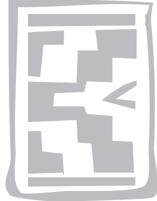

Prevalence of *Theileria equi* and *Babesia caballi* infections in horses belonging to resource-poor farmers in the north-eastern Free State Province, South Africa

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ABSTRACT

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The prevalence of *Theileria equi* and *Babesia caballi* infections in the north-eastern Free State Province of South Africa was determined by examination of thin and thick Giemsa-stained blood smears, IFAT and PCR. No parasites were detected by microscopy from any blood samples collected at five study sites, Qwaqwa, Kestell, Harrismith, Vrede and Warden. Of the tested serum samples, 28/29 (96.5 %), 20/21 (95.2 %) and 42/42 (100 %) were positive by IFAT for *T. equi* infections in Harrismith, Kestell and Qwaqwa, respectively, and 5/29 (17.2 %), 13/21 (61.9 %) and 30/42 (71.4 %) were sero-positive for *B. caballi* infections in Harrismith, Kestell and Qwaqwa, respectively. All DNA samples from the study sites were negative for *B. caballi* infections by PCR, but five samples, two from each of Kestell and Warden and one from Vrede, were PCR positive for *T. equi* infections. The high prevalence of antibodies against *T. equi* and *B. caballi* in the sampled horses indicates that the animals had been exposed to *T. equi* and *B. caballi* infections but the absence of parasitaemia and very low number of positive PCR samples, however, imply that *T. equi* and *B. caballi* are endemically stable in the north-eastern Free State Province.

Keywords: *Babesia caballi*, Free State, *Theileria equi*

INTRODUCTION

Equine piroplasmosis is a tick-borne disease affecting horses, mules, donkeys and zebras worldwide (De Waal 2000) caused by two haemoprotozoan parasites, *Theileria equi* and *Babesia caballi*. The disease can be acute, sub acute or chronic and is characterized by fever, anaemia, icterus, hepatomegally, splenomegally and, in some cases, death (Friedhoff & Soule 1996; Knowles 1996). Most clinical cases of equine piroplasmosis in southern Africa are caused by *T. equi*, which usually shows a high prevalence, while *B. caballi* infection is usually not clinically apparent and usually has a lower prevalence (De Waal 1995, 2000; Zweygarth, Lopez-Rebollar, Nurton & Guthrie 2002). Horses infected with *T. equi* and/or *B. caballi* may remain carriers for long periods and act as sources of infection for ticks, which in turn act as vectors of the disease (De Waal 2000; Zweygarth *et al*. 2002). In South Africa, the

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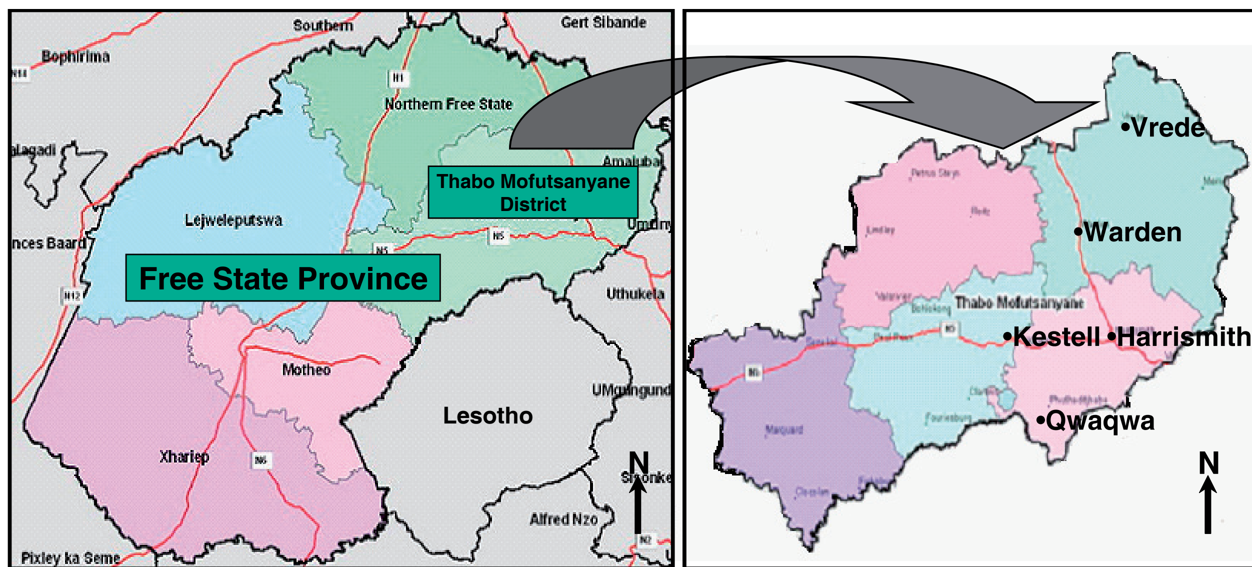

FIG. 1 Maps of the Free State Province and the Thabo Mofutsanyane District Municipality showing the location of the five study sites: Harrismith, Kestell, Qwaqwa, Vrede and Warden. The maps were obtained from the website of the Municipality Demarcation Board of the Republic of South Africa (www.demarcation.org.za)

ixodid tick, *Rhipicephalus evertsi evertsi* is the main vector of *T. equi* and *B. caballi* (De Waal & Potgieter 1987).

Theileria equi and *B. caballi* infections are usually diagnosed by microscopic examination of Giemsa- or Wright-stained blood films (Saal 1964), but these methods have poor sensitivity during very low parasitaemia. Serological assays, such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescent antibody test (IFAT) are also commonly used for detection of *T. equi* and *B. caballi* infections (Bose, Jorgensen, Dalgleish, Friedhoff & De Vos 1995; Bruning 1996; Weiland 1986) but cannot distinguish between current and past infections due to persistence of antibodies even when parasites are cleared from the animal. PCR is the commonly used molecular technique for the diagnosis of *T. equi* and *B. caballi* and has been reported to be highly specific and sensitive (Bashiruddin, Camma & Rebelo 1999; Rampersad, Cesar, Campbell, Samlal & Ammons 2003).

The prevalence of some of the tick-borne diseases, poultry diseases and helminth infections in domestic livestock of resource-poor farmers in the north-eastern Free State Province has recently been reported, as well as the ticks occurring in the region (Hlatshwayo, Mbati & Dipeolu 2002; Mbati, Hlatshwayo, Mtshali, Mogaswane, De Waal & Dipeolu 2002; Nyaile, Thekisoe, Bisschop & Mbati 2003; Thekisoe, Mbati & Bisschop 2003; Tsotetsi & Mbati 2003; Mtshali, De Waal & Mbati 2004). The current study was aimed at documenting *T. equi* and *B. caballi* infections in horses in the north-eastern Free State Province by conventional parasitological methods, IFAT and DNA amplification by PCR. A questionnaire survey in order to assess the perceptions, attitudes and expectations of farmers in the study area with regard to *T. equi* and *B. caballi* infections was also conducted.

MATERIALS AND METHODS

Description of the study area

The north-eastern Free State Province includes towns situated in the Thabo Mofutsanyane district municipality which borders Lesotho on the south, Motheo, Lejweleputswa and northern Free State district municipalities on the south-west, west and north, respectively, while Mpumalanga and KwaZulu-Natal provinces are on the north-east and eastern borders, respectively. Blood samples were collected between August 2003 and October 2005 from horses of 38 resource-poor farmers located in five study sites within the north-eastern Free State: Harrismith (four farms), Kestell (three farms), Qwaqwa (16 villages), Vrede (11 farms) and Warden (four farms). Fig. 1 shows the exact locations of the study sites within Thabo Mofutsanyane district.

Collection of blood samples

A total of 122 blood samples were collected from horses at the five localities, viz. 29 from Harrismith, 21 from Kestell, 42 from Qwaqwa, 19 from Vrede

and 11 from Warden. Ten milliliters of blood were collected in both silicone and EDTA-coated vacutainers by venipuncture of the jugular vein, and were kept in a cooler-box before transport to the laboratory. Blood samples were also collected from seven horses known to be infected with *T. equi* parasites from Kaalplaas farm of the Onderstepoort Veterinary Institute (OVI), Pretoria, and were used as controls for *T. equi*.

Preparation of thin and thick blood smears

Thin and thick blood smears were prepared from the unclotted blood in the EDTA-coated vacutainers and stained with Giemsa as described by De Waal (1999). The parasitaemia was estimated by counting parasitized red blood cells (RBC) in eight different fields consisting of an average of 800 red blood cells per field using a formula in which the number of infected RBC is divided by the total number of uninfected RBC x 100. A smear was considered negative based on the criterion described by De Waal & Potgieter (1987), i.e. smears that reveal no parasites after 5 min examination are judged to be negative.

Serum preparation and DNA extraction

The blood in silicone-coated vacutainers was left for 24 h at room temperature and then centrifuged at 200 x *g* for 5 min (De Waal & Potgieter 1987). The resulting sera were then placed in cryogenic vials and stored at –35 °C until used. Genomic DNA was extracted from horse blood by the phenol-chloroform method (Sambrook & Russel 2001) with minor modifications. Briefly, 400 µℓ of blood was treated with five units of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) buffer and then centrifuged at 14 000 x *g* for 2 min. The resulting pellet was washed five times in TE, removing the erythrocyte ghost layer with every wash, and re-suspended in 400 µℓ of distilled water. After incubation at 37 °C for 1 h with 100 µg/mℓ of proteinase K, 1 % SDS and 1 % sarcosine, DNA was extracted with phenol-chloroform and precipitated with ethanol at –34 °C, and was finally dissolved in 20 µℓ distilled water.

Indirect immunofluorescent test (IFAT)

Antigen slides, conjugate and control sera were obtained from the OVI. Batches of antigen slides were removed from storage at –20 °C, incubated at 37 °C for 10 min and then fixed in cold acetone at –20 °C. Two-fold dilutions, of the test and control sera, starting at 1/40, 1/80 and 1/160, were prepared using PBS (Tenter & Friedhoff 1986). A drop (~ 4 µℓ) of each diluted serum was pipetted into each respective circle of the antigen slide, incubated at 37 °C for 30 min and then washed off with PBS.

A second wash was done using distilled water and the slides were allowed to air dry, after which 2 µℓ of conjugate dilution was added and incubated at 37 °C for 30 min. The conjugate was washed in PBS for 10 min and after air-drying, mounting fluid consisting of 50 % glycerin and 50 % PBS was placed on each slide and covered with 24 x 50 mm cover slip. The slides were observed under a fluorescent microscope using a 50x water objective (Tenter & Friedhoff 1986). Test readings were ranked as negative for slides with no specific fluorescence when observed under the fluorescent microscope, positive for those that presented strong fluorescence at a serum dilution of 1/80 and positive/negative for those that showed a weak fluorescence (OVI 1999).

Polymerase chain reaction (PCR)

A primer pair targeting the EMA1 gene (Accession no. AY058899) of *T. equi* with a product of 743 bp was used for amplification of *T. equi* DNA, and a primer pair designed from the Bc48 gene (Accession no. AB017700) with a product of 179 bp were used for amplification of *B. caballi* DNA (Table 1). The PCR was conducted in a total volume of 50 µℓ containing PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 2 mM each of the 4 dNTPs, 5 pmol of each primer and five units of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction mixture was heated at 94 °C for 10 min (denaturation step) and subjected to 35 cycles each at 94 °C for 45 s, 60 °C for

TABLE 1 PCR primers used for amplification of *T. equi* and *B. caballi* DNA

Parasite	Target gene	Primer sequence
Theileria equi	EMA1	F: 5'GCATCCATTGCCATTTCGAG3' R: 5'TGCGCCATAGACGGAGAAGC3'
Babesia caballi	Bc48	F: 5'CGGCTGCTATGGTTATTCAG3' R: 5'AGAGTGCAACCGAGCAATGC3'

1 min (annealing), and 2 min at 72 °C with a final extension at 72 °C for 7 min. The PCR products were electrophoresed on a 1 % agarose gel, stained with ethidium bromide and visualized under UV light. The positive PCR products were purified using the QIA-quick gel extraction Kit (Qiagen, USA). The nucleic acid sequence was determined with the BigDye terminator cycle sequencing kit (Applied Biosystems, Japan).

Questionnaire survey

Questionnaires were completed by a total of 23 farmers in Harrismith, Kestell and Qwaqwa. The aim of the survey was to determine the level of knowledge of resource poor farmers in the north-eastern Free State Province concerning equine piroplasmosis, ranging from knowledge of its cause and transmission, whether they could recognize the clinical signs and whether they had knowledge on control measures for the disease.

RESULTS

Microscopy and IFAT

Neither *T. equi* nor *B. caballi* was detected in the thick and thin blood smears made from the blood on samples from Harrismith, Kestell, Qwaqwa, Vrede and Warden (Table 2). However, antibodies against *T. equi* and *B. caballi* were detected by IFAT in the blood of some of the horses in Harrismith, Kestell and Qwaqwa. Out of a total of 99 samples from these three study sites, 98 % were *T. equi* positive and 48 % were *B. caballi* positive (Table 2), while 58 % had mixed infections. *Theileria equi* was detected in three of the seven horses from Kaalplaas in both thick and thin smears, which were used as a positive control group for *T. equi*, while serology showed the presence of antibodies against *T. equi* in all of them (Table 2).

Gene amplification by PCR

All the blood samples from Kaalplaas were PCR positive for *T. equi*, while only five samples (two from Kestell, two from Warden and one from Vrede) were PCR positive for this parasite (Fig. 2). These PCR-positive products were sequenced and the sequence fragments were identical ranging from 83–100 % nucleic acid sequence identities, with other EMA1 genes in the NCBI GenBank database determined by BLASTn search (data not shown) (www.ncbi.nlm.nih.gov/BLAST). All the north-eastern Free State Province and Kaalplaas DNA samples were PCR negative for *B. caballi* using primers targeting the Bc48 gene.

Questionnaire survey

None of the farmers in the study sites who completed questionnaires had any scientific knowledge on equine piroplasmosis. Forty-eight percent of them admitted observing clinical signs such as nasal discharge where affected animals appeared to suffocate from accumulation of mucus with occasional

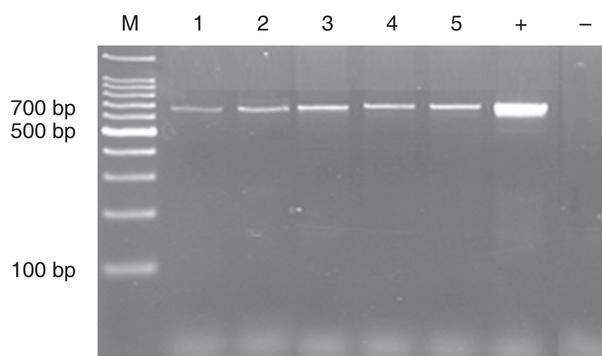

FIG. 2 Five DNA samples positive by PCR for *Theileria equi* using EMA1 primers. Lane M: 100 bp marker; Lanes 1–2: Kestell samples; Lane 3: Vrede sample; Lanes 4–5: Warden samples; Lane +: Positive control (*T. equi*—USDA strain); and Lane –: Negative control (*B. caballi*—USDA strain)

TABLE 2 Microscopic, IFAT and PCR results for detection of *T. equi* and *B. caballi* infections

Study site	No. of samples	Microscopy (%)		IFAT (%)		PCR (%)	
		T. equi	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*
Harrismith	29	0 (0)	0 (0)	28 (96.5)	5 (17.2)	0 (0)	0 (0)
Kestell	21	0 (0)	0 (0)	20 (95.2)	13 (61.9)	2 (9.5)	0 (0)
Qwaqwa	42	0 (0)	0 (0)	42 (100)	30 (71.4)	0 (0)	0 (0)
Vrede	19	0 (0)	0 (0)	ND*	ND	1 (5.2)	0 (0)
Warden	11	0 (0)	0 (0)	ND	ND	2 (18.1)	0 (0)
Kaalplaas	7	3 (43)	0 (0)	7 (100)	3 (43)	7 (100)	0 (0)

ND* – Not done

sneezing, but these were signs which could not necessarily be linked to equine piroplasmosis. All of them indicated that they believed ticks did have an effect on their livestock's health although they could not specifically single out the disease(s) they transmitted. A common inexpensive method of tick control used by those who owned a relatively small number of livestock included burning the grass of the grazing area in winter while dipping in an acaricide was done by those who owned a larger numbers of animals.

DISCUSSION

In this study, neither *T. equi* nor *B. caballi* was detected by microscopy in the samples from Qwaqwa, Harrismith, Kestell, Vrede and Warden. Beaver, Jung & Cupp (1984) stated that blood smears are useful for studying the morphological changes of blood cells and blood parasites. However, the main disadvantage of this method is that the volume of blood used for preparing the smears is small, making the detection of a low parasitaemia and of carrier animals difficult (Ambrosio & De Waal 1990). Demonstration of parasites by microscopy was only successful in three of the seven control horses from Kaalplaas which had a known history of *T. equi* infections.

Indirect immunofluorescent test is the most widely used serological diagnostic test. As shown in Table 2, the prevalence of antibodies against *T. equi* in the samples from Kestell, Harrismith and Qwaqwa was higher than that of *B. caballi*. Results of the current study correspond to those of a previous sero-epidemiological survey of *T. equi* and *B. caballi* in the Northern and Eastern Cape provinces in which it was found that the prevalence of *T. equi* infections was relatively higher than that of *B. caballi* (Gummow, De Wet & De Waal 1996). Moreover, *T. equi* parasites are reported to propagate faster than *B. caballi* (Holman, Frerichs, Chieves & Wagner 1993), which also explains why the prevalence and pathogenicity of *T. equi* is higher than those of *B. caballi* in endemic areas (Schein 1988; De Waal 2000; Alhassan, Pumidonming, Okamura, Hirata, Battsetseg, Fujisaki, Yokoyama & Igarashi 2005).

All the DNA samples from the study areas were negative for *B. caballi* infections by PCR. *Babesia caballi* generally produces a low parasitaemia (Potgieter, De Waal & Posnett 1992; Holman *et al.* 1993) which makes it extremely difficult to detect in blood smears and even DNA probes have been reported to detect *B. caballi*-specific DNA at irregular intervals during the clinical course of an infection (Posnett & Ambrosio 1991; Holman *et al.* 1993). The respective sequences of *T. equi* were identical to other EMA1 genes of *T. equi* in the Genbank database, thereby confirming that our results are not falsely positive. The two PCR-positive animals from Kestell also showed the presence of antibodies by IFAT but parasitaemia was not within detectable levels by microscopic examination.

The current study has demonstrated a high prevalence of antibodies against *T. equi* and *B. caballi* in the horses by IFAT. Although the presence of antibodies did not distinguish current from previous infections, these results do, however, indicate that the horses in the study region are exposed to *T. equi* and *B. caballi* infections. However, it can be concluded that five *T. equi* PCR-positive animals were still harbouring the parasites, although at a very low parasitaemia level, while the remainder might have recovered from the infections before the sampling period. Furthermore, the questionnaire survey revealed that these animals had never been treated for equine piroplasmosis, which suggests that they are possibly randomly re-exposed to *T. equi* and *B. caballi* infections several times during their lifetime as the vector tick (*R. evertsi evertsi*) is ever present in the region (Hlatshwayo *et al*. 2002; Mbati *et al*. 2002). The PCR method, with its high specificity, can be used in confirmatory diagnosis while IFAT is useful in large-scale epidemiological surveys.

In conclusion, this study has demonstrated the existence of *T. equi* and *B. caballi* in the north-eastern Free State Province which confirms previous reports that this tick-borne disease is widespread in South Africa (De Waal 1995; Gummow *et al*. 1996). However, the negative Giemsa-stained blood smears, high incidence of positive serological assay, presence of the tick vector (Hlatshwayo *et al*. 2002; Mbati *et al*. 2002) and absence of clinical signs from the horses imply that the study region is endemically stable for *T. equi* and *B. caballi* infections. Data from this study can serve as a basis for future large-scale epidemiological studies on equine piroplasmosis in this region. There is a need for scientific education for resource-poor farmers on diseases of economic and veterinary importance as this can improve their overall control.

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