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Detection and molecular characterization of tick-borne pathogens infecting sheep and goats in Blue Nile and West Kordofan states in Sudan

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ABSTRACT

Tick-borne pathogens (TBPs) are common in livestock of sub-Saharan Africa. However, information regarding TBPs in sheep and goats in Sudan is limited. In this study, 178 blood samples of sheep and goats in Blue Nile and West Kordofan states were investigated for TBPs using PCR. Overall, 110 (61.8%) samples were found to be infected with at least one of the following pathogens: *Anaplasma ovis*, *Theileria ovis*, and *Ehrlichia ruminantium*. *Babesia ovis* and *T. lestoquardi* were not identified. *A. ovis* was the most prevalent pathogen (n = 107, 60.1%), followed by *T. ovis* (n = 23, 12.9%) and *E. ruminantium* (n = 1, 0.6%). The prevalence rates of *A. ovis* and *T. ovis* were significantly higher in sheep than in goats. Phylogenetic analysis of *T. ovis* 18S rRNA and *A. ovis msp4, groEL*, and 16S rRNA, revealed that the pathogens identified in this study are clustered together, indicating similar molecular characteristics. Additionally, phylogenetic analysis of *E. ruminantium* pCS20 revealed that *E. ruminantium* in this study belong to the West Africa group, and different to *E. ruminantium* previously identified in ticks from Sudan. We concluded that TBPs are highly prevalent in the study area and continuous monitoring of TBPs in sheep and goats in Sudan is highly required.

1. Introduction

Ticks are important vectors of tick-borne pathogens (TBPs) and can transmit TBPs not only to animals but also to humans (Aouadi et al., 2017). Tick-borne diseases such as anaplasmosis, babesiosis, theileriosis, and heartwater are common in livestock of sub-Saharan Africa and cause health problems, decrease the milk and meat production, and negatively impact the welfare of livestock (Aouadi et al., 2017; El Hussein et al., 2004).

Anaplasma ovis is an obligate intracellular Gram-negative bacterium and is mainly transmitted by *Rhipicephalus bursa* (Chochlakis et al., 2010). *A. ovis* causes anaplasmosis in sheep and goats. This is considered as a benign condition, but when combined with other stress factors such as co-infection, hot weather, vaccination, and heavy tick infestation, *A. ovis* infection can show severe clinical symptoms (Renneker et al., 2013). Recently, Chochlakis et al. (2010) reported a case of human anaplasmosis caused by an *A. ovis*-variant in Cyprus. This report drew attention toward *A. ovis* in animals and humans.

Babesia ovis and *Theileria ovis* are protozoan parasites belonging to the phylum Apicomplexa, and cause babesiosis and theileriosis, respectively, in small ruminants (El Imam et al., 2016). Different species of parasites belonging to the piroplasms including *T. lestoquardi*, *T. annulata*, *T. luwenshuni*, *T. uilenbergi*, *B. ovis,* and *B. motasi* can infect small ruminants, and their pathogenicity varies (Aouadi et al., 2017; El Imam et al., 2016; Gebrekidan et al., 2014). Ixodid ticks including the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma*, and *Haemaphysalis* are responsible for the transmission of piroplasms (Gebrekidan et al., 2014).

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Ehrlichia ruminantium (formerly *Cowdria ruminantium*) is an obligate intracellular rickettsia and a causative agent of heartwater in cattle, goats, sheep, and wild ruminants. Heartwater is transmitted by ticks belonging to the genus *Amblyomma* and is prevalent in sub-Saharan Africa and some Caribbean islands. Although it causes a high mortality in sheep, an effective vaccine or treatment is currently not available (Allsopp and Allsopp, 2007).

In Sudan, the estimated sheep and goat populations in 2012 were about 39,484,000 and 30,837,000, respectively (Ali et al., 2013). Sheep and goats play an integral part in most traditional production systems, because they provide milk, meat, skin and contribute to Sudan's export earnings (Ageeb, 1992). The majority of these animals are owned by nomads who are moving freely, while minor farms are found only in large cities. This situation increases the contact between different animal species during pasture and watering (Ali et al., 2013).

More than 70 tick species, including *Amblyomma* spp. and *Hyalomma* spp., and different tick-borne diseases such as anaplasmosis, babesiosis, theileriosis, and heartwater are present in Sudan (El Hussein et al., 2004; El Imam et al., 2016; Morita et al., 2004). However, previous studies in Sudan focused on TBPs of cattle and thus minimal data are available on sheep and goats (El Imam et al., 2016; Renneker et al., 2013; Salih et al., 2003; Tageldin et al., 1992; Taha et al., 2011). In addition, these previous studies focused on the identification and differentiation of the TBPs, and as a result, limited molecular information on TBPs is available (Muramatsu et al., 2005; Schnittger et al., 2003). Therefore, the present study aimed at the detection and molecular characterization of the tick-borne pathogens (*A. ovis*, *T. ovis*, *T. lestoquardi*, *B. ovis*, and *E. ruminantium*) in sheep and goats in two states in Sudan, namely Blue Nile and West Kordofan.

2. Materials and methods

2.1. Ethical statements

Prior to conducting the experiments, permission for the use of experimental animals, DNA, and pathogens was obtained from Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan (Permission number: 29-2).

2.2. Study area and sample collection

This study was conducted in two southern states of Sudan, namely, Blue Nile (11°16′ N 34°4′ E) and West Kordofan (12°0′ N 28°9′ E) (Fig. 1). The two states are known for their free range breeding system of sheep and goats. Sheep and goats are reared strictly within the semi-desert belt of Sudan, including these two states. They are owned exclusively by nomadic tribes of Arab origin or others closely related to them in the region (Sulieman et al., 1990).

Overall, 178 animal blood samples were obtained after a consent of owners from Blue Nile (n = 112; 76 goats and 36 sheep) and West Kordofan (n = 66; 40 goats and 26 sheep) from April 2014 to December 2014. From Blue Nile, goat and sheep blood samples were collected from four and three different villages, respectively, which are about 100 km away from a town known as Foola. From West Kordofan, goat and sheep blood samples were collected from five and three different villages, respectively, which are about 100 km away from a town known as Damazin. Information on the sex of each animal was also recorded.

2.3. DNA extraction, molecular detection and characterization of TBPs

DNA was extracted using the phenol-chloroform method from blood loaded to filter papers. The quality and quantity of DNA were verified using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was stored at −30 °C until further use.

To detect TBPs in sheep and goats in the two states of Sudan, a PCR assay was used as previously described with primers listed in Table 1. The PCR reaction mixture was composed of the following: 0.1 µl *Taq* polymerase (0.5 U; New England BioLab, Ipswich, MA, USA), 1 µl each of forward and reverse primer (100 µM), 0.8 µl deoxynucleotide triphosphate (200 µM), 1 µl 10 × ThermoPol Reaction Buffer (New England BioLab), 1 µl DNA template, and distilled water up to 10 µl. For positive controls, DNA samples confirmed positive for *T. ovis*, *A. ovis*, *B. ovis* (Zhou et al., 2017), and *E. ruminantium* (Ringo et al., 2017) were used. No positive control was available for *T. lestoquardi*. For a negative control, double distilled water was used. After optimizing the PCR conditions using positive controls, positive controls were not included in the experiments to prevent contamination and only a negative control was included in each experiment.

Some *msp4*-positive samples were selected for molecular characterization of *A. ovis* based on the *groEL* and 16S rRNA as previously described (Ochirkhuu et al., 2017). In addition, for longer sequences of *A. ovis msp4*, the gene was amplified with a primer set of MSP45/MSP43 (Ochirkhuu et al., 2017). An additional portion of the *A. ovis groEL* was amplified and aligned with the former portion (Table 1).

2.4. Sequencing analysis

Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, New York, USA) and the results were analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. All the amplicons from the positive samples were directly sequenced bidirectionally and the obtained sequences were aligned using MUSCLE in MEGA 7.0 (Kumar et al., 2016). The obtained sequences were compared with the sequences deposited in GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Phylogenetic analysis

For molecular characterization, phylogeny was assessed based on the *A. ovis msp4*, *groEL*, and 16S rRNA, *T. ovis* 18S rRNA, and *E. ruminantium* pCS20 using MEGA 7.0. (Kumar et al., 2016). Only protein coding regions were included for the analysis of *A. ovis msp4* and *groEL*. The phylogenetic trees were constructed based on the maximum-likelihood method (Kimura 2-parameter model for nucleotide and Jones-Taylor-Thornton model for amino acids) and topology was supported by 500 replications. The inclusion of the sequences in the analysis was based on the host and country, and an appropriate outgroup was included.

2.6. Statistical analysis

Statistical analysis was performed to evaluate the relevance of prevalence according to the animals or sampling regions by the Chi-square test using R (https://www.r-project.org/). A P value less than 0.05 was considered to be statistically significant. In addition, 95% confidence intervals were calculated for all estimates. Some samples lacking information on the animal's sex (n = 8) were excluded from statistical analysis.

3. Results

3.1. Molecular detection of TBPs

Overall, 110 (61.8%) of 178 animals were found infected with at least one of the following pathogens: *A. ovis*, *T. ovis*, and *E. ruminantium* (Table 2). *B. ovis* and *T. lestoquardi* were not detected. *A. ovis* was the most prevalent pathogen (n = 107, 60.1%) followed by *T. ovis* (n = 23, 12.9%) and *E. ruminantium* (n = 1, 0.6%) (Table 3).

A. ovis was detected in 55 (47.4%) and 52 (83.9%) goats and sheep, respectively. According to the sampling regions, 70 (62.5%) and 37