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Bartonella Infection in Stray Dogs from Central and Southern Chile (Linares and Puerto Montt)

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Abstract

Bartonellae are emerging zoonotic vector-borne pathogens causing a broad spectrum of clinical symptoms in humans and animals, including life-threatening endocarditis. Dogs are infected with a wide range of *Bartonella* species and infection has been reported in free-roaming dogs from various South American countries. We report a high *Bartonella* seroprevalence in 82 Chilean stray dogs. More than half of the dogs from Linares (72.7%, $n=66$) and Puerto Montt (56.2%, $n=16$) were seropositive for *Bartonella henselae*, *Bartonella vinsonii* ssp. *berkhoffii*, or *Bartonella clarridgeiae* with antibody titers ranging from 1:64 to 1:512. Three dogs (3.6%) were PCR positive for *Bartonella* sp. Partial sequencing of the *glt*A gene indicated that two dogs were infected with *B. henselae*, and one with a strain close to *Bartonella vinsonii* ssp. *vinsonii*. Exposure to *Bartonella* species was common in stray Chilean dogs, as for other South American countries, likely associated with heavy ectoparasite infestation.

Keywords: *Bartonella*, serology, PCR, dogs, Chile

Introduction

Bartonella are vector-borne blood-borne pathogens that have been recognized within the last 25 years as a source of zoonoses, especially for *Bartonella henselae*, the agent of cat scratch disease (Chomel and Kasten 2010). These bacteria have been isolated from a wide range of mammalian species, including dogs (Chomel et al. 2009a). In both human and dogs, it can cause mild infection to severe and life-threatening endocarditis (Chomel et al. 2009b). In dogs, several *Bartonella* species have been identified, the most common ones being *B. henselae*, *B vinsonii* ssp. *berkhoffii*, and *B. rochalimae* (Breitschwerdt et al. 2010). Infection is usually more prevalent in stray dogs that are frequently infected by fleas and/or ticks (Chomel et al. 2009a). In South America, several studies have reported on the presence of *Bartonella* in dogs from Argentina (Mascarelli et al. 2016), Brazil (de Paiva Diniz et al. 2007, Diniz et al. 2007, Brenner et al. 2013, Malheiros et al. 2016), Colombia (Brenner et al. 2013), Peru (Diniz et al. 2013), and very recently in southern Chile (Valdivia) (Müller et al. 2018b).

Presence of *Bartonella* infection in domestic cats has been reported in Chile (Ferrés et al. 2005, Müller et al. 2017). One study revealed a seroprevalence of 85.6% (of 187 cats, including 92 from Santiago, 71 from Valdivia, and 24 from Coquimbo) and blood culture was positive for *B. henselae* in 41% of 60 cats from Santiago for which it was performed (Ferrés et al. 2005). Another study in cats from Valdivia (Southern Chile) reported 18.1% (67/370) prevalence of *Bartonella* infection (DNA detected by PCR from blood samples) (Müller et al. 2017). The first Chilean study of *Bartonella* in dogs was recently reported (Müller et al. 2018b), as 139 dog blood samples from rural localities of the Valdivia Province, Los Ríos region (southern Chile), were submitted to quantitative real-time PCR (qPCR) for *Bartonella* spp. based on *nuoG* gene, with a prevalence of 4.3% (6/139), including *B. henselae* and *B. vinsonii* ssp. *berkhoffii*.

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Bartonella infection in cat fleas has also been recently reported, with a *Bartonella* prevalence of 39.3% (59/150) (Müller et al. 2018a). Fleas collected from the same cat were tested individually ($n=92$) or pooled ($n=58$, pool of 2 to 7 fleas). From 19 sequenced *Bartonella* spp., 9 (47.3%) were identified as *Bartonella clarridgeiae*, 8 (42.1%) as *B. henselae*, and 1 (5.3%) as *B. koehlerae* (Müller et al. 2018a). In Chile, >200 human cases of cat scratch disease were detected serologically between 1997 and 2000 (Ferrés et al. 2005). The present study aimed to determine the seroprevalence and molecular prevalence of *Bartonella* in stray dogs from two cities in southern Chile (Linares and Puerto Montt).

Materials and Methods

Samples

Blood samples (2–3 mL) were collected in plastic EDTA tubes between October and December 2015 from impounded dogs kept at the municipal shelter of the city of Linares (35°51′S 71°36′W) located 303 km south from the Chilean capital, Santiago, and dogs captured on the campus of Santo Tomás University in the city of Puerto Montt (41°28′S 72°56′W), located 1055 km south from Santiago. This study was performed under the current regulations on animal welfare in Chile, after being authorized by shelter veterinarians and managers to collect blood samples. Blood samples were obtained by venous puncture of the cephalic or saphenous vein, and before needle puncture, the area was depilated and disinfected with 95% ethanol. The blood sample was divided into 2 mL within two tubes, one without additive, and the other with EDTA. From the blood sample collected on EDTA tube, 0.5 mL was transferred on filter papers (Nobuto strips) and air-dried, as previously described (Belkhiria et al. 2017). The rest of the whole blood was frozen at −20°C for storage and possible backup testing. Age of dogs was estimated according to the age of dental eruption and tooth wear of deciduous and permanent teeth (San Román, 1998).

Serology

Antibodies against *B. vinsonii* ssp. *berkhoffii*, *B. clarridgeiae*, and *B. henselae* in stray dog samples were detected using an indirect immunofluorescent antibody assay (IFA). These three antigens were selected, as *B. henselae* and *B. vinsonii* ssp. *berkhoffii* have been frequently detected in dogs, and *B. clarridgeiae*, which is also a good substitute for detection of *B. rochalimae* (Namekata et al. 2009, Schaefer et al. 2011, Brenner et al. 2013). The IFA procedure was similar to a procedure previously described (Brenner et al. 2013). A 90% confluent tissue culture flask (containing Vero cells) was, respectively, inoculated with a 4-day-old culture of *B. vinsonii* ssp. *berkhoffii* (ATCC51672), *B. clarridgeiae* (ATCC51734) or a mixture of *B. henselae* (ATCC 49882) and *B. henselae* U4 (strain; University of California, Davis) to detect both genotypes I and II, as lack of seropositivity between genotypes has been reported (Chomel et al. 2009a), resuspended in 0.5 mL saline. Serum samples were initially screened at 1:64 dilution. Fluorescein conjugated goat anti-dog immunoglobulin G (ICN Biomedicals, Inc.) was diluted in PBS (1:1400 for *B. vinsonii* ssp. *berkhoffii*, 1:3600 for *B. clarridgeiae*, and 1:2800 for *B. henselae*) with 5% skim milk containing 0.001% Evans Blue, and 20 mL of the dilution was applied to each well. The intensity of bacillus-specific fluorescence was scored subjectively from 1 to 4. Samples with a fluorescence score of ≥2 at a dilution of 1:64 were reported as positive and final titration was performed (last dilution with a score ≥2). The same two readers performed a double-blind reading of each slide. Negative and positive control samples were included on each slide.

DNA extraction and PCR

As previously reported by Belkhiria et al. (2017), each Nobuto strip was cut off with sterile scissors and placed in a tube containing 180 μL ATL buffer (Qiagen, Valencia, CA). Each vial was incubated at 90°C for 15 min; 20 μL of proteinase K solution (Qiagen) was then added and the vial was incubated at 56°C for 1 h. Extraction was continued using the manufacturer's (Qiagen) recommended procedure. Final elution volume was 100 μL in AE buffer heated to 70°C (Qiagen).

PCRs were carried out in a total volume of 50 μL, containing 0.5 μM primers, 200 μM of dNTP, 1.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), and 5 μL of template DNA or water (negative control). DNA from *B. henselae* strain U4 was used as a positive *Bartonella* control.

For the *glt*A gene, a 381 bp fragment was amplified using two oligonucleotides homologous to the *glt*A gene of *B. henselae* Houston I (BhCS781.p and BhCS1137.n) (Norman et al. 1995). All PCR amplifications were performed with a PTC-200 DNA Engine (Bio-Rad). PCR amplifications were performed with 5 μL sample in a mixture containing 50 mm KCl, 10 mm Tris-HCl, 1.5 mm $MgCl_2$, 0.001% gelatin, 0.1% Brij-35, 200 μm of each deoxynucleotide triphosphate, 0.5 μm of each primer, and 0.2 U thermostable Ampli-Taq DNA polymerase (ThermoFisher Scientific). This mixture was incubated at 95°C for 2 min and amplified for 40 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and subsequently at 72°C for 5 min.

For the PCR of the *fts*Z gene fragment, primers prAPT0257, forward GCCTTCAAGGAGTTGA TTTTGT TGTTGCCAAT and prAPT0258, reverse ACGACCCATT TCATGCATAACAGAAC were used (Veikkolainen et al. 2014). PCR run conditions were initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 1 min. Amplification was completed by 40 additional cycles and final extension at 72°C for 10 min. Amplified products were verified by gel electrophoresis on a 2% agarose gel in 0.5 × Tris-borate EDTA buffer followed by staining with ethidium bromide and visualization by a UV transilluminator.

Sequencing and phylogenetic analysis

Amplified PCR products were cleaned using a Qiagen QIAquick PCR Purification Kit according to the manufacturer's instructions. Purified PCR products (suspect bands) were sequenced at the College of Biological Sciences DNA Sequencing Facility at the University of California, Davis. Both directions of the *glt*A primers (forward and reverse) were combined after removing the primers sequences to form distinct consensus *glt*A sequences. Phylogenetic trees were constructed from *glt*A sequences using the MrBayes plugin in

TABLE 1. DESCRIPTIVE DATA FOR THE DOGS TESTED FOR *BARTONELLA* INFECTION, LINARES AND PUERTO MONTT, CHILE

	Linares, n (%)	*Puerto Montt*, n (%)	*Total*
Number of dogs	66 (80)	16 (20)	82 (100)
Sex			
Male	32 (48.5)	12 (75)	44 (53.6)
Female	34 (51.5)	4 (25)	38 (46.4)
Age			
<2 years old	8 (12.1)	2 (12.5)	10 (12.2)
2–5 years old	38 (57.6)	12 (75)	50 (60.1)
>5 years old	17 (25.7)	2 (12.5)	19 (23.2)
Not recorded	3 (4.5)	0	3 (3.6)
Ectoparasites			
Fleas only	1 (1.5)	9 (56.2)	10 (12.2)
Ticks only	60 (91)	0	60 (73.2)
Fleas and ticks	4 (6)	2 (12.5)	6 (7.3)
None	1 (1.5)	5 (31.2)	6 (7.3)

Geneious version 8.1.7 (www.geneious.com) with a 1,100,000 Markov Chain Monte Carlo (MCMC) and a 100,000 burn-in length (Huelsenbeck and Ronquist 2001, Kearse et al. 2012).

Statistical analysis

Odds ratio (OR) and 95% confidence interval (95% CI) were calculated using univariate analysis with $p < 5\%$ for potential risk factors associated with *Bartonella* seropositivity, such as age group or sex. Calculations were performed using MedCalc software bvba (BE) (https://www.medcalc.org/calc/odds_ratio.php).

Results

Blood samples were collected from 82 stray dogs, including 66 dogs from Linares and 16 dogs from Puerto Montt. Forty-four (53.6%) dogs were males, of which 32 were from Linares and 12 from Puerto Montt (Table 1). Most dogs (60%) were adults (2–5 years old). Age could not be properly estimated for three dogs from Linares. A vast majority of dogs were also infested with ectoparasites, especially ticks in Linares (97%, 64/66) and fleas (68.8%, 11/16) in Puerto Montt (Table 1). In Puerto Montt, the two dogs >5 years old were both seropositive for *Bartonella* (Table 2) but no ectoparasites were observed on them.

Overall, 48 (72.7%) dogs from Linares were seropositive for at least one of the three *Bartonella* species tested, including 43 (52.4%) dogs seropositive for *B. henselae*, 40 (48.8%) for *B. vinsonii* ssp. *berkhoffii*, and 30 (36.6%) for *B. clarridgeiae*. Of these 48 dogs, 24 (50%) were positive for all three antigens; 2 (4.2%) dogs were only seropositive for *B. henselae* and 2 (4.2%) dogs were only seropositive for *B. vinsonii* ssp. *berkhoffii*. Nine (56.2%) of 16 dogs from Puerto Montt were seropositive (Table 2), all of them being seropositive for all three antigens. Antibody titers ranged from 1:64 to 1:512, with most of the dogs having low antibody titers (1:64) for *B. clarridgeiae* and *B. vinsonii* ssp. *berkhoffii* (Table 3). In Linares, 29 females and 18 males were seropositive (Table 2). Therefore, female dogs were 4.5 times more likely to be seropositive than males (OR = 4.51; 95% CI = 1.39–14.66; $p = 0.012$). Such a difference was not observed in the Puerto Montt dogs where 7 males and 2 females were seropositive (Fisher exact test, $p > 0.05$). However, when considering dogs from both location, sex difference was still significant (OR = 3.36; 95% CI = 1.22–9.28; $p = 0.02$). No statistically significant differences were observed by age groups for *Bartonella* seropositivity, as prevalences were, respectively, 70% in dogs <2 years old, 66% in the 2- to 5-year-old group and 68% in dogs >5 years old. Despite the high prevalence of flea and/or tick infestation in dogs from both locations, seroprevalence was not significantly associated with presence of ectoparasites. In fact, seropositive dogs were more likely not to be flea infested than seronegative dogs (OR = 3.42; 95% CI = 1.11–10.6; $p = 0.032$).

Bartonella DNA was detected in three dogs (4.5%; 3/66) from Linares for the *glt*A gene, but only two of the three dogs were also PCR positive for the *fts*Z gene (Table 4). Partial sequencing of the *glt*A gene showed that two dogs were infected with *B. henselae* (Fig. 1). One dog (no. 46) was infected with a strain closer to *Bartonella vinsonii* ssp. *vinsonii* than *B. vinsonii* ssp. *berkhoffii* or *B. vinsonii* ssp. *yucatanensis*, as shown on the dendrogram (Fig. 1). Partial sequence homology was 97% (334/344 bp) with *B. vinsonii* ssp. *vinsonii* and 96% (330/344 bp) with *B. vinsonii* ssp. *berkhoffii*. Sequences were deposited in GenBank under the following numbers: MN233800 (dog no. 9), MN233801 (dog no. 22), and MN233802 (dog no. 46). One (no. 22) of these three dogs, infected *with B. henselae*, was

TABLE 2. NUMBER OF *BARTONELLA* SEROPOSITIVE AND SERONEGATIVE DOGS BY AGE GROUPS AND SEX, LINARES AND PUERTO MONTT, CHILE

Age	*Sex*	*Linares Pos*	*Linares Neg*	*Puerto Montt Pos*	*Puerto Montt Neg*	*Total Pos*	*Total Neg*
<2 years	M	3	0	0	1	3	1
<2 years	F	3	2	1	0	4	2
2–5 years	M	10	8	6	4	16	12
2–5 years	F	17	3	0	2	17	5
>5 years	M	4	6	1	0	5	6
>5 years	F	7	0	1	0	8	0
Not recorded[a]	M	1	0	0	0	1	0
Not recorded[a]	F	2	0	0	0	2	0
Total	M	18	14	7	5	25	19
Total	F	29	5	2	2	31	7

[a]Unable to properly evaluate age from dental examination.

Table 3. Number and Percentages of Dogs Seropositive for Each *Bartonella* Titer Dilution for Both Locations, Chile

	Bartonella henselae, n (%)		Bartonella clarridgeiae, n (%)		Bartonella vinsonii *ssp.* berkhoffii, n (%)	
Titer	*Linares*	*Puerto Montt*	*Linares*	*Puerto Montt*	*Linares*	*Puerto Montt*
Neg	19 (28.8)	7 (43.7)	30 (45.5)	7 (43.7)	22 (33.3)	7 (43.7)
<64	3 (4.5)	0	5 (7.6)	0	4 (6.1)	0
Pos						
64	14 (21.2)	0	23 (34.8)	3 (18.7)	22 (33.3)	3 (18.7)
128	14 (21.2)	4 (25)	2 (3)	2 (12.5)	5 (7.8)	1 (6.2)
256	14 (21.2)	4 (25)	4 (6.1)	2 (12.5)	7 (10.6)	3 (18.7)
512	1 (1.5)	1 (6.2)	1 (1.5)	2 (12.5)	5 (7.8)	2 (12.5)

seropositive (titer of 1:64) for *B. henselae* and *B. vinsonii* ssp. *berkhoffii*.

Discussion

As for several other South American countries, *Bartonella* exposure was common in stray dogs from Chile, with seroprevalence ranging from 56.2% in Puerto Montt to >70% in dogs from Linares. Of interest, most dogs were infested with ectoparasites, mainly ticks. Observation of flea infestation was less common, but these ectoparasites are not as easy to detect in the fur of dogs without a careful screening, which likely was not possible for time constraint. The lack of association between seropositivity and presence of ectoparasite is surprising. However, the level of infestation and the sample size, especially in Puerto Montt, may have restricted the validity of the data, as many of the seropositive dogs could have been exposed to fleas before the time of observation and blood sample collection. Our data support that heavy load of ectoparasites is common in dogs infected with *Bartonella* sp. The seroprevalence observed in dogs from Chile was higher than in dogs from Peru (Diniz et al. 2013) and much higher than in dogs from Bogota, Colombia (10.1%), Sao Paulo, Brazil (7.6%) or the Galapagos Islands, Ecuador (Levy et al. 2008). As in Colombia, many dogs were seropositive for all three antigens (Brenner et al. 2013).

Overall, three dogs (3.6%; 3/82) were PCR positive, two infected with *B. henselae* and one with a strain closer to *B. vinsonii* ssp. *vinsonii* than to *B. vinsonii* ssp. *berkhoffii*. Unfortunately, we were not able to confirm this result when targeting another gene. Because our data are based only on a short segment (~350 bp) of the *glt*A gene, further studies will be necessary to confirm our report of infection of dogs with a strain close to *B. vinsonii* ssp. *vinsonii*, which has mainly been reported in rodents (Rubio et al. 2014). The strain was also closer to *B. vinsonii* ssp. *vinsonii* than to *B. vinsonii* ssp. *yucatanensis*, isolated from *Peromyscus yucatanicus* from Mexico (Schulte Fischedick et al. 2016). However, *B. vinsonii* ssp. *vinsonii* has been detected a few years ago in two cat fleas in Thailand (Billeter et al. 2012) and the first human case was recently reported (Breitschwerdt and Maggi 2019). As cat fleas are commonly observed infesting dogs, they likely could be the vector of *B. vinsonii* ssp. *vinsonii* in that Chilean dog.

Table 4. PCR and Sequencing Results for Dog Blood Samples for *glt*A and *fts*Z *Bartonella* Genes

Dog ID no.	*gltA*		*ftsZ*	
	PCR	*Seq*	*PCR*	*Seq*
9	+	*Bartonella henselae*	+	NR
22	+	*B. henselae*	+	*B. henselae*
46	+	*Bartonella vinsonii*	Neg	NA

GenBank no.: dog no. 9: MN233800; dog 22: MN233801; dog 46: MN233802.

NA, not applicable; NR, not readable.

In Chile, Müller et al. (2018b) reported the detection of *Bartonella* DNA in 6 (4.3%) of 139 client-owned dogs from rural localities in the Valdivia Province (southern Chile), using qPCR testing for the *nuoG* gene. However, no information was available on ectoparasite load and for seroprevalence in that group of dogs. A *B. henselae* genotype I sequence was obtained from one dog (sequence 100% identical), using the *glt*A gene and *B. vinsonii* ssp. *berkhoffii* (sequence 97% identical to ATCC reference strain) for another dog using the *fts*Z gene. Similar to the study by Müller et al. (2018b), we were not able to detect many *Bartonella* PCR-positive dogs and samples were not all positive for several genes. Therefore, prevalence of bacteremia in stray dogs in south-central and southern Chile is quite low and indicative of a short-lived bacteremic phase in these dogs. However, we demonstrated a frequent exposure to these infectious agents with a seroprevalence ranging from 56.2% up to 72.7% in a population of dogs heavily infected with ectoparasites. These data are quite similar to what was reported from dogs in Peru, where seroprevalence was high for both *B. rochalimae* and *B. vinsonii* ssp. *berkhoffii*, but DNA detection quite low (<5%) (Diniz et al. 2013) and from stray dogs from northern California where seroprevalence was ~30% (54/182), but bacteremia was <3%, as only three dogs were infected with a *B. clarridgeiae*-like strain, now known as *B. rochalimae*, and one dog with *B. vinsonii* ssp. *berkhoffii* (Henn et al. 2007). Stray dogs are, therefore, important sentinels for the detection of these zoonotic agents in the human environment.

In Chile, cat fleas have also been identified as an important vector for *Bartonella* sp. In a recent study, 251 fleas (all *Ctenocephalides felis*) were collected from 150 cats in Valdivia city (Müller et al. 2018a). All fleas belonging to the same cat were pooled (2–7 fleas per pool) and a subset ($n = 19$) of *Bartonella* PCR positive fleas for the *glt*A gene were sequenced. Overall, 59 (39.3%) of pooled or single fleas

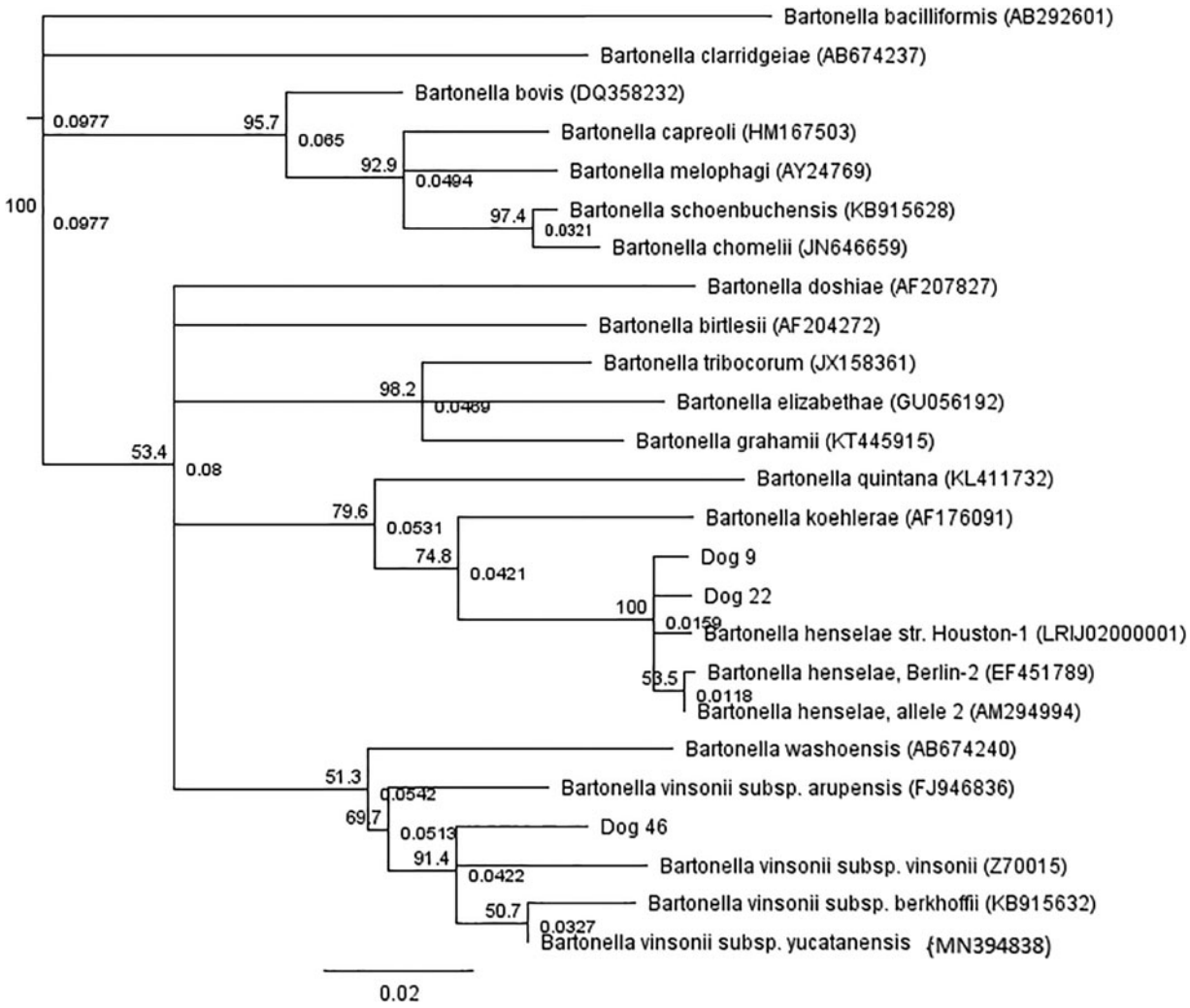

FIG. 1. Dendrogram of the partial sequence of the gltA gene for the three Chilean dogs and Bartonella reference strains.

were *Bartonella* PCR positive. For the 19 positive fleas sequenced, 9 (47.3%) were identified as *B. clarridgeiae*, 8 (42.1%) as *B. henselae*, 1 (5.3%) as *B. koehlerae*, and 1 could not be speciated. Cevidanes et al. (2018) detected *B. clarridgeiae* in one cat flea (*C. felis*) pool collected on dogs from Easter Island (Chile). Similar data were reported from Peru, where 238 fleas were collected from cats and dogs, with a *Bartonella* DNA prevalence ranging from 16.4% to 20.6% depending on the gene tested. *B. rochalimae* was the most common species detected followed by *B. clarridgeiae* and *B. henselae* (Rizzo et al. 2015).

This study confirms common *Bartonella* sp. exposure in Chilean dogs, at least in stray dogs from southern Chile, and reports the first detection of a strain close to *B. vinsonii* ssp. *vinsonii* in a domestic dog. Some limitations of this study are related to (1) the limited sample size in a restricted geographic zone of Chile and therefore it should be investigated in other areas in the country; (2) the short period of sample collection (October–December) that may not reflect the seasonality of *Bartonella* infection in dogs in southern Chile; and (3) analytical sensitivity of each assay was not determined and variation in such sensitivity may play a role in the lack of proper detection. Finally, we were not able to directly culture the blood samples, and using Nobuto strips may have limited our ability to detect *Bartonella* DNA and obtaining consistent DNA detection using different gene targets, some of which may require higher DNA concentration for positive detection.

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