PREVALENCE AND DIVERSITY OF BARTONELLA IN RODENTS OF NORTHERN THAILAND: A COMPARISON WITH BARTONELLA IN RODENTS FROM SOUTHERN CHINA

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Abstract. We report results of the first study to investigate the distribution and diversity of Bartonella in rodents from Thailand. Whole blood from 195 rodents, representing six species, was tested for the presence of Bartonella species using standard culture techniques. Isolates were obtained from 17 (8.7%) of the samples, and 14 of those isolates represented distinct strains, based upon partial sequencing of the citrate synthase (gltA) gene. Phylogenetic analysis of the isolates and other Bartonella species indicated that five unique isolates from Bandicota indica form a cluster that may represent a new Bartonella species. Two additional isolates from B. indica clustered together, and were nearly identical to an isolate from Apodemus draco collected in southern China. Importantly, a number of the isolates from Thailand rodents are closely related to B. grahamii and B. elizabethae, species which have been associated with human illness.

INTRODUCTION

Members of the genus Bartonella are gram-negative bacteria that infect erythrocytes of their mammalian hosts. Several species in the genus cause human illness, including: trench fever (B. quintana), Carrion's disease (B. bacilliformis), bacillary angiomatosis, and cat scratch disease (B. henselae). Other manifestations in humans that have been described recently include cardiovascular, central nervous system, and hepatosplenic disease.

In recent years, there has been an increase in the number of studies designed to investigate the distribution and prevalence of Bartonella species throughout many regions of the world, including southeastern Asia. Several studies have described the prevalence of Bartonella in cats in Japan, and others have reported the seroprevalence of Bartonella infections in Japanese citizens. Recently, B. quintana DNA was recovered from lice infesting homeless people in Tokyo. Ying and others investigated the prevalence of Bartonella in small mammals from southern China, and found that 43% of 131 animals were culture-positive. In Thailand, cats and dogs were bacteremic and/or seropositive for B. henselae, B. clarridgeiae, and B. claridgeaeae, and fleas were found to contain B. henselae or novel bartonellae. While human illness due to Bartonella infection has not been reported from Thailand, Maruyama and others found that 5.5% of 163 healthy humans were seropositive for B. henselae.

Along with increased interest in the worldwide distribution of known bartonellae, there has recently been a surge in the number of descriptions of novel members of this genus, with isolates being obtained from rodents, cats, dogs, and other domesticated and wild animals. The public health importance for many of these isolates remains undefined; however, some, such as B. vinsonii arupensis, B. elizabethae, B. grahamii, and B. washoenisis have been isolated from humans and linked to a rodent reservoir. The close association between rodents and humans throughout the world, especially in areas of high human population density, including southeastern Asia, makes the study of rodent-borne Bartonella essential to determine the extent to which rodents may serve as a source of human infections.

The objectives of this study were to determine the prevalence and diversity of Bartonella species in rodents of Thailand, to determine the level of Bartonella bacteremia in individual animals, to characterize Bartonella cultures obtained from those animals using genetic analyses, and to compare the results obtained from Thai rodents with published and unpublished data obtained from rodents in southern China.

MATERIALS AND METHODS

Animals. This research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council Publication, 1996 edition. All procedures involving animals were conducted under animal use protocols approved by the Institutional Animal Care and Use Committees of the Armed Forces Research Institute of Medical Sciences (Bangkok, Thailand) and the Centers for Disease Control and Prevention (Atlanta, GA).

Study site and sample collection. Wild rodents were collected during studies originally designed to investigate the epidemiology of scrub typhus. Trapping sites were located in and around the villages of Ban Mae Sad and Ban Pa Gook in Chiang Rai Province in Thailand. Rodents were captured by a number of methods: trapping with live-capture traps baited with bananas or dried fish, by digging, or by hand. Animals were collected from orchards, cultivated rice-fields, grassland areas, edges of dense forest, stream margins, and around houses. Rodents were humanely killed and identified to species. Blood samples were handled in the field and in the laboratory as previously described. Briefly, samples were collected post-mortem from each animal via cardiocentesis, and were stored on dry ice. Whole blood was frozen, then shipped on dry ice to the Centers for Disease Control and Prevention (Fort Collins, CO).

Culture technique. Whole blood that had been frozen at −70°C was thawed and diluted 1:4 in brain heart infusion media containing 5−10% amphotericin B to reduce the likeli-
hood that fungal contaminants would overgrow the fastidious and slow-growing \textit{Bartonella} colonies. Diluted blood samples (0.1 mL) were pipetted onto heart infusion agar plates containing 5% rabbit blood (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD). Plates were incubated aerobically at 35–37°C in an atmosphere of 5% CO$_2$ for up to four weeks. Plates were monitored for bacterial growth at least once per week after initial plating, and every 2–3 days after passages were made. Bacterial colonies were tentatively identified as \textit{Bartonella} spp. based upon colony morphology, bacterial size and shape, and Gram-staining characteristics. The number of colonies on the original plates was counted to allow calculation of colony-forming units (CFU)/mL of blood. Single colonies were harvested from initial plates and from subsequent passages; passages were continued until a pure culture, free from contamination, was obtained. Each isolate was collected after $\leq$ 3 passages. Colonies were harvested by adding 5 mL of brain heart infusion media plus 10% glycerol to each plate, gently scraping the layer of bacteria from the surface of the agar plate, and pipetting the material into individual vials.

\textbf{Extraction of DNA and polymerase chain reaction (PCR).} DNA extractions were performed on whole bacterial cells using a DNA extraction kit (Qiagen Inc., Valencia, CA). The oligonucleotide primers BhCS871.p (5'-GGGGACCGCTATGGTG-3') and BhCS1137.n (5'-AATGCAAAAAGACAGTAAACA-3') were used (100 $\mu$M) for amplification of a region of the \textit{Bartonella} citrate synthase (gltA) gene. Positive and negative controls were included in each PCR run to evaluate the presence of appropriately sized amplicons and contamination, respectively. Each PCR was conducted in a PTC 200 Peltier thermal cycler (MJ Research, Inc., Waltham, MA) using the following program parameters: an initial denaturing at 95°C for five minutes, and 35 cycles at 95°C for one minute, 56°C for one minute, and 72°C for one minute.

The PCR products were analyzed for the presence of amplicons of the correct size by electrophoresis of 5 $\mu$L of the products in 1.5% agarose gels containing ethidium bromide. Amplicons of the proper size were identified by comparison of products in 1.5% agarose gels containing ethidium bromide.

\textbf{Sequencing and analysis of DNA.} Sequencing reactions were carried out in a PTC 200 Peltier Thermal cycler using the BhCS871.p and BhCS1137.n primers at a concentration of 1–2 $\mu$M. Cycle parameters for the sequencing reactions were 45 cycles at 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for four minutes.

Sequences were analyzed using Lasergene (DNASTAR, Madison, WI) sequence analysis software to determine consensuses sequences for the amplified region of the gltA gene. The Clustal V program within Megalign (DNASTAR) was used to align and compare homologous \textit{Bartonella} gltA sequences from the present study and from the GenBank database. The resulting alignment was analyzed using the PAUP software program for parsimony analysis (Center for Biodiversity, Illinois Natural History Survey, Champaign, IL).

\textbf{Statistical analysis.} Prevalence of \textit{Bartonella} infections among rodent species and among regions was compared using Fisher’s exact test and chi-square tests. We compared the number of CFU found in Thailand rodent blood to the CFU found in rodents from Yunnan, China using analysis of variance (ANOVA) on log-transformed data. The Student’s $t$-test was used to compare individual species when the overall ANOVA was significant. For all analyses, significance was initially set at $P < 0.05$, and was corrected for multiple comparisons when necessary.

Comparisons between rodents collected in Thailand (this study) and China (Bai and others, unpublished data) are robust because samples were collected, handled, and analyzed using the same methods in both studies.\textsuperscript{12}

\section*{RESULTS}

\textbf{Animals and samples collected.} A total of 199 small mammal samples representing eight species in four families and three orders were examined over the course of this study. The majority (173 of 199) of rodents were collected from Ban Mae Sad Village; 26 rodents were from Ban Pa Gook Village. The predominant rodent species was the greater bandicoot rat (\textit{Bandicota indica}; 151 of 199, 76%); other rodent species collected were 25 black rats (\textit{Rattus rattus}), 12 lesser rice-field rats (\textit{R. losea}), 6 Ryukyu mice (\textit{Mus caroli}), 2 Polynesian rats (\textit{R. exulans}), and 3 Berdmore’s palm squirrels (\textit{Menetes berdmorei}). Additional small mammals collected included two common tree shews (\textit{Tupaia glis}) and one small Indian mongoose (\textit{Herpestes javanicus}).

\textbf{Cultures.} \textit{Bartonella} presence could not be ascertained for 4 of the 199 samples obtained because of repeated fungal and/or bacterial overgrowth on the blood agar plates. \textit{Bartonella} isolates were obtained from 17 (8.7%) of 195 samples, including 12 (8.2%) of 147 \textit{B. indica}, 2 (18.2%) of 11 \textit{R. losea}, and 3 (12.5%) of 24 \textit{R. rattus} (Table 1). The number CFU/mL of blood varied among individuals. The average CFU/mL was 2,329 for \textit{B. indica}, 240 for \textit{R. losea}, and 9,547 for \textit{R. rattus} (Table 2). The overall comparison of \textit{Bartonella} CFU/mL showed a significantly lower level of bacteremia in rodents from Thailand versus rodents from China ($P < 0.001$, by ANOVA; Table 3). Comparisons of individual species for which sufficient data exist indicate that \textit{B. indica} from Thailand had lower levels of bacteremia than \textit{A. javanicus} and \textit{R. tanezumi flavipes} collected from China ($P < 0.02$ for each comparison).

\textbf{Phylogenetic analysis.} Of 17 \textit{Bartonella} isolates obtained from \textit{B. indica}, \textit{R. losea}, and \textit{R. rattus}, 10 demonstrated unique \textit{gltA} sequences and were submitted to GenBank (Table 3). Four \textit{B. indica} shared the sequence represented by

\begin{table}[ht]  
\centering  
\caption{Number of small mammals tested for \textit{Bartonella}, the number of isolates obtained, and the prevalence of \textit{Bartonella} in each mammal species}  
\begin{tabular}{llll}  
\hline  
Species & Number tested & Number of isolates & Prevalence (%) \\
\hline  
\textit{Bandicota indica} & 147 & 12 & 8.1 \\
\textit{Herpestes javanicus} & 1 & 0 & 0 \\
\textit{Menetes berdmorei} & 3 & 0 & 0 \\
\textit{Mus caroli} & 5 & 0 & 0 \\
\textit{Rattus exulans} & 2 & 0 & 0 \\
\textit{Rattus losea} & 11 & 2 & 18 \\
\textit{Rattus rattus} & 24 & 3 & 12.5 \\
\textit{Tupaia glis} & 2 & 0 & 0 \\
Total & 195 & 17 & 8.7 \\
\hline  
\end{tabular}  
\end{table}
GenBank number AY269417, 2 shared the sequence represented by GenBank number AY264493, and 2 shared the sequence represented by GenBank number AY269418. Sequences obtained from the other four B. indica isolates were unique.

The phylogenetic relationship among the isolates obtained in the present study and five previously described Bartonella species is presented in Figure 1. Sequences of nine isolates from B. indica form a distinct cluster (similarity ranges from 98% to 100%), which also contains a single isolate from R. rattus. The other three isolates from B. indica cluster together and are nearly identical to an isolate from Apodemus draco collected in China (99.4 and 98.8% similarity, respectively). Two R. rattus isolates were identical to isolates from R. t. flavipectus collected in China. One R. losea isolate also clustered with that R. t. flavipectus group, while the other R. losea isolate was only distantly related to previously described Bartonella.

## DISCUSSION

This study is the first to report isolation of Bartonella bacteria from rodents in Thailand. However, the study area for this project was fairly narrow in scope, and additional studies are being conducted to determine the extent of rodent-associated Bartonella throughout the country. The overall prevalence of Bartonella in those rodents was 9%. Prevalence was highest in R. losea (18%) and R. rattus (12%); however, these rodents accounted for only 5 of the 17 isolates collected. The highest number of isolates (12) was obtained from B. indica, the most common rodent species captured. The presence of Bartonella in those rodents, especially B. indica, is significant because those species are often found in close contact with humans, who might therefore be at risk of exposure. Prevalence of Bartonella seen in the present study (9%) is low when compared with the prevalence reported from Yunnan, China.12 In the Yunnan province of China, overall prevalence was 43% (57 of 131); prevalence ranged from 27% to 48% among various study sites, and differed among species.12 Birtles and others isolated Grahemella (Bartonella) from 64% (23 of 36) of the woodland rodents captured in the United Kingdom.23 Kosoy and others isolated Bartonella from 43% (119 of 279) of the viable samples collected from rodents in the southeastern United States.22 In central Sweden, Holmberg and others cultured Bartonella from 17% (36 of 216) of the rodents collected.23

The reasons for the lower prevalence of Bartonella observed in the present study are not clear, but could include fewer potential vectors present in the study region, better host immunity, decreased intraspecific interactions, and/or greater ecologic separation of potential host species. Sample handling procedure might have caused some variation, although we have no evidence to suggest this. Sufficient data for an analysis of the latter two possibilities are not currently available; however, the former two can be addressed.

## Figure 1

Phylogenetic tree of Bartonella species and isolates based upon sequence analysis of the citrate synthase (gltA) gene. The scale bar represents five nucleotide changes. GenBank accession numbers for Bartonella isolated in the present study are given in parentheses. The program PAUP was used to create the phylogenetic tree.

### Table 2

Colony-forming units (CFU)/mL of Bartonella in the blood of rodents from Chiang Rai, Thailand and Yunnan, China*

<table>
<thead>
<tr>
<th>Region</th>
<th>Species</th>
<th>No.</th>
<th>Average (CFU/mL)</th>
<th>SE (CFU/mL)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yunnan, China</td>
<td>A. Chevrieri</td>
<td>21</td>
<td>94,621</td>
<td>18,468</td>
<td>160–320,000</td>
</tr>
<tr>
<td></td>
<td>A. draco</td>
<td>2</td>
<td>36,000</td>
<td>28,000</td>
<td>8,000–64,000</td>
</tr>
<tr>
<td></td>
<td>E. latens</td>
<td>6</td>
<td>55,493</td>
<td>33,063</td>
<td>1,600–160,000</td>
</tr>
<tr>
<td></td>
<td>E. milleus</td>
<td>2</td>
<td>27,253</td>
<td>26,377</td>
<td>160–80,000</td>
</tr>
<tr>
<td></td>
<td>R. flaviepectus</td>
<td>23</td>
<td>31,090</td>
<td>11,245</td>
<td>160–160,000</td>
</tr>
<tr>
<td></td>
<td>R. norvegicus</td>
<td>3</td>
<td>67,200</td>
<td>46,848</td>
<td>9,600–160,000</td>
</tr>
<tr>
<td>Chiang Rai, Thailand</td>
<td>R. indica</td>
<td>9</td>
<td>2,329</td>
<td>1,080</td>
<td>160–9,600</td>
</tr>
<tr>
<td></td>
<td>R. rattus</td>
<td>3</td>
<td>9,547</td>
<td>9,227</td>
<td>160–28,000</td>
</tr>
<tr>
<td></td>
<td>R. losea</td>
<td>2</td>
<td>240</td>
<td>80</td>
<td>160–320</td>
</tr>
</tbody>
</table>

* A. = Apodemus; E. = Eothenomys; R. = Rattus; B. = Bandicota.

### Table 3

GenBank accession numbers of Bartonella isolates obtained from Thailand rodents

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Isolate identification</th>
<th>Rodent species</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY264493</td>
<td>Bi5131tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY2777891</td>
<td>Bi3726tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY269417</td>
<td>Bi3778tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY269418</td>
<td>Bi3781tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY269421</td>
<td>Bi4251tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY2777893</td>
<td>Bi4271tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY269419</td>
<td>Bi4298tl</td>
<td>Bandicota indica</td>
</tr>
<tr>
<td>AY269420</td>
<td>RI5132tl</td>
<td>Rattus losea</td>
</tr>
<tr>
<td>AY269279</td>
<td>RI3725tl</td>
<td>Rattus losea</td>
</tr>
<tr>
<td>AY2777892</td>
<td>RR4252tl</td>
<td>Rattus rattus</td>
</tr>
</tbody>
</table>

* A. Chevrieri Yunnan, China; E. = Eothenomys; R. = Rattus; B. = Bandicota.
eastern Asia (Lerdthusnee K, unpublished data). Investigations of how climatic variables and ectoparasite densities influence the prevalence of Bartonella in rodent populations were outside the scope of this study, but would be worth pursuing in the future.

Our results suggest some evidence of host-specificity among Bartonella species. If certain Bartonella species exhibit host specificity among rodents and other mammals, one way such specificity may manifest is as variable levels of bacteremia within different host species, and that could explain some of the differences we detected among rodents from Thailand and China. Some hosts may be better able to tolerate a high level of bacteremia, without showing overt signs of illness. Alternatively, bacteria may be able to reproduce within host erythrocytes, but only to a finite level, which may be set by host or bacterial factors. Indeed, such finite infection has been reported in experimentally infected rats. Non host-adapted Bartonella may produce more of an immune response, in which case they may be cleared by host defenses, or may cause serious morbidity and/or mortality.

Phylogenetic analysis of rodents from Thailand indicates that the cluster of isolates from B. indica may represent a distinct Bartonella species. However, additional genotypic and phenotypic analyses must be completed before a new species can be described. The presence of a nearly identical isolate in R. rattus could represent spillover of the B. indica strain into R. rattus. Three B. indica isolates, represented by two unique sequences, were nearly identical to Bartonella isolated from A. draco and R. t. flavipes collected in China. Apodemus draco and R. t. flavipes were not collected in our study area, so the natural host of this Bartonella species remains unknown.

It is important to note the close genetic relationship of some Bartonella strains isolated from Thailand rodents to B. elizabethae, which has been isolated from a human patient with endocarditis. Subsequent studies have implicated B. elizabethae as a cause of additional cases of endocarditis, a case of Leber’s neuroretinitis, and have shown the presence of B. elizabethae-reactive antibodies in a high proportion of intravenous drug users.

Since 1996, numerous studies have implicated rats of the genus Rattus as potential rodent reservoirs for B. elizabethae. The current consensus is that B. elizabethae is one member of a cluster of closely related Bartonella spp. that includes isolates from R. norvegicus collected in France, Peru, and the United States, and from R. rattus collected in Portugal. Our results, along with those of Ying and others support the hypothesis that B. elizabethae-like strains may have originated in southeastern Asia.

Recently, Parola and others recovered B. henselae and B. claridgeiae DNA from cat fleas (Ctenocephalides felis) collected from cats, and a novel Bartonella isolate from a rat flea (Nosopsyllus fasciatus) collected from a yellow raja rat (Rattus surifer) trapped along the Thailand-Myanmar border. While transmission of Bartonella from rodents to humans in Thailand has not been documented, the possibility of such transmission exists.

Cat scratch disease or other known manifestations of Bartonella infections have not yet been reported from Thailand; however, one study found that 5% of healthy humans were seropositive for B. henselae. Bartonella henselae and B. claridgeiae have recently been isolated from the blood of cats from numerous regions in Thailand, and Sukwasath and others reported that 38% of 49 dogs tested in Thailand were positive for antibodies to Bartonella vinsonii subspecies berkhoufti. It is possible that the high number of feral and hand-fed communal cats in Thailand could increase the risk of Bartonella transmission from rodent reservoirs to humans, since cats could potentially become infected via fleas or rodent ingestion, or may serve as mechanical flea vectors. However, the existence of such a rodent-cat-human transmission cycle warrants further investigation since rodent bartonellae have not yet been isolated from domestic cats.

Future studies should investigate the modes of transmission of Bartonella among rodents and between rodents and other vertebrate hosts, including humans. In addition, the prevalence of rodent-associated Bartonella in humans exhibiting febrile illness of unknown origin throughout Thailand should be investigated. An understanding of Bartonella transmission cycles throughout southeastern Asia, along with studies of Bartonella prevalence in humans, will allow health care workers to assess the risks associated with exposure the bartonellae.

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REFERENCES


