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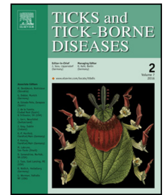

A novel ehrlichial agent detected in tick in French Polynesia

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ABSTRACT

Ticks are hematophageous arthropods that are known to host and transmit miscellaneous pathogens including zoonotic bacteria. The aim of this study was to investigate the presence of tick-associated microorganisms in Tahiti, French Polynesia with molecular tools. A total of 658 ticks from two species including *Rhipicephalus sanguineus s.l.* and *Rh. annulatus* were collected with forceps on dogs and cattle respectively, or with a flag on pasture in several locations of Tahiti in 2013. Two *Rickettsia* belonging to the spotted fever group different from *R. conorii* and *R. massiliae* were detected by qPCR in two *Rh. sanguineus s.l.* ticks, but sequencing failed. A *Rh. annulatus* tick was found positive for a new ehrlichial agent characterized by amplification and sequencing of fragments of the Anaplasmataceae 23S and *Ehrlichia* 16S genes. Phylogenetic analyses based on the 23S and 16S sequences reveals that this bacterium is a new genotype, genetically close to *Ehrlichia minasensis*, a recently described *Ehrlichia* sp. close to *Ehrlichia canis*.

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1. Introduction

Ticks are obligate hematophagous parasites of the Acari order. These arthropods can feed on every known class of vertebrate and can occasionally bite people. Ticks are currently the second leading vector of human infectious diseases after mosquitoes as they can carry bacterial (Parola and Raoult, 2001), viral (Hubálek and Rudolf, 2012), and protozoan pathogens (Gray et al., 2010). The past 30 years have seen an increased awareness of tick-borne diseases (Stanek et al., 2012), partly due to the growing number of rickettsioses discovered recently throughout the world. However, knowledge about tick-borne diseases remains very weak in some parts of the world (Parola et al., 2013).

Tahiti is the largest island in the Windward group of French Polynesia, an overseas territory of France, located in the archipelago of the Society Islands in the South Pacific Ocean. It is the economic, cultural and political center of French Polynesia.

Until now, two tick species have been described in Tahiti, *Rhipicephalus annulatus* and *Rhipicephalus sanguineus* s.l. (Rageau and Vervent, 1959; Raust and Legros, 1980). *Rh. annulatus*, previously known as *Boophilus annulatus*, was accidentally brought to Tahiti in the beginning of the twentieth century, probably through cattle importation. This tick is a cattle ectoparasite of significant importance for veterinary medicine, being involved in the transmission of bovine babesiosis (Texas Fever) due to *Babesia bigemina* and *Babesia bovis* (Smith and Kilborne, 1893).

Rh. sanguineus s.l, commonly known as the brown dog tick, is the most widespread tick in the world, transmitting several pathogens affecting humans. *Rh. sanguineus* is known as a vector of Mediterranean spotted fever (*Rickettsia conorii conorii*), Rocky Mountain spotted fever (*Rickettsia rickettsii*), and an emerging rickettsiosis caused by *Rickettsia massiliae* (Dantas-Torres, 2010). It is also a vector of dog pathogens including *Anaplasma platys, Babesia vogeli* and *Ehrlichia canis* (Dantas-Torres, 2010).

To date, no pathogen has yet been detected in ticks from Tahiti and the epidemiology of tick-borne diseases in humans and animals in this region is still unknown.

The objective of the present work was to investigate the presence of microorganisms including *Rickettsia* spp., *Bartonella* spp., *Babesia* spp., *Theileria* spp., *Anaplasma* spp. and *Ehrlichia* spp. in ticks collected in Tahiti.

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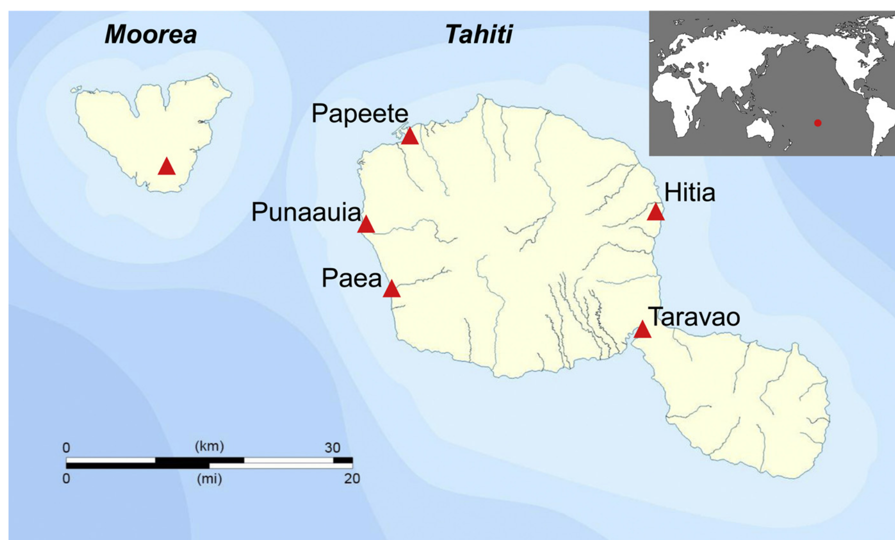

Fig. 1. Map of tick collection sites in Tahiti and Moorea. The different sampling sites are indicated by triangles.

2. Materials and methods

2.1. Tick collection and identification

Tick collections were performed in five localities of Tahiti: Papeete, Punaauia (veterinary clinics), Hitia, Paea and Taravao (bovine breeding areas), and in the southern part of Moorea Island from July to October 2013 (Fig. 1). Ticks were collected directly on cattle or in pasture by the use of a flannel flag (Parola and Raoult, 2001). Larvae were only collected in pasture using the flag technique while engorged adult and nymph ticks were collected directly on cattle with forceps. Ticks from a sick dog (Punaauia) with a clinical suspicion of ehrlichiosis were also included in the present study. Ticks were stored in 70% ethanol immediately after their collection and until their further use for molecular analysis. Each tick was identified morphologically using standard taxonomic keys (Rageau and Vervent, 1959; Pérez-Eid, 2007; Heath and Hardwick, 2011), and for positive infected tick specimens, a taxonomic confirmation was performed with molecular biology using primers targeting the 12S rRNA gene (Lv et al., 2014).

2.2. DNA extraction

Each tick specimen stored in 70% ethanol was rinsed with sterile water and air-dried on filter paper. Ticks were cut in two halves, using a new sterile surgical blade for each tick. One half of each specimen was stored at −80 °C as a backup sample and the other half was used for molecular analyses. Briefly, each half tick was crushed with a piston in a G2 buffer solution with proteinase K (Qiagen) and incubated at 56 °C overnight. DNA was then individually extracted from tick specimens using the EZ1 DNA tissue extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 μl of Tris EDTA (TE) buffer using the Qiagen EZ1 Advanced XL Robot with respective Qiagen kits as recommended by the manufacturers (Qiagen). DNA was then stored at −20 °C until use for molecular analysis.

2.3. Real-Time PCR screening

DNA samples were individually tested by genus-specific PCR using primers and probes targeting specific sequences of six tick-borne pathogens listed in Table 1. Real-time quantitative PCR was carried out according to the manufacturer's protocol using a CFX Connect™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA) with the Eurogentec Takyon qPCR kit (Eurogentec, Seraing, Belgium).

The *gltA* gene was used for screening for all *Rickettsia* species of the spotted fever group, as previously described (Rolain et al., 2009). An internally transcribed spacer (Angelakis et al., 2010) was used to target *Bartonella* spp. by qPCR, while the *rrl* gene of Anaplasmataceae species (Dahmani et al., 2015) was used to target *Anaplasma* and *Ehrlichia* spp. (Table 1).

For the detection of *Babesia* spp. and *Theileria* spp., primers and probes respectively targeting 18S rRNA and 28S rRNA gene sequences were designed using the online "Primer3" software. Primers were chosen with a size of 15–30 nucleotides, a GC% ranging from 40% to 60% and a melting temperature (Tm) of 60 °C. Probes were selected with a size of 20–40 nucleotides, a GC% ranging from 40% to 60% and a Tm of 10 °C higher than the primers' Tm. Taq-man® probes were labelled with the 6-FAM™ Fluorescein fluorophore.

Rickettsia montanensis DNA served as a PCR positive control for the primers and probes targeting the spotted fever group *Rickettsia*. *Bartonella elizabethae* DNA was used as a PCR-positive control for the primers and probes targeting all *Bartonella* species while *Anaplasma marginale* DNA served as a PCR positive control for the primers and probe targeting all Anaplasmataceae species. Sets of *Babesia* spp. and *Theileria* spp. synthetic positive controls were designed in association with the sets of primers and probes designed for these pathogens. They were designed by cloning the whole gene targeted by the associated primers and probe (18S rRNA and 28S rRNA genes respectively) and flanking sequences (from 210pb to 350pb) in a pUC57 plasmid (Eurogentec). For each run, a PCR mix without any DNA was taken as a negative control.

2.4. Sequencing and GenBank accession numbers

DNA samples that were positive in qPCR were submitted to conventional PCR amplifications using a Bio-Rad Thermocycler (Bio-Rad Laboratories, Hercules, CA) prior to sequencing. DNA amplification of the sample positive in Anaplasmataceae-specific qPCR was performed using two different systems, one targeting the *rrl* gene of the Anaplasmataceae family and the second targeting the 16S rRNA gene of *Ehrlichia* spp. (Table 1). *A. marginale* DNA and *Ehrlichia canis* DNA were respectively used as PCR positive controls. Amplification products were controlled by electrophoresis through a 1.5% agarose-tris-borate-EDTA gel containing SYBR Safe

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