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Short Communication

Evaluation of a TaqMan real-time PCR for the detection of *Theileria parva* in buffalo and cattle

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ABSTRACT

A real-time PCR assay based on TaqMan probe chemistry was developed for the detection of *Theileria parva* DNA in blood samples. It uses a *Theileria* genus-specific PCR primer set and a *T. parva*-specific probe to amplify and hybridize with a species-specific part of the 18S rRNA gene of the parasite. The test was evaluated using positive and negative reference blood samples and shown to be specific for *T. parva*. Analytical sensitivity was determined by testing a dilution series of *T. parva* positive blood. It was shown to be able to detect parasitaemia as low as 2×10^{-6}%. The Taqman assay results were also compared with that obtained with the real-time hybridization probe PCR assay, which is currently employed as the official test for the diagnosis of *T. parva* infections in buffalo and cattle and was shown to be equally sensitive. A panel of 1164 field samples was screened using both assays and 164 samples tested positive in both tests, indicating a good correlation.

1. Introduction

Theileria parva, a tick-borne protozoan parasite, is infective to cattle and the Cape buffalo (*Syncerus caffer*), the latter act as a reservoir host for the infection (Uilenberg, 1981). *T. parva* infections cause severe and fatal disease syndromes in cattle known as East Coast fever (ECF), Corridor disease (CD) and January disease (Zimbabwean theileriosis). It occurs predominantly in eastern, central and southern Africa (Uilenberg, 1981). In South Africa, *T. parva* is transmitted by *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* from carrier buffalo to cattle, causing Corridor disease (Neitz, 1955; Walker et al., 1981). Contact between infected buffalo and cattle in the presence of the vector ticks inevitably result in disease outbreaks. Corridor disease usually occurs in an acute form and was first reported in South Africa in 1953 where it caused the deaths of 300 cattle in a single outbreak (Neitz, 1955).

Since the eradication of cattle–cattle transmitted ECF in South Africa in 1955 (Anon, 1981), buffalo-derived Corridor disease became the only important form of cattle theileriosis. Outbreaks usually occur in the CD endemic disease areas where cattle are farmed in close proximity to game ranches and nature reserves where infected buffalo are found. Corridor disease is a controlled disease in South Africa and buffalo have to be certified *T. parva*-free before relocation to non-endemic areas will be allowed by veterinary authorities. Accurate diagnosis of *T. parva* in carrier buffalo is therefore crucial to prevent the spread of the

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disease to non-endemic areas where the vector ticks are present.

A recent study described the development of a hybridization probe real-time PCR assay for the detection of *T. parva* and it was reported to be more sensitive and faster to perform than conventional PCR (Sibeko et al., 2008). However, the interpretation of the results can be difficult due to the concurrent amplification of closely related *Theileria* species, such as *T.* sp. (buffalo) (Allsopp et al., 1993; Sibeko et al., 2008; Zweygarth et al., 2009). In this study we report the development and evaluation of a real-time TaqMan probe PCR assay for the detection of *T. parva* with a Roche LightCycler instrument. We show that this is a simple, sensitive, specific and rapid molecular test for the detection of *T. parva*.

2. Materials and methods

2.1. Control and field samples

Three positive control samples, referred to as the gold standards, included a naturally infected *T. parva* carrier buffalo and two experimentally infected splenectomised bovines. The *T. parva* carrier status of these animals was confirmed by xenodiagnosis (Sibeko et al., 2008). Negative control blood was obtained from cattle reared and maintained under tick-free conditions in the laboratory. The field samples consisted of 1164 blood samples collected in EDTA tubes by veterinary authorities countrywide and sent to the Onderstepoort Veterinary Institute (OVI) for diagnostic purposes.

2.2. Primer and probe design

Theileria genus-specific primers were selected from the sequence data of the 18S rRNA gene of *T. parva* (GenBank L02366). The Theileria S (5′-ATTGTTGCAGTTAAAAAGCTC GTA-3′) and Theileria B (5′-GCAAAAGCCTGCTTTGAGCAC-3′) primers generated a 147 bp PCR fragment. The TaqMan probe L1 FAM-TTCGGACGGAGTTCGC+T+T+TG-BBQ was designed to hybridize with a *T. parva*-specific sequence in the V4-hypervariable region of this gene fragment and allowed differentiation with *T.* sp. (buffalo), as the sequences differ at three positions within this region. The probe was 5′-terminal labeled with 6-carboxyfluorescein (FAM) as the reporter dye and contained a 3′-terminal black berry quencher (BBQ) dye. The "+" in the probe sequence denotes the location of locked nucleic acid (LNA) bases, enhancing probe binding and increasing the specificity for *T. parva*.

2.3. DNA extraction and TaqMan PCR

Genomic DNA was extracted from the blood samples using the MagNa Pure LC and the MagNa Pure Large Volume Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The extracted DNA samples were stored at −20 °C until further use. A PCR mixture consisting of 4 μl of 5× LightCycler FastStart DNA Master^{PLUS} Hybridization Probe reaction mix (Roche Diagnostics), 0.5 μM of each primer, 0.4 μM of the TaqMan probe, 1 U uracil deoxy-glycosylase (UDG) (Roche Diagnostics) and 2.5 μl of template DNA was prepared in a 20 μl final volume. The PCR amplification was performed on the LightCycler 2.0 instrument (Roche Diagnostics) under the following conditions: 40 °C for 10 min to activate UDG, 95 °C for 10 min to achieve optimal FastStart *Taq* DNA polymerase activity, followed by 50 cycles of 95 °C for 10 s, 62 °C for 5 s and 72 °C for 20 s. Fluorescence was monitored during extension in the 530 nm channel.

2.4. Specificity of the T. parva TaqMan PCR

DNA preparations from the three positive *T. parva* (gold standard) and negative control samples as well as from other *Theileria* species, including *T. buffeli*, *T. mutans*, *Theileria* sp. (buffalo) and *T. taurotragi*, were subjected to the *T. parva* TaqMan PCR to evaluate the analytical specificity. DNA from other microorganisms that are likely to be present in buffalo and/or bovine samples were also tested and include *Anaplasma centrale*, *Babesia bigemina*, *B. bovis*, *Ehrlichia ruminantium* and *Trypanosoma* spp. Bacteria that could occur as incidental contaminants or infections such as *Arcanobacterium pyogenes*, *Bacillus lactosporus*, *Staphyloccocus aureas*, *Escherichia coli*, *Salmonella typhimurium* and *Enterococcus facium* were also tested.

2.5. Sensitivity of the T. parva TaqMan PCR

In order to determine the analytical sensitivity of the TaqMan PCR, *T. parva* infected blood from the positive control buffalo with 0.002% parasitaemia was subjected to 10-fold serial dilutions with uninfected blood. The dilutions ranged from 2×10^{-3} to 2×10^{-6}% parasitaemia and were prepared in 30 replicates. The DNA was extracted and the PCR performed as described above. Efficiency of the reaction was determined from the slope of the regression curve obtained from the serial dilution as implemented in the LightCycler 2.0 Version 4.0 software (Roche) and described by Rebrikov and Trofimov (2006).

2.6. Comparison of the real-time T. parva TaqMan PCR with the hybridization PCR

The TaqMan probe test was compared with the *T. parva*-specific hybridization probe test that is the current, official diagnostic assay in use (Sibeko et al., 2008). This was done by testing a panel of 1164 blood samples that had been submitted to the laboratory for routine diagnoses. DNA extraction was performed as described above and subjected to the TaqMan real-time PCR. The DNA extracted from the dilution series of the parasite in blood were used for comparing the sensitivity of the two tests. The hybridization probe real-time PCR protocol was followed as described (Sibeko et al., 2008).

3. Results

3.1. Analytical specificity of the T. parva TaqMan PCR

An exponential increase in real-time fluorescence at 530 nm is indicative of a positive *T. parva* signal (Fig. 1), and