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Original article

New *Rickettsia* species in soft ticks *Ornithodoros hasei* collected from bats in French Guiana

Djamel Tahir[a], Cristina Socolovschi[a,e], Jean-Lou Marié[a,b], Gautier Ganay[a], Jean-Michel Berenger[a], Jean-Michel Bompar[c], Denis Blanchet[d], Marie Cheuret[d], Oleg Mediannikov[a], Didier Raoult[a], Bernard Davoust[a,b,*], Philippe Parola[a]

[a] *Research Unit of Emerging Infectious and Tropical Diseases (URMITE) UMR CNRS 7278 IRD 198 INSERM U1095, Aix-Marseille University, Marseille, France*
[b] *Animal Epidemiology Working Group of the Military Health Service, Toulon, France*
[c] *Chiroptérologue, Solliès-Pont, France*
[d] *Laboratoire Hospitalier Universitaire de Parasitologie et Mycologie, Centre Hospitalier A. Rosemon, Cayenne, France*
[e] *Service de pneumologie, Hôpital Saint Joseph, Marseille, France*

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ABSTRACT

In French Guiana, located on the northeastern coast of South America, bats of different species are very numerous. The infection of bats and their ticks with zoonotic bacteria, especially *Rickettsia* species, is so far unknown. In order to improve knowledge of these zoonotic pathogens in this French overseas department, the presence and diversity of tick-borne bacteria was investigated with molecular tools in bat ticks.

In the beginning of 2013, 32 bats were caught in Saint-Jean-du-Maroni, an area close to the coast of French Guiana, and the ticks of these animals were collected. A total of 354 larvae of Argasidae soft ticks (*Ornithodoros hasei*) from 12 bats (*Noctilio albiventris*) were collected and 107 of them were analysed. DNA was extracted from the samples and quantitative real-time PCR was carried out to detect *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp. and *Coxiella burnetii*. All tested samples were negative for *Bartonella* spp., *Borrelia* spp. and *Coxiella burnetii*. *Rickettsia* DNA was detected in 31 (28.9%) ticks. An almost entire (1118 base pairs long) sequence of the *gltA* gene was obtained after the amplification of some positive samples on conventional PCR and sequencing. A Bayesian tree was constructed using concatenated *rrs*, *gltA*, *ompA*, *ompB*, and *gene* D sequences. The study of characteristic sequences shows that this *Rickettsia* species is very close (98.3–99.8%) genetically to *R. peacockii*. Nevertheless, the comparative analysis of sequences obtained from *gltA*, *ompA*, *ompB*, *rrs* and *gene* D fragments demonstrated that this *Rickettsia* is different from the other members of the spotted fever group. The sequences of this new species were deposited in GenBank as *Candidatus* Rickettsia wissemanii. This is the first report showing the presence of nucleic acid of *Rickettsia* in *Ornithodoros hasei* ticks from South American bats.

1. Introduction

Rickettsiae are obligate intracellular gram-negative bacteria transmitted by blood-feeding arthropods, primarily ticks, which may act as vectors, reservoirs, and/or amplifiers in the life cycles of the bacteria (Raoult and Roux, 1997). Ixodid ticks, also called hard ticks, are the main vectors of Rickettsiae (Parola et al., 2013). There are at least six genera (*Rhipicephalus*, *Dermacentor*, *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Ixodes*) which are recognized as competent vectors (Parola and Raoult, 2001). Concerning soft ticks (Argasidae), *R. bellii* is found in both *Argas* and *Ornithodoros* genera, and it is the most common *Rickettsia* found in ticks in United States of America (Raoult and Roux, 1997; Ogata et al., 2006). Recently described, the spotted fever group *Rickettsia hoogstraalii* is regularly identified in *Ornithodoros* spp. and *Haemaphysalis* spp. ticks (Duh et al., 2010; Dietrich et al., 2014). In humans, rickettsiae are the causative agents of the tick-borne rickettsioses, characterized by clinical features including fever, headache, rash, and occasional eschar formation at the site of the tick bite (Parola et al., 2013).

French Guiana is an overseas territory situated between Brazil and Suriname, and its size (84,000 km^2) is equivalent to about one

* Corresponding author at: Research Unit of Emerging Infectious and Tropical Diseases (URMITE), Faculté de medicine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France.
E-mail address: bernard.davoust@gmail.com (B. Davoust).

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fifth of the territory of mainland France; it is a sparsely populated department. Its climate is equatorial (hot and humid) and the Amazon rainforest covers 90% of its territory. Its ecosystem is characterized by a rich fauna and flora (5500 species of plants, 700 species of birds, and 177 species of mammals) and a dense river network. To our knowledge, in French Guiana only one *Rickettsia*, *Candidatus* R. amblyommii, has been detected in *Amblyomma coelebs* ticks (Parola et al., 2007). This rickettsia, with unknown pathogenic potential, is very common in North and Central America (Parola et al., 2013).

In French Guiana, bats of different species are very numerous. It is estimated that there are approximately 110 species. The infection of bats and their ticks with bacterial pathogens such as *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp. and *Coxiella burnetii* is so far unknown. In order to improve the knowledge of these zoonotic pathogens in this country, the presence and diversity of these tick-transmitted pathogens were investigated with molecular tools in bat ticks.

2. Materials and methods

2.1. Study site and bat samples

In January 2013, 32 bats (Chiroptera) were caught in Saint-Jean-du-Maroni (05°23′95″N–54°04′72″W), an area close to the coast of French Guiana, and the ticks (N = 354) of these animals were collected. Bats were caught with mist nets. Catches occurred in unoccupied buildings. The identification of bats was performed using previously described conventional morphological keys (Brosset and Charles-Dominique, 1990).

2.2. Collection and identification of ticks

A total of 32 apparently healthy bats were captured; species and sex were identified. The entire body of each bat was thoroughly examined for ticks by visual inspection. All visible ticks were removed from the body using tweezers. Ticks of each bat were then transferred to 5 mL vials containing 70% ethanol. All ticks were sent to our laboratory at the Reference Center for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases (Marseille, France). Tick larvae were identified at the genus level using previously described morphological identification keys (Barros-Battesti et al., 2013). Molecular identification of 16 randomly selected ticks was carried out using standard PCR assays by targeting the 16S and 12S rRNA mitochondrial genes, which amplify a fragment of 460 bp and 420 bp respectively (Norris et al., 1996). Subsequently, PCR products were subjected to sequencing as described in a previous study (Norris et al., 1996). Finally, the sequences obtained were compared with those available in GenBank for species identification.

2.3. DNA extraction from ticks

Prior to DNA extraction, all ticks were examined individually by optical microscope observation (ZEISS Axio Observer, Germany). Specimen images were taken for each tick for possible subsequent morphological identification. Then each tick was rinsed in sterile water for 10 min, then dried on sterile filter paper. Each tick was cut longitudinally into two parts using a scalpel. One-half of each tick was crushed in a buffered solution (G2) with proteinase K (Qiagen Hilden, Germany) and incubated at 56 °C overnight. The total genomic DNA was extracted in 50 μL of Tris EDTA (TE) buffer using the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was stored at −20 °C under sterile conditions to preclude contamination until the sample was used for PCR. The remaining half of each tick was kept at −20 °C for further analysis. Simultaneously, DNA was extracted according to the same protocol from our laboratory colonies of uninfected ticks, to be used as a negative control.

2.4. Real time PCR detection of bacteria in ticks

All DNA samples were individually screened for the presence of *Rickettsia* spp. by quantitative real-time PCR for the entire spotted fever group according to the Rickettsiae-specific *glt*A gene-based RKND03 system previously described (Socolovschi et al., 2010). Positive results were confirmed by another real-time PCR based on the RC0338 membrane phosphatase gene (Socolovschi et al., 2010). In addition, all DNA samples were tested by qPCR for other tick-borne bacteria such as *Bartonella* spp. by targeting an internally transcribed spacer (Angelakis et al., 2010); *Coxiella burnetii* using IS30A spacers (Mediannikov et al., 2010), and *Borrelia* spp. by targeting a fragment of the 16S rRNA gene (Parola et al., 2011). Briefly, the real-time PCR experiment was performed in a total reaction volume of 20 μL, containing 10 μL master mix Takyon® (Eurogentec France, Angers, France), 3.5 μL distilled water, 0.5 μL (20 μM) of each primer, 0.5 μL probe (5 μM), and 5 μL DNA template. All amplifications in real-time PCR were performed on the thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France). For each reaction, DNA-free water and DNA from uninfected ticks were used as negative controls; *Rickettsia montanensis*, *Bartonella elizabethae*, *Coxiella burnetii*, and *Borrelia burgdorferi* DNAs were used as positive controls. The samples were considered positive when the threshold cycles (Ct) were inferior to 35.

2.5. Sequencing and phylogenetic analysis

Positive results were confirmed by standard PCR using the following gene primers: *glt*A (Roux et al., 1997), *rOmpA* (Fournier et al., 1998), *rOmpB* (Roux and Raoult, 2000), *sca4* ("gene D") (Sekeyova et al., 2001) and 16S rRNA (Roux and Raoult, 1995), which amplify sequences of 1177 bp, 611 bp, 4346 bp, 3026 bp and 1466 bp, respectively. Subsequently, all PCR products obtained after the DNA amplification were purified using the PCR filter plate Millipore NucleoFast 96 PCR kit following the manufacturer's recommendations (Macherey-Nagel, Düren, Germany). The sequence reactions were carried out using the BigDye® terminator v3.3 cycle sequencing kit DNA according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). The sequence reaction program contains the following steps: initial denaturation at 96 °C for one min, followed by 25 cycles of denaturation at 96 °C for10 s, annealing at 50 °C for 5 s and extension at 60 °C for 3 min. Sequencing was performed with an ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems®). Finally, all obtained sequences were assembled and corrected on ChromasPro 1.7 software (Technelysium Pty Ltd., Tewantin, Australia) and then were compared to sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic trees were constructed using the neighbor-joining method tree algorithm in the MEGA6 program (http://megasoftware.net/) based on the protein-coding genes gltA, ompA, ompB, gene D and on the 16S rRNA-coding gene rrs sequences. Support for the tree nodes was calculated with 100 bootstrap replicates.

2.6. Accession numbers

The *rrs*, *gltA*, *ompA*, *ompB*, and *gene* D sequences determined in this study were submitted to the Genbank database under the following accession numbers: LT558851, LT558852, LT558853, LT558854 and LT558855, respectively.