Identification of *Bartonella* Infections in Febrile Human Patients from Thailand and Their Potential Animal Reservoirs

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Abstract. To determine the role of *Bartonella* species as causes of acute febrile illness in humans from Thailand, we used a novel strategy of co-cultivation of blood with eukaryotic cells and subsequent phylogenetic analysis of *Bartonella*-specific DNA products. *Bartonella* species were identified in 14 blood clots from febrile patients. Sequence analysis showed that more than one-half of the genotypes identified in human patients were similar or identical to homologous sequences identified in rodents from Asia and were closely related to *B. elizabethae*, *B. rattimassiliensis*, and *B. tribocorum*. The remaining genotypes belonged to *B. henselae*, *B. vinsonii*, and *B. tamiae*. Among the positive febrile patients, animal exposure was common: 36% reported owning either dogs or cats and 71% reported rat exposure during the 2 weeks before illness onset. The findings suggest that rodents are likely reservoirs for a substantial portion of cases of human *Bartonella* infections in Thailand.

INTRODUCTION

Over 30 species and subspecies of *Bartonella* have been identified, and it is clear from ongoing research that many more remain to be described. Globally, they reside in diverse ecological niches; many cause infections in animals, and at least 10 species have been linked to human disease. *Bartonella* infections are remarkable in the wide range of pathological manifestations that they cause in animals and humans, ranging from self-limiting regional lymphadenitis to severe endocarditis. *Bartonellae* are also unique in the manner of their association with mammalian reservoir hosts, in which they can cause a chronic bacteremia with no or few symptoms.

Commonly used detection methods for *Bartonella* include serology for identifying specific antibodies, culture techniques to recover viable organisms, and polymerase chain reaction (PCR)-based detection of *Bartonella*-specific sequences from DNA in blood and tissue samples. Serological assays are the most commonly used diagnostic approach for *Bartonella* infections, although conclusive identification of the *Bartonella* species causing a rise antibody titer is limited because of the apparent cross-reactivity between *Bartonella* species. Culturing on solid medium, although considered the gold standard, has its own limitations, including very low isolation rate of *Bartonella* from clinical specimens, especially human blood, and the time-consuming nature of the methodologies.

Molecular assays offer the potential of improved sensitivity and timeliness of *Bartonella* detection. One of the most impressive applications of *in situ* nucleic acid techniques to the study of an infectious process was direct detection of *Rochalimae* (later *Bartonella*) microorganisms in bacillary angiomatosis tissue lesions by Relman and others.¹ There is a growing trend to reliance on genotyping for microbial characterization.² Usually, genotypes are more specific and are more easily

quantified and standardized among different bacterial species than are the phenotypic markers used traditionally. This is especially true for identification of *Bartonella* species with their fastidious nature in terms of culture and a low capacity for discrimination by morphology or nutrient requirements. There is, however, a challenge, because PCR amplification of *Bartonella*-specific DNA targets directly from whole blood, likely because of the inhibitory effect of blood. Both conventional and real-time PCRs directly from human or specific animal-blood specimens lack the sensitivity to detect bacteria, sometimes even from a bacteremic blood.

In Thailand, little is known about the burden of *Bartonella* infections in humans and potential zoonotic reservoirs. Data on human infections have been limited to culturing strains of two *Bartonella* species (*B. henselae*, agent of cat scratch disease and *B. tamiae*, a novel human pathogen recently described from cases of febrile illness^{3,4}) and two serological investigations.⁵ *Bartonella* species have been isolated in Thailand from many mammalian hosts, including rodents, cats, and dogs.^{6–8} No studies, however, have systematically assessed the scope of *Bartonella* species isolated from humans in Thailand or examined linkages to zoonotic sources. Such investigations are needed, especially in Thailand, where even rigorous etiologic studies have failed to identify the cause of febrile illnesses in up to 60% of cases.^{9–12} Recently, investigations using serological assays have suggested a high disease burden attributable to *Bartonella* infections (S. Sutthirattana and others, unpublished data).

As part of a prospective study to determine causes of acute febrile illness in northern and northeastern Thailand, we evaluated data from two provinces in rural Thailand to examine the etiologies of acute febrile illness, including the role of *Bartonella* species during 2002–2003. To address the limitations of common diagnostic approaches for *Bartonella*, we used a novel strategy of co-cultivation of blood clots with eukaryotic cells, DNA amplification of positive cultures, and subsequent phylogenetic analysis of *Bartonella*-specific products. To investigate potential animal reservoirs for *Bartonella* bacteria causing human illnesses in Thailand, genotypes from human infections were compared with *Bartonella* strains

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isolated from animals in Thailand and globally. Here, we report the findings based on this multifaceted laboratory approach.

MATERIALS AND METHODS

Patients and samples. This study was a part of an Institutional Review Board (IRB)-approved investigation to determine etiologies of acute febrile illnesses among patients in rural Thailand. We enrolled febrile patients presenting to outpatient and inpatient departments of two community hospitals (ranging in size from 30 to 90 beds), which served as entry points for medical evaluation in each of two Thai provinces (Chiang Rai and Khon Kaen) from February 2002 to March 2003. Chiang Rai province is in northern Thailand, borders Laos and Myanmar, and has a population of 1,167,780. Khon Kaen is in northeastern Thailand with a population of 1,801,016. Eligible patients were those ≥ 7 years old with a documented fever $> 38^\circ\text{C}$. Patients were excluded if they had a history of fever ≥ 2 weeks, an apparent focal infection (e.g., pharyngitis, meningitis, or urinary tract infection) or an infection that could be definitively diagnosed clinically (e.g., mumps, croup, varicella, parvovirus, measles, or rubella), received an immunization in the preceding 48 hours, or received blood products in the previous 6 months. All patients completed a questionnaire with multiple epidemiological and clinical questions. Blood specimens were collected from all patients at the time of enrollment, stored at -70°C , and later shipped frozen to the Bartonella Laboratory at the Centers for Disease Control and Prevention (CDC) in Fort Collins, Colorado.

Culturing. Vero E6 cell monolayers were grown in T150 tissue-culture flasks (Corning, Corning, NY) containing M199-E growth medium with 10% fetal bovine serum and L-glutamine until they became confluent. Immediately before the addition of the patient blood, the growth medium was removed. Blood clots (100 μL) were mixed with 2 mL of the medium. The suspension was used to inoculate the cell monolayers in flasks, and inoculated cells were incubated on a rocking platform at room temperature for 30 minutes. After the original incubation, 50 mL of the Dulbecco's modified eagle medium (DMEM) maintenance medium with 2% fetal bovine serum and without L-glutamine was added to the flasks. The inoculated cells were incubated at 35°C in 5% CO_2 and were harvested with sterile glass beads after 7 days of incubation.

PCR. DNA extractions from Vero-E6 cells inoculated with human blood were performed using the Qiagen extraction kit (Qiagen, Valencia, CA) using the blood protocol provided by the manufacturer. PCR was performed using published primers BhCS781.p and BhCS1137.n to generate a 379-bp amplicon of the *Bartonella* citrate synthase gene (*gltA*).¹³ PCR amplifications were performed in a 25- μL reaction mixture containing 5 μL of $5\times$ PCR buffer, 300 nM of each primer, 400 μM of premixed deoxynucleotide triphosphate (dNTP) solution (Promega, Madison, WI), 1 U Taq DNA polymerase (Promega, Madison, WI), and 5 μL DNA template. PCR was carried out in a PTC 200 Peltier thermal cycler (MJ Research, Waltham, MA) using the following program parameters: a 5-minute denaturation at 95°C followed by 40 cycles of 1 minute denaturation at 95°C , 1 minute annealing at 56°C , and 1 minute elongation at 72°C . Amplification was completed by holding the reaction mixture at 72°C for 10 minutes. PCR products were visualized for the presence of amplicons of

the correct size by electrophoresis in a 1.5% agarose gel with ethidium bromide staining.

Sequencing. Amplicons with the proper size were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and both strands were sequenced using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing reactions were carried out in a PTC 200 Peltier Thermal cycler using the same primers for the PCR assay with a final concentration of 160 nM. Cycle parameters for the sequencing reactions were 45 cycles at 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes.

Phylogenetic analysis. PCR-positive co-cultures from human patients were characterized by DNA sequence analysis of the *gltA* fragment to confirm their belonging to the *Bartonella* genus and to determine their phylogenetic relatedness to known *Bartonella* species according to La Scola and others.¹⁴ Relatedness was also assessed to animal isolates using > 300 amplicons from rodents, insectivores, dogs, cats, ruminants, and other mammals from Thailand and neighboring Asian countries. Sequences were analyzed with Lasergene 8 (DNASTAR, Madison, WI) to determine sequence consensus for the *gltA* amplicons. Unique sequences were submitted to GenBank. The Clustal V program within the Megalign module of Lasergene was used to compare homologous *Bartonella gltA* sequences from the present study and GenBank. The neighbor-joining method by Kimura's two-parameter distance method and bootstrap calculation was carried out with 1,000 resamplings. A criterion of $\geq 96\%$ homology to *gltA* was used to define species.

RESULTS

Detection and identification of *Bartonella* genotypes in human blood. *Bartonella* DNA was detected in 20 of 261 (7.7%) blood clots co-cultivated with Vero E6 cells. Sequence analysis of the *gltA* amplicons was completed for 14 of the positive samples. For this study, only amplicons with unambiguous sequences aligned within the range of the *Bartonella* genus were considered to be confirmed positives; further analysis was restricted to these 14 identified sequences. The analysis showed that these sequences were heterogeneous and represented 13 unique genotypes. Each *gltA* sequence submitted to GenBank was assigned an accession number (Table 1).

Sequence analysis of *Bartonella* DNA detected in human blood. Genotypes obtained from the blood of two patients

TABLE 1
Identified *gltA* genotypes, GenBank accession number, and recognized *Bartonella* species name

Genotype	GenBank accession	<i>Bartonella</i> species relatedness	Sequence similarity (%)
45-00032	GQ200855	<i>B. rattimassiliensis</i>	97.9
45-00015	GQ200856	Unknown	< 95.0
45-00398	GQ200857	<i>B. vinsonii</i> subsp. <i>arupensis</i>	98.7
45-00439	GQ200858	<i>B. henselae</i>	100.0
45-00515	GQ200860	Unknown	< 95.0
45-00135	GQ200861	<i>B. vinsonii</i> subsp. <i>vinsonii</i>	97.9
45-00239	GQ200862	<i>B. tamiae</i>	100.0
45-00518	GQ225705	Unknown	< 95.0
45-00534	GQ225706	<i>B. tribocorum</i>	97.0
45-00551	GQ225707	<i>B. elizabethae</i>	95.4
45-00559	GQ225708	<i>B. vinsonii</i> subsp. <i>arupensis</i>	99.3
45-00249	GQ225709	<i>B. henselae</i>	100.0
45-00311	GQ225710	<i>B. elizabethae</i>	97.9
45-00517	GQ225712	Unknown	< 95.0

(45-00249 and 45-00439) were identical to *B. henselae*, the agent of cat scratch disease. All other 12 genotypes represented unique sequences with different degrees of relatedness to described *Bartonella* species. Two genotypes (45-00311 and 45-00551) were relatively close (identity 97.9% and 95.4%) to *B. elizabethae*, a bacterium originally isolated from a human patient with endocarditis from Massachusetts, United States.¹⁵ One genotype (45-00534) exhibited its closest phylogenetic relationship (97.0%) to *B. tribocorum*, originally isolated from a domestic rat in France¹⁶; another genotype (45-00032) was closer (similarity of 97.9%) to *B. rattimassiliensis* that was recovered from a European rat *Rattus norvegicus*.¹⁷ One genotype (45-00015) was similar but not identical to *B. tribocorum* and *B. rattimassiliensis* (similarity of 94.3% and

91.3%, respectively). A group of four genotypes (45-00515, 45-00517, and 45-00518) were similar to each other (identity 97.0 to 98.2%) and represented a distinct phylogenetic lineage in the genus *Bartonella*. Two genotypes (45-00398 and 45-00559) were related (identity 98.7% and 99.3%, respectively) to *B. vinsonii* subsp. *arupensis*, an organism characterized from a cattle rancher in Wyoming, United States.¹⁸ Another genotype (45-00135) found in the blood of Thai patients was similar (identity 97.9%) to *B. vinsonii* subsp. *vinsonii*, which had been originally isolated from a Canadian vole by Baker.¹⁹ Finally, one genotype (45-00239) was obtained from a cell culture co-cultivated with a patient blood that yielded pure culture isolate, which has been described as a novel species—*B. tamiae* (Figure 1).³

FIGURE 1. Phylogenetic relationships between the *gltA* sequences of *Bartonella* genotypes detected in 16 human patients from Thailand, previously described *Bartonella* species, and selected genotypes detected in mammals in Asia. The phylogenetic tree was constructed by the N-J method and bootstrap values with 1,000 replicates. Only bootstrap replicates > 70% are noted. The name and accession number of genotypes detected in human patients are outlined in the text box. Each unique *Bartonella* species or genotype detected in mammals is provided with the name of the type strain and its GenBank accession number.

***Bartonella*-positive febrile patients.** Among the 14 febrile patients with *Bartonella* infection, the median age was 28 years, 57% were male, 79% lived in Chiang Rai, and 43% were rice farmers. Common clinical characteristics of *Bartonella*-infected patients included headache (100%), lethargy (86%), and muscle pain (79%). Four patients had conjunctival suffusion noted on physical exam. Five (36%) patients were hospitalized, and 50% had anemia defined as hemoglobin < 12 mg/dL. One patient had hemoglobin < 10 mg/dL. Animal exposure was common; 36% reported owning either dogs or cats, and 71% report rat exposure during the 2 weeks before illness onset, defined as having seen a rat around their home or field or having participated in a local rat-control program (Table 2). However, the frequency of animal ownership and rat exposure did not differ from that reported by febrile patients without *Bartonella* infection or a group of afebrile controls (S. Sutthirattana and others, unpublished data).

Phylogenetic relations between *Bartonella* genotypes identified in human and animals. To gain additional information on potential animal reservoirs for human infections, we compared the homologous sequence fragments of *Bartonella*

genotypes, which were different from *B. henselae* and probably associated with domestic cats, with those derived from diverse animal sources from Thailand and other Asian countries.

The genotype 45-00032 identified as *B. rattimassiliensis* was completely identical to the homologous sequence of the strain Rr19192th (GenBank accession FJ655409) obtained from a black rat (*Rattus rattus*) from Surat Thani province in Thailand⁸ and was more distantly related (99.0% similarity) to a group of *Bartonella* strains (type strain Bi3781th; GenBank accession AY269418) obtained from bandicoot rats (*Bandicota indica*) from Chiang Rai province, Thailand.⁷ The genotype 45-00551 identified as *B. elizabethae* was very similar by the corresponding fragment to the isolates from a tree squirrel (*Tupaia glis*) from Chiang Mai, Thailand and from a Norway rat (*R. norvegicus*) from Ubon Ratchathani province, Thailand (similarity 99.7% with a1-bp-mismatch).⁸ Both animal isolates were also identical to the strain from a rat *R. norvegicus* from Beijing, China (GenBank accession EF213769).²⁰ The genotype 45-00311, which also was relatively close to *B. elizabethae*, was 100% identical to two isolates (type strain Bb7699bgl; GenBank accession AY589562) obtained from *B. bengalensis* from Dhaka, Bangladesh.²¹ The group of closely related genotypes (45-00515, 45-00517, and 45-00518), which were different from all known *Bartonella* species, were most similar (98.5%) to an isolate from a rat *R. norvegicus* from Phra Nakhon Si Ayutthaya province, Thailand.⁸ Another previously unidentified genotype (45-00015) was 96.1% similar to an isolate (strain Rr18771th; GenBank accession) from a rat *R. rattus* from Chiang Mai, Thailand.⁸

Genotype 45-00534 was close to several *Bartonella* strains that are common among rats of the genera *Bandicota* and *Rattus* in Thailand: 99.1% similarity with strain Bs19435th (GenBank FJ655395) from *B. savilei* from Nan province, Thailand, and 99.4% similarity with 18 isolates from *R. exulans* (1 isolate), *R. norvegicus* (12 isolates), and *R. rattus* (5 isolates) found in many Thai provinces.⁸

Genotypes similar to *B. vinsonii* subsp. *arupensis* have not been found in the blood of rodents from any Asian country but surprisingly, were identified in stray dogs from Bangkok and Khon Kaen (Y. Bai, unpublished data). The genotype from a human patient, which was relatively close to *B. vinsonii* subsp. *vinsonii*, did not exhibit a good match with any strains detected in Thai rodents; the closest but still distant relationship was found between this genotype and two strains from the voles *Eothenomys miletus* from southwestern China (Yunnan province).²⁰

Genotype 45-00239 belongs to the strain that, along with two more isolates from human patients, was described under the name of *B. tamiae*. Similar sequences have not been found in rodents or other mammals; however, the *gltA* and internal transcribed spacer (ITS) fragments specific to *B. tamiae* were detected in chigger mites from Thailand (H. Kabeya and others, unpublished data), and similar ITS sequences were also reported from ixodid ticks from Virginia, United States.²²

DISCUSSION

Our study found that the spectrum of *Bartonella* species associated with human infections in Thailand is likely much wider than expected based on reports from Europe and the United States, which identified a causative association for human infections with only two well-recognized *Bartonella*

TABLE 2

Characteristics of 14 febrile patients with culture-confirmed *Bartonella* infection in Khon Kaen and Chiang Rai, Thailand (2002–2003)

Patients	N	Percentage
Median age in years (range)	28 (7–56)	
Median fever duration in days (range)	3 (1–14)	
Male gender	8	57.1
Province		
Chiangrai	11	78.6
Khonkaen	3	21.4
Occupation		
Farmer	6	42.9
Student	4	28.6
Shopkeeper	2	14.3
Day laborer	1	7.1
Clinical characteristics		
Headache	14	100
Lethargy	12	85.7
Muscle pain	11	78.6
Cough	10	71.4
Chest pain	6	42.9
Joint pain	4	28.6
Conjunctival suffusion	4	28.6
Lymphadenopathy	4	28.6
Illness required hospitalization	5	35.7
Laboratory results		
Elevated alkaline phosphatase (> 121 IU/dL)	9	64.3
Anemia (hemoglobin < 12 mg/dL)	7	50
Elevated aspartate aminotransferase (> 36 IU/dL)	6	42.9
Elevated alanine aminotransferase (> 36 IU/dL)	4	28.6
White blood cell count > 10,000 cells/ μ L	6	42.9
White blood cell count < 5,000 cells/ μ L	1	7.1
Thrombocytopenia (platelet count < 150 $\times$ 10 ³ / μ L)	3	21.4
Elevated blood urea nitrogen ($\geq$ 21 mg/dL)	4	28.6
Animal exposure		
Owned any animal	10	71.4
Owned dog	4	28.6
Owned cat	3	21.4
Rat exposure during 2 weeks before illness onset*	8	57.1

*Reported having seen a rat at the patient's home or field or having participated in a local rat-control program that rewards participants for documented rat extermination.

pathogens: *B. henselae* and *B. quintana*. Although we used only a portion of the citrate synthase gene for phylogenetic analysis, this gene has been shown to be a reliable tool for distinguishing between *Bartonella* species and closely related *Bartonella* genotypes.¹⁴ An identification of genotypes based on sequencing of one gene fragment presents an evident limitation for a characterization of etiological agents for human illnesses; however, our results indicated that most identified human cases (12 of 14) were associated with species other than *B. henselae* and *B. quintana*.

Our study also showed that the cell-culture technique combined with a subsequent PCR amplification of a specific marker and sequencing of the amplicons can be an effective tool for identification of genotypes of *Bartonella* infections associated with the human cases. All attempts to apply a single-step PCR-based assay directly in the blood of the 261 patients targeting the fragment of the *gltA* gene were unsuccessful (unpublished data). Successful identification of the *Bartonella* bacteria on cells rarely leads to successful propagation of the isolates on solid agar. Among 14 infected cells in our study, only 1 cell was successfully adapted to agar, and this cell has also been used for description of a novel *Bartonella* species (*B. tamiae*). Strictly speaking, detection of *Bartonella* genotypes in human patient blood co-cultivated with a cell line cannot reliably prove a role of the detected agents as causes of specific cases. We are early in our experience with interpreting such findings. Nevertheless, a potential etiologic association is supported by three facts: (1) the clinical specimens were derived from patients with evident pathology, (2) nearly all of the detected genotypes were symbionts of specific mammalian species beyond *Homo sapiens*, and (3) a high rate of cases with a greater than or equal to four times increase of titers to one of *Bartonella* antigens was observed in the same cohorts of the patients (S. Sutthirattana and others, unpublished data).

Comparative phylogenetic analysis based on comparison of *Bartonella* genotypes detected in blood of human patients and strains detected in rodents and their ectoparasites has already better defined a range of potential zoonotic sources of human bartonellosis in Thailand. Among sequences obtained from 14 human patients, at least 8 sequences were closely related or identical to homologous sequences identified in small mammals from Thailand. These findings suggest that rodents and other small mammals are likely reservoirs for a substantial portion of cases of human *Bartonella* infections in rural Thailand. More likely, the proportion of rodent-borne bartonellosis among human cases in an urban setting, such as the Bangkok metropolitan area, may be quite different compared with rural areas. The identification of diverse and previously uncharacterized *Bartonella* species from patients with undifferentiated febrile illnesses in Thailand suggests the need to assess the public-health impact posed by these novel bacterial agents.

Another outcome of our study was the identification of two *gltA* genotypes that resemble *B. vinsonii* subsp. *arupensis* in human blood. This finding is unexpected because of the well-documented host-specific association between this bacterium and deer mice (*Peromyscus maniculatus*). Strains identical or closely related to *B. vinsonii* subsp. *arupensis* were prevalent among deer mice and other mice of *Peromyscus* genus throughout the United States and Canada.^{23–25} However, these new findings support previous reports where *B. vinsonii* subsp. *arupensis* was implicated as a source of human endocarditis

in France²⁶ and febrile illness in two patients in Moscow, Russia.²⁷ Because *B. vinsonii* subsp. *arupensis* has never been detected in rodents from the Old World and dogs are the only Old World animals from which this subspecies was identified (Y. Bai, unpublished data), it is logical to suggest that this bacterium was acquired by dogs from wild rodents in North America and then, relocated to other continents through the translocation of infected dogs. Regardless, further investigations are needed to define the role of domestic animals as potential sources for human bartonellosis in Thailand. It is also important to continue efforts to identify additional *Bartonella* strains and genotypes among human patients in Thailand to better estimate the range of putative *Bartonella* pathogens that can infect people.

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