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RESEARCH ARTICLE
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First report of three novel *Bartonella* species isolated in rodents and shrews from nine provinces of Thailand

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Abstract

Background and Aim: *Bartonella* spp. are Gram-negative zoonotic bacteria that are transmitted to humans by several types of animal hosts, including rodents. Several studies have been conducted on the prevalence of *Bartonella* infections in rodents. However, the risk of rodent-associated *Bartonella* spp. infection in humans remains unclear. This study aimed to estimate the prevalence and genetic heterogeneity of *Bartonella* spp. in rodents and shrews from nine provinces of Thailand using culture and molecular techniques.

Materials and Methods: A total of 860 blood samples from rodents and shrews across nine provinces of Thailand were collected from January 2013 to June 2016. *Bartonella* spp. were isolated from all samples using conventional culture techniques and polymerase chain reaction. Phylogenetic tree analysis was used to align the *Bartonella* sequences obtained from this study.

Results: The prevalence of *Bartonella* spp. in rodents and shrews was 11.5% (99/860, 95% confidence interval: 9.38–13.64%). The following nine species of *Bartonella* were detected: *Bartonella tribocorum*, *Bartonella rattimassiliensis*, *Bartonella queenslandensis*, *Bartonella elizabethae*, *Bartonella chanthaburi* spp. nov., *Bartonella satun* spp. nov., *Bartonella coopersplainsensis*, *Bartonella ranong* spp. nov., and *Bartonella henselae.* The prevalence of *Bartonella*-positive animals differed significantly among provinces.

Conclusion: To the best of our knowledge, the three novel *Bartonella* spp. isolated from rodents and shrews across Thailand were detected for the first time in this study. Further studies on the epidemiology of *Bartonella* infection in rodents and its interaction with human health should be conducted in accordance with the Thai government's "One Health" approach to humans, animals, and the environment.

Keywords: *Bartonella* spp., phylogenetic analysis, polymerase chain reaction, rodents.

Introduction

Bartonella spp. are Gram-negative intraerythrocytic bacteria including more than 40 species and subspecies [1]. Several *Bartonella* spp. have been confirmed as zoonotic pathogens, such as *Bartonella elizabethae*, *Bartonella tribocorum*, *Bartonella henselae*, *Bartonella vinsonii.* Sub spp. *arupensis*, and *Bartonella tamiae*, most of which are transmitted by reservoir hosts and blood-sucking arthropods [2].

Rodents are known to be the main reservoir hosts for different *Bartonella* spp.; however, some species involve other animals as well. *B. henselae* utilizes cats and *Bartonella bovis* and *Bartonella chomelii* utilize cattle as reservoirs [3]. Several *Bartonella* spp. have been isolated from rodents in several countries, including Thailand [4–9]. These pathogens are associated with various human diseases, such as cat scratch disease (*B. henselae*), trench fever (*Bartonella quintana*), Oroya fever (*Bartonella bacilliformis*), and endocarditis (*B. tamiae*) [10–12]. In particular, past exposure to rats has been reported in three patients from Thailand with fever, myalgia, and headache [13]. Several reports of these infections in rodents in Thailand have been described [6–9, 12–15]. However, the risk of rodent-associated *Bartonella* spp. infection in humans remains unclear.

The study aimed to estimate the prevalence and genetic heterogeneity of *Bartonella* spp. in rodents and shrews from nine provinces of Thailand using culture and molecular techniques and phylogenetic analysis.

Materials and Methods

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of the National Institute of Health (NIH), Thailand.

Study period and location

The study was conducted from January 2013 to June 2016. The blood samples were collected from nine provinces of Thailand; Khon Kaen, Nakhon Phanom, Tak, Chon Buri, Chanthaburi, Ranong, Phuket, Songkhla, and Satun (Figure-1). The samples were processed at the Department of Medical Sciences, NIH Laboratory, Ministry of Public Health, Nonthaburi, Thailand.

Sample collection

We calculated the minimum sample size based on a previous study by Pangjai *et al.* [6] using the Epitools program (www.epitool.net) with 95% confidence interval (CI) and 2.5% precision. Based on the results, 401 samples should have been collected. Overall, 860 small mammals, comprising 800 rodents and 60 shrews, were captured using traps from nine provinces of Thailand. Animal species were identified by their morphological characteristics before they were euthanized using a Carbon oxide (CO_2) chamber. A total of 0.5–2 mL of blood samples were aseptically collected through cardiopuncture and immediately placed in sterile ethylenediamine tetra-acetic acid tubes. The samples were transported to the Department of Medical Sciences, NIH Laboratory under chilled conditions and stored at −20°C until further processing.

Isolation of *Bartonella*

Bartonella was isolated according to a previously described method [16] with slight modifications.

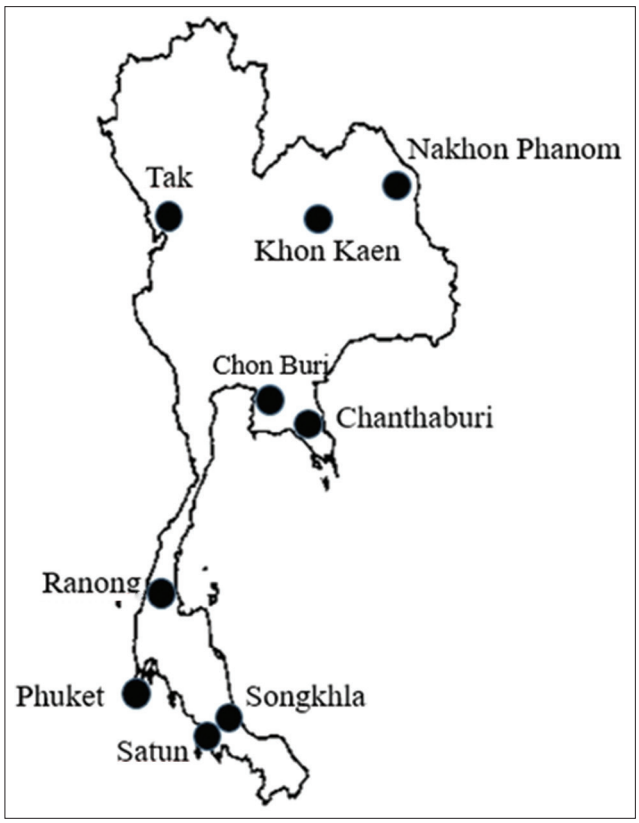

Figure-1: Geographic locations of nine provinces in Thailand where rodents and shrews were captured for this study [Source: http://geosurin.blogspot.com/2009/09/blog-post-17.html].

Briefly, frozen blood samples were thawed at 25°C, and 200 µL of each sample was centrifuged at 1,800x*g* for 70 min. The sediment was mixed with an equal volume of Medium 199 (Life Technologies, USA) supplemented with sodium pyruvate and fetal bovine serum (Life Technologies, United States). The mixture was then inoculated onto brain heart infusion agar (BHIA, Difco, United States) plates containing 5% defibrinated rabbit blood. The plates were incubated at 35°C under 5% CO_2 for 2–4 weeks. Consequently, Gram-negative coccobacilli grew as small, rough, and grayish colonies and required long culture periods, which were tentatively considered as *Bartonella* species. The bacteria were subcultured in fresh media and all isolates were maintained in Trypticase Soy Broth with 20% glycerol (v/v) for further characterization.

DNA extraction and polymerase chain reaction (PCR) amplification

The genomic *Bartonella* DNA was detected using specific PCR primers as described previously by Boonmar *et al*. [16]. Genomic DNA was extracted from each isolate using InstaGene Matrix (BioRad, Hercules, United States). Primers targeting the β-subunit of RNA polymerase (*rpoB*) [17] (primer pair sequences, 5′-CGCATTGGCTTACTTCGTATG-3′ and 5′-GTAGACTBATTAGAACGCTG-3′) and citrate synthase (*gltA*) [18] (primer pair sequences, 5′-AATGCAAAAAGAACAGTAAACA-3′ and 5′-GGGGACCAGCTCATGGTGG-3′) were used for PCR. PCR was performed using 20 µL of reaction mixtures containing 20 ng of extracted DNA, 200 µmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L of $MgCl_2$, 0.5 U of Go-Taq DNA polymerase (Promega, Madison, Wisconsin, United States), and 1 pmoL of each primer. The thermal cycling conditions of PCR included a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 7 min. Positive and negative controls were included in each experiment. Finally, 10 µL of each PCR product was subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized on an ultraviolet transilluminator. The expected length of PCR products was 825 bp (*rpoB* primers) and 379 bp (*gltA* primers).

Phylogenetic analysis

The Clustal X program [19] was used to align *Bartonella* sequences obtained from this study. The data will be deposited in the GenBank/EMBL/DDBJ databases. A phylogenetic tree was drawn based on the aligned sequences of *gltA* and *rpoB* genes using the neighbor-joining method with Kimura's two-parameter distance method in MEGA 11 [20]. Bootstrap analysis was conducted using 1,000 resamples. The *Brucella melitensis* strain 16M sequence was used as an out-group.

Statistical analysis

Pearson's Chi-square test and Fisher's exact test were used to comparatively analyze the prevalence of

animal species among provinces using the IBM SPSS Statistics software. The differences observed were considered statistically significant at $p \leq 0.05$.

Results

A total of 860 small mammals were captured from nine provinces of Thailand, including 399 *Rattus* spp., 50 *Bandicota* spp., 351 other spp., and 60 *Suncus murinus* (shrews). Overall, 11.5% of blood samples from rodents (99/860, 95% CI: 9.38–13.64%) were positive for nine *Bartonell*a species; the rodents included 86/399 *Rattus* spp. (21.5%), 5/50 *Bandicot*a spp. (10%), 3/299 *Mus musculu*s (1.0%), and 5/60 *Suncus murinus* (8.3%). The incidence and identities of the nine *Bartonella* spp. were as follows: 27.3% of *B. tribocorum*, 20.2% of *Bartonella rattimassiliensis*, 15.2% of *Bartonella queenslandensis*, 10.1% of *B. elizabethae*, 8.1% of *Bartonella chanthaburi* spp. nov., 6.1% of *Bartonella satun* spp. nov., 6.1% of *Bartonella coopersplainsensis*, 5.1% of *Bartonella ranong* spp. nov., and 2.0% of *B. henselae* (Table-1).

Table-2 shows the geographic distribution of the nine *Bartonella* spp. isolated from rodents and shrews. Of all animals carrying these pathogens, 22/414 (5.31%) were captured in the northeastern region of Thailand, 1/41 (2.5%) in the northern region, 5/40 (12.5%) in the central region, 25/148 (16.9%) in the eastern region, and 46/217 (21.2%) in the southern region. The prevalence of the nine *Bartonella* spp. in the nine provinces was as follows (in descending order): 35.1% (40/114 animals) in Ranong, 31.0% (14/45) in Nakhon Phanom, 16.9% (25/148) in Chanthaburi, 12.5% (5/40) in Chonburi, 7.7% (3/39) in Phuket, 5.4% (2/37) in Songkhla, 3.7% (1/27) in Satun, 2.5% (1/41) in Tak, and 2.2% (8/369) in Khon Kaen. Among the northeastern provinces, *Bartonella* prevalence in Nakhon Phanom was significantly higher than that in Khon Kaen ($p < 0.001$). Further, among the southern provinces, the prevalence in Ranong was significantly higher than that in Satun ($p < 0.001$). The phylogenetic tree of the 99 *Bartonella*-positive sequences of gltA and rpoB fragments is shown in Figure-2. Table-3 shows the GenBank accession numbers of the nucleotide sequences obtained from this study, which were deposited in the GenBank.

Discussion

The prevalence of rodent-associated *Bartonella* spp. has shown high diversity, with more than 20 such species reported worldwide. It is known that more than one *Bartonella* spp. can circulate in rodent communities, and the presence of multiple *Bartonella* genotypes in the same host has been reported [3, 5, 21], leading to emerging bartonellosis, particularly in Southeast Asia [22, 23]. The prevalence of these pathogens was reported to be 6% in Indonesia [24], 9.3–42.9% in China [25], 10.1–30.4% in Lao PDR [26], and 13.5–13.8% in Malaysia [27], depending on the diagnostic method, location, environmental conditions, presence

Table-1: Prevalence of nine *Bartonella* species from 860 rodents and shrews of nine provinces in Thailand.

Host species	Number of examined	Number of positive (%)	Number of animals infected with *Bartonella* species							
			Bartonella coopersplainsensis	*Bartonella elizabethae*	*Bartonella henselae*	*Bartonella queenslandensis*	*Bartonella rattimassiliensis*	*Bartonella tribocorum*	*Bartonella chanthaburi* spp. nov.	*Bartonella satun* spp. nov.	*Bartonella ranong* spp. nov.
Bandicota indica	46	5 (11.1)	1	0	0	2	1	1	0	0	0
Bandicota savilei	4	0	0	0	0	0	0	0	0	0	0
Berylmys berdmorei	5	0	0	0	0	0	0	0	0	0	0
Callosciurus notatus	3	0	0	0	0	0	0	0	0	0	0
Crocidura fuliginosa	23	0	0	0	0	0	0	0	0	0	0
Maxomys rajah	5	0	0	0	0	0	0	0	0	0	0
Maxomys surifer	1	0	0	0	0	0	0	0	0	0	0
Menetes berdmorei	3	0	0	0	0	0	0	0	0	0	0
Mus caroli	10	0	0	0	0	0	0	0	0	0	0
Mus musculus	299	3 (1.0)	0	0	0	0	0	3	0	0	0
Rattus argentiventer	5	0	0	0	0	0	0	0	0	0	0
Rattus exulans	125	8 (6.4)	0	0	0	0	0	8	0	0	0
Rattus norvegicus	59	28 (47.5)	0	8	1	8	2	9	0	0	0
Rattus rattus	171	48 (28.1)	5	2	1	5	17	6	7	5	0
Rattus tenezumi	39	2 (5.1)	0	0	0	0	0	0	1	1	0
Tupaia glis	2	0	0	0	0	0	0	0	0	0	0
Suncus murinus	60	5 (8.3)	0	0	0	0	0	0	0	0	5
Total	860	99 (11.5)	6 (6.1)	10 (10.1)	2 (2.0)	15 (15.2)	20 (20.2)	27 (27.3)	8 (8.1)	6 (6.1)	5 (5.1)

Table-2: Geographic distribution of nine *Bartonella* species isolated from rodents and shrews in nine provinces, Thailand.

	Province	Number examined	Number of positive (%)	Number of animals infected with *Bartonella* species								
				Bartonella coopersplainsensis	***Bartonella elizabethae***	***Bartonella henselae***	***Bartonella queenslandensis***	***Bartonella rattimassiliensis***	***Bartonella tribocorum***	***Bartonella chanthaburi* spp. nov.**	***Bartonella satun spp.* nov**	***Bartonella ranong* spp. nov**
North-Eastern	Khon Kaen	369	8 (2.2)*	0	0	0	3/(Rr = 1, Bi = 2)	2/(Bi = 1, Rr = 1)	3/(Mm = 3)	0	0	0
	Nakhon Phanom	45	14 (31)*	0	0	0	0	3/(Rr = 3)	11/(Re = 8, Rn = 1, Rr = 2)	0	0	0
	subtotal	414	22 (5.31)	0	0	0	3/(Rr = 1, Bi = 2)	5(Bi = 1, Rr = 4)	14 (Mn = 3, Re = 8, Rn = 1, Rr = 2)	0	0	0
Northern	Tak	41	1 (2.5)	0	0	0	0	0	1/(Bi = 1)	0	0	0
Central Eestern	Chon Buri	40	5 (12.5)	2/(Bi = 1, Rr = 1)	0	0	0	2/(Rr = 2)	0	1/(Rr = 1)	0	0
	Chanthaburi	148	25 (16.9)	4/(Rr = 4)	0	0	2/(Rr = 2)	8/(Rr = 8)	2/(Rr = 2)	5/(Rr = 5)	4/(Rr = 4)	0
Southern	Ranong	114	40 (35.1)**	0	10/(Rn = 8, Rr = 2)	2/(Rn = 1, Rr = 1)	10/(Rn = 8, Rr = 2)	3/(Rn = 2, Rr = 1)	10/(Rn = 8, Rr = 2)	1/(Rr = 1)	0	4/(Sm = 4)
	Phuket	39	3 (7.7)	0	0	0	0	2/(Rr = 2)	0	0	1/(Rr = 1)	0
	Songkhla	37	2 (5.4)	0	0	0	0	0	0	1/(Rt = 1)	0	1/(Sm = 1)
	Satun	27	1 (3.7)**	0	0	0	0	0	0	0	1/(Rt = 1)	0
	Subtotal	217	46 (21.2)	0	10/(Rn = 8, Rr = 2)	2/(Rn = 1, Rr = 1)	10/(Rn = 8, Rr = 2)	5/(Rn = 2, Rr = 3)	10/(Rn = 8, Rr = 2)	2/(Rr = 1, Rt = 1)	2/(Rr = 1, Rt = 1)	5(Sm = 5)
	Total	860	99 (11.5)	6 (6.1)	10 (10.1)	2 (2.0)	15 (15.2)	20 (20.2)	27 (27.3)	8 (8.1)	6 (6.1)	5 (5.1)

*Prevalence in NakhonPhanom was significantly higher than in KhonKaen ($p < 0.001$), **Prevalence in Ranong was significantly higher than in Satun ($p < 0.001$)

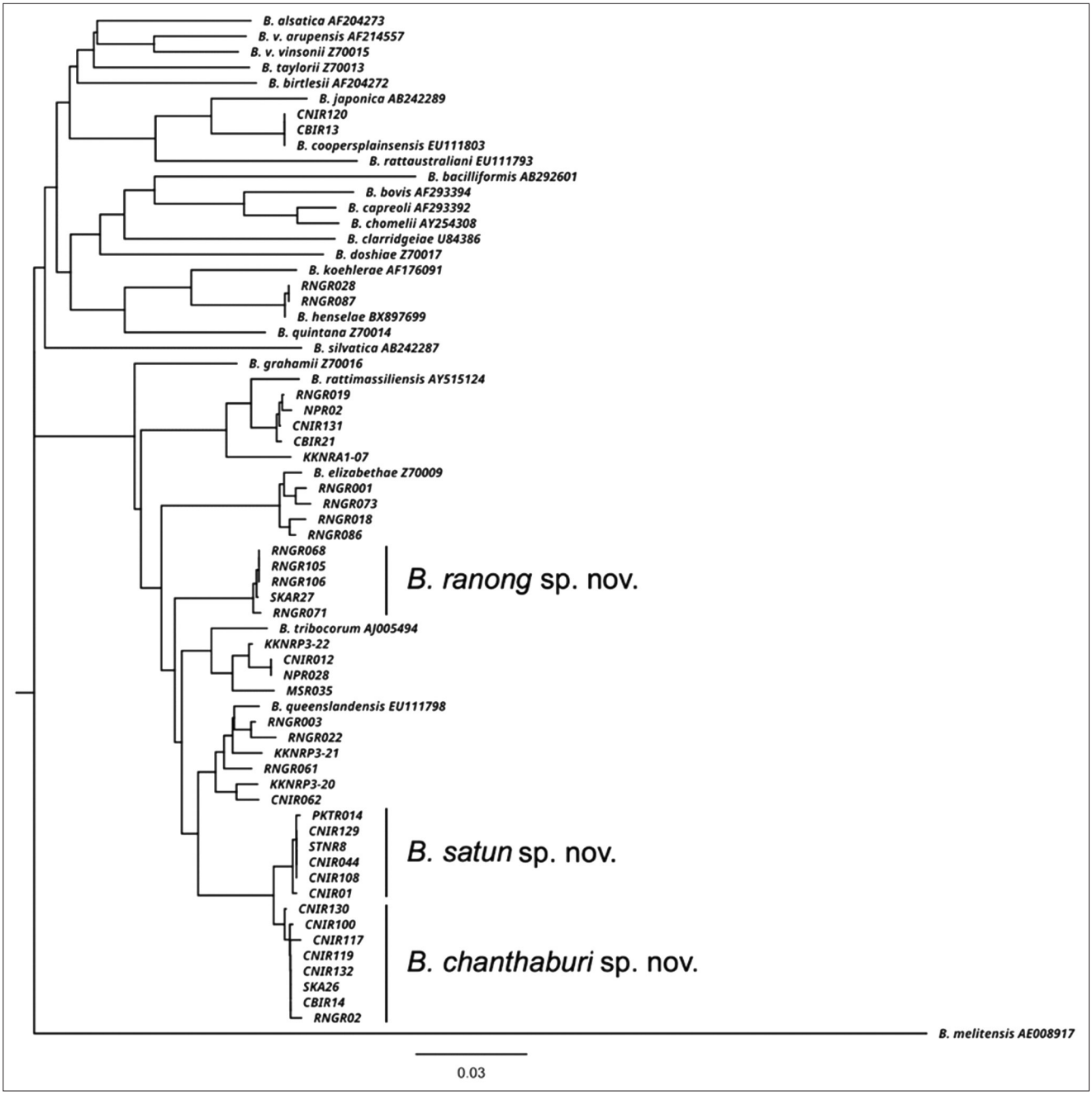

Figure-2: Neighbor-Joining tree based on the concatenated sequences of 2 loci (*gltA* and *rpoB*) of *Bartonella* species. The phylogenetic tree was reconstructed using the Kimura 2-parameter substitution model. The tree was rooted using *Brucella melitensis* as an out-group. Bootstrap values resulting from 1000 bootstrap trials are indicated for each branch. Bar represents 0.03 estimated nucleotide substitutions per site. gltA=Citrate synthase, rpoB=RNA polymerase.

of vectors, and animal host species and their habitats. The prevalence of 11.5% reported in this study is similar to that reported in a study conducted in Malaysia [27]. In line with the previous studies, *Bartonell*a spp. in our study was also frequently isolated from *Rattus rattu*s [7, 8]; however, other studies showed contrasting results [24–26]. The previous studies from Thailand have identified the presence of *B. elizabethae*, *B. henselae*, *Bartonella clarridgeae*, and *B. tamiae*, which were known to cause infections in humans [6–9, 12–13]. Of these, we did not detect *B. tamiae* and *B. clarridgeae* in this study but we found the other two species in addition to *B. tribocorum*, *B. rattimassiliensis*, *B. coopersplainsensis*, *B. queenslandensis*, and three novel *Bartonella* spp.

B. henselae is a well-known pathogen in wild and domestic cats and causes cat-scratch disease [28, 29]. It has been isolated from rodents in Thailand in a previous study [7, 8] and is associated with febrile Thai patients [13, 30]. This pathogen was detected in approximately 2% of the rodents from the Ranong Province. Notably, the three novels *Bartonella* spp. were also found in this province and were isolated from shrews.

B. elizabethae is widely distributed in Asian countries [7, 8, 25]. It is known to be associated with

Table-3: GenBank accession numbers for nucleotide sequences.

Nucleotide sequences	GenBank accession numbers	
	gltA	rpoB
B. elizabethae_THRNGR001	MF105784	MF105860
B. queenslandensis_THRNGR003	MF105785	MF105861
B. tribocorum_THRNGR006	MF105786	MF105862
B. tribocorum_THRNGR012	MF105787	MF105863
B. elizabethae_THRNGR018	MF105788	MF105864
B. rattimassiliensis_THRNGR019	MF105789	MF105865
B. tribocorum_THRNGR020	MF105790	MF105866
B. queenslandensis_THRNGR022	MF105791	MF105867
B. henselae_THRNGR028	MF105792	MF105869
B. elizabethae_THRNGR029	MF105793	MF105870
B. rattimassiliensis_THRNGR032	MF105794	MF105871
B. tribocorum_THRNGR033	MF105795	MF105872
B. queenslandensis_THRNGR034	MF105796	MF105873
B. elizabethae_THRNGR036	MF105797	MF105874
B. queenslandensis_THRNGR037	MF105798	MF105875
B. elizabethae_THRNGR043	MF105799	MF105876
B. tribocorum_THRNGR044	MF105800	MF105877
B. elizabethae_THRNGR045	MF105801	MF105937
B. queenslandensis_THRNGR061	MF105802	MF105878
B. ranong_THRNGR068	MF105803	MF105879
B. ranong_THRNGR071	MF105804	MF105880
B. elizabethae_THRNGR073	MF105805	MF105881
B. queenslandensis_THRNGR074	MF105806	MF105882
B. tribocorum_THRNGR077	MF105807	MF105884
B. tribocorum_THRNGR079	MF105808	MF105885
B. tribocorum_THRNGR080	MF105809	MF105886
B. queenslandensis_THRNGR081	MF105810	MF105887
B. tribocorum_THRNGR083	MF105811	MF105888
B. elizabethae_THRNGR084	MF105812	MF105889
B. elizabethae_THRNGR086	MF105813	MF105890
B. queenslandensis_THRNGR091	MF105814	MF105892
B. tribocorum_THRNGR094	MF105815	MF105893
B. ranong_THRNGR105rpoB	MF105816	MF105894
B. ranong_THRNGR106rpoB	MF105817	MF105895
B. rattimassiliensis_THPKTR006	MF105818	MF105896
B. satun_THPKTR014	MF105819	MF105897
B. rattimassiliensis_THCTIR99	MF105820	MF105898
B. chanthaburi_THCTIR100	MF105821	MF105899
B. coopersplainsensis_THCTIR101	MF105822	MF105900
B. rattimassiliensis_THCTIR103	MF105823	MF105901
B. rattimassiliensis_THCTIR105	MF105824	MF105902
B. rattimassiliensis_THCTIR106	MF105825	MF105903
B. rattimassiliensis_THCTIR107	MF105826	MF105904
B. satun_THCTIR108rpoB	MF105827	MF105905
B. chanthaburi_THCTIR119	MF105829	MF105906
B. coopersplainsensis_THCTIR120	MF105830	MF105907
B. coopersplainsensis_THCTIR128	MF105831	MF105908
B. chanthaburi_THCTIR129	MF105832	MF105909
B. chanthaburi_THCTIR130	MF105833	MF105910
B. rattimassiliensis_THCTIR131	MF105834	MF105911
B. chanthaburi_THCTIR132	MF105835	MF105912
B. rattimassiliensis_THCTIR135	MF105836	MF105913
B. rattimassiliensis_THCTIR141	MF105837	MF105914
B. queenslandensis_THKKNRP3-20	MF105838	MF105915
B. queenslandensis_THKKNRP3-21	MF105839	MF105916
B. tribocorum_THKKNRP3-22	MF105840	MF105917
B. tribocorum_THKKNRP3-24	MF105841	MF105918
B. tribocorum_THKKNRP3-25	MF105842	MF105919
B. queenslandensis_THKKNRP3-29	MF105843	MF105920
B. satun_THSTNR8	MF105844	MF105921
B. chanthaburi_THSKAR26	MF105845	MF105922
B. ranong_THSKAR27	MF105846	MF105923
B. tribocorum_THMSR035	MF105847	MF105924
B. satun_THCTIR01	MF105848	MF105925
B. tribocorum_THCTIR012	MF105849	MF105926
B. tribocorum_THCTIR043	MF105850	MF105927
B. satun_THCTIR044	MF105851	MF105928
B. queenslandensis_THCTIR062	MF105852	MF105929
B. queenslandensis_THCTIR064	MF105853	MF105930
B. coopersplainsensis_THCBIR13	MF105854	MF105931
B. chanthaburi_THCBIR14	MF105855	MF105932
B. coopersplainsensi_sTHCBIR20	MF105856	MF105933
B. rattimassiliensis_THCBIR21	MF105857	MF105934
B. rattimassiliensis_THCBIR23	MF105858	MF105935
B. rattimassiliensis_THKKNRA1-07	MF105859	MF105936

(Contd...)

Table-3: *(Continued).*

gltA=Citrate synthase, rpoB=RNA polymerase*, B. tribocorum=Bartonella tribocorum, B. rattimassiliensis=Bartonella rattimassiliensis, B. queenslandensis=Bartonella queenslandensis, B. elizabethae=Bartonella elizabethae, B. chanthaburi=Bartonella chanthaburi, B. satun=Bartonella satun, B. coopersplainsensis=Bartonella coopersplainsensis, B.ranong=Bartonella ranong, B. henselae=Bartonella henselae*

endocarditis [31] and human neuroretinitis [32]. We found this species in approximately 10% of the rodents in the Ranong province near the Myanmar border, where there are several markets, which are visited by business travelers and workers. The Ranong Province showed the highest prevalence of *Bartonella* infection in animals (35.1%) among all provinces. Thus, the epidemiology of this infection in febrile patients with rodent exposure should be considered.

In this study, *B. tribocorum* was the most prevalent *Bartonella* spp. in rodents (27.3%) followed by *B. rattimassiliensis* (20.2%), both of which had been detected in febrile patients in Thailand in a previous study [13]. Almost all incidences of these species were in *Rattus* rodents, similar to that reported in the previous studies [4, 26, 30, 33].

The other two *Bartonella* spp.; *B. queenslandensis* and *B. coopersplainsensis* were also isolated from *Rattus* rodents. They have been isolated from rodents and fleas in Taiwan in a previous study [34].

We found three novel *Bartonella* spp. in the Chanthaburi, Satun, and Ranong provinces. The public health information concerning *Bartonella* infections in these three provinces remains unknown. Further collaboration between human and animal sectors can help investigate the possibility of new *Bartonella* spp. infections in febrile patients with rodent exposure in these three provinces.

Conclusion

To the best of our knowledge, this is the first study that reported the detection of three novel *Bartonella* spp. isolated from rodents and shrews in Thailand. In this study, nine different *Bartonella* spp.

were detected and most of them were potentially zoonotic, using rodents as reservoir hosts. Further studies on the risk of this infection among humans, rodents, and the environment are needed to advance public health information.

Authors' Contributions

DP, BN, WP, WW, and NC: Collected the samples. DP and WR: Provided technical help during the experiments. PW and MB: Did the statistical analysis. SB: Designed the study and drafted and revised the manuscript. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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