

## ***Bartonella* infection in urban and rural dogs from the tropics: Brazil, Colombia, Sri Lanka and Vietnam**

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### **SUMMARY**

Dogs can be infected by a wide range of *Bartonella* spp., but limited studies have been conducted in tropical urban and rural dog populations. We aimed to determine *Bartonella* antibody prevalence in 455 domestic dogs from four tropical countries and detect *Bartonella* DNA in a subset of these dogs. *Bartonella* antibodies were detected in 38 (8·3%) dogs, including 26 (10·1%) from Colombia, nine (7·6%) from Brazil, three (5·1%) from Sri Lanka and none from Vietnam. DNA extraction was performed for 26 (63%) of the 41 seropositive and 10 seronegative dogs. Four seropositive dogs were PCR positive, including two Colombian dogs, infected with *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii*, and two Sri Lankan dogs harbouring sequences identical to strain HMD described in dogs from Italy and Greece. This is the first detection of *Bartonella* infection in dogs from Colombia and Sri Lanka and identification of *Bartonella* strain HMD from Asia.

**Key words:** *Bartonella*, Brazil, Colombia, Sri Lanka, stray dogs, Vietnam.

### **INTRODUCTION**

*Bartonella* spp. are vector-borne, haemotropic, Gram-negative, rod-shaped bacteria in the family

Bartonellaceae which can cause long-lasting intra-erythrocytic bacteraemia [1]. *Bartonella* invade erythrocytes, endothelial cells, CD34+ progenitor cells, and dendritic cells of their hosts and subsequently induce persistent infection [1, 2]. *Bartonella* bacteria have been isolated or detected increasingly for the last two decades [3, 4] in a wide range of mammals, including humans, and in various arthropods [5, 6].

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At least 30 *Bartonella* spp. and subspecies have been identified [7] which are mainly transmitted by haematophagous arthropods such as fleas, ticks, lice and possibly mites and flies [3]. Domestic animals and wildlife represent major reservoirs of *Bartonella* spp., especially in relation to human infection [8]. The distribution of *Bartonella* spp. varies between countries and their domestic and wildlife reservoir species [3]. *Bartonella* infections have been reported more frequently in humans [9] and domestic animals in warm and humid climates [10, 11] and tropical countries [6, 12–15].

Domestic dogs can be infected with several *Bartonella* spp. or subspecies, including *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. vinsonii* subsp. *arupensis*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, *B. quintana*, *B. koehlerae*, *B. bovis*, *B. rochalimae*, and several new candidate species, such as *B. volans*-like and a strain similar to *B. bovis* called HMD [14, 16, 17]. In infected dogs, the clinical signs are similar to those seen in human patients [7, 18]. Therefore dogs represent excellent epidemiological sentinels for human exposure yet their role as reservoirs requires further investigation [19].

Several studies have shown a high prevalence of antibodies in stray dogs but very low rates in pet dogs in various parts of the world, including the USA [20–22], Europe [21, 23, 24] and North Africa [25]. Seropositivity was shown to be associated with outdoor activities, heavy tick or flea infestation, and rural environment [22].

The aims of this study were to determine the seroprevalence of antibodies against *B. clarridgeiae*, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* in stray dogs living in four tropical countries and to detect and identify by molecular techniques the presence of *Bartonella* DNA in the subset of seropositive and seronegative dogs.

## MATERIALS AND METHODS

### Dog samples

Whole blood (1–2 ml) or serum samples (0.5–1 ml) used for this study were initially collected from a convenient sample of stray dogs from four tropical countries, two in the Americas (Colombia, Brazil), and two from Asia (Sri Lanka, Vietnam), for studies investigating *Toxoplasma gondii* infection in domestic dogs [26–29].

Dogs of different breeds and age groups from Colombia (Bogota), Brazil (São Paulo) and Sri Lanka

(Peradeniya) were caught by the respective municipalities and euthanized by intravenous injection of sodium thiopental [26, 27, 29]. For the dogs from Vietnam (provinces in southern Vietnam, near Ho Chi Min City: Bin Phuoc (10 dogs), Dong Nai (five dogs) and Long An (five dogs), each dog (aged 1–4 years) was from a different home and the houses were at least 2 km apart. These dogs, not considered as pets but raised to be sold for human consumption, were purchased from individual homes and euthanized by a veterinarian with an overdose of sodium thiopental (30–40 mg/kg i.v.) [28].

### Serology

Antibodies against *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and *B. henselae* in stray domestic dog samples were detected using an indirect immunofluorescent antibody assay (IFA). These three antigens were selected, as *B. henselae* and *B. vinsonii* subsp. *berkhoffii* have been frequently detected in dogs as well as *B. clarridgeiae*, which is also a good substitute for detection of *B. rochalimae* [30, 31]. The IFA procedure was similar to a procedure previously described [19], with the following modifications. A 90% confluent tissue culture flask (containing MDCK cells) was inoculated with a 4-day-old culture of *B. vinsonii* subsp. *berkhoffii* (ATCC 51672) resuspended in 0.5 ml saline. Similarly, flasks containing Vero tissue cultures were inoculated with either *B. clarridgeiae* ATCC 51734 or a mixture of *B. henselae* ATCC 49882 and *B. henselae* U4 (University of California, Davis, strain). Serum samples added to the test wells were screened at 1:64 dilution in PBS with 5% milk. Slides were incubated at 37 °C for 30 min, followed by three washes in PBS. Fluorescein-conjugated goat anti-dog immunoglobulin G (IgG; ICN Biomedicals Inc., USA) was diluted in PBS (1:1400 for *B. vinsonii* subsp. *berkhoffii*, 1:3600 for *B. clarridgeiae*, and 1:2800 for *B. henselae*) with 5% milk containing 0.001% Evans Blue, and 20 µl of the dilution was applied to each well. The slides were incubated at 37 °C for 30 min and again washed in PBS three times. The intensity of bacillus-specific fluorescence was scored subjectively from 1 to 4. Samples with a fluorescence score of  $\geq 2$  at a dilution of 1:64 were reported as positive and final titration was performed (last dilution with a score  $\geq 2$ ). The same two readers performed a double-blind reading of each slide. Negative and positive control samples were included on each slide.

### ***Bartonella* PCR procedures**

To allow for specific identification of *Bartonella* isolates, DNA was extracted from the blood of seropositive dogs (dogs for which only serum was available were excluded) and a sampling of seronegative dogs (three dogs from Sri Lanka, three dogs from Brazil, four dogs from Colombia) and analysed by PCR, gel electrophoresis, and DNA sequencing of the 16S–23S intergenic spacer (ITS) region [32], the citrate synthase *glta* [33] gene and the *rpoB* gene [34]. The DNA was extracted as described previously [35] using a Qiagen DNeasy blood and tissue kit (Qiagen Sciences, USA). The primers used for the ITS region were 325s 5'-CTTCAGATGATGATCCCAAGC-CTTYTGGCG-3' and 1100as 5'-GAACCGACGACCCCCT GCTTGCAAAGCA-3' [36]. The run conditions were 95 °C for 2 min and then 54 cycles of 94 °C for 15 s, 66 °C for 15 s, and 72 °C for 18 s, followed by a final step at 72 °C for 1 min. The primers used for the *glta* gene were Bhcs.781p 5'-GGGG-ACCAGCTCATGGTGG-3' and Bhcs.1137n 5'-AATGCAAAAAGAACAGTAAACA-3' [33]. Run conditions were 95 °C for 10 min and then 40 cycles of 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 2 min, followed by a final step at 72 °C for 5 min. The primers used for the *rpoB* gene were primers 1615s 5'-ATYACYCATAARCGYCGTCTTTCTGCTCT-TGG-3' [36] and 2300R 5'-GGATCTAAATCTT-CYGTYGCACGRATACG-3' [34]. Following a first denaturation step of 94 °C for 10 min, a three-step cycle of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min was repeated 45 times. The PCR programme was ended by a single 5-min extension step at 72 °C [34].

An approximately 400-bp fragment of the *glta* gene, 670-bp fragment of the ITS region and 700-bp fragment of the *rpoB* gene were amplified and then verified by gel electrophoresis. Amplified PCR products were stained with GelRed™ (Phenix Research Products, USA) and visualized by ultraviolet light after electrophoresis on 2% agarose gels (SeaKem LE agarose; Cambrex Bio Science Rockland Inc., USA).

### ***Bartonella* DNA sequencing and alignment**

PCR products from the *glta* and *rpoB* genes and ITS region were used for DNA sequencing. Products were purified with the QIAquick PCR purification kit (Qiagen Sciences, USA), and the sequencing of both DNA strands was done using a fluorescence-based automated sequencing system (Davis Sequencing, USA).

A consensus sequence for each amplification product was obtained after raw sequences were imported into MEGA 5 [37]. Blastn (<http://ncbi.nih.gov/BLAST/>) was utilized to compare sequences with entries in GenBank. MEGA 5 was then used to align sequence variants of each gene with one another and with relevant sequences available through GenBank.

### **Statistical tests**

Exact  $\chi^2$  contingency table analysis was performed to evaluate any differences in seroprevalence by geographical region and antigen response, using StatXact 8 (Cytel Corporation, USA). A *P* value <0.05 was considered statistically significant. Epi Info version 3.5.1 was used to calculate *P* values,  $\chi^2$ , and Fisher's exact test.

## **RESULTS**

A total of 455 urban stray dogs or rural owned dogs from four tropical countries on two continents (South America, Asia) were enrolled in the study, including 118 (25.9%) dogs from Brazil (São Paulo), 258 (56.7%) from Colombia (Bogota), 59 (13.0%) from Sri Lanka (Peradeniya) and 20 (4.4%) from Vietnam. Overall, 376 (82.6%) dogs were from South America and 79 (17.4%) from Asia.

### **Serology**

*Bartonella* spp. antibodies at a titre of at least 1:64 were detected by IFA in 38 (8.3%) of these 455 dogs, including 26 (10.1%) dogs from Colombia, nine (7.6%) dogs from Brazil, and three (5.1%) dogs from Sri Lanka (Table 1). None of the 20 dogs from Vietnam had detectable *Bartonella* antibodies. Overall, 30 (6.6%) dogs were seropositive for *B. henselae*, 25 (5.5%) for *B. clarridgeiae*, and 21 (4.6%) for *B. vinsonii* subsp. *berkhoffii* (Table 1). In Colombia, the antibody titres ranged from 64 to 4096 with a mode of 256 for the three antigens. In Brazil, the titres ranged from 64 to 512, with dogs seropositive for *B. clarridgeiae* having the highest titres (256–512). Dogs from Sri Lanka had titres ranging from 64 to 1024.

Many dogs were seropositive to more than one antigen. Overall, 53.8% (14/26) of the seropositive dogs from Colombia and 25% (1/4) of the seropositive dogs from Sri Lanka were positive for the three antigens. Of the seropositive dogs, a smaller

Table 1. Number of dogs seropositive (+) and by respective final titres for *Bartonella henselae*, *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* by country of origin

Antigen	+	64	128	256	512	1024	2048	4096
Colombia (n=258)								
<i>B. henselae</i>	21	6	5	6	2	1	1	
<i>B. clarridgeiae</i>	21	2	4	6	6	1	1	1
<i>B. v. berkhoffii</i>	18	4	4	5	3	2		
Brazil (n=118)								
<i>B. henselae</i>	7	6		1				
<i>B. clarridgeiae</i>	3			2	1			
<i>B. v. berkhoffii</i>	1		1					
Sri Lanka (n=59)								
<i>B. henselae</i>	2	1				1		
<i>B. clarridgeiae</i>	1			1				
<i>B. v. berkhoffii</i>	2	1				1		
Vietnam (n=20)								
<i>B. henselae</i>	0							
<i>B. clarridgeiae</i>	0							
<i>B. v. berkhoffii</i>	0							

number of dogs were seropositive for two antigens, including four dogs from Colombia and one dog from Brazil which were seropositive for *B. henselae* and *B. clarridgeiae*, and one dog each from Brazil and Colombia which were seropositive for *B. henselae* and *B. vinsonii* subsp. *berkhoffii*. Finally, one dog from Colombia was seropositive for *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii*.

### Statistical analysis

A significant association between seropositivity for all three *Bartonella* spp. and country of origin was found [ $\chi^2=8.16$ , 2 degrees of freedom (D.F.),  $P=0.017$ ]. Similarly, there was a significant association between seropositivity for *B. clarridgeiae* ( $\chi^2=9.50$ , 2 D.F.,  $P=0.009$ ), *B. vinsonii* subsp. *berkhoffii* ( $\chi^2=9.41$ , 2 D.F.,  $P=0.009$ ) and *B. henselae* ( $\chi^2=6.11$ , 2 D.F.,  $P=0.047$ ) and country of origin. Dogs from Colombia were more likely to be seropositive for *B. vinsonii* subsp. *berkhoffii* (Fisher's exact test:  $P=0.005$ ) and for *B. clarridgeiae* (Fisher's exact test:  $P=0.014$ ) than dogs from Brazil.

### Molecular testing

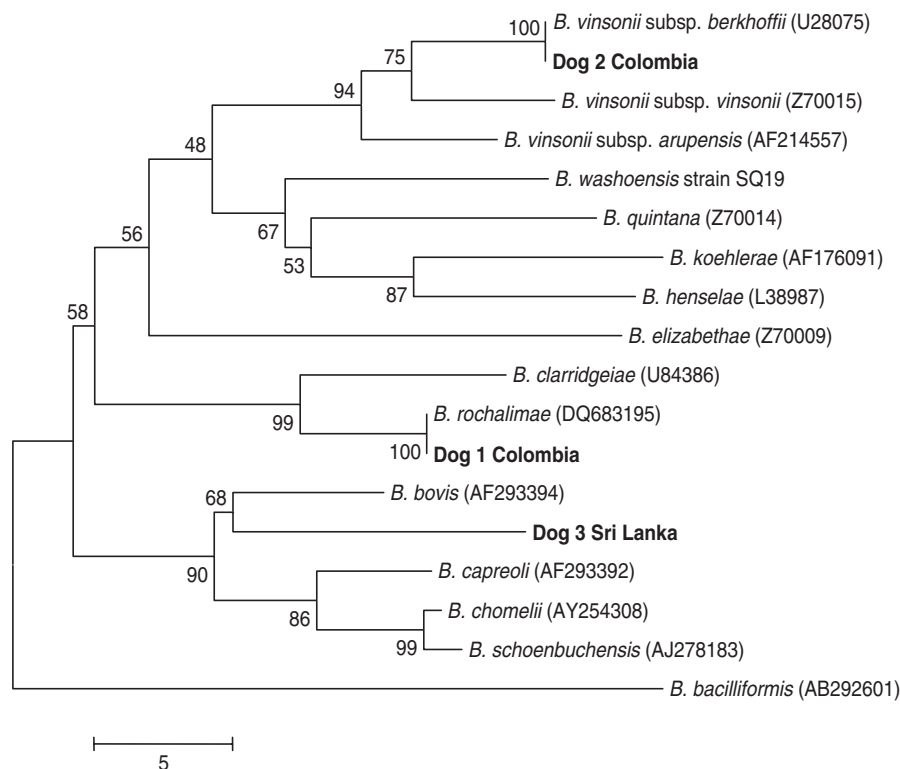
Whole blood for DNA extraction was available for 26 (63%) of the 41 seropositive dogs, including the four seropositive dogs from Sri Lanka and 22/28 seropositive dogs from Colombia. DNA was also extracted from the whole blood of 10 seronegative dogs

(Colombia: four samples, Brazil: three samples, Sri Lanka: three samples) and were all negative by PCR. Of these 26 DNA extracts, four had suspect bands on PCR gel electrophoresis. After purification and sequencing in both directions, the *Bartonella* strains could be identified as *B. rochalimae* for a dog (dog 1) from Bogota, Colombia and *B. vinsonii* subsp. *berkhoffii* for another dog (dog 2) from Bogota (Figs 1–3). The two other samples were from dogs from Sri Lanka and both were identified as identical to strain HMD, previously described in dogs from southern Italy and Greece [16]. For one of the two Sri Lanka dogs, a possible mixed *Bartonella* infection was detected, but the second sequence could not be interpreted.

The *B. rochalimae* strain (dog 1) was confirmed by PCR and sequencing on all three genes or interspacer segment (Figs 1–3). The HMD strain from the dog with mixed infection (dog 4) was confirmed by both PCR and sequencing of the ITS region and the *rpoB* gene (Figs 2 and 3). The *gltA* sequence could not be interpreted because of the mixed infection. The *B. vinsonii* subsp. *berkhoffii* (dog 2) and the other HMD strain (dog 3) were both confirmed only on the sequence of the *gltA* gene (Fig. 1).

### DISCUSSION

We report the investigation of *Bartonella* seroprevalence in stray and domestic dogs from four

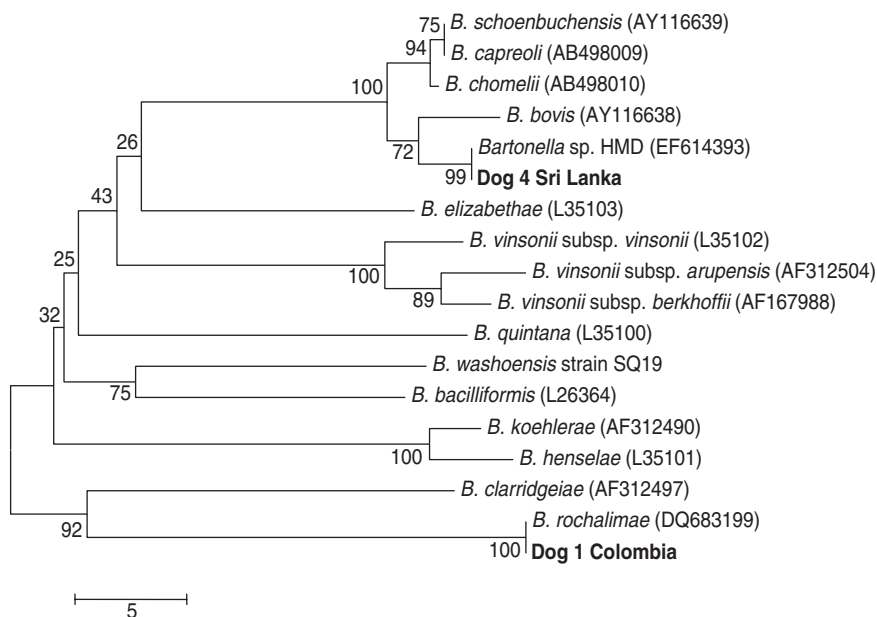


**Fig. 1.** Dendrogram of *Bartonella* strains identified by partial sequencing of the *gltA* gene. The tree shown is based on the neighbour-joining method. Bootstrap values are based on 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 272 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.

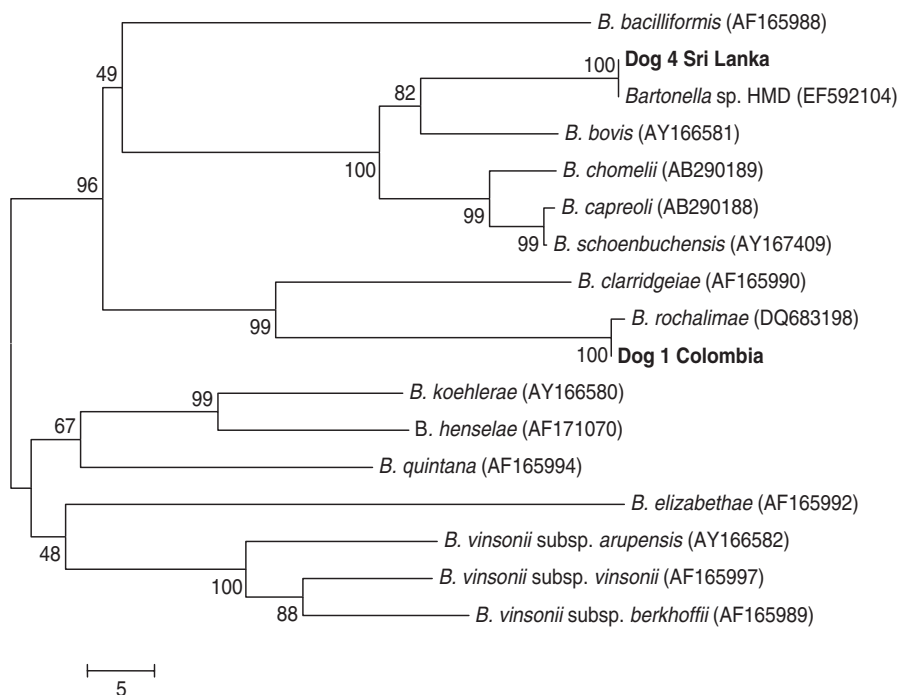
different countries within the tropics, including two countries (Colombia, Sri Lanka) for which no information on *Bartonella* infection in dogs has been previously reported. Furthermore, we were able to identify from extracted DNA belonging to a subset of seropositive dogs the *Bartonella* spp. involved in these infections. Rural dogs from Vietnam were surprisingly all seronegative, as *Bartonella* infection is common in dogs from neighbouring countries, such as Thailand, where 38% of 49 dogs presented at the veterinary teaching hospital of Kasetsart, Bangkok were seropositive for *B. vinsonii* subsp. *berkhoffii* [15] and 31.3% (60/192) of dogs were bacteraemic from Bangkok and Khon Kaen [14]. However, the limited sample size for each province (5–10 dogs) may explain these negative results. Of note, approximately 7% of the tested dogs from Sri Lanka were *Bartonella* seropositive, including two dogs which were PCR positive. Sequencing indicated that these strains were identical to a strain previously identified in shelter dogs from southern Italy and sick dogs from northern Greece [16]. This is the first report of presence of this new *Bartonella* sp., similar to *B. bovis*, from dogs from Asia.

Prevalence of *Bartonella* antibodies was low in stray dogs in São Paulo, Brazil. One dog was seropositive for *B. vinsonii* subsp. *berkhoffii* (0.8%) and seven (5.9%) were seropositive for *B. henselae*, which are values close to those previously reported for 197 sick dogs seen at the São Paulo State University Veterinary Teaching Hospital (2% for *B. vinsonii* subsp. *Berkhoffii*, 1.5% for *B. henselae*) [36]. Unfortunately, none of the nine seropositive dogs could be tested by PCR, due to a lack of whole blood.

By contrast, the highest seroprevalence for the three antigens was identified in stray dogs from Bogota, Colombia, including a much higher prevalence for *B. clarridgeiae* antibodies than for the dogs from Brazil or Sri Lanka. Of the two Colombian dogs that were PCR positive, one was infected with *B. vinsonii* subsp. *berkhoffii* and the other with *B. rochalimae*, which are *Bartonella* spp. previously isolated or detected in dogs or their fleas in the Americas, including California [19, 38], Chile [39], and Peru [40], as well as from Europe [16]. Several dogs were detected to be seropositive for *B. clarridgeiae*, which is a species closely related to *B. rochalimae* [38]. Using



**Fig. 2.** Dendrogram of *Bartonella* strains identified by partial sequencing of the 16S–23S genic interspacer. The tree shown is based on the neighbour-joining method. Bootstrap values are based on 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 160 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.



**Fig. 3.** Dendrogram of *Bartonella* strains identified by partial sequencing of the *rpoB* gene. The tree shown is based on the neighbour-joining method. Bootstrap values are based on 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 570 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.

*B. clarridgeiae* as a substitute for the detection of *B. rochalimae* has been reported previously [30, 31]; therefore, it is likely that most of these dogs could have been infected with *B. rochalimae* rather than *B. clarridgeiae*, as shown by molecular analysis.

In conclusion, we report the first detection of *Bartonella* infection in stray dogs from Colombia, South America and Sri Lanka in the Indian subcontinent as well as the first detection of HMD strain from Asia. In addition, we confirmed the low prevalence of *Bartonella* infection in stray dogs from São Paulo, Brazil and the lack of infection in rural dogs from Vietnam.

#### DECLARATION OF INTEREST

None.

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