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# Presence of *Borrelia* spp. DNA in ticks, but absence of *Borrelia* spp. and of *Leptospira* spp. DNA in blood of fever patients in Madagascar

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## ABSTRACT

The occurrence of tick-borne relapsing fever and leptospirosis in humans in Madagascar remains unclear despite the presence of their potential vectors and reservoir hosts.

We screened 255 *Amblyomma variegatum* ticks and 148 *Rhipicephalus microplus* ticks from Zebu cattle in Madagascar for *Borrelia*-specific DNA. *Borrelia* spp. DNA was detected in 21 *Amblyomma variegatum* ticks and 2 *Rhipicephalus microplus* ticks. One *Borrelia* found in one *Rhipicephalus microplus* showed close relationship to *Borrelia theileri* based on genetic distance and phylogenetic analyses on 16S rRNA and *flaB* sequences. The borreliae from *Amblyomma variegatum* could not be identified due to very low quantities of present DNA reflected by high cycle threshold values in real-time-PCR. It is uncertain whether these low numbers of *Borrelia* spp. are sufficient for transmission of infection from ticks to humans.

In order to determine whether spirochaete infections are relevant in humans, blood samples of 1009 patients from the highlands of Madagascar with fever of unknown origin were screened for *Borrelia* spp. − and in addition for *Leptospira* spp. − by real-time PCR. No target DNA was detected, indicating a limited relevance of these pathogens for humans in the highlands of Madagascar.

## 1. Introduction

Data on the prevalence of the spirochaetes *Borrelia* spp. and *Leptospira* spp. in Madagascar and their health-related relevance on the human population are scarce. Previous reports suggested a complete absence of leptospirosis and tick-borne relapsing fever in humans in Madagascar (Rodhain and Fontenille, 1989, Desvars et al., 2013). However, most recently, we have identified *Rickettsia* spp. in Madagascan ticks (Keller et al., 2016) and *Brucella* spp. in human blood samples of Madagascan fever patients (Boone et al., 2017), although little was known on the local occurrence of these pathogens before.

Concerning *Leptospira* spp., small mammals that can serve as reservoirs are present in Madagascar and rodents have been shown to be infected with *Leptospira* spp. (Rahelinirina et al., 2010; Ralaiarijaona et al., 2001; Lagadec et al., 2012; Desvars et al., 2013; Dietrich et al., 2014). Data on humans are extremely scarce (Desvars et al., 2013).

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Concerning *Borrelia* spp., suitable vectors like *Ornithodoros moubata* ticks are present in Madagascar (Colas-Belcour et al., 1952; Rodhain and Fontenille, 1989). There is no reliable information on the frequency of tick bites in humans in Madagascar. However, tick bites in humans are reported frequently, especially in farmers (personal correspondence of the authors). Recent data on the frequency of relapsing fever in humans in Madagascar are lacking (Rodhain and Fontenille, 1989).

In the here described investigation, we assessed tick samples that were collected previously from Zebu cattle in Madagascar for another study (Keller et al., 2016) on the presence of DNA of *Borrelia* spp. and identified borrelia DNA. In a next step, we investigated human blood samples, that were collected in the scope of a study on fever of unknown origin (FUO) (Boone et al., 2017; Marks et al., 2017) for the presence of DNA of the spirochaetes *Borrelia* spp. and *Leptospira* spp.

## 2. Materials and methods

Four hundred and three ticks were included in the study. Sixteen ticks (*A. variegatum*, 6 males; *R. microplus*, 10 females) were collected in 2013 from 8 free-ranging Zebu cattle in the region Atsimo-Andrefana while working on a project on turtle ticks (Ehlers et al., 2016) and screened for borrelia as a proof-of-principle approach. In addition, 387 ticks were collected in 2012 at slaughter-houses located in Antananarivo, the capital of Madagascar, from 83 Zebu cattle from the regions of Bongolava, Haute Masiatra, Itasy, Menabe, Mampikony, and Vakinkaratra, Madagascar (Keller et al., 2016). Identification was performed according to the guides and keys of Voltzit & Keirans and Walker et al. (Voltzit and Keirans, 2003; Walker et al., 2003). Tick species included *Amblyomma variegatum* (n = 249, 81 females, 150 males, and 18 nymphs) and *Rhipicephalus microplus* (n = 138, 111 females, 27 males).

DNA from ticks was extracted as previously described using QIAamp DNA® Mini Kits (Qiagen) (Keller et al., 2016). All nucleic acid extractions of ticks were screened for *Borrelia* spp.-specific DNA as described (Parola et al., 2011).

Samples with positive results for *Borrelia* spp. were further characterized by PCR and Sanger sequencing of the target genes *flaB* (P41), *uvrA*, *pepX*, *gyrB* and a longer fragment of the *16S* rRNA gene as described previously (Barbour et al., 1996; Schwan et al., 2005; Margos et al., 2009; Margos et al., 2015; Assous et al., 2016; Venczel et al., 2016). PCRs for *p41*, *uvrA*, and *pepX* were only performed if sequencing of *flaB*, the *16S* rRNA gene and *gyrB* led to inconclusive results. In case the PCRs for *flaB*, *p41*, the *16S* rRNA gene, *uvrA*, and *pepX* failed or led to inconclusive sequencing results, the less sensitive *gyrB* PCR was not performed (Table 1).

Sequencing was conducted by Eurofins Genomics (Ebersberg, Germany). Sequence alignment, genetic distance analyses and construction of phylogenetic trees was done in MEGA5 (Kimura, 1980; Tamura et al., 2011). Basic local alignment search tool (BLAST) searches in GenBank were conducted. Genetic distance analyses were based on the Kimura 2-parameter model (Kimura, 1980). The evolutionary history was inferred using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar, 2000). Initial trees for the heuristic search were obtained by applying Neighbour-Joining and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach, followed by selecting the topology with superior log likelihood value. To calculate node support values, 1000 bootstrap repeats were chosen. A discrete Gamma distribution was used to model evolutionary rate differences among sites [+G]. The rate variation model allowed for some sites to be evolutionarily invariable [+I]. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding for *flaB* and *gyrB* sequences. All positions containing gaps and missing data were eliminated. Further information is provided in the figure legends.

Human EDTA-blood samples were collected between 09/2011 and 06/2013 from Madagascan patients with FUO (≥38.5 °C) (von Kalckreuth et al., 2016; Boone et al., 2017; Marks et al., 2017). Collection took place at paediatric wards as well as in inpatient or outpatient departments of the basic health centers Centre de Santé de Base (CSB) II Imerintsiatosika, Itasy Region, CSB II Fenoarivo and CSB II Isotry, Antananarivo, and of the clinic Centre Hospitalier Universitaire (CHU) Tsaralalana, Antananarivo, and included into the study. The median age of included patients was 21 years (IQR 11, 34). The age-distribution analysis indicated 48 (4.8%) patients between 0 and 1 year of age, 73 (7.2%) patients between 2 and 4 years of age, 220 (21.8%) patients between 5 and 14 years of age, and 668 (66.2%) patients older than 14 years. Written informed consent was obtained from the patients or the next-of-kin in case of minors. All samples were stored at −20 °C. We included all available samples of patients with body temperature above 38.5 °C, that contained more than 1 ml 1009 of 1251 samples could be included, each sample representing one patient. 242 samples were not used, because of insufficient sample volume. The same samples were in parallel used for another study on brucellosis, Q fever and melioidosis (Boone et al., 2017). In 174 (17.2%) cases, the corresponding patients had received an antibiotic drug before acquisition of the sample. Patients of all ages presenting with current fever or self-reported history of fever within the past 72 h were eligible for enrolment. Blood was sampled from all patients meeting inclusion criteria and enrolled in the study. All health facilities recruiting patients represented primary level health services, therefore patients enrolled were assumed to present with early onset disease. Unfortunately no serum samples were collected for this study.

Nucleic acid was extracted from 1 ml EDTA blood per patient sample using FlexiGene DNA® kits (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

Ethical clearance was obtained from the Malagasy Ethical Committee; for patients recruited at the CSB-II in Imerintsiatosika and CSB-II Isotry ethical clearance was additionally obtained from the Institutional Review Board of the International Vaccine Institute.

## 3. Results

In a very first step, we investigated 16 ticks (*A. variegatum*, 6 males; *R. microplus*, 10 females) that were collected in 2013 from 8 free-ranging Zebu cattle in the region Atsimo-Andrefana while working on a project on turtle ticks (Ehlers et al., 2016). This was done as a proof-of-principle approach. Unexpectedly, one *R. microplus* tick turned out to be positive for borreliae.

In a next step, we subsequently assessed 387 further ticks that were collected for a study on rickettsiae in a slaughter house in the capital Antananarivo (Keller et al., 2016) (Table 1) and performed additional PCRs and sequencing of the positive samples for species identification.

A total of 23 out of 403 ticks were positive for *Borrelia* spp. The positive ticks comprised 21 *A. variegatum* (21/255; 8.2%, 18 males, 2 females, 1 nymph) and 2 *R. microplus* (2/148; 1.4%, 2 females). Only real-time PCR curves with characteristic sigmoid shapes in the screening PCR (Parola et al., 2011) were considered positive. The mean cycle-threshold (Ct) value was 38.2 ( ± standard deviation (SD) 4.1). If questionable PCR curves were taken into account, the number of positive results would rise to 57 out of 255 *A. variegatum* ticks (22.4%, 56 adults (8 females, 48 males), 1 nymph) and 5 out of 148 *R. microplus* ticks (3.4%, all female). The Ct-values would be similar with 37.9 ± 2.9 (mean value ± standard deviation). PCRs of 23 ticks showed no atypical curves and were considered truly positive for *Borrelia* spp. DNA and were thus further analyzed (Table 1). One of these was the above mentioned tick from Southern Madagascar, all other positive ticks were from the slaughter-house of Antananarivo.

Species identification of *Borrelia* spp. by typing PCRs with subsequent Sanger sequencing failed for 22 out of 23 samples as shown in Table 1. Although 8 out of 110 typing PCRs were positive in these 22 ticks, low sequence quality did not allow further analyses. DNA

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