" buffalo and it is compulsory to test all buffalo for *T. parva* and other infectious diseases prior to translocation in order to protect cattle and non-infected buffalo from infection (Collins et al., 2002). The animals undergo a series of parasitological, serological and molecular diagnostic tests before they are certified "disease-free" and fit for translocation. Conventional parasitological methods include microscopic examination of blood smears for the presence of piroplasms and/or lymph node biopsies in suspected clinical cases for schizonts. Serological assays such as the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) detect specific antibodies to parasite schizonts or sporozoites (Katende et al., 1998; Ogden et al., 2003; Oura et al., 2004). However, these tests sometimes lack specificity and sensitivity especially in the case of low level infections, as is the case with carrier animals (Allsopp et al., 1993; Billiouw et al., 2005). A molecular test based on PCR amplification of the *Theileria* 18S rRNA gene followed by probing with species-specific oligonucleotide probes (Allsopp et al., 1993), has recently been superseded by a real-time PCR test for the diagnosis of *T. parva* in cattle and buffalo in South Africa (Sibeko et al., 2008).

Due to the similarities in the 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo), the real-time PCR amplification primers currently in use also amplify *Theileria* sp. (buffalo) DNA. The hybridization probe set is specific for *T. parva*, and although an amplicon is obtained from *Theileria* sp. (buffalo) DNA, a melting curve is generated for *T. parva* DNA only (Sibeko et al., 2008). Preliminary evidence obtained in our laboratory suggests that *T. parva* 18S rRNA gene sequence variants exist, although to date, no sequences with differences in the probe region have been identified. The specificity of the real-time PCR test could be compromised if strains of *T. parva* with such sequence differences exist, or if there are strains of *Theileria* sp. (buffalo) with sequences similar to *T. parva* in the probe region. Therefore the aim of this study was to investigate the extent of sequence variation in the 18S rRNA gene within and between these two species in South Africa.

2. Materials and methods

2.1. Blood samples and DNA extraction

Ninety-eight blood samples spotted on filter-paper, collected from buffalo in the KNP, and 100 whole blood samples (in EDTA) collected from buffalo in the Hluhluwe-iMfolozi Park, South Africa, were investigated. Five buffalo blood samples from a private game ranch located in the Hoedspruit area bordering the KNP and 49 samples from the Greater Limpopo Transfrontier Park (Mozambique) close to the KNP border were also included in the study. Genomic DNA was extracted from the filter paper blood spots using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany) and from whole blood using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocols. DNA was eluted in 100 μl elution buffer and stored at −20 °C pending further analysis.

2.2. PCR amplification and reverse line blot (RLB) assay

The V4 hypervariable region of the piroplasm 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (5′-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3′) (Nijhof et al., 2003, 2005). Platinum Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group,