Large-Scale Survey for Tickborne Bacteria, Khammouan Province, Laos

Andrew J. Taylor, Khamsing Vongphayloth, Malavanh Vongsouvath, Marc Grandadam, Paul T. Brey, Paul N. Newton, Ian W. Sutherland, Sabine Dittrich

We screened 768 tick pools containing 6,962 ticks from Khammouan Province, Laos, by using quantitative real-time PCR and identified *Rickettsia* spp., *Ehrlichia* spp., and *Borrelia* spp. Sequencing of *Rickettsia* spp.–positive and *Borrelia* spp.–positive pools provided evidence for distinct genotypes. Our results identified bacteria with human disease potential in ticks in Laos.

*Rickettsia*, *Borrelia*, *Ehrlichia*, *Anaplasma*, and *Coxiella* spp. are tick-associated bacteria and well-described human pathogens. All of these bacteria, except *Coxiella* spp., are primarily transmitted through tick bites and cause febrile disease with a wide spectrum of severity. Tickborne bacterial pathogens are believed to be an underrecognized cause of acute febrile illness in Southeast Asia (1).

In Laos, spotted fever group *Rickettsia* have been shown to cause undifferentiated fever in 2% of febrile hospitalized adult patients (2). However, data on bacteria in ticks in Laos are sparse. To date, 1 *Rickettsia* sp. has been identified in a *Boophilus* sp. tick from Luang Namtha Province; this species showed 99.8% similarity with the *Rickettsia* sp. FUJ98 ompA gene (3). No other tickborne bacteria have been reported from Laos. Therefore, we investigated *Rickettsia*, *Borrelia*, *Ehrlichia*, *Anaplasma*, and *Coxiella* spp. in ticks from Khammouan Province, Laos.

The Study

We collected ticks in Nakai District, Khammouan Province, during the dry seasons (December–April) during 2012–2014, as previously described (4) (online Technical Appendix Figures 1, 2, http://wwwnc.cdc.gov/EID/article/22/9/15-1969-Techapp1.pdf). A total of 6,692 ticks were pooled (n = 768 pools, 1–10 ticks/pool) according to genus, sex, developmental stage, collection period, and site. One *Amblyomma testudinarium* nymph that contained a blood meal was processed separately.

We extracted DNA by using the NucleoSpin 8 Virus Extraction Kit (Macherey-Nagel, Düren, Germany). Pools were screened by using single quantitative real-time PCRs specific for *Rickettsia* spp. (17-kDa gene), *Borrelia* spp. (23S rRNA gene), *Anaplasma* spp. (major surface protein 2 gene), *Ehrlichia* spp. (16S rRNA gene), and *Coxiella* spp. (IS1111) (5–8) (online Technical Appendix Table 1). Five microliters of diluted (1:10) template containing 1× Platinum Supermix-UDG (Invitrogen, Carlsbad, CA, USA) and bovine serum albumin (40 mg/mL) were used for each assay. Positive and nontemplate controls were included in each run. Screening by PCR was performed once per sample. In concordance with published guidelines, results were considered positive if they had a cycle quantitation (Cq) value <40 and likely positive if they had a Cq value 40–45 (9).

Sequencing was attempted for pools with Cq values <40 (online Technical Appendix Table 2) and performed by Macrogen (Seoul, South Korea). Consensus sequences were analyzed by using CLC Main Workbench 7 (http://www.clcbio.com/products/clc-main-workbench/) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank. Phylogenetic trees were constructed by using the Kimura 2-parameter model and the neighbor-joining method. Bootstrap values were determined by using 1,000 replications.

A total of 768 tick pools containing 6,692 ticks were screened. Pools contained 3 genera of ticks: 59.9% (460/768) *Amblyomma* spp., 36.3% (279/768) *Haemaphysalis* spp., 3.8% (29/768) *Dermacentor auratus*. Of the pools, 3% (23/768) contained adults, 36.5% (279/768) contained nymphs, and 60.5% (465/768) contained larvae (Table 1).

*Rickettsia* spp. were identified in 5.7% (44/768) of pools, and an additional 2.3% (18/768) of pools were likely positive for *Rickettsia* spp. Sequences consistent with 5 described *Rickettsia* species or genotypes were identified: *R. tamurae*, *R. japonica*, *Rickettsia* sp. ATT, *Rickettsia* sp. Kagoshima6, and *Rickettsia* sp. TwKM01 (Table 2; Figure 1).

Three novel genotypes (Table 2) were identified that might be new species. *Candidatus* Rickettsia laoensis (pool 447) was identified in 1 *Haemaphysalis* sp. pool. Phylogenetic analysis of 2845–2920-bp concatenated sequences of gltA, sca4, and ompB genes suggested that this bacteria

*These senior authors contributed equally to this article.*
belonged to the *R. massiliae* group of rickettsiae (online Technical Appendix Figure 3). *Candidatus Rickettsia mahosotii* (pools 81 and 372) was identified in *Haemaphysalis* spp. and *A. testudinarium* pools. Phylogenetic analysis of gltA, *sca4*, and *ompB* genes suggested that this bacteria belonged to the *R. rickettsii* group (online Technical Appendix Figure 3). *Candidatus Rickettsia khammouanensis* was identified in 1 *Haemaphysalis* sp. nymph pool (pool 120). Phylogenetic analysis of gltA, 17-kDa, and *ompB* genes suggested a relationship with the *R. helvetica* group (online Technical Appendix Figure 4).

In addition, 15 *A. testudinarium* pools showed dual peaks for 17-kDa gene sequences, which suggested the presence of *R. tamurae* and *Rickettsia* sp. ATT. Sequencing of *sca4*, *ompA*, and *ompB* genes from 1 of these pools (pool 239) identified unique sequences (Table 2; online Technical Appendix Figure 4).

*Borrelia* spp. were identified in 1.6% (12/768) of pools (Table 1). Two unique sequences obtained from *Haemaphysalis* spp. pools showed 99.3% (298/300) (GenBank accession no. KR733069) and 98.7% (296/300) (accession no. KR733068) identity with Shiretoko *Haemaphysalis Borrelia* sp. (AB897888). Phylogenetic analysis confirmed that both bacteria were closely related to Shiretoko *Haemaphysalis Borrelia* sp. (accession no. B897888) and belong to the relapsing fever group of *Borrelia* (Figure 2).

Twelve (1.6%) of 768 pools were positive for *Ehrlichia* spp. (Table 1); an additional 6 pools (0.8%) were likely positive. One short sequence from a *Haemaphysalis* sp. nymph pool (pool 357) was obtained, and this sequence showed 100% identity (116/116 bases) with the genus *Ehrlichia*.

No pools were positive for *Anaplasma* spp., but 2 were likely positive (Table 1). Although not all pools were tested for *Coxiella* spp. (n = 511), 1 pool (0.2%) was positive, and 4 pools were likely positive for *C. burnetti*. No confirmatory sequences were obtained from these pools. The 1 tick that contained a blood meal (*A. testudinarium* nymph) showed negative results by screening PCRs.

### Conclusions

This study provides evidence that *Rickettsia* spp., *Borrelia* spp., and *Ehrlichia* spp. are present in ticks in Laos. Several *Rickettsia* spp. identified in this study are human pathogens. Infections with *R. tamurae* (2) and *R. japonica* are well described in Southeast Asia (10). However, the pathogenicity of *Rickettsia* sp. TwkM01 (11), *Rickettsia* sp. ATT (12), *Rickettsia* sp. kagoshima6 genotypes (13) and potential novel *Candidatus Rickettsia laoensis*, *Candidatus Rickettsia mahosotii*, and *Candidatus Rickettsia khammouanensis* is unknown. *Candidatus Rickettsia khammouanensis* is phylogenetically related to *R. helvetica*, for which there is serologic evidence for its role as a human pathogen in Laos (2). Unique *ompA*, *ompB*, and *sca4* sequences identified in this study (Table 2) might indicate the presence of...
**Table 2.** Sequence data for *Rickettsia* species isolated from ticks, Khammouan Province, Laos*

<table>
<thead>
<tr>
<th>Tick pool</th>
<th>Tick stage and species</th>
<th><em>Rickettsia</em> spp. gene, GenBank accession no., and % similarity (no. matching nucleotides/total)</th>
<th>ompB</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td><em>Amblyomma testudinarium</em> nymph</td>
<td>Unclear sequence NS Unclear sequence KT753264, 100.0 (529/529) with <em>Rickettsia</em> sp. TwKM01 EF219467</td>
<td>NS</td>
</tr>
<tr>
<td>177-180</td>
<td><em>A. testudinarium</em> nymphs, A. testudinarium nymph (17 kDa only)</td>
<td>KT753277, 98.4 (360/361) with <em>Rickettsia</em> sp. ATT AF483196</td>
<td>KT753279, 98.4 (794/807) with <em>Rickettsia</em> sp. JL-57499 to <em>R. japonica</em> AF155055</td>
</tr>
<tr>
<td>315</td>
<td><em>A. testudinarium</em> nymph</td>
<td>KT753267, 98.8 (407/412) with <em>R. raoultii</em> JX885457</td>
<td>KT753269, 98.6 (795/821) with <em>Rickettsia</em> sp. AUS 118, KF666473</td>
</tr>
<tr>
<td>239</td>
<td><em>A. testudinarium</em> nymph</td>
<td>KT753272, 99.7 (367/370) with <em>Rickettsia</em> sp. hhmj7 KC566999</td>
<td>KT753274, 97.1 (759/782) with <em>Rickettsia</em> sp. AUS 118, KF666473</td>
</tr>
<tr>
<td>76-7, 337</td>
<td>*Haemaphysalis G1 nymphs (3), A. testudinarium nymph (1)</td>
<td>KT753277, 98.4 (417/423) with <em>R. raoultii</em> JX885457</td>
<td>KT753279, 98.4 (794/807) with <em>Rickettsia</em> sp. JL-57499 to <em>R. japonica</em> AF155055</td>
</tr>
<tr>
<td>81, 372</td>
<td>*Haemaphysalis G1 nymphs, A. testudinarium nymph (17 kDa only)</td>
<td>KT753277, 99.0 (408/412) with <em>R. raoultii</em> JX885457</td>
<td>KT753285, 98.5 (838/851) with <em>R. japonica</em> AF150555</td>
</tr>
<tr>
<td>120</td>
<td>*Haemaphysalis G1 nymph</td>
<td>KT753287, 96.1 (391/407) with <em>R. helvetica</em> GU827073</td>
<td>KT753288, 97.1 (370/381) with <em>Candidatus Rickettsia rara</em> DQ365805</td>
</tr>
<tr>
<td>407</td>
<td><em>Haemaphysalis hystrix</em> adult</td>
<td>KT753307, 98.4 (413/413), <em>R. japonica</em> CP001533</td>
<td>KT753321, 100.0 (1,063/1,063), <em>R. japonica</em> AP011533</td>
</tr>
<tr>
<td>447</td>
<td>*Haemaphysalis G1 nymph</td>
<td>KT753291, 98.6 (407/413) with <em>R. massiliae</em> CP006683</td>
<td>KT753290, 99.6 (981/985) with <em>R. raoultii</em> JX885455</td>
</tr>
</tbody>
</table>

*New sequences were compared with reference sequences. NS, not sequenced.

*Rickettsia* sp. ATT (12), which was previously believed to be identical to *R. tamurae* (14), and suggests that it might be a distinct species. Further studies, including whole-genome sequencing, are required to identify and confirm these novel genotypes and understand their role in human disease.

*Borrelia* spp. sequences identified in *Haemaphysalis* spp. pools were shown to have high concordance with the Shiretoko *Haemaphysalis Borrelia* isolated from *Haemaphysalis* spp. ticks and deer in Japan (15). The species belongs to the relapsing fever group of *Borrelia* and is related to *B. lonestari*.

Sequence data for *Ehrlichia* spp. indicated the presence of these bacteria but were not sufficient to identify them to the species level. The Cq values were high (40–45) for *Anaplasma* spp., but no sequence data were obtained. *Coxiella* spp. were screened by using primers for IS1111, which are not specific for *C. burnetii*, and no confirmatory sequence data were obtained. Because of limited reagents, screening of all 768 pools for *Coxiella* spp. was not completed. Further work is required to investigate the presence of these bacteria in Laos.

Our study had several limitations. First, pooling of ticks precludes an accurate assessment of prevalence of bacterial pathogens. Second, sequences obtained from some *A. testudinarium* pools had dual peaks, suggestive of multiple infections, and could therefore not be interpreted. Third, ticks were collected only from 1 area in Laos.
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(Khammouan Province); thus, extrapolating findings to the entire country must be done cautiously.

Our results highlight the frequency of tickborne bacterial infections in Laos. These findings emphasize the need for further research of tick-associated bacteria and their role in human disease.

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Figure 1. Phylogenetic analysis of Rickettsia spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (350 bp) of the 17-kDa gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.

Figure 2. Phylogenetic analysis of Borrelia spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (299–323 bp) of the flaB gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.
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Dr. Taylor is a research physician at the Centre for Tropical Medicine and Global Health, University of Oxford, Oxford, UK. His primary research interest is infectious diseases.

References


Address for correspondence: Andrew J. Taylor; Center for Tropical Medicine and Global Health, Nuffield Department of Medicine, Research Building, University of Oxford, Oxford OX3 7FZ, UK; email: andrewtaylor9@gmail.com

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