Massive infection of seabird ticks with Coxiella burnetii related species

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Seabird ticks are known reservoirs of bacterial pathogens of medical importance; however, ticks parasitizing tropical seabirds have received less attention than their counterparts from temperate and subpolar regions. Recently, *Rickettsia africae* was described to infect seabird ticks of the western Indian Ocean and New Caledonia, constituting the only available data on bacterial pathogens associated with tropical seabird tick species. Here, we combined a pyrosequencing-based approach with a classical molecular analysis targeting bacteria of potential medical importance in order to describe the bacterial community in two tropical seabird ticks, *Amblyomma loculosum* and *Carios (Ornithodoros) capensis*. We also investigated the patterns of prevalence and host specificity within the biogeographical context of the western Indian Ocean islands. The bacterial community of the two tick species was characterized by a strong dominance of *Coxiella* and *Rickettsia*. Our data support a strict *Coxiella*-host tick specificity, a pattern resembling the one found for *Rickettsia* spp. in the same two seabird tick species. Both the high prevalence and stringent host tick specificity suggest that these bacteria may be tick symbionts with probable vertical transmission. Detailed studies of the pathogenicity of these bacteria will now be required to determine whether horizontal transmission can occur and to clarify their status as potential human pathogens. More generally, our results show that the combination of next generation sequencing with targeted detection/genotyping approaches proves to be efficient in poorly investigated fields where research can be considered to be starting from scratch.
lands, respectively, and investigated the prevalence of *Coxiella* bacteria in soft and hard ticks of the region.

**MATERIALS AND METHODS**

**Tick collection.** Ticks were collected from different seabird species in 2011 and 2012 on six islands of the WIO: La Réunion Island, Bird and Aride Islands (the Seychelles), and Europa, Juan de Nova, and Tromelin Islands (the Eparses Islands). Ticks were collected either directly from adults and chicks or in the nest environment, as follows: soft ticks were sampled on red-footed tropicbirds (*Phaethon rubricauda*) on Europa Island, on red-footed booby (*Sula sula*) specimens and nests on Tromelin Island, within reproductive colony boundaries of sooty terns (*Onychoprion fuscatus*) on Juan de Nova Island, and in wedge-tailed shearwater (*Puffinus pacificus*) nests on Petite Ile (La Réunion Island); hard ticks were collected on the ground of Petite Ile (La Réunion Island). Both soft and hard ticks were collected from a sooty tern colony at Bird Island. On Aride Island, ticks were collected from sooty terns (*Onychoprion fuscatus*), tropical shearwaters (*Puffinus bailloni*), brown noddies (*Anous stolidus*), lesser noddies (*Anous tenuirostris*), white-tailed tropicbirds (*Phaethon lepturus*), and wedge-tailed shearwaters (*Puffinus pacificus*). All ticks were morphologically identified as *A. loculosum* or *C. capensis* using standard taxonomic keys (21, 22). Depending on the access to liquid nitrogen tanks, ticks were either conserved by dry flash freezing or by placing them in 70% ethanol until the samples were brought back to the laboratory and frozen at −80°C before analyses.

Sampling in La Réunion Island and in the Eparses Islands (Europa, Juan de Nova, and Tromelin Islands) was conducted under the approval of the Direction de l’Environnement, de l’Aménagement et du Logement and the Terres Australes et Antarctiques Françaises. Sample collection at Aride and Bird Islands and sample export to La Réunion Island were performed with the approval of the Seychelles Bureau of Standards and the Ministry of Environment.

**Bacterial metabarcoding design.** Ticks analyzed through metabarcoding were randomly selected from samples of 3 distinct and geographically distant sites, i.e., Juan de Nova, Tromelin, and La Réunion Islands. Pooled tick samples consisted of 5 adult ticks for both *C. capensis* and *A. loculosum*. For each pool, ticks were placed in a single 1.5-ml Eppendorf tube and crushed for 2 min at 25 Hz using two 3-mm tungsten beads in a Tissuelyser apparatus (Qiagen, Valencia, CA). Total nucleic acids were extracted using a V2 minikit (Qiagen, Valencia, CA), and each pool was prepared for bacterial 16S rRNA metabarcoding (23) with the use of the GSFLX technology (Genoscreen, Lille, France). Briefly, 16S rRNA V3 and V4 variable regions were amplified via specific PCR primers targeting the upstream and downstream regions of the V3–V4 segment: the 3′ ends of forward (TACGGRAGGCAGCAG) and reverse (GGACTACCAGGGTA TCTAAT) bacterium-specific primers were associated at the 5′ end with *Coxiella burnetii* using the ClustalW program and subsequently trimmed, and the alignment was eventually refined over 100 iterations using the MUSCLE algorithm implemented in the Geneious Pro (v5.6.0) software package (24). A maximum likelihood phylogenetic tree with 1,000 bootstrap iterations was generated using the PhyML plugin from Geneious Pro (v5.6.0) and the optimal substitution model (GTR+I+G), as selected using the jModelTest (v2.1.2) tool (28). The appearance of the tree was edited in the FigTree (v1.4) program.

**Nucleotide sequence accession numbers.** Partial 16S rRNA gene sequences have been submitted to GenBank and may be found under accession numbers KP913893 to KP913928.

**RESULTS**

**Bacterial identification by pyrosequencing.** A total of 125,880 sequences were obtained from the four tick pools. The lengths of the sequences ranged from 30 to 568 bp, and 12,851 (10%) were shorter than 250 bp. The average and median read lengths were 401 and 442 bp, respectively, and sequence reads were evenly distributed across each multiplexed pool. Sequences were filtered using quality criteria to exclude reads containing ambiguities and/or sequences with length of less than 250 bp.

Independent sequencing repeats generated highly similar taxonomic representations, whereas samples from different islands or tick species generated characteristic diversity profiles (Fig. 1A). The overall levels of bacterial diversity measured in samples from *A. loculosum* from La Réunion Island and *C. capensis* from La Réunion and Tromelin Islands were comparable, whereas a greater level of diversity was obtained from *C. capensis* ticks from Juan de Nova Island (Fig. 1B and Table 1). However, rarefaction curves based on the leaves of a fully expanded taxonomic tree in MEGAN (v5) plateaued for each of the obtained samples, suggesting that the biodiversity of each sample had been exhaustively categorized.

Of the sequence reads assigned with MEGAN to a minimum taxonomic level of order, the bacterial orders *Legionales, Rickettsiales, Bacillales, Actinomycetales*, and *Rhodobacterales* were the most prominently represented across the studied tick samples (Fig. 1C).

**Core and shared biomes and genera of possible medical importance.** Characterization of the core bacterial biome constituted by combining data from all pooled samples identified *Coxiella, Bacillus*, and *Rickettsia* as the three most prominent bacterial genera present in the tested ticks of the region (Fig. 2D).

Interestingly, two of the most abundant genera identified in the tested ticks included known human pathogens: *Rickettsia*, the causative agent of rickettsioses such as African tick bite fever, has previously been identified to be abundant in ticks of the region.
and was observed to be prevalent within samples of *A. loculosum* from La Réunion Island. *Rickettsia* bacteria were also present but were less well represented in some samples of *C. capensis*. In contrast, *Coxiella* bacteria were present in large quantities in samples of *C. capensis* but at low levels in *A. loculosum*. *Coxiella* sequences exhibited between 98.4 and 98.7% genetic identities with *Coxiella burnetii*, the causative agent of Q fever (29). Interestingly *Borrelia* spp., the causative agents of Lyme disease and relapsing fever, were also identified in *C. capensis* from Tromelin Island; however, they were present only in trace quantities (13 of 24,797 reads). Additionally, *Kocuria* bacteria, which are increasingly associated with human pathologies (30), were identified in *A. loculosum*.

**Coxiella prevalence in seabird ticks.** Although *Coxiella* spp. were found to be ubiquitous from 454 pyrosequencing, precise species identification and measures of population prevalence were impossible to obtain from these data. Thus, we set out to characterize the regional *Coxiella* prevalence by targeted PCR and classical Sanger sequencing using *Coxiella*-specific primer pairs that target the 16S rRNA gene (31).

Targeted PCR was performed on the full bank of available individual tick samples, which consisted of *C. capensis* and *A. locu-
losum specimens collected from seabirds and their nests in Juan de Nova, Tromelin, Europa, Bird, Aride, and La Réunion Islands (Fig. 3). The Coxiella detection prevalence ranged from 46% to 100% (Table 2). Of all C. capensis ticks tested, 65.7% were positive for Coxiella, whereas this figure was 71.2% for A. loculosum. The prevalence of Coxiella bacteria was significantly lower in C. capensis ticks from La Réunion Island than in C. capensis ticks from the other locations (P < 0.001 by Fisher’s exact test in all pairwise comparisons).

Coxiella diversity shows a strict tick-host specificity. Phylogenetic analyses demonstrated that all Coxiella strains originating from the WIO were closely related to Coxiella burnetii and demonstrated nearly perfect species specificity for their tick hosts (Fig. 4). Two well-supported, separate bacterial clades were identified in hard ticks (e.g., A. loculosum, Amblyomma americanum, Amblyomma cajennense, and Haemaphysalis longicornis) and soft ticks (C. capensis and Ornithodoros moubata). Additionally, distinct Coxiella clades were identifiable for C. capensis and A. loculosum hosts. Furthermore, two distinct Coxiella haplotypes were identified in C. capensis ticks: one in a single P. pacificus nest in Petite Ile and the other in all other sequenced samples from the WIO. Genetic distances revealed that Coxiella strains from C. capensis were the most closely related to Coxiella burnetii. The mean ± standard deviation genetic identities to the C. burnetii group of the phylogenetic groups presented in Fig. 4 were as follows: 98.7% ± 0.19% for C. capensis symbiont group 1, 98.9% ± 0.31% for C. capensis symbiont group 2, 96.4% ± 0.21% for the Amblyomma species symbiont group, and 96.2% ± 0.20% for the Hemaphysalis longicornis symbiont group.

DISCUSSION

The present investigation shows that a combination of nontargeted 454 pyrosequencing with a subsequent specific investigation of a taxon of interest quickly provides a powerful set of data for

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Tick species</th>
<th>No. of ticks</th>
<th>Replicate no.</th>
<th>MID used</th>
<th>No. of raw reads</th>
<th>No. of reads after quality control</th>
<th>Shannon index</th>
<th>Simpson index</th>
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<tbody>
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<td>5</td>
<td>1</td>
<td>MID1</td>
<td>14,743</td>
<td>13,652</td>
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<td></td>
<td></td>
<td></td>
<td>2</td>
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<td>14,243</td>
<td>1.61</td>
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<td>1</td>
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<td></td>
<td></td>
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</tr>
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<td>1.79</td>
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<td></td>
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<td>1.88</td>
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<td>1</td>
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<td>1.47</td>
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<td>13,418</td>
<td>1.54</td>
<td>2.35</td>
</tr>
</tbody>
</table>

**FIG 2** Bacteriome data from ticks of the WIO. Bars indicate the number of raw sequence reads assigned to named bacterial taxa. The numbers of sequences assigned to the named taxa are indicated above the bars. Bars corresponding to Coxiella spp. and Rickettsia spp. are colored red and green, respectively.
Rickettsia africae has shown that these ticks are infected with the human pathogen these bacterial species. Our results corroborate recent work that ticks are a frequent and possibly amplifying reservoir host for obtained from the majority of tested tick samples, suggesting that bacterial species substantially differ from the other Rickettsia spp. infecting the WIO sample and were strongly related to Rickettsia bellii, considered the earliest diverging Rickettsia species (34, 35). These two specimens may descend from a distinct tick population or lineage, as recently described for C. capensis (19), and thus be infected by distinct Rickettsia and Coxella strains. Such a pattern would suggest that “a single bird with reservoir ticks may serve to seed a new geographic area,” as previously suggested (36). A population genetics investigation of these ticks at a global scale may allow testing of this tempting but still speculative hypothesis.

**Coxiella burnetii** is the causative agent of Q fever, which can cause illness in many domesticated mammals, birds, and humans (37). *Coxiella*-like bacteria have now been identified in a number of distinct tick species (31, 38–42) and are considered symbionts with probable vertical transmission. Whether *Rickettsia* may be primarily considered an invertebrate symbiont or a vertebrate pathogen is still an open question (34, 43) and can be subjectively related to the main scientific interest of each researcher. Given the growing number of *Coxiella* species described in several distinct tick species and the prominent prevalence of this taxon in the poorly investigated biological material, such as tropical seabird ticks. Products from two independent PCRs carried out on DNA from four independent pooled tick samples were sequenced, and sequencing indicated little intrasample variability, which may have occurred due to the experimental variability in deep sequencing and/or PCRs. This suggests that increasing the number of samples may be more relevant than duplicating PCRs and sequencing when designing such approaches.

The depth of the analysis provided by this next generation sequencing methodology was sufficient to identify bacterial species of *Kocuria* (an emerging human pathogen) and other genera that were present in minute trace quantities, such as *Borrelia* spp., which are known to be transmitted by seabird ticks (5) and which are the causative agents of Lyme disease and relapsing fever. Furthermore, high read numbers of *Rickettsia* and *Coxiella* spp. were obtained from the majority of tested tick samples, suggesting that ticks are a frequent and possibly amplifying reservoir host for these bacterial species. Our results corroborate recent work that has shown that these ticks are infected with the human pathogen *Rickettsia africae* (20). The investigation presented herein reveals massive infection of both hard and soft ticks with *Coxiella*-like organisms, with the bacteria infecting soft ticks presenting up to 99% identity with *C. burnetii*. The strict host specificity reported herein supports the vertical transmission of this bacterial species and the previously proposed long coevolution of both *Coxiella* lineages following an ancient infection in the ancestor of both main families of ticks: the Argasidae (soft ticks) and the Ixodidae (hard ticks) (31). We did not observe any bird species-associated specificity or any spatial structure, which suggests that the presented *Coxiella* spp. are likely transmitted among islands and bird species. However, the resolution of a 16S rRNA gene marker is not sufficient to completely address these points (O. Duron, personal communication), and the use of additional markers will be necessary to resolve the regional structure of this bacterial population.

Another important point raised by the 454 approach is that the results provide information not only on bacterial diversity but also on bacterial abundance, as previously discussed (32). Indeed, 29.5% of all bacterial 16S rRNA gene haplotypes were related to *Coxiella*, and another 19.0% were related to *Rickettsia*. Thus, although a quantitative PCR study of individual ticks will be required to determine bacterial copy numbers per host, our data show that these two bacterial genera are clearly prominent in *C. capensis* and *A. loculosum*.

The absence of some symbionts in the sample is also of interest. For instance, the absence of *Wolbachia* in these seabird ticks is coherent with the proposition of Plantard et al. that *Wolbachia* isolates that were previously detected in ticks had a parasitoid origin (33).

Two *C. capensis* ticks from the same nest of a wedge-tailed shearwater (*P. pacificus*) were infected with a clearly distinct *Coxiella* haplotype. Interestingly, the same two tick specimens were shown to be infected with two *Rickettsia* spp., substantially different from the other *Rickettsia* spp. infecting the WIO sample and were strongly related to *Rickettsia bellii*, considered the earliest diverging *Rickettsia* species (34, 35). These two specimens may descend from a distinct tick population or lineage, as recently described for *C. capensis* (19), and thus be infected by distinct *Rickettsia* and *Coxiella* strains. Such a pattern would suggest that “a single bird with reservoir ticks may serve to seed a new geographic area,” as previously suggested (36). A population genetics investigation of these ticks at a global scale may allow testing of this tempting but still speculative hypothesis.

### TABLE 2 Rate of detection of *Coxiella* spp. in ticks from WIO region

<table>
<thead>
<tr>
<th>Tick species</th>
<th>Origin</th>
<th>No. of ticks:</th>
<th>Prevalence (%) of <em>Coxiella</em> detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Tested</td>
</tr>
<tr>
<td><em>C. capensis</em></td>
<td>Tromelin Island</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Juan de Nova Island</td>
<td>42</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Europa Island</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>La Réunion Island</td>
<td>87</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>Bird Island</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td><em>A. loculosum</em></td>
<td>Aride Island</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Tromelin Island</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>La Réunion Island</td>
<td>37</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Bird Island</td>
<td>28</td>
<td>39</td>
</tr>
</tbody>
</table>
seabird ticks investigated herein, it seems that a proper assessment of the pathogenicity of these *Coxiella* species should today be considered an important issue.

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