International Journal for Parasitology: Parasites and Wildlife 4 (2015) 239–243

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Brief Report

Identification of novel *Theileria* genotypes from Grant's gazelle

Janis Hooge [a], Laryssa Howe [a], Vanessa O. Ezenwa [b,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North 4442, New Zealand
[b] Odum School of Ecology and Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, USA

ARTICLE INFO

Article history:
Received 15 February 2015
Revised 22 April 2015
Accepted 23 April 2015

Keywords:
Theileria
Hemoparasite
18S rRNA
Nanger granti
Kenya

ABSTRACT

Blood samples collected from Grant's gazelles (*Nanger granti*) in Kenya were screened for hemoparasites using a combination of microscopic and molecular techniques. All 69 blood smears examined by microscopy were positive for hemoparasites. In addition, *Theileria/Babesia* DNA was detected in all 65 samples screened by PCR for a ~450-base pair fragment of the V4 hypervariable region of the 18S rRNA gene. Sequencing and BLAST analysis of a subset of PCR amplicons revealed widespread co-infection (25/39) and the existence of two distinct Grant's gazelle *Theileria* subgroups. One group of 11 isolates clustered as a subgroup with previously identified *Theileria ovis* isolates from small ruminants from Europe, Asia and Africa; another group of 3 isolates clustered with previously identified *Theileria* spp. isolates from other African antelope. Based on extensive levels of sequence divergence (1.2–2%) from previously reported *Theileria* species within Kenya and worldwide, the *Theileria* isolates detected in Grant's gazelles appear to represent at least two novel *Theileria* genotypes.

1. Introduction

Theileria and *Babesia* (Phylum: Apicomplexa) are two of the most economically important hemoparasites of livestock. Species in both genera are vectored by ticks and infect host erythrocytes. The diversity and distribution of *Theileria* and *Babesia* species in cattle have been studied in detail (Bishop et al., 2004; Bock et al., 2004), and with the advent of new molecular techniques, an increasing number of studies are characterizing these organisms in wild herbivores. For example, recent studies on African ungulates have examined *Theileria* or *Babesia* genotype diversity in buffalo (*Syncerus caffer* (Chaisi et al., 2011, 2014; Mans et al., 2011)), sable and roan antelope (*Hippotragus niger and H. equinus* (Oosthuizen et al., 2008, 2009)), tsessebe (*Damaliscus lunatus* (Brothers et al., 2011)), waterbuck (*Kobus defassa* (Githaka et al., 2014)), giraffe (*Giraffa camelopardalis* (Oosthuizen et al., 2009; Githaka et al., 2013), and zebras (*Equus quagga* and *E. zebra* (Bhoora et al., 2010)). Novel parasite genotypes were reported in several of these hosts, suggesting that the full diversity of *Theileria* and *Babesia* species infecting wild African herbivores is only beginning to be understood.

In this study, we evaluated the occurrence of *Theileria* and *Babesia* species in Grant's gazelle (*Nanger granti*), a common antelope species found in East Africa. Grant's gazelle occupy similar habitat as a range of plains herbivore species, and in some regions they also overlap extensively with livestock. A serological survey of Kenyan wildlife and livestock for the rickettsial hemoparasite, *Anaplasma*, found that four of five Grant's gazelles tested were positive for *Anaplasma* antibodies (Ngeranwa et al., 2008). Beyond this report, there is very little information on the hemoparasites infecting Grant's gazelle. Thus, we used a combination of microscopic and molecular techniques to estimate hemoparasite infection prevalence in this species and to identify target *Theileria* and/or *Babesia* parasites.

2. Materials and methods

2.1. Animal sampling

Grant's gazelles ≥2 years of age were captured at the Mpala Research Center (MRC), Laikipia, Kenya (0°17′N, 37°52′E) in January, February, and August 2009. Animals were captured using drive nets (Jan-Feb) or by helicopter using a hand-held net gun fired from the aircraft (August). Blood was collected from the jugular vein into 10 ml heparin tubes. Samples were kept on ice until transported to the laboratory where they were stored at –20 °C in the lab until processing. A 5-mm biopsy sample was also taken from the ear of each animal, and a residual drop of blood from the ear vein was collected into a capillary tube and used to make blood smears. Hematocrit (HCT) levels were used to estimate the possible impact of hemoparasite infection on anemia. To measure HCT, capillary tubes filled with heparin blood were spun in a microhematocrit centrifuge for 5 min and HCT values were estimated using a microhematocrit reader card. Animal protocols were approved by the University of

* Corresponding author. Odum School of Ecology and Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, USA. Tel.: +1 706 542 2288; fax: 1-706-542-4819.
E-mail address: vezenwa@uga.edu (V.O. Ezenwa).

http://dx.doi.org/10.1016/j.ijppaw.2015.04.004

Montana (#023-09VEDBS-051509) and the University of Georgia (#A2010 10–188) Animal Care and Use Committees.

2.2. Microscopic analysis

Immediately after collection of peripheral blood into capillary tubes, a thin smear was prepared on a glass slide, air-dried, and fixed with 100% methanol. Fixed slides were transported back to the laboratory where all slides were stained with Giemsa for 45 minutes. Presence or absence of infection was determined by examining each smear under oil immersion (×1000) for up to 90 minutes concentrating on the monolayer. For each smear, the intensity of infection (i.e. the number of hemoparasites) was quantified by counting all organisms observed in the first 10 fields of view. Smears without any parasites in the first 10 fields were classified as having low infection intensities, and smears with parasites were classified as having high intensities.

2.3. Molecular analysis

DNA was extracted from 100 µl of heparinized blood samples using the Qiagen DNeasy Kit (Qiagen, CA, USA) following the manufacturer's instructions. All extracted DNA samples were kept at –20 °C until further analysis was performed. A ~450-base pair (bp) fragment of the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene was amplified using primers RLB F2 [5′-GAC ACA GGG AGG TAG TGA CAA G-3′] and RLB R2 [5′-CTA AGA ATT TCA CCT CTA ACA GT-3′ to identify *Babesia* and *Theileria* species as described by Gubbels et al. (1999) with the following modifications. Briefly, a PCR amplification was performed in 50 µl reactions containing approximately 50 ng of template genomic DNA, 0.2 µM of each of the forward and reverse primer, 0.2 µM of dNTP, 5 µl 10X PCR buffer, 1.5 mM of MgCl2, and 0.2 µl (1 unit) of Platinum Taq DNA polymerase (Invitrogen, CA, USA). For negative controls, an equivalent volume of purified water was substituted for DNA. PCR reaction conditions involved a 4 minute initial denaturation at 94 °C, followed by 35 cycles of 94 °C for 20 seconds, 57 °C for 30 seconds, and 72 °C for 30 seconds and a final 10 minute extension at 72 °C. PCR products were run on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen) containing ethidium bromide at 100V for 45 minutes and visualized under UV light on a transilluminator. A subset of positive PCR amplicon samples was purified (PureLink PCR purification kit, Invitrogen) and subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems, CA, USA) using both forward and reverse primers. Sequencing results were compared to the NCBI BLAST database to confirm *Theileria* or *Babesia* DNA amplification and identify relatedness.

Sequences from three gazelle isolates (GG1, GG2, and GG3) were aligned using Clustal W (Thompson et al., 1994) with 33 representative *Theileria* sp. sequences and a *Toxoplasma gondii* sequence available in GenBank and trimmed to the same length (400 base pairs) using Geneious Pro 4.8.5™ (Biomatters, Auckland, New Zealand). A Bayesian phylogenetic tree was generated in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) using a general time-reversible model including invariable sites (GTR +I). The Bayesian phylogeny was obtained using 1 cold and 3 hot Monte Carlo Markov chains, which were sampled every 1000 generations over 2 million generations. Of these trees, 25% were discarded as burn-in material. The remaining trees were used to construct a majority consensus tree. Bootstrap percentages from the Bayesian analysis were added to the tree at the appropriate nodes. The sequence divergence between and within the different lineages was calculated using a Jukes–Cantor model of substitution implemented in the program PAUP* 4.0 Beta version (Swofford, 2002). Sequences reported in this paper are available in GenBank under accession numbers: KP641655, KP641656 and KP641657.

3. Results

The blood smears of 69 individuals (41 males, 28 females) were examined for hemoparasites and at least one organism was detected in all of the smears. Some positive samples contained single organisms while others had paired or multiple organisms (Fig. 1); however, the parasites could not be distinguished taxonomically with this method. A smaller number of samples (12 of 69) had quantifiable levels of infection. A total of 83.3% (10 of 12) of these samples were males and 16.4% (2 of 12) were females. Infection intensity in these samples ranged from 1 to 15 parasites per 10 fields of view (mean = 5.2). There was no evidence that infection intensity was associated with differences in hematocrit (2-way ANOVA controlling for host sex: mean HCT (high) = 47.3 [n = 10], mean HCT (low) = 47.2 [n = 55], p = 0.93).

Of the 69 samples examined by microscopy, 60 were available for molecular analysis. Five additional samples, for which we did not have smears, were available for PCR so in total we examined 65 samples by PCR. As with the smears, 100% of the blood samples tested by PCR were positive for hemoparasites (*Babesia/Theileria* spp.). Sequencing a random subset of the PCR amplicons revealed that the majority of samples (25 of 39 sequenced samples) had conspicuous overlapping peaks suggestive of co-infection. The conserved nature of the first 100 base pairs of the amplicons from co-infected animals confirmed the presence of multiple *Theileria* spp. Of the 14 non-coinfected amplicons, a BLAST search revealed a group of 11 isolates (represented by Grant's Gazelle [GG]1 and GG2 in Fig. 2) which were most similar (99%) to a large number of previously identified *Theileria ovis* isolates including those from Spain (GenBank DQ866845), Egypt (GenBank AB986194), Turkey, Tanzania, Sudan (GenBank AY260172, AY260173, AY260171 respectively) and China (GenBank FJ603460). A comparison of 18S rRNA sequences derived from these 11 gazelle isolates showed minor levels of heterogeneity (0.0–0.3%) at the nucleotide level (GG1 and GG2: Fig. 2, Table 1). Sequence divergence analysis also revealed extensive heterogeneity of 1.2–1.7% when compared to representative *T. ovis* isolates within

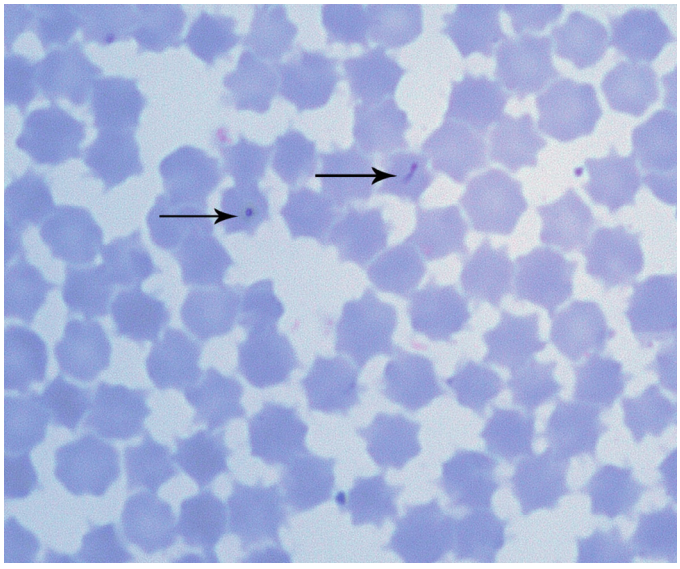

Fig. 1. Light microscopy of a blood smear stained with Giemsa showing single and paired hemoparasites (highlighted by arrows).