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Molecular analysis of tick-borne protozoan and rickettsial pathogens in small ruminants from two South African provinces

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ABSTRACT

Tick-borne protozoan and rickettsial diseases are a major threat to livestock in tropical and sub-tropical regions of Africa. In this study we investigated the presence and distribution of *Theileria* spp., *Babesia ovis*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium* and SFG *Rickettsia* in sheep and goats from Free State and KwaZulu-Natal provinces. A total of 91 blood samples were screened in this study, 61 from goats and 30 from sheep. PCR assay was conducted using primers based on *Theileria* spp. 18S rRNA, *Babesia ovis* (BoSSU rRNA), *Anaplasma ovis* (AoMSP4), *Anaplasma phagocytophilum* epank1, *Ehrlichia ruminantium* pCS20 and SFG *Rickettsia* OmpA. Overall infection rates of *Theileria* spp., *Anaplasma ovis* and *Ehrlichia ruminantium* were 18 (19.8%), 33 (36.3%) and 13 (14.3%), respectively. The co-infection of two pathogens were detected in 17/91 (18.7%) of all samples, goats having higher rates of co-infection compared to sheep. Phylogenetic tree analysis sequence of pCS20 gene of *E. ruminantium* of this study was found to be in the same clade with Kumm2 and Riverside strains both from South Africa. The phylogram of SSU rRNA of *Theileria ovis* had longer branch length compared to all other sequences most of which were from Asia and Middle East. This study provides important data for understanding the tick-borne diseases occurrence in the study area and it is expected to improve the approach for the diagnosis and control of these diseases.

1. Introduction

Tick-borne diseases (TBDs) are significant constraint to livestock production in tropical and subtropical regions. These diseases lead to losses of meat, milk, wool, skin and manure [1]. Babesiosis, theileriosis, anaplasmosis and ehrlichiosis are TBDs which cause significant threats to small ruminant's health. Several *Babesia* species have been described in small ruminants including *Babesia ovis*, *B. motasi* and *B. crassa* [2]. However, only *B. ovis* and *B. motasi* are known to cause babesiosis [3,4]. The *B. ovis* is highly pathogenic for sheep and normally has mortality rates ranging from 30 to 50% [4].

Theileria species known to infect small ruminants includes *T. lestoquardi*, *T. ovis* and *T. separata* [5]. The *T. lestoquardi* is known to be the most pathogenic particularly in sheep [6] while *T. ovis* is has moderate pathogenicity and normally causes subclinical infections although animals subjected to stressful conditions may develop significant illness [6]. Meanwhile, *T. separata* is considered non-pathogenic but can be fatal to immunocompromised animals or those newly introduced to endemic areas [7].

Anaplasma ovis and *A. phagocytophilum* are the two species of *Anaplasma* found in small ruminants [8,9]. The *A. ovis* normally cause subclinical infection but under stressful condition the animal may develop a disease [10,11]. Ehrlichiosis is caused by *Ehrlichia ruminantium* and normally occurs with a sudden onset of clinical signs and death follows thereafter if not attended.

The presence of the above-mentioned tick-borne pathogens has been

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well documented in Europe [12], Middle East [13] and Asia [14]. In Africa, however, very little information is available. Tick- borne pathogens in small ruminants have been reported in Tunisia [15], Egypt [16] and Morocco [17]. In sub-Saharan Africa the information available is negligible compared to the importance of small ruminants. To the best of our knowledge the only molecular studies to have been done in sub-Saharan Africa were in Ethiopia [18,19] and South Africa [20].

Small ruminants are very important in many African communities as they play great role in poverty alleviation and as source of protein [21]. To improve their health and production, effective control measures of tick-borne diseases are required, and these will be achieved if the detailed and precise information of these diseases is available. Therefore, this study was carried out to broaden the available data on tick-borne pathogens of small ruminants in sub-Saharan Africa. The occurrence of tick-borne pathogens, namely, *Theileria* spp., *B. ovis*, *A. ovis*, *A. phagocytophilum* and *E. ruminantium* was investigated in sheep and goats sampled in the KwaZulu-Natal and Free State provinces of South Africa and their phylogenetic analysis was conducted as well.

2. Material and methods

2.1. Study area

Samples were collected in two provinces of South Africa named KwaZulu-Natal which is in the east coast and Free State which is located centrally as shown in Fig. 1. The Global Positioning System codes of the sample collection sites were as follows: Glen Gweni farm (S27°59′22.2″E032°17′08.4″), Hlambanyathi diptank (S28°10′13.1″E031°48′59.0″), Mvutshini diptank (S28°07′10.3″E032°07′55.0″), Suderpracht farm (S27°56′08.4″E032°20′12.1″), Ekuphindisweni diptank (S-28°020′38.89″E32°078′52.78″) and Seotlong (QwaQwa) (S28. 533°28.8167°E″) (Fig. 1).

2.2. Sample collection and DNA extraction

Blood samples were collected from clinically healthy sheep and goats, using sterile needles and vacutainer tubes which were coated with EDTA. Blood (3–5 ml) was drawn from the jugular vein of the animal, kept in cool boxes and then kept frozen (− 20 °C) in the laboratory until DNA extraction was conducted. A total number of 91 samples were collected from different farms, 61 from goats and 30 from sheep. KZN had a total number of 67 samples while Free State had 24. Samples were collected from animals of around two years of age and above. DNA was extracted from 200 μl of blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, German), following manufacturer's protocol, and stored at − 20 °C until used.

Fig. 1. Map of South Africa indicating sample collection sites (red cycles with numbers) in both provinces of KwaZulu-Natal (KZN) and Free State. Seotlong (QwaQwa) (1), Hlambanyathi diptank (2), Suderpracht farm (3), Glen Gweni farm (4), Ekuphindisweni diptank (5), and Mvutshini diptank (6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Ethical statement

The owners of the selected farms were informed of the study and provided their approval for sampling of their sheep and goats. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Permit for animal experiment: 280,080, DNA experiment 1219-2; Pathogen: 2015727).

2.4. Molecular detection of Theileria spp., Anaplasma, Babesia, Ehrlichia and Rickettsia

Polymerase Chain Reaction (PCR) was used to screen all the samples with species-specific primers (Table 1) obtained from previous studies for *A. ovis*, *A. phagocytophilum*, *B. ovis*, *E. ruminantium* and SFG *Rickettsia* detection. *Theileria* group was screened by using the genus specific 18S rRNA primers and positive samples were sequenced to confirm the species. The thermocycling conditions were set as described previously (Table 1). The reaction mixture had a final volume of 10 μl, containing 0.5 mM of each primer, 1 μl of 10 × standard *Taq* buffer, 1 μl of dNTP mix and 0.1 μl of Ex *Taq* polymerase (Takara – Japan), 1 μl of DNA template and 5.9 μl of double distilled water. The positive controls were positive samples from previous studies [22], while double distilled water (DDW) was used as negative control. PCRs were run in a thermal cycler (Bio rad, USA). The PCR products were electrophoresed on a 1.5% agarose gel and later stained with ethidium bromide and viewed under UV transilluminater.

2.5. Cloning and sequencing

For sequencing, 1–3 positive samples were randomly selected per detected pathogens. Amplicons were extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, German). The concentration of the extracts was checked using NanoDrop 2000 Spectrophotometer. The template (6 μl) was ligated into a pGEM-T Easy vector (2 μl) (Promega, USA) with T4 DNA ligase and restriction buffer (each 1 μl) added and incubated at 16 °C for 3 h then at 4 °C overnight. Transformation of the plasmid into *Escherichia coli* DH5α competent cells was performed. Lysogeny broth (LB) was added and incubated at 37 °C in a shaker incubator for 1 h, then inoculated on LB agar with antibiotic (Ampicillin) and incubated at 37 °C overnight. Colonies were picked and put in LB broth with antibiotic (Ampicillin) incubated at 37 °C overnight in a shaker incubator. Plasmid was extracted using NucleoSpin® Plasmid QuickPure (Macherey-Nagel-German) kit and sequencing PCR of the plasmid was done with dye Terminator Cycle Sequencing Kit (applied Biosystems, USA). For each agar plate a number of four clones were sequenced. Nucleotide sequences were produced using 3100 genetic analyzers (Applied Biosystems, USA). The nucleotide sequence identities and similarities were determined by performing NCBI GenBank BLASTn analysis.

2.6. Phylogenetic analysis

The sequences obtained in this study were compared to sequences of the same pathogens from other regions of the world by phylogenetic analysis using MEGA version 7.0 program [23]. Neighbor-joining