BARTONELLA SPECIES IN RODENTS AND SHREWS IN THE GREATER JAKARTA AREA

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Abstract. In February 2004, we captured 221 rodents and shrews in the Greater Jakarta area as part of a study to determine the prevalence of rodent-associated vector-borne infections. Microscopic examination of blood smears revealed 6% (13/218) to be positive for *Bartonella* spp. The corresponding DNA samples, either from blood blots or frozen spleen pieces and from fleas collected on these animals, were tested for evidence of *Bartonella* infection by PCR, targeting the portions: 378bp and 930bp of the citrate synthase gene (*glt*A). The sequences from our sample clusters with a Peruvian entity, *B. phoceensis*, *B. rattimassiliensis* and *B. elizabethae*, the latter species has been associated with endocarditis and neuroretinitis in humans. As previous analyses have shown, there appears to be little geographic or host consistency with phylogenetic placement. The public health significance of these findings remains to be determined.

INTRODUCTION

Bartonella is a gram-negative bacteria transmitted by fleas, lice and sand flies. It is related to rickettsiae, but because *Bartonella* is not an obligatory intracellular organism, the Bartonellaceae family is no longer classified under the order Rickettsiales. *Bartonella* spp are the causative agents of forms of bartonellosis, namely: *B. henselae* (cat-scratch disease), *B. quintana* (trench fever), *B. bacilliformis* (Carrion's disease) and *B. elizabethae.* Recently, however, other species have also been implicated in human infection, *eg B. grahamii* (Kerkhoff *et al*, 1999), particularly as a cause of neuroretinitis.

Animal surveys demonstrate a great prevalence and diversity of *Bartonella* worldwide. In Asia, information on the presence of *Bartonella* in animals, ectoparasites and as a pathogen in humans is rapidly accumulating (Ying *et al*, 2002; Parola *et al*, 2003; Castle *et al*, 2004; Maruyama *et al*, 2004). We conducted a survey of small mammals in the Greater Jakarta area to determine the prevalence of various rodent-associated vector-borne infections. Here, we report the results of phylogenetic analyses of *Bartonella* spp detected in these animals, as well as in the fleas that infested them.

MATERIALS AND METHODS

From 19-27 February 2004, we trapped rodents and shrews in three areas in and around Jakarta, namely: the village of Bambu Apus in Tangerang (south of Jakarta); the village of Penjaringan in the harbor area of North Jakarta, and at Ragunan Zoo in South Jakarta. The protocol was approved by NAMRU-2 Institutional Animal Care and Use Committee (IACUC) and the National Institute of Health Research and Development, Indonesian Ministry of Health.

Small mammal trapping

Locally made live box traps designed for capturing small mammals, as well as some

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Sherman traps, were baited with roasted coconut. Traps containing captured animals were put in individual cloth bags and transported to a nearby specimen processing site/lab, where the animals were euthanized with isoflurane anesthesia, ectoparasites were removed, placed in labeled vials with 70% ethanol and a site voucher record was completed for each animal trapped.

Specimen collection

Captured animals were processed according to NAMRU-2 standard operating procedures. Blood samples obtained by cardiac puncture were collected in sterile tubes for sera separation. Thin blood smears were made, and in some, blood was also blotted on filter paper. Pieces of tissue were taken from the spleen, liver and lungs and placed in sterile tubes. Both tissue and aliquots of sera were stored at -80ºC.

Microscopy

Thin blood smears were stained with Giemsa and examined under light microscopy at 1,000x magnification for hemoparasites.

PCR and sequencing

Frozen pieces of spleen (or blood blots when available) from animals found positive for *Bartonella* spp by microscopy, as well as fleas that were collected from these animals, were extracted for DNA using DNeasy® tissue Kit (Qiagen GmbH, Hilden Germany). These DNA samples were then tested for evidence of *Bartonella* infection by Polymerase Chain Reaction (PCR) targeting a 378bp portion of the citrate synthase gene (gltA) using primers BhCS.781p and BhCS.1137n as described previously (Norman *et al*, 1995). *Taq* polymerase (Qiagen) and buffers were used as recommended by the manufacturer. Amplification products were run on a 1% agarose gel and visualized using ethidium bromide staining. All positive amplicons were sequenced, while bands were excised from the gel and purified using a spin column (Qiagen) and sequenced (both strands) at the University of Maine Sequencing Facility (Orono, ME, USA).

For phylogenetic analysis, *Bartonella* sequences from this work and those retrieved from GenBank were aligned using ClustalX and then adjusted by eye using GeneDoc (Nicholas and Nicholas, 1997). The 370bp sequences were analysed in MEGA by the neighbor-joining method with the Kimura 2-parameter model. Some 500 bootstrap replicates were run to determine the robustness of the resulting tree topology (Kimura *et al*, 1993). However, the tree topology from this short portion of the citrate synthase gene (*glt*A) was unsatisfactory, with bootstrap support for major nodes rarely achieving 50% consensus. Therefore, a larger piece of the *glt*A was amplified. Primer BvCS.205p, TTTATCGYGGTTA TCCTATYG, was designed to amplify a 930bp piece when paired with BhCs.1137n. Cycling conditions used were the same as above. All resulting amplicons were sequenced as described above. The longer 930bp pieces were analysed in PAUP (Swofford, 1998) using a maximum likelihood model. The statistically best model, general time reversible with a gamma distribution of rate variation among sites and the proportion of invariable sites estimated from the data (GTR+I+G), was determined using Modeltest (Posada and Crandall, 1998). All analyses used *B. bacilliformis*, a species previously shown to be divergent from all others, as the outgroup (Houpikian and Raoult, 2001).

Statistical analysis

Chi-square test and Fisher's exact test were used for comparative analyses of animal species prevalence by site, host-specific *Bartonella* infection prevalence and prevalence of flea infestation by animal species.

RESULTS

A total of 1,040 traps were set; 221 animals belonging to 5 species were caught in 219 traps with the percentages of success varying from 16.3 in Bambu Apus to 32.4 in Penjaringan (Table 1). *Suncus murinus* was the most common species (85/221; 38.5%, 95% CI 32.0-44.9%) and accounted for 65.5% (95% CI 52.9-78.0%; 36/55) of animals trapped at Ragunan Zoo. There was a statistically significant difference in the distribution of animal species by site (χ^2 = 42.4 df = 8, p < 0.001). The majority of animals trapped in the Bambu Apus village were *R. tanezumi* (Asian house rat) (39/82; 47.6%,), while *Rattus tanezumi*, *R. norvegicus* and *S. murinus* were about equally common in Penjaringan.

Table 1
Number of animals trapped in the three study sites.

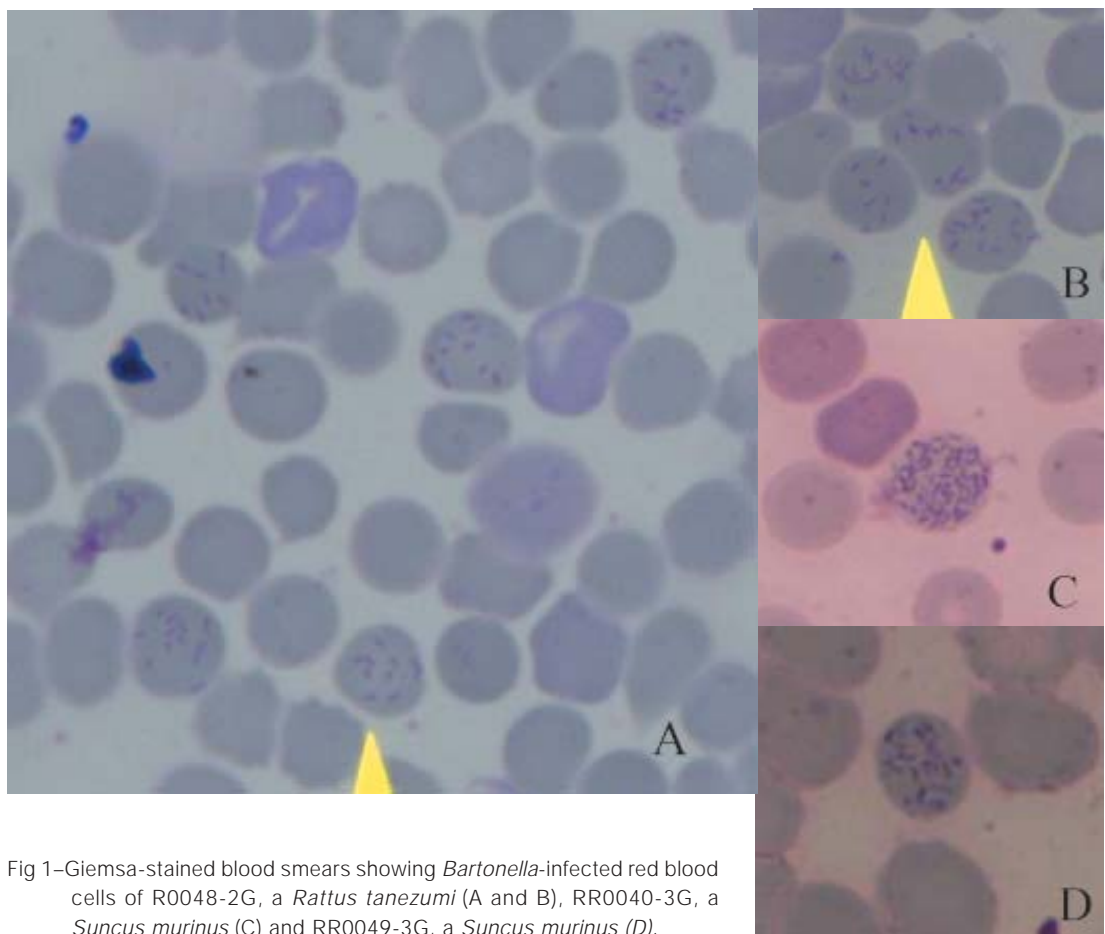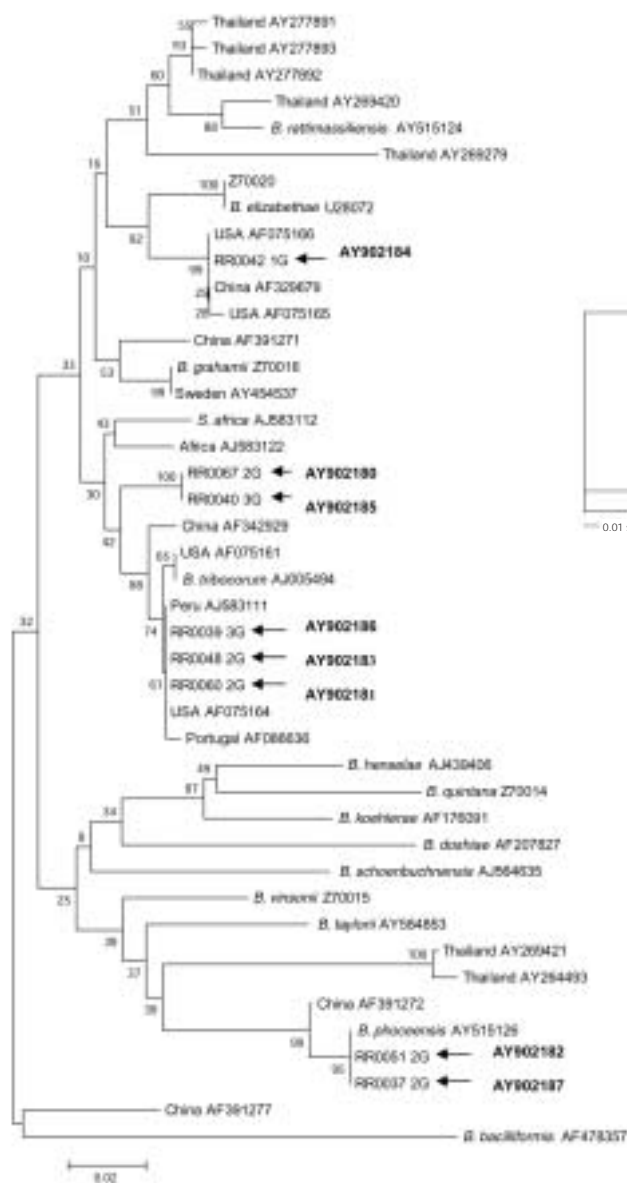| Study site	No. of traps set	No. (%) of traps with animals	No. of animals					
			Total	R. tanezumi	R. norvegicus	M. musculus	S. murinus	R. exulans
Bambu Apus	486	79 (16.3)	82	39	17	-	26	-
Penjaringan (Harbor)	253	82 (32.4)	84	27	27	7	23	-
Ragunan Zoo	300	55 (18.3)	55	13	5	-	36	1
			221	79	49	7	85	1

Fig 1–Giemsa-stained blood smears showing *Bartonella*-infected red blood cells of R0048-2G, a *Rattus tanezumi* (A and B), RR0040-3G, a *Suncus murinus* (C) and RR0049-3G, a *Suncus murinus (D).*

Thirteen of 218 available blood smears (6%) examined were found positive for *Bartonella* spp (Table 2, Fig 1). No blood blots or spleen samples were available for two of them. Among the other 11, PCR detected *Bartonella* from blood blots i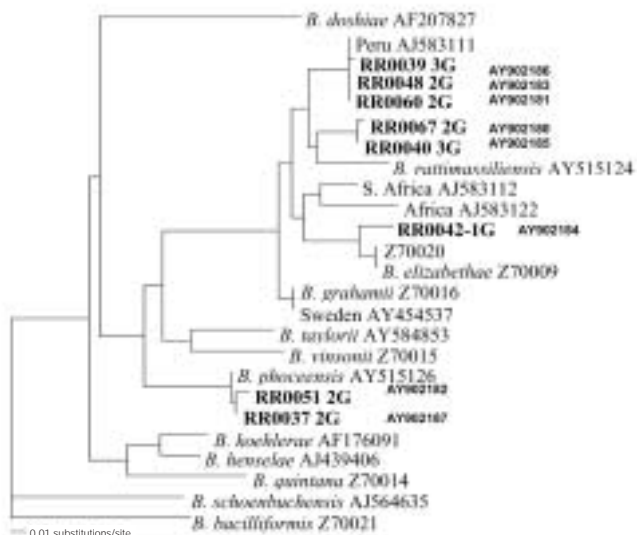n three, and from spleens in the other eight. Two of the 3 flea pools derived from animals with *Bartonella*-positive blood smear were positive for *Bartonella* by PCR.

Host-specific *Bartonella* infection prevalences were: 10.3% (8/78) for R. *tanezumi*, 2.0% (1/49) for *Rattus norvegicus*, and 4.8% (4/83)

RR0002-1G, RR0002-1E, RR0038-2G, RR0040-2G, and RR0042-1E are not showed on the tree because RR0042-1E was identical to RR0042-1G and the rest were identical to RR0039-3G, RR0048-2G and R0060-2G.

Fig 3–Maximum likelihood phylogenetic tree of the *Bartonella* species. The 930bp pieces of the citrate synthase gene (*glt*A) were analyzed using the maximum likelihood model in PAUP.

for *S. murinus*. There was no statistically significant difference in *Bartonella* infection among animal species (χ^2 = 3.9, df =2, p=0.139). However, the proportion of animals with fleas were significantly different among the species (χ^2 = 35.7, df = p < 0.001). *R. tanezumi* was significantly more commonly infested with fleas than *S. murinus* [40.5% (32/79) *vs* 3.5% (3/85)] (χ^2 = 33.4, df=1, p< 0.001). In *R. norvegicus*, the overall flea infestation was 16.3% (8/49).

The identity of bartonellae detected in this study was determined by phylogenetic analysis of citrate synthase gene (*glt*A) sequences. Neighbor-joining analysis (Fig 2) using the small portion of *glt*A placed our samples with those that had been previously described, with the exception of samples RR0067 and RR0040, which appear to comprise a group that has not yet been reported. A maximum likelihood analysis (Fig 3) using a larger portion of the *glt*A allows more precise placement of the sequences, although

RR0002-1G, RR0002-1E, RR0038-2G, RR0040-2G, and RR0042-1E are not showed on the tree because RR0042-1E was identical to RR0042-1G and the rest were identical to RR0039-3G, RR0048-2G and R0060-2G.

Fig 2–Neighbor joining tree (using Kimura 2-parameter model) of the *Bartonella* species. The phylogenetic tree was created by analyzing the 370bp sequences of the citrate synthase gene (*glt*A) in MEGA.

Table 2
Number of blood smears found positive for *Bartonella* spp.

No.	Study site	Host species	Type of sample	Sample ID	GenBank Accession No.
1	Pamulang	*Rattus tanezumi*	blood blot	RR0002-1G	AY902191
			Xenopsylla cheopis male	RR0002-1E	AY902188
2	Pamulang	*Rattus tanezumi*	Spleen	RR0042-1G	AY902184
			Xenopsylla cheopis male	RR0042-1E	AY902179
3	Penjaringan (Harbor)	*Rattus tanezumi*	Spleen	RR0037-2G	AY902187
4	Penjaringan (Harbor)	*Rattus norvegicus*	blood blot	RR0038-2G	AY902190
5	Penjaringan (Harbor)	*Rattus tanezumi*	blood blot	RR0040-2G	AY902189
6	Penjaringan (Harbor)	*Rattus tanezumi*	Spleen	RR0048-2G	AY902183
7	Penjaringan (Harbor)	*Rattus tanezumi*	Spleen	RR0051-2G	AY902182
8	Penjaringan (Harbor)	*Rattus tanezumi*	Spleen	RR0060-2G	AY902181
			Xenopsylla cheopis female	RR0060-2E*	*(PCR Negative)*
9	Penjaringan (Harbor)	*Suncus murinus*	Spleen	RR0067-2G	AY902180
10	Ragunan Zoo	*Rattus tanezumi*	Spleen	RR0039-3G	AY902186
11	Ragunan Zoo	*Suncus murinus*	Spleen	RR0040-3G	AY902185
12	Ragunan Zoo	*Suncus murinus*	Not available	RR0049-3G	-
13	Ragunan Zoo	*Suncus murinus*	Not available	RR0051-3G	-

fewer informative taxa are available from GenBank for comparison. These unique RR0067/RR0040 sequences are most closely related to *Bartonella rattimassiliensis* and RR0051/RR0037 to *Bartonella phoceensis* isolated from *Rattus* spp in Marseilles, France (Gundi *et al,* 2004).

All fleas collected on *Bartonella*-positive animals were identified as *Xenopsylla cheopis* (Table 2). PCR and sequencing of the DNA samples from the flea pools revealed the *Bartonella* sequence obtained from a flea collected on a *R. tanezumi* (RR0002-1G) was identical to that from the blood sample of its rodent host (RR0002-1E). A similar situation was found for the sequence of flea sample RR0042-1G and

that of the DNA from the spleen of its *R. tanezumi* host (RR0042-1E). Because it would be difficult to attribute the evidence of infection in this flea to a prior bloodmeal, as opposed to simply having ingested the blood from a bacteremic host, we cannot conclude whether *X. cheopis* is a vector of this agent.

DISCUSSION

Bartonella in rodents on Java Island was first described over 70 years ago. In 1929, Soewandi reported that 19% of rats trapped in Jakarta and a district just west of Jakarta were infected with *Bartonella muris* (Soewandi, 1929). *Bartonella* transmission by *Xenopsylla cheopsis*

and *Haemotopinus spinulosus* (rat louse) was described in 1930 by Timmerman in his study in Bandung, where he found 10.7% of the rats sampled to be microscopically positive for *Bartonella* (Timmerman, 1930). The genus *Bartonella* is known for its genetic diversity, extent of animal hosts and worldwide distribution. In Asia, isolation of *Bartonella* spp from small mammals has been reported in southern China and northern Thailand (Ying *et al,* 2002; Castle *et al,* 2004). Prevalence varies from less than 10% to over 60% depending on diagnostic methods, location, environmental conditions, presence of vectors, animal species and their habitats (Kosoy *et al*, 1997; Engbaek and Lawson, 2004; Pretorius *et al*, 2004). In this study of urban and suburban rodents and shrews, we detected *Bartonella* in 6% of blood smears by microscopy. This is comparable to that of a rodent survey in northern Thailand, which reported 8.7% prevalence by culture (Castle *et al*, 2004). Cultivation of blood samples is significantly more efficient at detecting infection than microscopy (Tyzzer, 1941). It is likely that our prevalence estimate would be much greater had we cultured or performed PCR on all the blood samples that were collected.

As previous analyses have shown, there appears to be little geographic or host consistency with phylogenetic placement; our sequences group with those reported from Peru, USA, France and China. The significance of such sequence differences between those from Marseilles as described by Gundi *et al*, (2004) and those from our study (RR0067/RR0040 and RR0051/RR0037) remains speculative without sequencing other genes or obtaining phenotypic data. Other sequences that we detected cluster with a Peruvian entity and with *B. elizabethae*. It is likely that the wide distribution of related *Bartonella* spp detected within *Rattus rattus* or closely related rats reflects the human transport of rats and their fleas by ship for many centuries. Although there is much speculation about the vectors for bartonellae that infect rodents, the few published reports suggest an intricate relationship with fleas (Telford and Goethert, 2005). The detection of bartonellae common to both *Suncus* spp and *Rattus* spp would thus be attributable to shared infestation through common generalist fleas, such as *X. cheopis,* by these small mammals, which are the most common human housing commensals throughout Asia.

Our study demonstrates that *Bartonella* infection is endemic in rodents and shrews in the Greater Jakarta area. A previous study by culturing blood of cats from Jakarta demonstrated the presence of *B. henselae* and *B. clarridgeiae,* which are known to be pathogenic in humans (Marston *et al*, 1999). We did not detect *B. henselae* or *B. clarridgeiae*, perhaps because of our limited sample size or because they are maintained by fleas that do not commonly infest the small mammals we trapped. The citrate synthase gene (*glt*A) sequence of one sample from our study shows a close similarity with *B. elizabethae*, previously described in a rodent reservoir from Peru (Birtles, 1999) and known to be associated with endocarditis (Daly *et al*, 1993) and neuroretinitis in humans (O'Halloran *et al*, 1998). *B. elizabethae*-like strains have been detected in *Rattus norvegicus* and *Rattus rattus* from geographically diverse areas, including Portugal (Birtles and Raoult, 1996), USA (Ellis *et al*, 1999), southern China (Ying, 2002) and Thailand (Castle *et al*, 2004).

Both the previous cat study and this one suggest the possibility that Jakarta residents may be exposed to *Bartonella* infection. Serosurveys of the inhabitants and domestic cats will be useful for assessing these risks because cats are common pets in Indonesia. Because of diverse geographic and climatic regions within the Indonesian archipelago, animal surveys in other parts of the country would be useful to better understand the epidemiology and genetic characterization of bartonellae and to determine whether a public health burden currently exists or may emerge.

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