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Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania

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ABSTRACT

Tick-borne diseases cause significant losses to livestock production in tropical and subtropical regions. In Tanzania, detailed studies on tick-borne pathogens in cattle using sensitive molecular detection methods are scarce. In this study, we investigated the occurrence of *Theileria* spp., *Babesia* spp., *Anaplasma* spp. and *Ehrlichia* spp. in 245 blood samples collected from cattle on Pemba Island, Tanzania. We used polymerase chain reaction (PCR) and gene sequencing to detect and identify pathogens. PCR screening revealed overall infection rates of 62.4% for *Theileria* spp., 17.6% for *Babesia bigemina*, 15.9% for *Anaplasma marginale*, 7.4% for *Ehrlichia ruminantium* and 4.5% for *Babesia bovis*. Further analysis using sequences of *Theileria* spp. *18S rRNA* revealed infection of cattle with *Theileria mutans* (68.6%), *Theileria taurotragi* (48.4%), *Theileria parva* (41.2%), and *Theileria ovis* (1.9%). Co-infections of cattle, with up to six tick-borne pathogens, were revealed in 46.9% of the samples. Sequence analysis indicated that *T. parva p104*, *E. ruminantium pCS20* and *A. marginale MSP-5* genes are conserved among cattle blood samples in Pemba, with 99.3%–100%, 99.6%–100% and 100% sequence identity values, respectively. In contrast, the *B. bigemina RAP-1a* and *B. bovis SBP-2* gene sequences were relatively diverse with 99.5%–99.9% and 66.4%–98.7% sequence identity values respectively. The phylogenetic analyses revealed that *T. parva p104*, *E. ruminantium pCS20* and *A. marginale MSP-5* gene sequences clustered in the same clade with other isolates from other countries. In contrast, the *B. bigemina RAP-1* and *B. bovis SBP-2* gene sequences showed significant differences in the genotypes, as they appeared in separate clades. This study provides important data for understanding the epidemiology of tick-borne diseases, and is expected to improve the approach for diagnosis and control of tick-borne diseases in Tanzania.

1. Introduction

Tick-borne pathogens (TBPs) pose a major threat to livestock in tropical and sub-tropical regions (Jongejan and Uilenberg, 1994; Jensenius et al., 2006; Gomes et al., 2013). In Africa, infection of cattle and other ruminants with TBPs cause significant economic losses to livestock farmers (Young et al., 2009). The main tick-borne diseases impacting cattle include east coast fever, babesiosis, anaplasmosis and ehrlichiosis. East coast fever is caused by *Theileria parva* while *Babesia bovis* and *B. bigemina* are the major causes of bovine babesiosis. Infections of cattle with *Anaplasma marginale* and *Ehrlichia ruminantium* cause anaplasmosis and ehrlichiosis, respectively (Simuunza et al., 2011). Bovine cerebral theileriosis (turning sickness) is another important tick-borne disease affecting cattle. *Theileria taurotragi,* previously considered a pathogen of Eland (Martin and Brocklesby, 1960), *T. parva* and occasionally *Theileria annulata* are considered main causes of this disease (Devos and Roos, 1981).

Tanzania has approximately 25 million herds of cattle, representing the third largest cattle population in Africa (Engida et al., 2015). However, cattle productivity (milk and meat) is ranked low among African countries (Kivaria, 2006). One of the key obstacles contributing to low productivity is prevailing infectious diseases, notably, tick-borne

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diseases (Kivaria et al., 2006). Notable tick species in Tanzania includes *Rhipicephalus appendiculatus, R. microplus, R. evertsi evertsi, R. pravus, Amblyomma variegatum, A. lepidum, A. gemma*, and *Hyalomma albiparmatum* (Swai et al., 2005; Mamiro et al., 2016; Kerario et al., 2017). Accordingly, several important tick-borne diseases have been reported in Tanzania. East coast fever associated with *R. appendiculatus* is the most common disease and constitutes 78% and 46% threats to cattle health in northern and southern-highland regions of Tanzania, respectively (Kiswaga et al., 2014; Kerario et al., 2018). *Rhipicephalus appendiculatus* is also a vector of *T. taurotragi*, a parasite which has recently been suspected to cause massive losses to livestock farmers keeping indigenous East African Zebu cattle in Tanzania (Catalano et al., 2015). This pathogen has frequently been reported to occur in co-infections with *Theileria mutans*, causing turning sickness mainly among indigenous breeds of cattle predominantly owned by the pastoralists (Catalano et al., 2015).

Babesia spp., transmitted by *R. microplus*, causes considerable economic losses indirectly by reduced cattle weight gain and milk production or directly through veterinary costs and mortalities (Swai et al., 2007). It is estimated that babesiosis constitutes cattle health threats by 38% in northern and 5% in southern-highland Tanzania (Kiswaga et al., 2014; Kerario et al., 2018).

Anaplasma marginale is another important pathogen transmitted by *R. microplus,* which is regarded as the principal vector of pathogens affecting cattle in Tanzania (Swai et al., 2007). This tick is mainly found in the coastal areas of Tanzania (Mamiro et al., 2016). However, other forms of transmission, including mechanical transfer of contaminated blood through haematophagous arthropods, fomites and placental transmission during pregnancy (Kocan et al., 2004; Costa et al., 2016; Rjeibi et al., 2017), also contribute to the occurrence of anaplasmosis in Tanzania. The disease is rarely severe in calves or young stocks but is generally fatal in older cattle, and on recovery the animal remains carrier of the disease agent and hence, the source of infection (Kocan et al., 2010). In Tanzania, anaplasmosis constitutes 53% of threats to cattle health in the northern highlands and 34% in the southern highlands (Kiswaga et al., 2014; Kerario et al., 2018).

Amblyomma variegatum is known to be the main vector for *E. ruminantium* in cattle (Swai et al., 2009a,b) in Tanzania.

Ehrlichiosis is another major obstacle to introducing high producing cattle (exotic breeds) in endemic areas (Jane et al., 1987; Allsopp, 2010). The indigenous breeds are fairly resistant to this disease (Faburay et al., 2005). In the southern-highland of Tanzania, ehrlichiosis constitutes 15% of threats to cattle health (Kerario et al., 2018).

Despite the significance of these tick-borne pathogens in cattle, little information is available in terms of their occurrence, distribution and the degree of enzootic stability in Tanzania. Therefore, this study was conducted to help fill the information gap regarding the occurrence and genetic diversity of tick-borne protozoan and rickettsial pathogens infecting cattle on Pemba Island, Tanzania. The results of this study are expected to improve the approach for diagnosis and control of tick-borne diseases in Tanzania.

2. Materials and methods

2.1. Study area

Pemba Island is located in the Indian Ocean, 50 km from the mainland of Tanzania (Fig. 1). The island is located between latitude 39.606019 °S and 39.86269 °S and longitude -4.907837 °E and -5.419286 °E. The size of the island is 988 square km, with four districts: Mkoani, Chake, Wete and Micheweni. The topography is characterized by numerous small valleys and hills. Pemba experiences bimodal rainfall pattern. The average rainfall per annum is 1860 mm, which falls mostly between March and May (long rains) and October to December (short rains). The climate is tropical sub-humid with average temperatures of 26 °C annually. The vegetation can be classified into four types, which are tropical moist forest, coastal rag shrubs, clove plantations and farmlands (Catry et al., 2000).

2.2. Sample collection and DNA extraction

Blood samples were collected from clinically healthy cattle in April and May 2017, using sterile needles and vacutainer tubes coated with EDTA. Sampled cattle were mostly indigenous breeds and their crosses with Friesian, Jersey and Brown Swiss. Approximately 3–5 ml of blood was drawn from the jugular vein of the animal, transported to the laboratory in cool boxes and then kept at 4 °C in the laboratory for no more than a few days until DNA extraction was conducted. A total of 245 blood samples were collected randomly from different farms in all four districts of Mkoani, Micheweni, Chake and Wete, whereby the number of samples collected were 65, 60, 60 and 60 respectively. Samples were collected from animals of around one and a half years of age and above. DNA was extracted from 200 µl of blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and stored at -30 °C until used.

2.3. Ethical statement

The owners of the selected farms were informed of the study and provided their approval for sampling of their cattle. 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 (Approval ID: 29–68).

2.4. Molecular detection of Theileria, Anaplasma, Babesia and Ehrlichia species

Nested PCR was used to screen all the samples with species-specific primers (Table 1) for *A. marginale, B. bovis, B. bigemina* and *E. ruminantium* detection. *Theileria* species were screened using the genus specific *18S rRNA* primers and positive samples were sequenced to confirm the species. After species confirmation, *T. parva*-positive samples were screened again and sequenced using species-specific primers for *p104* gene. The thermo-cycling 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 dNTP mix and 0.1 µl of Ex *Taq* polymerase (Takara Bio, Otsu, Japan), 1 µl of 10 × standard *Taq* buffer, 1 µl of DNA template and 5.9 µl of double distilled water. The positive controls were positive samples from previous studies (Adjou Moumouni et al., 2015; Jirapattharasate et al., 2016; Ringo et al., 2018), while double-distilled water (DDW) was used as negative control. PCRs were run in a thermal cycler (Bio rad, Hercules, CA, USA). The PCR products were electrophoresed on a 2% agarose gel and stained by ethidium bromide and then viewed under UV transilluminater.

2.5. Cloning and sequencing

For sequencing, 1–5 positive samples were randomly selected per detected pathogens. Amplicons were extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen). The concentration of the extracts was measured using a NanoDrop 2000 spectrophotometer (Thermofisher, Waltham, MA, USA). The extracted PCR product (6 µl) was ligated into a pGEM-T Easy vector (2 µl) (Promega, Fitchburg, WI, USA) with a T4 DNA ligase (1 µl) in a restriction buffer (1 µl). Thereafter, the ligation mixture was incubated at 16 °C for 3 h and then kept at 4 °C overnight. The plasmid construct was transformed into *Escherichia coli* DH5α competent cells and then extracted using NucleoSpin® Plasmid QuickPure (Machery-Nagel, Düren, Germany) kit". The PCR product, ligated to the plasmid, was sequenced with Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using a 3100 Genetic Analyzer (Applied Biosystems). Four clones were sequenced for each amplicon. The sequencing primers used