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Small Indian mongooses and masked palm civets serve as new reservoirs of *Bartonella henselae* and potential sources of infection for humans

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Abstract

The prevalence and genetic properties of *Bartonella* species were investigated in small Indian mongooses and masked palm civets in Japan. *Bartonella henselae*, the causative agent of cat-scratch disease (CSD) was isolated from 15.9% (10/63) of the mongooses and 2.0% (1/50) of the masked palm civets, respectively. The bacteraemic level ranged from 3.0×10^1 to 8.9×10^3 CFU/mL in mongooses and was 7.0×10^3 CFU/mL in the masked palm civet. Multispacer typing (MST) analysis based on nine intergenic spacers resulted in the detection of five MST genotypes (MSTs 8, 14, 37, 58 and 59) for the isolates, which grouped in lineage 1 with MST genotypes of isolates from all CSD patients and most of the cats in Japan. It was also found that MST14 from the mongoose strains was the predominant genotype of cat and human strains. This is the first report on the isolation of *B. henselae* from small Indian mongooses and masked palm civets. The data obtained in the present study suggest that these animals serve as new reservoirs for *B. henselae*, and may play a role as potential sources of human infection.

Keywords: *Bartonella henselae*, cat-scratch disease, masked palm civet, mongoose, multispacer typing
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Introduction

Bartonella bacteria are small, fastidious, gram-negative, vector-transmitted pathogens. Since the early 1990s, more than 20 species including three subspecies of *Bartonella* have been identified and at least 13 species are known to be zoonotic agents [1,2]. Cat-scratch disease (CSD) is one of the most common zoonoses caused by *Bartonella henselae* and the cat (*Felis catus*) is recognized as the main reservoir for *B. henselae*. The prevalence of the organism in cats varies from 0% in Norway to 68% in the Philippines, and varies according to the housing status of cats (pet or stray) and the geographical location [3]. Except for domestic cats, *B. henselae* was isolated from wild African lions and cheetahs [4]. Antibody to *B. henselae* was also detected in free-ranging and captive wild felids such as bobcats, leopards, jaguars, pumas and tigers [5,6]. These data suggest that wild Felidae are reservoir hosts of *B. henselae* in nature.

Both the small Indian mongoose (*Herpestes auropunctatus*) and the masked palm civet (*Paguma larvata*) belong to the suborder Feliformia of the order Carnivora along with the felids. Since small Indian mongooses were introduced in 1910 from Bangladesh to Okinawa Prefecture, Japan, they have readily adapted to the new environment and have been having serious effects on the unique ecosystem and causing extensive damage to agricultural crops and the poultry industry in the

area [7]. Masked palm civets are widely distributed from Northern India to Southeast Asia and China, and the introduced individuals have also expanded their habitat and caused serious damage to agricultural products and intrusion into human dwellings in Japan [8].

Hence, the increased populations of small Indian mongooses and masked palm civets have resulted in many opportunities for these species to appear in the peridomestic environment and come into contact with either residents or animal control workers. Although these animals present serious risks as sources of zoonoses such as leptospirosis, rabies, severe acute respiratory syndrome, salmonellosis, yersiniosis and campylobacteriosis [9–12], no epidemiological studies on *Bartonella* infection in mongooses and masked palm civets have been conducted.

Several genotyping methods have been developed and applied for the characterization of *Bartonella* isolates. It is reported that multispacer typing (MST) using nine variable intergenic spacers is the most discriminatory genotyping method for *B. henselae* isolates and is used to investigate the relationships between human and cat isolates [13,14].

The aim of the present study was to investigate the prevalence of *Bartonella* species in small Indian mongooses and masked palm civets in Japan. Furthermore, we evaluated the possibility that these animals serve as a source of CSD for humans by MST of the isolates.

Material and Methods

Sample collection

During the period from 2009 to 2012, blood samples were collected from 63 small Indian mongooses in Okinawa Prefecture and 50 masked palm civets in Chiba ($n = 26$) and Kanagawa ($n = 24$) Prefectures, Japan. Blood samples from the mongooses and masked palm civets were collected by cardiopuncture after euthanasia following the guidelines for invasive alien species prepared by the Japanese Veterinary Medical Association, and then transferred into EDTA-containing collection tubes. Blood samples from the mongooses were immediately stored at − 70°C, whereas those from the masked palm civets were stored at − 20°C for 2–12 months after collection. The samples were sent to the Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University for examination of *Bartonella*.

Isolation and identification of *Bartonella* bacteria

Frozen blood samples were thawed at room temperature and submitted for the isolation of *Bartonella* species following previously reported procedures [15]. Bacterial colonies were tentatively identified as *Bartonella* species based on colony morphology and the long culture period (>1 week), and subsequently the CFU/mL of blood were calculated by additional quantitative culture. For further characterization, five colonies were picked from each sample and subcultured on fresh blood agar plates using the same conditions as the primary culture.

The genomic DNA of each isolate was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA, USA). Identification of *Bartonella* was performed using *Bartonella*-specific PCR for six housekeeping genes including the 16S ribosomal RNA gene (16S rRNA), the cell division protein gene (*ftsZ*), the citrate synthase gene (*gltA*), the heat-shock protein gene (*groEL*), the riboflavin synthase alpha chain gene (*ribC*) and the RNA polymerase beta subunit-encoding gene (*rpoB*). The primers and PCR conditions used for the PCR amplification of 16S rRNA [16], *ftsZ* [17], *gltA* [18], *groEL* [19], *ribC* [20] and *rpoB* [18] have been previously published.

For DNA sequencing of 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*, the PCR products were purified using a Spin Column PCR product purification kit (Bio Basic Inc., Markham, Ontario, Canada), and then sequenced directly by using dye terminator chemistry and a Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer's instructions. The sequence alignments were assembled and edited using the AUTOASSEMBLER program in GENETYX-WIN software, version 9 (Genetyx Corp., Tokyo, Japan), and compared with those of other known *Bartonella* species deposited in the GenBank/EMBL/DDBJ database by using the BLAST program.

Multispacer typing and phylogenetic tree based on nine intergenic spacers

Internal fragments of approximately 300–500 bp of nine intergenic spacers (S1–S9) were amplified by PCR as described previously [13]. Positive and negative controls were prepared using DNA from *B. henselae* Houston-1^T and nuclease-free distilled water, respectively. The PCR products of S1–S9 were purified and sequenced directly. Vector sequencing was applied only when obtaining extra bands for S1. The band with the expected size was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), subcloned using the plasmid pGEM-T Easy vector system (Promega), and sequenced using the same protocol as described for direct sequencing [15]. MST genotypes were determined for ten strains from the mongooses and one strain from the masked palm civet. Ten strains from cats were also subjected to MST analysis. Out of ten cat strains, seven are derived from Okinawa Prefecture where the mongooses were

captured. The other three cat strains are derived from near the areas of Chiba, Kanagawa and Tokyo Prefectures where the masked palm civet was captured. The MST genotype of each strain was defined by the combination of the S1–S9 genotypes. The genotypes of the intergenic spacers and MSTs were assigned numbers according to the previous reports [13,21,22]. When new combinations of intergenic spacers were found for the first time, the genotypes were assigned as novel MST genotypes in the order of detection.

Multiple alignment of the spacer sequences was carried out using the CLUSTALW program. A phylogenetic tree of the concatenated sequences of the nine spacers (S1–S9) was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA4 [23]. Fifty-seven MST genotypes from cat and human strains described in previous reports [13,21,22] were also included in the phylogenetic analysis.

Results

Prevalence and bacteraemic levels of *Bartonella* in small Indian mongooses and masked palm civets

The prevalences of *Bartonella* were 15.9% (10/63) in the small Indian mongooses and 2.0% (1/50) in the masked palm civets. Five isolates from each bacteraemic animal were applied to the genetic characterization of *gltA* because a large number of *Bartonella*-suspected colonies were found in the primary isolation from the animals. Finally, a total of 55 *Bartonella* isolates were obtained from ten mongooses and one masked palm civet. Since all of the isolates were identical in the nucleotide sequence of *gltA*, a representative isolate randomly selected from each bacteraemic animal was used for further genetic characterization. Nucleotide sequence identities of the 11 representative isolates to those of *B. henselae* Houston-1^T were 100% for the 16S rRNA gene, 99.9–100% for *ftsZ*, 100% for *gltA*, 100% for *groEL*, 100% for *ribC*, and 99.6–99.8% for *rpoB*, respectively. Therefore, all of the isolates were identified as *B. henselae*.

Quantitative cultivation indicated that the bacteraemic level varied from 3.0×10^1 to 8.9×10^3 CFU/mL in seven of ten mongooses and was 7.0×10^3 CFU/mL in the masked palm civet (Table 1).

Genotyping and phylogenetic analysis based on MST

The 21 strains (from ten mongooses, one masked palm civet and ten cats) formed eight MST genotypes (MSTs 8, 14, 33, 35, 37, 38, 58 and 59). The strains from the mongooses and the masked palm civet were classified into MSTs 8, 14, 37 and 58 and MST 59, respectively (Table 2). The cat strains from Okinawa, from Chiba, and from Kanagawa and Tokyo Prefectures were classified into MSTs 35, 38 and 58, MST 33, and MST 35, respectively. MST 58, from two mongooses and two cat strains, derived from Okinawa Prefecture and MST 59, from one masked palm civet strain, were novel genotypes. Two distinct S1 bands (nos. 5 and 8) were detected from the four strains of MST 58. All of the MST data obtained in this study were deposited in the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

All of the MST genotypes (MSTs 8, 14, 37, 58 and 59) from the small Indian mongoose and the masked palm civet strains belonged to lineage 1 with the MST genotypes of cat strains from Japan, the Philippines and Thailand, and of CSD patient strains in Japan. Similarly, the MST genotypes (MSTs 33, 35, 38

TABLE 1. Sequence similarities of the genes from the small Indian mongoose and the masked palm civet isolates to those of *Bartonella henselae* Houston-1^T and the bacteraemic levels in the host animals

Animal ID	Strain name	Sequence similarities (%) to *B. henselae* Houston-1^T in 16S rRNA (1348 bp)[a]	*ftsZ* (788 bp)[a]	*gltA* (312 bp)[a]	*groEL* (1185 bp)[a]	*ribC* (621 bp)[a]	*rpoB* (825 bp)[a]	Bacteraemic level (CFU/mL)[b]
Mongoose								
53	HJ53	100	99.9	100	100	100	99.6	NC[c]
54	HJ54	100	100	100	100	100	99.8	NC[c]
58	HJ58	100	100	100	100	100	99.8	NC[c]
90	HJ90	100	100	100	100	100	99.8	8.0×10^1
91	HJ91	100	99.9	100	100	100	99.6	3.0×10^1
106	HJ106	100	100	100	100	100	99.8	3.0×10^2
107	HJ107	100	99.9	100	100	100	99.6	5.9×10^3
108	HJ108	100	100	100	100	100	99.8	8.9×10^3
109	HJ109	100	99.9	100	100	100	99.6	5.0×10^2
111	HJ111	100	99.9	100	100	100	99.6	1.2×10^3
Civet								
18	PL18	100	100	100	100	100	99.8	7.0×10^3

[a]Length of the sequenced portion of the gene.
[b]Colony forming units/mL of blood.
[c]NC, not countable due to the lack of blood.

TABLE 2. Multispacer typing (MST) genotyping of 21 *Bartonella henselae* isolates from ten small Indian mongooses, one masked palm civet and ten cats

Strain name	Animal source	Prefecture	Genotypes									
			S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
HJ53	Mongoose	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
HJ54	Mongoose	Okinawa	8	2	5	4	1	2	1	1	3	8
HJ58	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ90	Mongoose	Okinawa	4	2	5	4	1	2	1	1	3	14
HJ91	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ106	Mongoose	Okinawa	4	2	5	4	1	2	1	1	3	14
HJ107	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ108	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ109	Mongoose	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
HJ111	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
Oki.cat17	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
Oki.cat26	Cat	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
Oki.cat38	Cat	Okinawa	5	2	6	5	2	2	2	2	1	38
Oki.cat41	Cat	Okinawa	5	2	6	5	2	2	2	2	1	38
Oki.cat48	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
Oki.cat49	Cat	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
Oki.cat50	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
PL18	Civet	Chiba	7	2	5	4	1	2	2	3	3	59
Chi.cat11	Cat	Chiba	4	2	5	4	1	2	2	1	3	33
Kan.cat37	Cat	Kanagawa	5	2	6	5	2	2	2	1	1	35
Tok.cat1	Cat	Tokyo	5	2	6	5	2	2	2	1	1	35

[a]S1 genotype 5+8 indicates that the strain had two different copies of intergenic spacer S1 in its genome.

and 58) of cat strains from four Prefectures were also classified in lineage 1 with the strains from the mongooses and the masked palm civet (Fig. 1).

Discussion

The present study demonstrates for the first time that small Indian mongooses and masked palm civets harbour *B. henselae* in their blood. The prevalence of *B. henselae* was 15.9% (10/63) in the mongooses and 2.0% (1/50) in the masked palm civets. The prevalence of *B. henselae* in masked palm civets was lower than in mongooses. Blood samples from the masked palm civets used in this study were stored at − 20°C, whereas those from the mongooses were stored at − 70°C. It has been suggested that *Bartonella* viability may decrease over time as the result of inadequate conservation of blood samples, and may result in the underestimation of *Bartonella* prevalence based on culturing [4]. Therefore, fresh blood samples or those stored at − 70°C should be used for the cultivation of *Bartonella*.

The levels of bacteraemia in cats experimentally infected with *B. henselae* have ranged from 1×10^1 to 1.7×10^5 [24] or 1.2×10^5 CFU/mL of the blood [25]. In the present study, mongoose and masked palm civet also showed relatively high bacteraemic levels of *B. henselae*: 8.9×10^3 CFU/mL and 7.0×10^3 CFU/mL, respectively. Furthermore, no clinical or pathological abnormalities due to the agent were observed in any of the infected animals, as in bacteraemic cats. These results suggest that the suborder Feliformia composed of mongooses and masked palm civets along with felids serves as a reservoir of *B. henselae*.

Previous studies of the MST genotype of *B. henselae* have shown that all the strains derived from patients with CSD in Japan were categorized in lineage 1 [22]. In the present study, all of the strains from the small Indian mongooses (MSTs 8, 14, 37 and 58) and the masked palm civet (MST 59) also grouped in lineage 1. Yanagihara *et al.* [22] have reported that MSTs 14 and 35 were the predominant genotypes of cat and human strains in Japan. These findings suggest that some of the mongoose strains have similar potential to infect humans as cat strains.

Two mongoose strains (HJ53 and HJ109) and two cat strains (Oki.cat26 and Oki.cat49) from Okinawa Prefecture showed the same genotype and were designated as a novel genotype, MST 58. Furthermore, the prevalence of the bacteria in mongooses was similar to that in cats (18%; 9/50) in Okinawa Prefecture [26]. The main vector of *B. henselae* among cats has been confirmed to be cat fleas (*Ctenocephalides felis*) [27] and a previous study showed that 9.3% (224/2,406) of mongooses in Okinawa Prefecture were infested with cat fleas that have low host specificity [28]. These findings suggest that the *B. henselae* strain with MST 58 may be transmitted between mongooses and cats in the area by direct contact of both animals or by some arthropod vectors such as cat fleas.

Interestingly, MSTs 8 and 37 from mongooses were also reported in cat strains from the USA and the Philippines, respectively [13]; however, strains with those genotypes have not been identified from cats or humans in Japan. It is unclear whether the mongooses were indigenously infected with MSTs

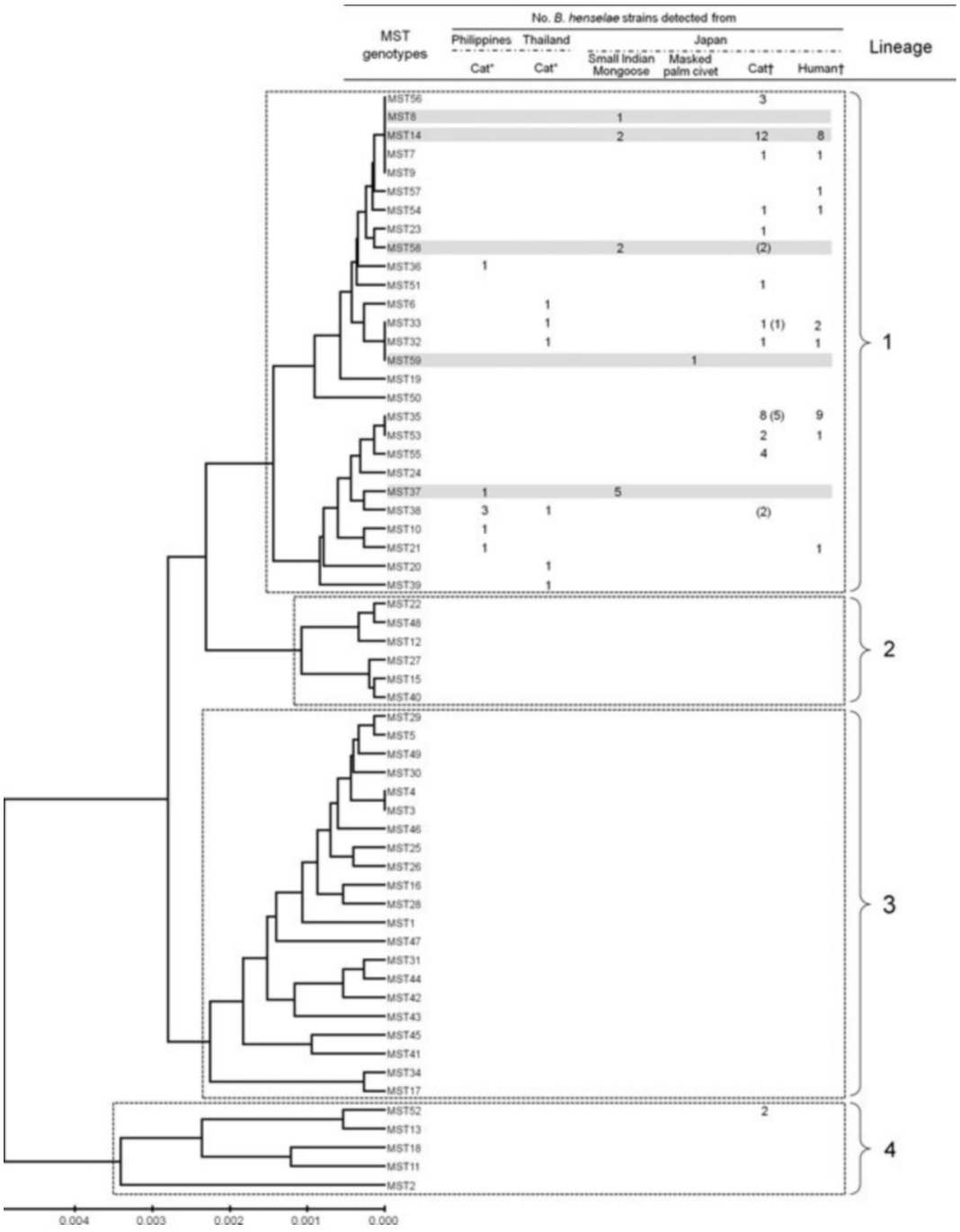

FIG. 1. Phylogenetic tree of *Bartonella henselae* strains from mongooses, masked palm civet, cats and patients with cat-scratch disease (CSD) in Japan based on nine concatenated intergenic spacer sequences. The tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA4 software. The *B. henselae* strains isolated from mongooses, a masked palm civet, cats and humans with 57 MST genotypes were included in the analysis. Hatching highlights multispacer typing (MST) genotypes and the numbers of strains from the mongooses, a masked palm civet, cats and humans. The cat strains from the Philippines (*) and Thailand (*) and the cat and human strains from Japan (†) were analysed in the previous reports [13,21,22] and added to this figure. The number of cat strains examined in the present study is shown in parentheses. Dotted rectangles show four lineages of MST genotypes. The scale bar indicates nucleotide substitutions per site.

8 and 37 in the area, so further epidemiological investigations on native mongooses in other Asian countries will allow us to understand the origin of those genotypes of *B. henselae* among Feliformia.

MST 59, detected from the masked palm civet strain, showed a unique genotype. Though the prevalence of cats in Chiba Prefecture where the masked palm civet was captured was 5.0% (1/20, data not shown in the result), the same genotype has not been found in any *B. henselae* strains of the cats from the same area and other prefectures. As only one masked palm civet harbouring the MST 59 genotype was detected in the present study, more samples should be examined to determine whether the genotype is prevalent in animals and humans.

Our investigation showed for the first time that small Indian mongooses and a masked palm civet harboured *B. henselae* and the isolates were grouped into lineage 1 of MST genotypes with strains derived from cats and from patients with CSD in Japan. Programmes to eradicate introduced mongooses are being carried out in Japan and other countries [29]. Masked palm civets have been sold for human consumption at wild live markets in China [11] and other Asian countries. Interestingly, a CSD case caused by a masked palm civet was reported in 2001 in Japan. In this case, the patient, who was scratched in the left leg by a pet masked palm civet, developed fever and left inguinal lymphadenopathy with high antibody titre (1 : 1024) to *B. hensleae* [30]. Taking into account the similar prevalence to cats in the examined areas, the high bacteraemic levels with no clinical and pathological abnormalities, similar MST genotypes to the cat and human strains of *B. henselae*, and the close contact between humans and these animals, the small Indian mongoose and the masked palm civet in the suborder Feliformia appear to serve as new reservoirs for *B. henselae*, and may play a role as potential sources of human infection.

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Transparency Declaration

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