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Short Report: Isolation and Molecular Identification of Bartonellae from Wild Rats (*Rattus* Species) in Malaysia

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Abstract. This study describes our investigation on the prevalence and molecular identification of bartonellae from *Rattus diardii* and *R. norvegicus* in the urban areas of Malaysia. Of 95 rats investigated, *Bartonella tribocorum*, *B. rattimassiliensis*, *B. coopersplainsensis*, *B. elizabethae*, and *B. queenslandensis* were isolated from kidney and spleen homogenates of four rats. Bartonellae DNA was amplified from the rat organ tissues by using primers specific for the bartonellae RNA polymerase beta subunit (*rpo*B) gene in nine other rats. Sequence analysis of the *rpo*B gene fragments shows the identification of *B. queenslandensis* in five rats, *B. elizabethae* in three rats, and *B. tribocorum* in one rat. Combining the results of isolation and molecular detection of bartonellae, we found that the prevalence of *Bartonella* infection in the *Rattus* spp. investigated in this study was 13.7%. Implementation of effective rat control program in the urban areas is necessary to prevent the spillover of bartonellosis from rats to humans.

Bartonellae are bacterial parasites that infect mammalian erythrocytes and endothelial cells.[1] These small gram-negative and fastidious bacteria are transmitted usually through the bites of hematophagous arthropods, such as fleas, lice, flies, and ticks. Bartonellosis is an emerging and reemerging zoonotic infection responsible for a variety of clinical syndromes in humans and animals.[2] Members of the genus *Bartonella* exhibit high degrees of genetic diversity and ecologic plasticity.[3] Since the first description of *Bartonella bacilliformis,* the type species of the genus, 29 *Bartonella* species have been reported (http://www.bacterio.net/b/bartonella.html). The increasing reports of new *Bartonella* species potentially causing human infections have spurred extensive investigations to identify the reservoir mammalian hosts and the arthropod vectors.[4,5]

The occurrence of bartonellae in rodents and other small mammals has been reported in several countries in Asia, including Bangladesh, China, Indonesia, Japan, Laos, Cambodia, Taiwan, Nepal, and Thailand.[6–17] *Bartonella tribocorum*, B. *rattimassiliensis*, *B. coopersplainsensis*, *B. elizabethae*, and *B. queenslandensis* are among those that have been identified from the wild rats (*Rattus* species). Arthropod vectors, particularly fleas (*Ctenocephalides felis*) and ticks (*Ixodes*, *Haemaphysalis*), are often implicated in the natural maintenance of various species of bartonellae.[16] A recent study in our laboratory reported the detection of *B. henselae* and *B. clarridgeiae* in *C. felis* fleas.[17] However, no information is available on the presence of bartonellae in rodents in the urban areas of Malaysia. Thus, the main objective of this study was to determine the occurrence and type of bartonellae circulating in the urban wild rat population in Malaysia.

A total of 95 rodents were captured as part of rodent management program conducted by the pest control sections of the municipal councils, i.e., Kuala Lumpur (n = 59) and Pulau Pinang (n = 36), Malaysia during January 2008–December 2011. Rats were trapped by using live traps with tapioca and dried fish as baits. Rats were identified as *Rattus diardii* (n = 58) and *R. norvegicus* (n = 37) (Table 1), brought to our laboratory, and anesthetized by using an ether-charged chamber. Postmortem examination was conducted and organs (kidney, liver, and spleen) were harvested aseptically. A total of 295 tissue samples were obtained and kept at −80°C before processing.

A 20% homogenized tissue was prepared by grinding approximately 0.4 grams of rat tissue in 2 mL of Schneider's liquid medium (Sigma, St. Louis, MO) by using a mortar and pestle. Two hundred microliters of the tissue homogenate were then inoculated on a commercially available Columbia agar plate (Isolabs Sdn. Bhd, Selangor, Malaysia) supplemented with 5% sheep blood, and incubated at 35°C in 5% CO_2 incubator for at least one month. Bacterial growth was monitored at least once per week after initial plating. Suspected colonies were streaked on a fresh agar plate and subjected for amplification targeting citrate synthase (*glt*A)[18] and RNA polymerase beta subunit (*rpo*B)[19] genes. Sequence determination of the amplified fragments was performed in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA) with primers for *glt*A (BhCS.781p and BhCS.1137n) and *rpo*B (1400D and 2300R). Resulting sequences were compared with known *Bartonella* sequences deposited in GenBank by using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, Bethesda, MD). Based on *rpoB* sequences, a dendrogram was constructed by using the neighbor-joining method in MEGA software and bootstrap analysis with 1,000 resamplings.[20]

The bartonellae isolation rate from the wild rats was low. Only five *Bartonella* isolates were successfully recovered from four rats caught in Kuala Lumpur after an incubation period of 4–8 days. One isolate was obtained from the kidney homogenate of a *R. norvegicus* rat, and the remaining four isolates were obtained from kidney, liver, and spleen homogenates of three *R. diardii* rats (Table 2). Mixed infection of *Bartonella* spp. was detected in an *R. diardii* rat (DKK4) by isolation of different *Bartonella* species in kidney and spleen homogenates. Based on the BLAST analysis of the *glt*A (276 nuleotides) and *rpo*B sequences (750 bp), the isolates obtained in this study were identified as *B. tribocorum*, *B. rattimassiliensis*, *B. coopersplainsensis*, *B. elizabethae*, and *B. queenslandensis*. All isolates had ≥ 97% sequence similarities with their respective type strains. Thus, they are regarded

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TABLE 1
Source and details of *Rattus* spp. investigated for bartonellae, Malaysia

Rat species	Location	Sex	No. positive rats*
R. norvegicus (n = 37)	Kuala Lumpur (n = 12)	Male (n = 5)	1
		Female (n = 7)	1
	Pulau Pinang (n = 25)	Male (n = 19)	3
		Female (n = 6)	0
R. diardii (n = 58)	Kuala Lumpur (n = 47)	Male (n = 28)	5
		Female (n = 19)	3
	Pulau Pinang (n = 11)	Male (n = 6)	0
		Female (n = 5)	0
Total (n = 95)	Kuala Lumpur (n = 59) and Pulau Pinang (n = 36)	Male (n = 58) Female (n = 37)	13

*Combining results of isolation and direct amplification from rat tissues.

as members of the same species, in accordance with a proposal by La Scola and others,[21] that bartonellae should be considered as the same species if the sequence similarities for their *glt*A and *rpo*B genes are > 95.4% and 96.0%, respectively.

Direct amplification of bartonellae DNA from rat tissues was performed to improve the detection of bartonellae from the rat tissue homogenates in this study. DNA was extracted from 200 μL of each rat tissue homogenate by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Polymerase chain reactions (PCRs) specific for *glt*A[18] and *rpo*B[19] genes were performed as described above. Amplification of the bartonellae *glt*A gene did not show a positive result. However, bartonellae DNA was detected from spleen and kidney samples of nine rats (four *R. norvegicus* and five *R. diardii*) by using *rpo*B PCRs (Table 2). Sequence analysis of the amplified *rpo*B genes identified *B. queenslandensis* in five rats, *B. elizabethae* in three rats, and *B. tribocorum* in one rat (Table 2). The species status of bartonellae in this study was confirmed when isolates clustered with known *Bartonella* species (Figure 1).

The prevalence of bartonellae in rodents varied in different geographic regions, ranging from 8.7% in Thailand[15] to 64.2%, in United Kingdom.[22] Combining the results of isolation and direct amplification from the rat organ tissues, we found that the overall prevalence of *Bartonella* infection in wild rats in this study was 13.7% (13 of 95 rats were positive). This finding suggests that *R. rattus* (13.5% positive) and *R. norvegicus* (13.8% positive) in cities may serve as a main reservoir for several *Bartonella* species in Malaysia.

Three *Bartonella* species (*B. rattimassiliensis*, *B. tribocorum*, and *B. elizabethae*) identified in this study are of public health significance in Southeast Asia because these species have been isolated from febrile patients in Thailand.[5] *Bartonella elizabethae* has been identified as a causative agent of human endocarditis and neuroretinitis in Indonesia.[8] These species have also been reported from small mammals in different regions e.g., France,[23,24] the United States, and Portugal.[25]

Bartonella queenslandensis was the predominant *Bartonella* species identified in 6 of 13 (5 *R. diardii* and 1 *R. norvegicus*) rats in this study. The *Bartonella* species was originally isolated from *R. fuscipes* rats in Australia,[26] and has been isolated from small mammals in Bangladesh,[6] Nepal[13] and three countries in southeastern Asia (Cambodia, Laos, and Thailand).[11] *Bartonella coopersplainsensis* was first isolated from the blood of *R. leucopus* in Coopers Plains, Queensland, Australia,[26] and later, from *Rattus* spp. and *Bandicota* spp. in Thailand.[14,16] Nevertheless, the zoonotic potential of *B. queenslandensis* and *B. coopersplainsensis* has not been reported.

This study demonstrated the prevalence and genetic heterogeneity of *Bartonella* organisms in the urban wild rat population in Malaysia. Because identification of *Bartonella* species by using conventional microbiologic tests is difficult, PCR, followed by sequence analysis of specific target genes (*glt*A and *rpo*B), assisted our investigation. A confirmed case of bartonellosis has not been documented in Malaysia. However; in view of the identification of several bartonellae of medical importance in this study, implementation of effective

TABLE 2
Sequence analysis of bartonellae identified from *Rattus* spp., Malaysia*

Rat	Tissue, rat species, location	Gene	Closest relative (gene accession no., % similarity)
Isolation (n = 4 rats)			
DKK5	Kidney, *R. norvegicus*, KL	*rpo*B	*B. tribocorum* strain IBS 506 (AF165996, 96.9)
		*glt*A	*B. tribocorum* strain IBS 506 (AJ005494, 99.6)
DKK4-(1)	Kidney, *R. diardii*, KL	*rpo*B	*B. rattimassiliensis* strain 15908 (AY515130, 98.2)
		*glt*A	*B. rattimassiliensis* strain 15908 (AY515124, 97.8)
-(2)	Spleen, *R. diardii*, KL	*rpo*B	*B. coopersplainsensis* AUST/NH20 (EU111792, 100.0)
		*glt*A	*B. coopersplainsensis* AUST/NH20 (EU111803, 99.6)
KL16	Kidney, spleen and liver, *R. diardii*, KL	*rpo*B	*B. queenslandensis* AUST/NH12 (EU111787, 97.6)
		*glt*A	*B. queenslandensis* AUST/NH12 (EU111798, 97.8)
KL20	Kidney, spleen and liver, *R. diardii*, KL	*rpo*B	*B. elizabethae* F9251 (AF165992, 100.0)
		*glt*A	*B. elizabethae* F9251 (Z70009, 100.0)
Direct amplification from rat organ tissue (n = 9 rats)			
KL4, KL7, KL8	Spleen, *R. diardii*, KL	*rpo*B	*B. queenslandensis* AUST/NH12 (EU111787, 97.1)
KL12	Spleen, *R. diardii*, KL	*rpo*B	*B. queenslandensis* strain AUST/NH12 (EU111787, 99.8)
KL5	Spleen, *R. diardii*, KL	*rpo*B	*B. elizabethae* (AF165992, 100.0)
KL11	Spleen, kidney, *R. norvegicus*, KL	*rpo*B	*B. queenslandensis* AUST/NH12 (EU111787, 97.1)
RP4	Spleen, kidney, *R. norvegicus*, PP	*rpo*B	*B. tribocorum* (AF165996, 97.6)
RP31, RP33	Spleen, kidney, *R. norvegicus*, PP	*rpo*B	*B. elizabethae* (AF165992, 100.0)

*KL = Kuala Lumpur; *rpoB* = RNA polymerase beta subunit; *gltA* = citrate synthase; PP = Pulau Pinang.

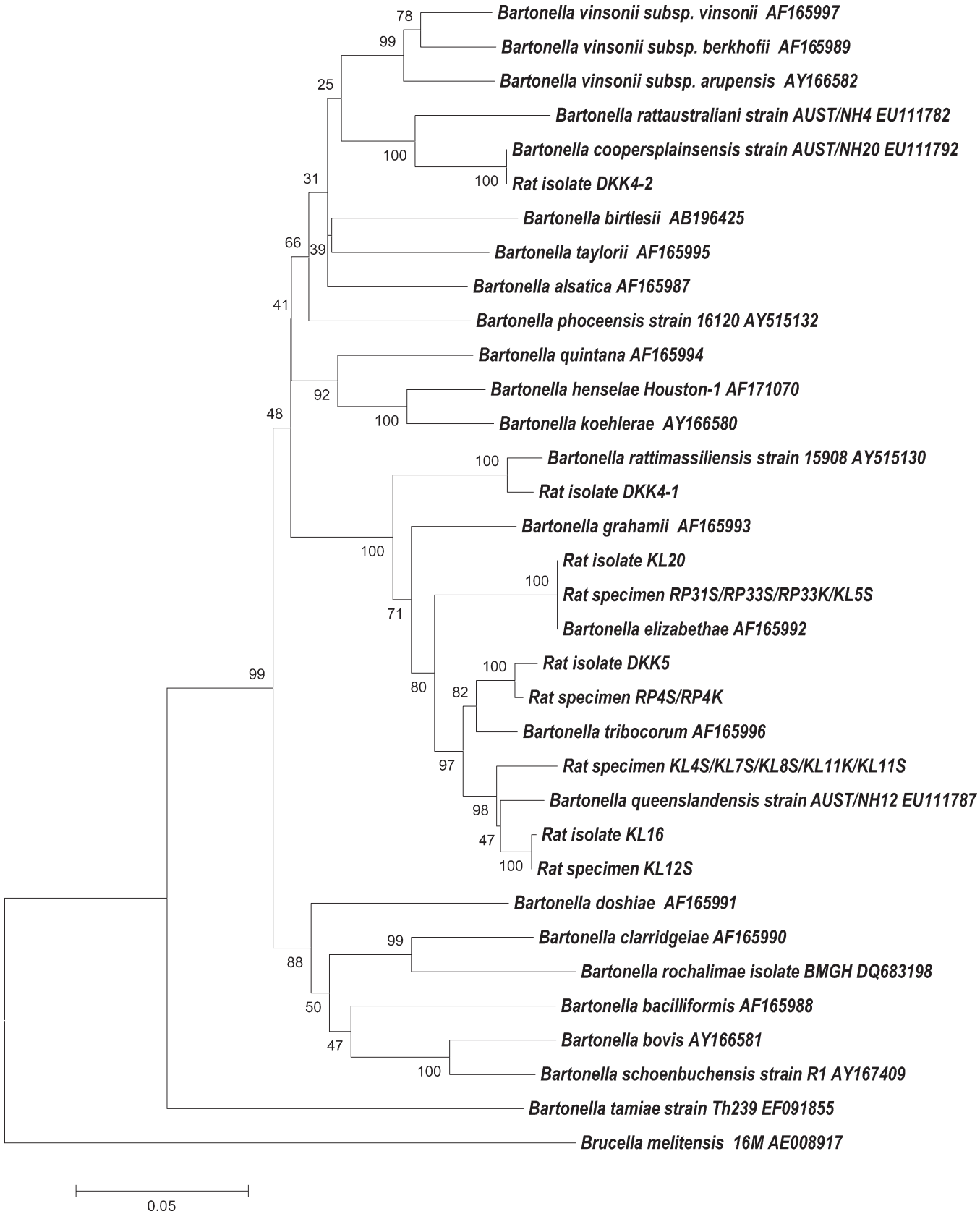

FIGURE 1. Identification of Bartonellae by comparing the sequences of RNA polymerase beta subunit gene fragments of known *Bartonella* species. The dendrogram was constructed using the neighbor-joining method in MEGA software and bootstrap analysis with 1,000 resamplings. Source of the isolates and specimens is indicated in Table 1. S = spleen; K = kidney. Scale bar indicates nucleotide substitutions per site.

rat control program in the urban areas is necessary to prevent the spilling over of bartonellosis from rats to human population.

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