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Full Length Research Paper

The detection and characterization of multiple tick-borne pathogens in cattle at Ficksburg and Reitz (Free State Province, South Africa) using reverse line blot hybridization

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Ticks and tick-borne diseases are widely distributed in southern Africa and limit livestock production of commercial farmers and the subsistence economy of the resource-poor farmers. Reverse line blot hybridization was used to survey the prevalence of tick-borne pathogens in cattle of commercial and small scale resource-poor farmers within the north-eastern region of the Free State Province, South Africa. Blood was collected from 74 clinically healthy cattle in five farms at Ficksburg and Reitz. DNA was extracted and subjected to two different polymerase chain reactions (PCRs): an 18S rRNA-based PCR for amplification of *Theileria* and *Babesia* species and a 16S rRNA-based PCR for amplification of *Ehrlichia* and *Anaplasma* species. Reverse line blot (RLB) assay was then performed with family and species-specific DNA probes. Forty nine percent of the samples were positive for *Anaplasma marginale*, 35.1% for *Theleria taurotragi*, 18.9% for *Ehrlichia* sp. (Omatjenne), 2.7% for *Babesia bigemina* and 1.4% for both *Anaplasma bovis* and *Babesia bovis*. The phylogenetic analysis of three representative strains tested in this study also indicated that *Anaplasma* sp. clone SAFS01 and *Anaplasma* sp. clone SAFS02 clustered with their corresponding species, while the *Ehrlichia* sp. clone SAFS03 clustered separately from another strain of the same species published in the GenBank database.

Key words: Reverse line blot hybridization, *Anaplasma*, *Babesia*, *Ehrlichia*, *Theileria*, 16S rDNA, phylogeny.

INTRODUCTION

Animal diseases in general and tick and tick-borne diseases in particular are among the many factors which directly and indirectly hamper the growth of the livestock sector (Sansoucy, 1995). Tick-borne diseases of major economic importance in southern Africa which affect cattle are heartwater caused by *Ehrlichia ruminantium*, babesiosis caused by *Babesia bigemina* and *Babesia bovis*, anaplasmosis caused by *Anaplasma marginale* and theileriosis caused by *Theileria parva.* Of much lesser importance in cattle are the generally non-pathogenic mild theileriosis caused by *Theileria mutans*,

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Theileria velifera and *Theileria taurotragi*, spirochaetosis (borreliosis) caused by *Borrelia theileri*, benign babesiosis caused by *Babesia occultans,* and bovine ehrlichiosis caused by *Ehrlichia bovis* (Horak, 1982).

Several techniques for the detection of these blood-borne pathogens have been developed separately for a number of species (Figueroa and Buening, 1995). Polymerase chain reaction (PCR) detection of *T. parva* using repetitive DNA sequences or gene-specific primers has been developed (Bishop et al., 1992). Species-specific PCR assays are available for *B. bovis* and *B. bigemina* (Calder et al., 1996; Figueroa et al., 1992). Figueroa et al. (1993) reported the first integrated approach for the sensitive detection and differentiation of *B. bigemina*, *B. bovis* and *A. marginale* in a 16S-18S rRNA gene multiplex PCR. There is need for a universal test to simultaneously detect and differentiate all protozoan parasites and bacteria that could be present in the blood of carrier cattle. A reverse line blot (RLB) technique fulfilling these criteria was developed to detect four different *Borrelia* species in ticks (Rijpkema et al., 1995), as well as for the simultaneous detection of bovine *Theileria* and *Babesia* species (Gubbels et al., 1999).

RLB was developed from a reverse dot blot assay for the diagnosis of sickle cell anaemia (Saiki et al., 1988). The essence of both assays is the hybridization of PCR products to specific probes immobilized on a membrane in order to identify differences in amplified sequences. In the “line” approach, multiple samples can be analyzed against multiple probes to facilitate simultaneous detection (Gubbels et al., 1999).

Most epidemiological studies have confirmed that RLB assay is very practical, since only one PCR is required for simultaneous detection of several different blood parasites. When cattle are screened for their infection status, information can be collected concerning the epidemiology of pathogenic and non-pathogenic parasites (Gubbels et al., 1999). In this study, the RLB hybridization assay was used to detect multiple tick-borne pathogens infecting cattle in Ficksburg and Reitz regions (Free State Province, South Africa). We also studied the phylogenetic relationship between the representative bacterial strains tested in this study based on 16S rDNA sequences.

MATERIALS AND METHODS

Sample collection

Cattle from small-scale resource-poor farmers are kept under small-scale farming systems at Ficksburg and Reitz in the Free State province, South Africa. During the day, the cattle graze on communal pastures, and the usual grazing time is 7 to 9 h per day, the duration being determined by the season of the year. At night, the cattle are herded, milked and kept in livestock enclosures.

Cattle (n = 74) were individually restrained in a mobile facility, and blood samples were collected from the tail vein into 7-ml EDTA-coated vacuum tubes (Becton Dickinson Vacutainer Systems Europe, Plymouth, UK), transported to the laboratory on ice and stored at -20°C until further processing.

DNA extraction

DNA isolated from 1 ml of blood was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The extracted DNA was eluted in 50 µl of elution buffer and stored at 4°C until analysis by PCR. The concentration of DNA was measured spectrophotometrically using the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, USA).

Amplification of 16S rDNA gene

A 16S rDNA-based PCR assay amplifying a fragment spanning the V1 region of *Anaplasma* and *Ehrlichia* species was performed using the forward primer Ehr-F (5′-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3′) as previously described by Schouls et al. (1999) and the biotinylated reverse primer Ehr-R (5′-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3′) as modified by Bekker et al. (2002). The primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa). PCR conditions for amplifying the 16S rDNA gene were similar to those described previously (Bekker et al., 2002).

Reverse line blot hybridization

The RLB hybridization assay used another set of primers to amplify a 460- to 520-bp fragment of the 18S rRNA gene spanning the variable region conserved for *Theileria* and *Babesia* species. The forward primer RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and biotin-labelled reverse primer RLB-R2 (5′-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3′) were designed to hybridize with regions conserved for the genera *Theileria* and *Babesia* (Nijhof et al., 2003, 2005). The sequences of specific oligonucleotide probes that were hybridized onto a reverse line blot membrane contained a N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl, *N,N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (Isogen). The PCR products were allowed to hybridize with species-specific oligonucleotide probes applied onto a Biodyne C blotting membrane. Hybridization and subsequent stripping of the membrane were performed as described by Gubbels et al. (1999) with minor modifications. Briefly, the species-specific oligonucleotides were diluted in 150 µl of 0.5 M $NaHCO_3$ (pH 8.4). PCR products were diluted to a final volume of 150 µl in 2 x SSPE, 0.1% SDS. The post-hybridization washes were carried out at 42°C for 10 min in 2 x SSPE, 0.5% SDS.

Cloning and sequencing of PCR amplicons

Purified PCR fragments of 16S rDNA genes were cloned into the pGEM®-T Easy vector (Promega, Madison, USA) as recommended by the manufacturer. Subsequently, plasmids harbouring the cloned fragments were purified using a standard alkaline lysis method and digested with *Eco*RI to confirm the size of the insert by gel electrophoresis. The 16S rDNA inserts were sequenced using an ABI Prism 3100 Automatic DNA Sequencer (BigDye Terminator Cycler Sequencing Kit, Perkin Elmer, Applied Biosystems, USA).

Phylogenetic analysis

The nucleotide sequence data were assembled, aligned and analyzed using Clustal X (Staden et al., 2000) and MEGA v4.1

Table 1. Identities between the determined 16S rDNA sequences and those retrieved from GenBank.

GenBank strain (accession no.)	*Anaplasma* sp. clone SAFS01 (JN004066)	*Anaplasma* sp. clone SAFS02 (JN004067)	*Ehrlichia* sp. clone SAFS03 JN004068)
A. marginale, Florida (CP001079)	99.4	93.1	95.3
A. marginale, China (HM538192)	99.4	93.1	95.3
A. marginale, Uruguay (AF414877)	99.4	93.1	95.3
A. marginale, Zimbabwe (AF414878)	99.4	93.1	95.3
A. marginale, Israel (AF414876)	99.4	93.1	95.3
A. marginale, Australia (AF414874)	99.4	93.1	95.3
A. marginale, South Africa (AF414873)	99.4	93.1	95.3
A. marginale, St. Maries (CP000030)	99.4	93.1	95.3
A. ovis, OVI (AF414870)	97.8	92.8	94.4
A. ovis, Chende (AJ633052)	97.5	93.1	94.0
A. ovis, CGX (EF587237)	97.2	93.4	93.7
A. centrale, Israel (CP001759)	98.1	93.4	95.0
A. centrale, CC (EF520686)	98.1	93.2	94.8
A. centrale, 16 (EF520690)	98.0	93.2	94.8
A. phagocytophilum, c3a (EU839849)	92.9	94.4	95.3
A. bovis, G41 (HQ913646)	92.8	99.1	95.0
A. bovis, ES1090 (HQ913645)	93.2	98.4	95.0
A. platys, Hd83 (GQ395385)	92.8	95.9	96.6
A. platys, Okinawa (AY077619)	92.8	95.9	96.6
Ehrlichia sp. Omatjenne (U54806)	92.5	95.6	97.2
E. platys, 10 (AY040851)	92.8	95.6	96.2
E. phagocytophila (AY035312)	92.9	94.4	95.3
E. bovis (U03775)	92.8	99.7	95.0

Numbers indicate percentage (%) identity of 16S sequences of our isolates with GenBank strains.

software (Kumar et al., 2008). The phylogenetic tree based on nucleotide sequences of 16S rDNA genes was constructed using the neighbour-joining algorithm of MEGA v4.1 software, with Kimura's 2-parameter distance correction model. Bootstrapping analysis was performed to evaluate the reliability of the topologies by using 1000 bootstrap replications (Felsenstein, 1985).

Nucleotide sequence accession numbers

The 16S rDNA nucleotide gene sequences determined in this study for *Anaplasma* sp. clone SAFS01, *Anaplasma* sp. clone SAFS02 and *Ehrlichia* sp. clone SAFS03 were deposited in the GenBank database under the accession numbers JN004066 to JN004068, respectively.

RESULTS

16S rDNA sequence analysis

In order to amplify the 16S rDNA genes of the isolates tested in this study, universal primers targeting the partial 16S rDNA gene of *Anaplasma* and *Ehrlichia* species were employed for PCR amplifications. PCR assays generated single fragments of an expected size (*ca.* 319 bp). For comparative analysis of sequences, PCR-amplified fragments from representative strains of *A. marginale*, *A. bovis* and *Ehrlichia* sp. (Omatjenne) were cloned and sequenced. The BLAST program of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for homologous sequences.

The percentage identities of 16S rDNA sequences with the sequenced strains in comparison with those published in GenBank database are shown in Table 1. From the 16S rDNA sequence analysis of *Anaplasma* sp. clone SAFS01 strain tested in this study, the highest sequence identity (>99%) was recorded with other *A. marginale* strains published in GenBank (Table 1). The same trend of sequence identity was observed with *Anaplasma* sp. clone SAFS02 strain, which showed 99.1% identity with *A. bovis* G41 (HQ913646), 98.4% identity with *A. bovis* ES1090 (HQ913645) and 99.7% identity with *E. bovis* (U03775). The BLAST search was also performed with the 16S rDNA sequence of *Ehrlichia* sp. clone SAFS03, which revealed the highest identity (97.2%) with that of *Ehrlichia* sp. strain Omatjenne (U54806) originating from sheep.

RLB hybridization results

Primers RLB-F2 and RLB-R2 amplified fragments of approximately 460 to 520 bp corresponding to the

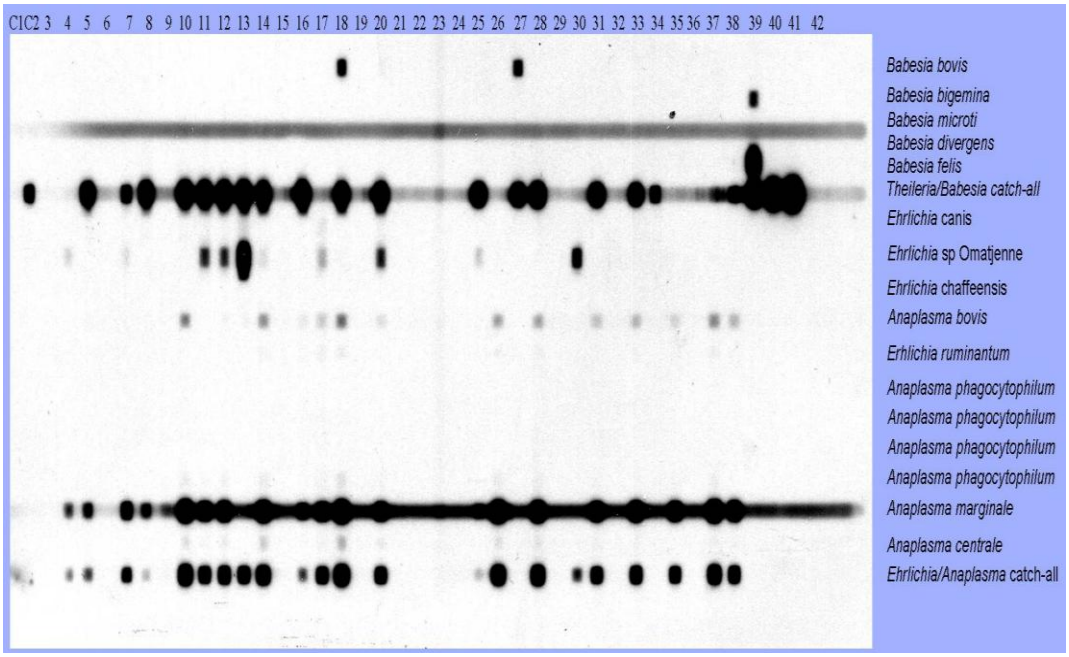

Figure 1. RLB hybridization of PCR products obtained from samples collected in the field. PCR products are applied in vertical lanes. Species-specific oligonucleotide probes are applied in horizontal rows with positive control C1 and negative control C2.

Table 2. Prevalence of tick-borne pathogens {SE = 5.817; SD = 14.250; CI (95%) = 14.954} found in cattle blood samples from Ficksburg and Reitz (Free State Province, South Africa).

Tick-borne pathogen	Percentage (%)
Anaplasma marginale	47.2
Theileria taurotragi	32.4
Ehrlichia sp. Omatjenne	14.9
Babesia bigemina	2.7
Anaplasma bovis	1.4
Babesia bovis	1.4

variable region spanning the 18S rRNA gene specific for *Theileria* and *Babesia* species. These PCR products were hybridized onto the membrane and were shown to bind with specific oligonucleotide probes (Figure 1). All samples positive for PCR showed positive reactions with their corresponding specific probes.

The occurrence of tick-borne pathogens identified in blood samples collected from cattle is shown in Table 2. A total of 47.2% of the studied animals were positive for *A. marginale*, indicating a significantly large number of animals exposed to anaplasmosis. The second most commonly found pathogen belonging to the *Anaplasma* group was *Ehrlichia* sp. (Omatjenne) with a percentage of 14.9. Other tick-borne pathogens that were identified from the blood samples included *T. taurotragi* (32.4%), *B. bigemina* (2.7%), *A. bovis* (1.4%) and *B. bovis* (1.4%).

Phylogenetic analysis

To study the phylogenetic relationship between the strains studied and those published in GenBank, a neighbour-joining tree was constructed based on partial 16S rDNA nucleotide sequences of the three identified strains of *Anaplasma* sp. clone SAFS01 (accession no. JN004066), *Anaplasma* sp. clone SAFS02 (accession no. JN004067) and *Ehrlichia* sp. clone SAFS03 (accession no. JN004068). The corresponding sequences of closely related species were retrieved from the GenBank database.

Phylogenetic analysis showed two distinct groups of pathogens as shown in Figure 2. The first group consisted of *Anaplasma* species, including *A. marginale*, *A. centrale* and *Anaplasma ovis*. The *Anaplasma* sp clone SAFS01 sequence described in this study clustered with other *A. marginale* sequences, but was clearly distinct from *A. centrale* and *A. ovis*. The second group comprised both *Anaplasma* and *Ehrlichia* species. The sequence of *Erhlichia* sp. clone SAFS03 strain determined in this study did not form a cluster with that of another *E.* sp. Omatjenne strain (accession no. U54806) originating from the sheep blood. On the other hand, *Anaplasma* sp. clone SAFS02 sequence formed a clade with that of other *A. bovis* strains published in the GenBank database.

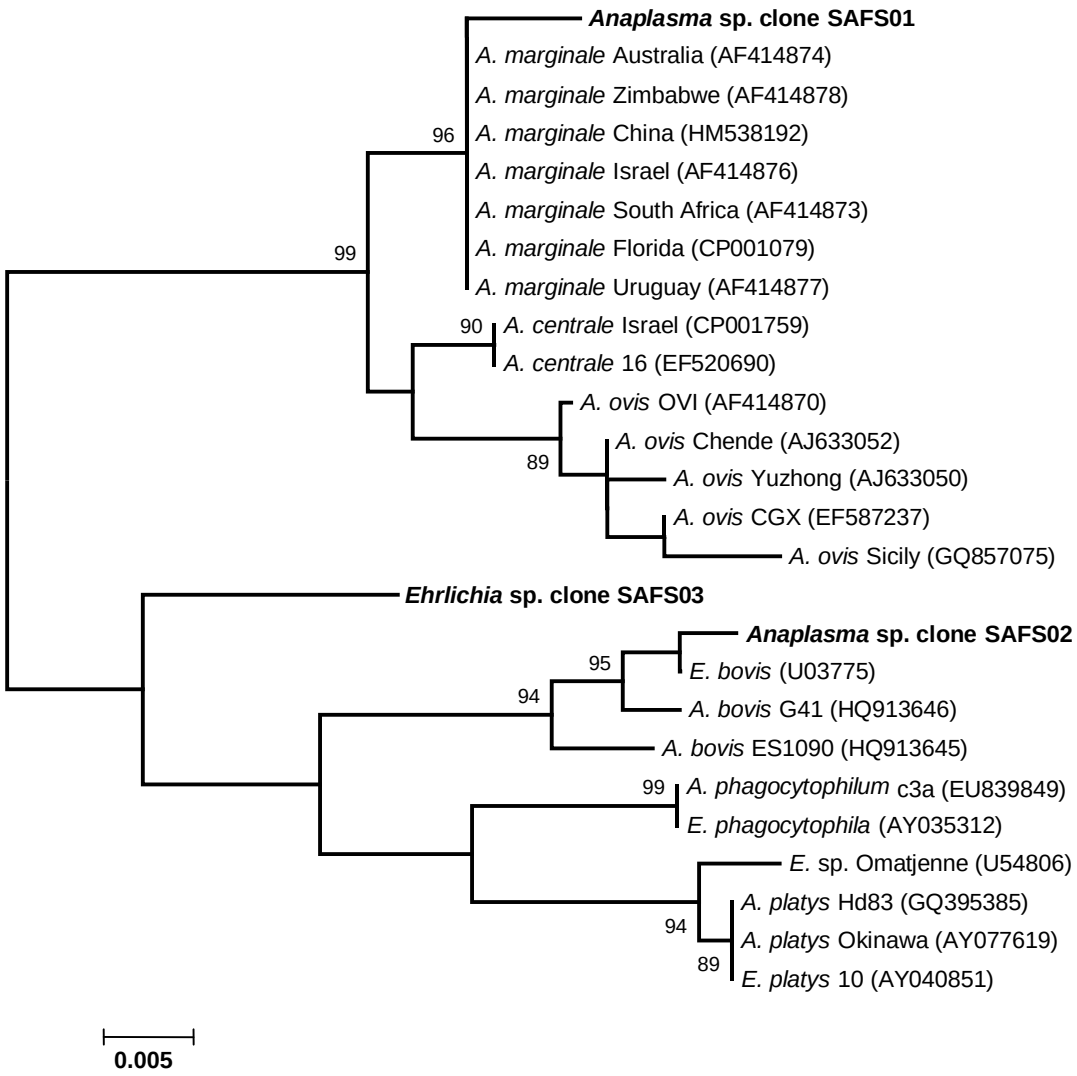

Figure 2. Neighbour-joining tree constructed with partial sequences of 16S rDNA genes from three representative strains of *Anaplasma* sp. clone SAFS01 (accession no. JN004066), *Anaplasma* sp. clone SAFS02 (accession no. JN004067) and *Ehrlichia* sp. clone SAFS03 (accession no. JN004068) tested in this study (indicated in bold), together with closely related taxa whose sequences were retrieved from the GenBank database (accession numbers in parentheses). Numbers at branching points (>80%) represent the results of bootstrap analyses and are expressed as percentages of 1000 replicates. The scale bar represents the number of base substitutions per site.

DISCUSSION

The aim of this study was to detect multiple tick-borne pathogens infecting cattle in Ficksburg and Reitz. The lack of a simple *in vitro* bacteriological culture system, the absence of a suitable laboratory animal model and the inability to distinguish *Anaplasma* species microscopically, all contributed towards the difficulty in diagnosing different species of this group from field samples. To date, there is a variety of conventional methods employed for the identification of pathogens. However, species identification with these methods is rather difficult. As an alternative, the use of PCR-based techniques allows for a quick and sensitive identification of pathogenic microorganisms and protozoa. The RLB method has been shown to be a powerful tool for a simultaneous detection of *Theileria* and *Babesia* species (Gubbels et al., 1999). In this study, an RLB hybridization assay targeting the 18S rRNA region of *Theileria* and *Babesia* species was employed for detecting multiple tick-borne pathogens in field samples collected from Ficksburg and Reitz regions, South Africa.

The RLB hybridization facilitated the detection of a novel *Ehrlichia* sp. strain Omatjenne in the field-collected samples. This species was initially isolated from sheep at Omatjenne region, Namibia (Allsopp et al., 1997). This

study is therefore the first to report on the detection and identification of this microorganism in cattle. There is still little known about the pathogenicity of *Ehrlichia* sp. Omatjenne in cattle. Therefore, this area warrants further studies aimed at defining the pathogenicity of this *Ehrlichia* species in cattle.

When the inoculation rate of haemoparasites is adequate to ensure that all young animals are infected while they are protected by innate and/or colostral immunity, clinical disease is minimal and endemic stability is achieved. Comparing these data with serological data collected in the same region by Mtshali et al. (2004), the results indicate a situation approaching endemic stability with moderate risk of clinical anaplasmosis occurring in the study farms (Ficksburg and Reitz). On these farms, there is a fairly high tick challenge or a combination of tick and biting flies challenge, which means that there are low levels of tick control and possibly little use of acaricides.

The phylogenetic results recorded in this study demonstrated that there is a phylogenetic separation between *Anaplasma* and *Ehrlichia* species. This was particularly the case for the newly identified *Ehrlichia* sp. clone SAFS03, which appeared intermediate between *Anaplasma* and *Ehrlichia* clades. In addition, this strain did not cluster with another *Ehrlichia* sp. Omatjenne strain previously isolated from sheep blood (Allsopp et al., 1997). These findings therefore suggest that there is a genetic heterogeneity amongst the geographical strains of pathogenic parasites.

In conclusion, this work has shed more light on the occurrence of multiple tick-borne pathogens in Ficksburg and Reitz regions. The RLB assay was also employed for pathogen species detection and it revealed the existence of *Ehrlichia* sp. clone SAFS03 from the collected field samples. Finally, the phylogenetic analysis of three representative strains tested in this study indicated that, while *Anaplasma* sp clone SAFS01 and *Anaplasma* sp. clone SAFS02 clustered with their corresponding species, *Ehrlichia* sp. clone SAFS03 strain clustered separately from another strain of the same species published in the GenBank database (U54806).

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