Identification of *Bartonellae* in the Soft Tick Species *Ornithodoros sonrai* in Senegal

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Abstract

Ticks, belonging to the soft ticks species *Ornithodoros sonrai*, have been collected from six sites in Senegal and were tested for the presence of *Bartonella* spp. Initial screening by PCR revealed the presence of these bacteria in ticks from two villages, Soukhou Thissé (5/8, 62.5%) and Maka Gouye (1/24, 4.2%). Three bacterial strains were isolated from live ticks, and the genetic characterization of these strains suggests that they belong to two previously unknown species. The pathogenicity of these two new species of *Bartonella* is not yet known. The new isolates described here are the first strains of *Bartonella* spp. from soft ticks and the first isolates from any arthropod species in Africa.

Key Words: *Ornithodoros sonrai*—Soft ticks—*Bartonella*—Senegal.

Introduction

Bartonella is the monotypic genus of the Bartonellaceae family of Alphaproteobacteria. Twenty-nine species of this genus have been officially recognized to date, and other isolates are yet to be described (www.bacterio.cict.fr/b/bartonella.html; Sato et al. 2012). *Bartonella* species are facultative intracellular bacteria; many members of this family have been found to infect erythrocytes (Birtles 2005). *Bartonella bacilliformis* causes Carrion's disease, *B. quintana* is the causative agent of trench fever, and most of the cat scratch disease cases in humans are caused by *B. henselae*. Furthermore, at least 10 other members of the Bartonellaceae family have been associated with human pathologies, such as chronic bacteremia and/or endocarditis, bacillary angiomatosis, peliosis hepatitis, retinitis and uveitis, and myocarditis (Angelakis et al. 2010a).

The associations between the *Bartonellae* and their primary hosts are relatively specific and stable, i.e., most of *Bartonella* species may be found only in the blood of the specific mammal. For example, *B. henselae* is commonly found in domestic and wild felids worldwide, including in Africa (Pretorius et al. 2004b, Chomel et al. 2006, Chomel and Kasten 2010), and *B. alsatica* is associated with rabbits (Angelakis et al. 2008). Other animal hosts of *Bartonella* species include dogs, coyotes, foxes, cattle, deer, elk, and multiple species of rodents (Breitschwerdt and Kordick 2000, Minnick and Anderson 2006, Chomel and Kasten 2010). For most pathogenic *Bartonella* (except

for *B. bacilliformis* and *B. quintana*), humans are an accidental (secondary) host that may, however, develop pathology (Chomel and Kasten 2010).

Insects are often implicated in the transmission of the *Bartonella*. The vector for *B. quintana* is the *Pediculus humanus* louse, and the vector for *B. bacilliformis* is the sandfly *Lutzomyia verrucarum*. The role of ticks in transmission of bartonellosis, however, remains unclear (Angelakis et al. 2010a, Angelakis et al. 2010b, Telford III and Wormser 2010). The presence of *Bartonella* DNA in the tick samples does not mean that bacteria are still alive and transmissible to the next host. Recently, however, a study has reported the transmission of *Bartonella* by a tick bite (Angelakis et al. 2010b). Although evidence of the *Bartonella* has been found in hard ticks, no evidence of the presence of these bacteria in soft ticks exists.

The *Ornithodoros sonrai* soft tick is the only vector transmitting *Borrelia crocidurae*, the causative agent of relapsing fever (Hoogstraal 1985, Trape et al. 1996, Vial et al. 2006, Parola et al. 2011). The average incidence of tick-borne relapsing fever in West Africa may be as high as 25 out of every 100 people per year, and the average infection rate of the vector with *B. crocidurae* is 31% (Vial et al. 2006). Therefore, humans are regularly subjected to tick attacks, and this tick species is a successful vector for at least one known human pathogen. Recently, *O. sonrai* was also reported to be a potential reservoir of *Coxiella burnetii*, the causative agent of Q fever in Senegal (Mediannikov et al. 2010).

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TABLE 1. *ORNITHODOROS SONRAI* TICKS COLLECTED FOR MOLECULAR STUDIES IN RURAL SENEGAL IN SEPTEMBER, 2008: TICK COLLECTION SITES IN SENEGAL AND STUDY RESULTS

No.	Collection site	Geographic coordinates	No. of ticks collected in 70% ethanol	No. of ticks positive for <i>Bartonella</i> by PCR	No. of ticks collected alive
1	Soulkhoul Thissé	14°03'N 15°31'W	8	5/8 (62.5%)	15
2	Maka Gouye	13°48'N 14°56'W	24	1/24 (4.2%)	5
3	Didé	13°58'N 12°20'W	112	0/112 (0%)	0
4	Diana	13°32'N 12°51'W	84	0/84 (0%)	0
5	Dielmo	13°43'N 16°24'W	29	0/29 (0%)	0
6	Dakar	14°43'N 17°25'W	75	0/75 (0%)	0

In this work, we investigated the possible role of epidemiologically important soft tick as a host for *Bartonella*. We report the identification of *Bartonellae* species in *O. sonrai* from Senegal and the isolation of these bacterial strains.

Materials and Methods

Ticks collection and identification

Adult *O. sonrai* were collected from six sites (three households in each) in rural Senegal in September, 2008 (ticks in alcohol) and May, 2009 (live ticks) (Table 1, Fig. 1), in continuance of a prospective study on tick-borne relapsing fever in West Africa (Vial et al. 2006). Sampling was conducted in human dwellings; most of the habitats had no cement floor, or the floor was severely degraded, facilitating the colonization of rooms by rodents. During a detailed inspection of the households, rodents' burrows were observed in all cases. The *O. sonrai* species is a nidicolous tick that inhabits the burrows of small mammals. Ticks were collected by introducing a flexible tube into these burrows and aspirating their contents using a portable, petrol-powered aspirator (STIHL® SH56 27,2cc, Marne-La-Vallée, France). The contents were exposed to sunlight on sorting trays

to stimulate the ticks to move, and the recovered ticks were stored either in absolute ethanol (332 ticks total) or kept alive (20 ticks total) in hermetic plastic bottles until determination of the species and either DNA extraction or bacterial culture could be performed. All of the collected ticks exhibited morphological features similar to those previously described by Sautet and Witkowski and were identified as *O. sonrai*, the only *Ornithodoros* species that is known to live in rodent burrows in West Africa (Sautet and Witkowski 1944).

Molecular studies

The 332 ticks that had been designated for molecular studies were washed in three sterile water baths and were then crushed individually inside clean 1.5-mL plastic tubes using sterile scalpels. DNA from these ticks was then isolated and purified using the QIAamp® DNA Mini Kit Tissue Extraction Kit (Qiagen, Hilden, Germany). For screening purposes, the primers URBARTO.1 (5'-CTTCGTTTCTCTTCTTCAA-3') and URBARTO.2 (5'-CTTCTCTTCACAATTTCAAT-3') were used to amplify the 16S–23S ribosomal DNA internal transcribed spacer (ITS), with an annealing temperature of 48°C, as previously described (Rolain et al. 2003). The molecular

FIG. 1. Map of Senegal with the tick collection sites marked according to the numbering in Table 1.

characterization of the isolated strains was performed by sequence analysis of this intergenic spacer region, the 16S rRNA gene, and the *ftsZ*, *rpoB*, and *gltA* genes, according to the proposed gene sequence-based criteria for the species definition of the *Bartonella* genus (La Scola et al. 2003) and previously described methods (Birtles et al. 1995, Birtles and Raoult 1996, Renesto et al. 2001).

The amplified products were detected by electrophoresis, using 2% agarose gels in Tris/borate EDTA (TBE) buffer and staining with ethidium bromide. The remaining reaction mixtures were stored at -20°C for direct sequencing. The amplified DNA was sequenced using an ABI PRISM 3730xl DNA analyzer (Genome Express, Grenoble, France).

The evolutionary history of the sequenced samples was inferred using Bayesian phylogenetic analysis (Ronquist and Huelsenbeck 2003), performed on the TOPALi 2.5 software (Biomathematics and Statistics Scotland) with the integrated MrBayes application (<http://mrbayes.csit.fsu.edu>). One substitution model was used for the compiled alignments, based on the concatenated *gltA* and *rpoB* sequences. The sequences of the *rrs* (16S rDNA), *gltA*, *ftsZ*, and *rpoB* genes, as well as the ITS that was used for comparison, were obtained from the GenBank database (www.ncbi.nlm.nih.gov). The sequences were aligned and corrected manually for conserved motifs. Sites with ambiguous alignments were removed before the phylogenetic analysis.

Strain isolation

The period between the collection and treatment was 40 days. During this period, 20 live ticks were stored for in a plastic ventilated container at a temperature of $20\text{--}24^{\circ}\text{C}$ and 80% relative humidity. Each of 20 ticks was washed in a 10% solution of a commercial disinfectant/detergent (Amphomousse, Hydenet S.A., Sainghin-en-Melantois, France) in water, rinsed in sterile water, and placed in a 1% solution of sodium hypochlorite for 10 min. After the ticks were rinsed with distilled water, they were incubated in 70% ethanol for 15 min. A final rinse in sterile

phosphate-buffered saline (PBS) preceded the inoculation step. Each tick was then placed in a sterile 1.5-mL plastic tube, where they were triturated with a sterile micropestle in $300\text{ }\mu\text{L}$ of minimum essential medium (MEM), supplemented with 4% fetal bovine serum (FBS). Crushed ticks were distributed on Columbia agar supplemented with 5% sheep blood (BioMérieux, Marcy l'Etoile, France), and the isolation of the *Bartonella* strains was performed as described previously (Heller et al. 1998). Cultures were then frozen at -80°C in brain-heart infusion broth containing 20% of glycerol.

Electron microscopy

Negative staining for transmission electron microscopy was performed for the size determination and flagella studies. Bacteria were gently collected in their culture medium in a biosafety cabinet. An initial centrifugation was performed at 1000 rpm for 10 min, and a drop of the supernatant was collected on a piece of parafilm. A nickel 400-mesh Formvar carbon grid was deposited on top of the drop for 10 min at 37°C . The samples were exposed to a solution of 1% ammonium molybdate for 10 s for contrast. Before their removal from the biosafety cabinet grid, the samples were exposed to ultraviolet (UV) light for 1 h. Samples were examined using a Morgagni 268D (Philips) transmission electron microscope.

This work is an integral part of the IDEPATH project, which consists of investigations into the causes of nonmalarial febrile diseases in rural Senegal. The project was approved by the National Ethics Committee of Senegal (No. 0-00.87).

Results

Despite the large number (332) studied, ticks from only two of the villages (Soukhouth Thissé and Maka Gouye) were infected with *Bartonella* spp. in 5/8 (62.5%) and 1/24 (4.2%), respectively (Table 1). A BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of ITS amplicon sequences obtained from the ticks stored in alcohol showed that the *Bartonella* that were identified from

TABLE 2. SEQUENCE SIMILARITY OF THE ISOLATES FROM THE PRESENT STUDY TO THE MOST CLOSELY RELATED *BARTONELLA* SPECIES

Strain	Gene	GenBank accession number	Closest species with standing in nomenclature or incompletely described isolates; GenBank accession numbers (similarity, %)
"<i>Bartonella senegalensis</i>"			
OS02	<i>rrs</i>	HM636442	<i>Bartonella henselae</i> BX897699 (99.3)
	ITS	HM636451	<i>Bartonella henselae</i> AF312495 (86.2)
	<i>rpoB</i>	HM636454	<i>Bartonella henselae</i> BX897699 (93.7)
	<i>gltA</i>	HM636448	<i>Bartonella henselae</i> BX897699 (95.2)
	<i>ftsZ</i>	HM636445	<i>Bartonella henselae</i> BX897699 (94.5)
"<i>Bartonella massiliensis</i>"			
OS09	<i>rrs</i>	HM636440	<i>Bartonella queenslandensis</i> EU111758 (99.5)
	ITS	HM636449	<i>Bartonella elizabethae</i> JF766264 (79.5)/ <i>Bartonella</i> sp. EU218552 (82.1)
	<i>rpoB</i>	HM636452	<i>Bartonella tribocorum</i> JF766251 (93.2)/ <i>Bartonella</i> sp. GU143488 (93.7)
	<i>gltA</i>	HM636446	<i>Bartonella grahamii</i> CP001562 (94.5)/ <i>Bartonella</i> sp. FJ851110 (96%)
	<i>ftsZ</i>	HM636443	<i>Bartonella grahamii</i> CP001562 (96.6)
OS23	<i>rrs</i>	HM636441	<i>Bartonella queenslandensis</i> EU111758 (99.5)
	ITS	HM636450	<i>Bartonella elizabethae</i> JF766264 (81.3)/ <i>Bartonella</i> sp. EU218552 (82.4)
	<i>rpoB</i>	HM636453	<i>Bartonella tribocorum</i> JF766251 (93.2)/ <i>Bartonella</i> sp. GU143488 (93.7)
	<i>gltA</i>	HM636447	<i>Bartonella grahamii</i> CP001562 (94.6)/ <i>Bartonella</i> sp. FJ851110 (95.9)
	<i>ftsZ</i>	HM636444	<i>Bartonella grahamii</i> CP001562 (96.8)

The similarity to incompletely described isolates is presented only in cases when the total BLAST score for these isolates is higher than for species with officially validated names.

ticks from the Soukhou Thissé and Maka Gouye villages differed from each other insignificantly (0.3–3%), as well as from any other officially recognized species. The most closely related sequences found were *B. tribocorum* CIP 105476 (AM260525.1) and *B. elizabethae* F9251 (L35103.1), which had approximately 80–83% identity to the sequences that were obtained from the ticks.

The repeatedly (May, 2009) collected ticks from two above-mentioned villages were subjected to bacterial culture. We successfully isolated three strains of *Bartonella*: Strains OS02 and OS23 were recovered from the ticks found in Soukhou Thissé, and strain OS09 was recovered from the ticks found in Maka Gouye. The first bacterial colonies appeared 5–7 days after inoculation.

Two strains, OS09 and OS23, were found to be almost genetically identical. The identities between them are as follows: 100% for the *rrs* gene, 100% for the *rpoB* gene, 99.8% for the *ftsZ* gene, and 99.9% for the *gltA* gene. We found no mutations in the ITS; however, a 5-bp deletion was present in the ITS of strain OS23. Remarkably, the ITS sequences of strains OS09 and OS23 shared a degree of identity (80–83%) with some amplicons from ticks; however, they were not the same. The spacer sequences of the OS02 strain differed significantly from both the OS09 and OS23 strains. Both genotypes (*i.e.*, OS02 provisionally called here “*Bartonella senegalensis*” and OS09/OS23 provisionally called here “*Bartonella massiliensis*”) differed from any other officially recognized

FIG. 2. Phylogenetic tree highlighting the position of the *Bartonella* isolates from the *Ornithodoros sonrai* ticks (named OS02, OS09, and OS23) relative to other strains within the genus *Bartonella*. The evolutionary history was inferred using a Bayesian phylogenetic analysis with a codon position model, on the basis of the concatenated *gltA* and *rpoB* gene sequences. Numbers at the nodes are the bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree. The tree is drawn to scale, with branch lengths shown using the same units as those of the evolutionary distances that were used to infer the phylogenetic tree. There were a total of 1044 positions in the final dataset. The scale bar indicates a 10% nucleotide sequence divergence.

Bartonella spp. and from other uncultured *Bartonellae* when these genotypes were compared with data from GenBank.

The identities in the sequences from isolate OS02 to the closest member of the *Bartonella* family (*B. henselae* strain Houston-1) for the 16S rRNA gene, ITS, *ftsZ*, *rpoB*, and *gltA* were 99.3%, 86.2%, 94.5%, 93.7%, and 95.2%, respectively (Table 2). The identities in the sequences from isolates OS09/OS23 to different closest members of the *Bartonella* family for the 16S rRNA gene, ITS, *ftsZ*, *rpoB*, and *gltA* were 99.5%, 79.5–81.3%, 96.8–96.9%, 93.2%, and 94.5–94.6%, respectively (Table 2). These data may be sufficient to identify the OS02 and OS09/OS23 strains as two new species (La Scola et al. 2003).

A phylogenetic tree, based on the sequences from the concatenated protein-coding housekeeping *gltA* and *rpoB* genes (Fig. 2) demonstrates that the isolated strains form well-defined branches with high bootstrap values (97–100%). OS02 branches strictly with the *B. henselae*, *B. koehlerae*, *B. washoensis*, and *B. quintana* species. OS09 and OS23 branch consistently with the *B. rattimassiliensis*, *B. grahamii*, *B. tribocorum*, and *B. elizabethae* species (Fig. 2).

Bartonella sp. strain OS02

Bartonella sp. strain OS23

FIG. 3. Transmission electron micrograph of cells of the *Bartonella* strain OS02 and *Bartonella* strain OS23. Bars, 500 nm.

Electron microscopy indicated that the cell morphologies of all of the strains were similar, and no flagella or pili were observed (Fig. 3). Cells were found to be 1254.4 ± 329.3 nm long and 533.3 ± 100.5 nm wide for strain OS02 and 1337.4 ± 264.8 nm long and 490.8 ± 127.8 nm wide for strain OS23.

Discussion

We have identified *Bartonella* in 6/332 (2%) of studied soft ticks and isolated two new genotypes that might represent new species. In Africa, *Bartonella* species have been isolated only in humans or other mammals. These include *B. henselae* in cats, dogs, lions, cheetahs, and humans (Pretorius et al. 1999, Gundi et al. 2004, Pretorius et al. 2004b), *B. quintana* in humans (Thiam et al. 2002, Znazen et al. 2005), *B. clarridgeiae* in dogs (Gundi et al. 2004), multiple isolates of *Bartonella* spp. in small rodents in South Africa (Pretorius et al. 2004a), *B. bovis* in cattle in Côte d'Ivoire (Raoult et al. 2005), and several uncharacterized *Bartonella* species in felids (Molia et al. 2004). In Senegal, the only published data concerning the presence of *Bartonella* is a case report of *B. quintana*-related endocarditis in an immunocompetent patient (Thiam et al. 2002), so here we report the first isolates of *Bartonella* in Senegal.

The *Bartonella* isolates were first identified in African ticks. To the best of our knowledge, the presence of the *Bartonellae* has never been reported in ticks in Africa, even when a large number (1019 ticks) were studied (Loftis et al. 2006). However, *B. quintana* has been identified in lice in Burundi (Raoult et al. 1998), Ethiopia (Angelakis et al. 2011), and Senegal (Boutellis et al. 2012). New *Bartonellae* genotypes have been identified in *Pulicidae* fleas in Congo and Egypt (Sackal et al. 2008; Loftis et al. 2006) and in rat mites (Acari: Macro-nyssidae) in Egypt (Reeves et al. 2007). *B. rattimassiliensis*, *B. phoceensis*, and a *Bartonella* sp. similar to *B. tribocorum* has been found in lice (Hemiptera: Polyplacidae families) in Egypt (Reeves et al. 2006). Finally, *B. quintana* has been found in *Pulex irritans* fleas in Gabon (Rolain et al. 2005).

Also here we report the first isolation of the *Bartonellae* in the soft ticks. Moreover, *Bartonella* has been reported in the soft tick only once, when a fragment of DNA sharing 100% identity with *B. henselae* was amplified from 1 of 31 bat-associated soft ticks of the species *Carios kelleyi* that had been collected in the United States (Loftis et al. 2005).

The identification of live *Bartonella* bacteria in a well-established vector for relapsing fever suggests that *Bartonella* are well-adapted to *O. sonrai* and that soft ticks may represent hosts and, probably, even vectors. The unintentional period between collection and isolation was more than 40 days; therefore, ticks did not receive blood that could potentially contain mammalian-associated *Bartonella* for at least 40 days. Bacteria of the genus *Bartonella* are extremely fastidious and die easily (Brouqui and Raoult 2001) when exposed to improper culture conditions. The isolation of live bacteria from ticks in at least 40 days of hunger suggests a stable relationship between bacteria and tick. Nothing, however, is currently known about the pathogenicity of these two species in humans. Both bacteria morphologically (absence of pili and flagella, small size) resembled other species of *Bartonella* genus, pathogenic or not.

Our data reveal the presence of two arthropod-associated bacteria in the epidemiologically important soft tick *O. sonrai*. Morphologically similar to other species of *Bartonella*, genetic

differences among OS02 and OS09/OS23 isolates and other species of the genus *Bartonella* are enough to represent two new species. Because of the epidemiological and phylogenetic circumstances, further studies are needed to clarify the role of these new species in human and animal pathology and identification of reservoirs (rodents, insectivores, and others).

Acknowledgments

We are grateful to Lise Gern for her valuable advice and for her organization of the Master's degree for K.K., and to Denis Pyak and Audrey Borg for their technical help.

This work was supported in part by the Agence Nationale de Recherche grant 2010 MALEMAF (research on emergent pathogens in Africa) and the IDEPATH project of IRD. The funders had no role in the study design, the data collection and analysis, the decision to publish, or the preparation of the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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