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Molecular surveillance of novel tick-borne organisms in Madagascar's lemurs

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ABSTRACT

The discovery and characterization of emerging tick-borne organisms are critical for global health initiatives to improve animal and human welfare (One Health). It is possible that unknown tick-borne organisms underlie a subset of undiagnosed illness in wildlife, domesticated species, and humans. Our study lends support to the One Health concept by highlighting the prevalence of three blood-borne organisms in wild lemurs living in close proximity to domesticated species and humans. Previously, our team identified three novel, presumably tick-borne, intravascular organisms, belonging to the genera *Babesia*, *Borrelia*, and *Neoehrlichia*, circulating in two of Madagascar's lemur species. Here, we extend our previous observation by developing a targeted molecular surveillance approach aimed at determining the prevalence of these organisms in lemurs. Using quantitative PCR, we provide *Babesia*, *Borrelia*, and *Neoehrlichia* prevalence data for 76 individuals comprising four lemur species located in eastern Madagascar. Our results indicate a high prevalence (96%) of *Babesia* across sampled individuals with lower prevalences for *Neoehrlichia* (36%) and *Borrelia* (14.5%). In light of our results, we recommend additional studies of these tick-borne organisms to determine pathogenicity and assess zoonotic potency to other animals and humans in Madagascar.

1. Introduction

Approximately 75% of emerging infectious diseases are zoonotic and, of these, tick-borne organisms (TBOs) are of increasing concern to global human health initiatives (Moutailler et al., 2016). TBOs are known to cause significant illness in multiple mammalian species around the globe, yet information regarding their impact on human and animal health is limited in many countries (Moutailler et al., 2016). Borreliosis, spotted fever rickettsioses, tick-borne encephalitis, neoehrlichiosis and other diseases caused by TBOs are increasingly reported in domestic animals and humans (Jones et al., 2008; Daszak et al., 2000; Vayssier-Taussat et al., 2015; Silaghi et al., 2012). Additionally, it is hypothesized that TBO-related diseases may be responsible for unexplained syndromes observed in human patients (Briggs et al., 2011; Daszak et al., 2000; Jones et al., 2008; Silaghi et al., 2012; Vayssier-Taussat et al., 2015). A cross-disciplinary 'One Health' approach that examines disease ecology across wildlife, domestic animals, and humans enables a better understanding of emerging zoonotic diseases and helps mitigate the medical impact and spread of diseases caused by TBOs.

Madagascar provides a unique setting to examine evolutionary relationships among emerging and reemerging TBOs. As an island nation, Madagascar has a complex colonization history of humans and their associated pests (e.g., *Rattus rattus*, *R. norvegicus*, *Mus musculus*), the importations of domestic animals (e.g., cattle, pigs, horses, dogs, cats), and subsequent interactions between humans, pests and domestic animals with the indigenous lemur population. The fauna of Madagascar evolved in isolation for approximately 88 million years, with colonization by humans and associated species beginning approximately 5000 years ago (Crowley, 2010; Dewar et al., 2013; Gommery et al., 2011). Therefore, TBOs circulating among endemic wildlife, domestic animals, and/or humans may have unique genetic signatures to Madagascar or

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signatures shared with TBOs originating from invasive reservoir and/or vector species (e.g., imported cattle, pigs, dogs and associated ticks).

The tick fauna of Madagascar is comprised of at least 34 species, of which five have colonized in conjunction with humans and domestic animal migrations (*Ornithodoros porcinus*, *Otobius megnini*, *Amblyomma variegatum*, *Rhipicephalus sanguineus* sensu lato, and *Rhipicephalus microplus* (formerly *Boophilus microplus*) (Klompen, 2003; Uilenberg et al., 1979). A number of pathogens are associated with these invasive ticks on Madagascar, including the causative agents of African swine fever (Roger et al., 2001), anaplasmosis (Matysiak et al., 2016; Uilenberg et al., 1979), babesiosis (Bardsley and Thrusfield, 2014; Matysiak et al., 2016; Uilenberg et al., 1979), and Crimean-Congo hemorrhagic fever (Mathiot et al., 1988). However, relatively little research has been done with respect to TBO discovery and surveillance in putative wildlife reservoirs on the island (Larsen et al., 2016; Springer et al., 2015). It is within this framework that we investigate the prevalence of three newly discovered TBOs circulating within Madagascar's endemic lemur population.

Previously, we used next-generation high-throughput sequencing (NGS) of whole blood RNA transcriptomes to discover novel *Babesia*, *Borrelia*, and *Neoehrlichia* spp. circulating in wild lemurs in Madagascar (Larsen et al., 2016). These bacterial and protozoan organisms share close genetic relationships to pathogenic species known to cause disease in humans, domestic animals and wildlife, yet, if or the extent to which these organisms cause disease in Madagascar is unknown. Phylogenetic analyses of the novel *Babesia* and *Borrelia* revealed close genetic relationships with TBOs from both wildlife and domestic species occurring in Africa (e.g., *Babesia leo* and *Borrelia theileri*) (Larsen et al., 2016). This observation, combined with recent reports of babesiosis in ruminants in Madagascar (Bardsley and Thrusfield, 2014), underscore a critical need to further characterize TBOs in Madagascar, including the prevalence of these newly discovered TBOs within endemic lemurs, other animals and humans. To determine the molecular prevalence, we designed quantitative PCR (qPCR) assays to screen for these novel TBOs in a large set of archived blood samples collected from four species of lemurs (*Avahi laniger*, *Indri indri*, *Lepilemur mustelinus*, and *Propithecus diadema*) in eastern Madagascar between 2012 and 2015. For this study, we aimed to 1) design targeted qPCR assays using data generated from non-targeted whole transcriptome sequencing; 2) screen for and determine the prevalence of three novel TBOs in a population of wild lemurs living in close proximity to domestic animals and humans; and 3) identify potential associations and seasonal variations with TBO prevalence in lemurs. We hypothesized that there would be no difference in the prevalences of TBOs based upon the sex or age of the lemurs, and that TBOs would have a lower prevalence in lemurs during the winter seasons (August, September, October), when tick activity is decreased due to cooler temperatures (Rahajarison et al., 2014).

2. Material and methods

2.1. Sample collection

Whole blood (n = 102) was collected from wild lemurs (n = 76) comprising four species: *A. laniger* (n = 7), *I. indri* (n = 15), *L. mustelinus* (n = 3), and *P. diadema* (n = 51). Lemurs were sampled between September 2012 and October 2015 from mid-altitude (1000–1200 m) disturbed, primary forest approximately 80 km northeast of Moramanga, Madagascar (Fig. 1). Archived blood samples were stored in anticoagulant ethylenediamine tetraacetic acid (EDTA) and preserved at −80 °C prior to testing. Twenty-five lemurs, comprising *A. laniger* (n = 1), *I. indri* (n = 3), and *P. diadema* (n = 21) were sampled at two or three time points, allowing for temporal measures of infection. Ages were estimated for each lemur at time of capture and categorized as juveniles (aged 6 months–2 years) or adults (aged > 2 years). Seasonal changes in prevalence were determined with respect to sampling during the summer months (January and March; n = 35) and winter months (August, September and October; n = 67). Archived ticks found on 12 of the 76 lemurs were collected and stored in ethanol for identification. Ticks were morphologically identified using published taxonomic keys (Hoogstraal, 1953). Sampling was performed as part of an ongoing Lemur Biomedical Health Assessment project that monitors the health status of lemurs surrounding the Ambatovy Minerals nickel and cobalt mining operation in eastern Madagascar (Fig. 1). All samples were acquired in accordance with IACUC protocol A028-14-02 from Duke University and permits secured through the Ministère de l'Environnement, des Eaux et Forêts et du Tourisme, Madagascar.

2.2. Molecular methods

The *de novo* blood-borne parasite discovery methods described by Larsen et al. (2016) were confirmed by constructing and screening cDNA templates from the same six lemur blood samples (3 *P. diadema* and 3 *I. indri*) used in that study. In brief, approximately 3 µg of total RNA extracted from whole blood was converted to cDNA using random hexamers (50 ng/µl) and the SuperScript III First-Strand cDNA Synthesis System (ThermoFisher Scientific, Grand Island, NY, U.S.A.). Resulting cDNA was screened using established genus-specific vector-borne disease PCRs including an *Anaplasma/Ehrlichia* 16S conventional PCR (cPCR), *Babesia* 18S cPCR and *Borrelia* 16S rRNA quantitative PCR (qPCR) (Table 1). Sanger sequencing of PCR products was performed by GENEWIZ Inc. (Research Triangle Park, NC, U.S.A.) using standard methods. Additional sequence data generated herein from the lemur *Neoehrlichia* 16S rRNA and *Babesia* 18S rRNA genes are available on GenBank (see Results) and were used for primer design.

Primers targeting regions of nucleotide hypervariability that are specific to the novel *Babesia*, *Borrelia*, and *Neoehrlichia* organisms were designed using ribosomal 16S and 18S alignments of the sequence data presented herein. The following GenBank sequences were used in the alignments to improve specificity of the new primers to the novel TBOs and to avoid amplification of DNA from other TBOs: *Babesia* sp. (KT722781- KT722786), *Babesia felis* (AF244912, AY452706), *Babesia* sp. (AY452709), *Babesia leo* (AY452708), *Babesia* sp. (AF244913), *Babesia* sp. (GQ225744, FJ897741), *Babesia canis* (HQ148664, AY962186, DQ111760); *Borrelia* sp. (KT722787), *Borrelia* sp. (AB897891), *Borrelia* sp. (EF488992), *Borrelia coriaceae* (M60970), *Borrelia theileri* (KF569941), *Borrelia lonestari* (AY166715), *Borrelia miyamotoi* (NR025861); *Candidatus* Neoehrlichia sp. (KT722788, KT722789), *Candidatus* Neoehrlichia *mikurensis* (JQ359045), *Candidatus* Neoehrlichia lotoris (EF633744), *Candidatus* Ehrlichia khabarensis (FJ966352), *Ehrlichia* sp. (DQ324367), and *Candidatus* Ehrlichia shimanensis (AB074459). Alignments were constructed using the AlignX software package (Vector NTI Suite 6.0, InforMax, Inc., Bethesda, MD, U.S.A.).

PCR conditions were established for each new primer set using cDNA template from the six lemurs with known TBO infections and lemur genomic DNA negative for the targeted TBOs. Assay conditions were determined based on gradients performed with primer concentrations and annealing temperatures to identify a combination that efficiently amplified target DNA, but did not amplify lemur genomic DNA. The three qPCR assays were then used to screen archived lemur blood (n = 102).

DNA extraction was performed on QIAsymphony^{SP} (Qiagen, Hilden, Germany) with QIAsymphony® DNA Mini Kit (192) (Qiagen) or Qiagen BioRobot® M48 Robotic Workstation with MagAttract® DNA Mini M48 kit (Qiagen). DNA was stored at −20 °C prior to qPCR testing. The absence of PCR inhibitors was demonstrated by an internal control qPCR where the host GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene is amplified (Birkenheuer et al., 2003). Amplification reactions contained 12.5 µl SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA) for qPCRs or MyTaq HS Mix (2X) (Bioline, London, U.K. cat: BIO-25046) for cPCRs, 5 µl gDNA or 1 µl cDNA template, primers at either 0.4 µM or 0.6 µM (Table 1) and molecular grade water to a final