Prevalence, hematological findings and genetic diversity of *Bartonella* spp. in domestic cats from Valdivia, Southern Chile

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(*Received 30 October 2016; revised 12 November 2016; accepted 14 November 2016; first published online 12 December 2016*)

SUMMARY

The present study determined the prevalence, hematological findings and genetic diversity of *Bartonella* spp. in domestic cats from Valdivia, Southern Chile. A complete blood count and *nuoG* gene real-time quantitative PCR (qPCR) for *Bartonella* spp. were performed in 370 blood samples from cats in Valdivia, Southern Chile. *nuoG* qPCR-positive samples were submitted to conventional PCR for the *gltA* gene and sequencing for species differentiation and phylogenetic analysis. Alignment of *gltA* gene was used to calculate the nucleotide diversity, polymorphic level, number of variable sites and average number of nucleotide differences. *Bartonella* DNA prevalence in cats was 18·1% (67/370). Twenty-nine samples were sequenced with 62·0% (18/29) identified as *Bartonella henselae*, 34·4% (10/29) as *Bartonella clarridgeiae*, and 3·4% (1/29) as *Bartonella koehlerae*. *Bartonella*-positive cats had low DNA bacterial loads and their hematological parameters varied minimally. Each *Bartonella* species from Chile clustered together and with other *Bartonella* spp. described in cats worldwide. *Bartonella henselae* and *B. clarridgeiae* showed a low number of variable sites, haplotypes and nucleotide diversity. *Bartonella clarridgeiae* and *B. koehlerae* are reported for the first time in cats from Chile and South America, respectively.

Key words: *Bartonella henselae*, *Bartonella clarridgeiae*, *Bartonella koehlerae*, cat scratch disease, qPCR, South America.

INTRODUCTION

The *Bartonella* genus includes fastidious haemotropic Gram-negative bacteria mainly transmitted by arthropod-vectors (Chomel *et al*. 2009). Over the last 20 years, the number of *Bartonella* species or subspecies identified from a wide range of mammals has increased considerably (Chomel *et al*. 2009). Among the species or subspecies known or suspected to be pathogenic for humans, three have the domestic cat as their natural reservoir, namely *Bartonella henselae, Bartonella clarridgeiae* (Boulouis *et al*. 2005) and *Bartonella koehlerae* (Mogollon-Pasapera *et al*. 2009). *Bartonella henselae* and *B. clarridgeiae* are associated with cat-scratch disease (CSD) and other syndromes in humans and are the most commonly identified *Bartonella* species in cats, worldwide (Boulouis *et al*. 2005; Breitschwerdt *et al*. 2010*b*). *Bartonella koehlerae* was previously reported as a cause of human endocarditis (Avidor *et al*. 2004; Chomel *et al*. 2009).

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Considering that bacteria from the *Bartonella* genus are fastidious to grow *in vitro*, serological [Indirect Fluorescent Antibody Test (IFAT) and enzyme-linked immunosorbent assay (ELISA)] and molecular [conventional and real-time quantitative polymerase chain reaction (cPCR and qPCR), respectively] techniques are widely used for the diagnosis of *Bartonella* infection (Mogollon-Pasapera *et al*. 2009). Serological tests have limited specificity due to cross-reactions and inconsistent results (Maggi *et al*. 2011). Molecular techniques are sensitive and allow species identification (Fenollar and Raoult, 2004). cPCR assays have limited sensitivity compared with qPCR (André *et al*. 2016). A pre-enrichment liquid culture medium ('*Bartonella* Alpha Proteobacteria Growth Medium', BAPGM) prior to PCR was suggested to improve the sensitivity of molecular techniques, mainly for detecting *Bartonella* species in biological samples from non-reservoir hosts, including humans (Maggi *et al*. 2005, 2011; Breitschwerdt *et al*. 2010*a*; Pérez *et al*. 2011).

The prevalence rates of *Bartonella* spp. detected by PCR vary considerably among cat populations, with an increase from cold (0% in Norway) (Bergh

Parasitology (2017), **144**, 773–782.
doi:10.1017/S003118201600247X

et al. 2002) to warm and humid climates (61% in the Philippines) (Chomel *et al*. 1999). Although *Bartonella* spp. infection in cats can vary between areas, it is often associated with flea infestation (Boulouis *et al*. 2005). *Bartonella* spp. infection is associated with extended, often subclinical (Kordick *et al*. 1999), asymptomatic, with long-lasting intraerythrocytic bacteremia in domestic cats (Chomel *et al*. 2009). Understanding the potential associations between *Bartonella* spp. infection and clinical disease in cats is complicated. There is little information about haematological abnormalities in naturally infected cats (Breitschwerdt, 2008).

In Chile, few reports have assessed the prevalence of *Bartonella* in cats. An overall *B. henselae* seropositivity of 85% was found in cats sampled in three cities (Coquimbo, Santiago and Valdivia) (Ferrés *et al*. 2005). Another study in Valdivia, Southern Chile, described a *B. henselae* serosurvey of 71% (Zaror *et al*. 2002). Additionally, 41·7% (25/60) of blood samples from cats in Santiago, Central Chile, were culture positive for *Bartonella*, and confirmed as *B. henselae* by *16S* RNA gene sequencing (Ferrés *et al*. 2005). Nevertheless, the molecular prevalence of *Bartonella* spp. in cats from Southern Chile and its strain diversity are not yet known. *Bartonella* DNA was detected in 10·8% (4/37) of *Ctenocephalides felis* fleas collected from cats sampled in Chilean animal pounds (Pérez-Martínez *et al*. 2009). After PCR amplification and sequencing of *rpoB, gltA* genes and the 16–23S rRNA intergenic transcribed spacer, the species involved were identified as *B. clarridgeiae* and *B. henselae* (Pérez-Martínez *et al*. 2009). Increased exposure to cats, particularly kittens and cat-related trauma were associated with a higher prevalence of *Bartonella*-associated disease (Boulouis *et al*. 2005; Breitschwerdt *et al*. 2010*a*). In Chile, the disease is not of mandatory reporting. Nevertheless, more than 200 human cases of bartonellosis were diagnosed between 1997 and 2000 (Ferrés *et al*. 2005). According to previous studies, cats play a major role as *B. henselae* reservoirs in Chile; consequently, humans who have contact with those animals are at risk (Ferrés *et al*. 2005). The present study aimed at determining the prevalence, haematological findings and genetic diversity of *Bartonella* spp. in domestic cats from Valdivia, Southern Chile.

MATERIALS AND METHOD

Animals and area of study

The study was approved by the Universidad Austral de Chile (UACh) bioethics committee under the protocol number UACh 142/2013.

To accurately determine *Bartonella* spp. prevalence in Valdivia (39 48 30 S, 73 14 30 W), Southern Chile, the required sample size was estimated considering a prevalence of 50%, which fits the criteria when prevalence is unknown (Thrusfield, 2007), and corrected according to the cat population of Valdivia (Zuñiga, 2007), providing a sample of 370 cats. A 5% precision and 95% confidence interval were used (Thrusfield, 2007). Over a 15-month period (August 2013–November 2014), 370 client-owned cats had their blood sampled by a veterinary team. The cats came from all Valdivia city locations in order to acquire balanced and representative sampling. Samples were taken from: (1) cats during home visits to pet-owning households; and (2) cats admitted to the Veterinary Hospital of UACh, Valdivia (Fig. 1). Cats were sampled regardless of age, gender, health and reproductive status. Each owner signed a consent form before samples were taken.

Haematological analysis

Blood samples were collected aseptically by cephalic or jugular venipuncture, divided into two EDTA collecting plastic tubes (Vacutainer®), and sent to the UACh Veterinary Clinical Pathology Laboratory. One EDTA anticoagulated blood sample was stored at −20 °C until DNA extraction/purification. The other EDTA anticoagulated blood was used to perform a complete blood count (CBC). The following parameters were analysed: red blood cell, white blood cell (WBC) and platelet counts; haemoglobin concentration; packed red cell volume; mean corpuscular volume (MCV); and mean corpuscular haemoglobin concentration. An automated haematology analyser, KX-21N (Sysmex©, Japan), was used. The blood smears were stained with rapid staining (Hemacolor®, Merck) for a differential WBC count.

DNA extraction/purification

Frozen EDTA blood samples were thawed at room temperature and vortexed. DNA extraction from 100 μL of blood was performed using a DNeasy® Blood & Tissue Kit (QIAGEN®, Valencia, CA, USA) and was eluted with 100 μL of elution buffer, according to the manufacturer's instructions. Concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific©, USA). The absorbance ratio 260 and 280 nm (OD_{260}/OD_{280}) provided an estimate of sample purity, accepting a ratio of $1{\cdot}8 \pm 0{\cdot}2$ as 'pure'.

Endogenous control real-time PCR

The *28S* rDNA gene was used as an internal control for a PCR assay for feline genomic DNA (Helps *et al*. 2005) using primers feline-28S rDNAFw (5′-AGCAGGAGGTGTTGGAAGAG-3′) and feline-28S rDNARv (5′-AGG GAGA GCCTAAATCAAAGG-3′) to discard the presence

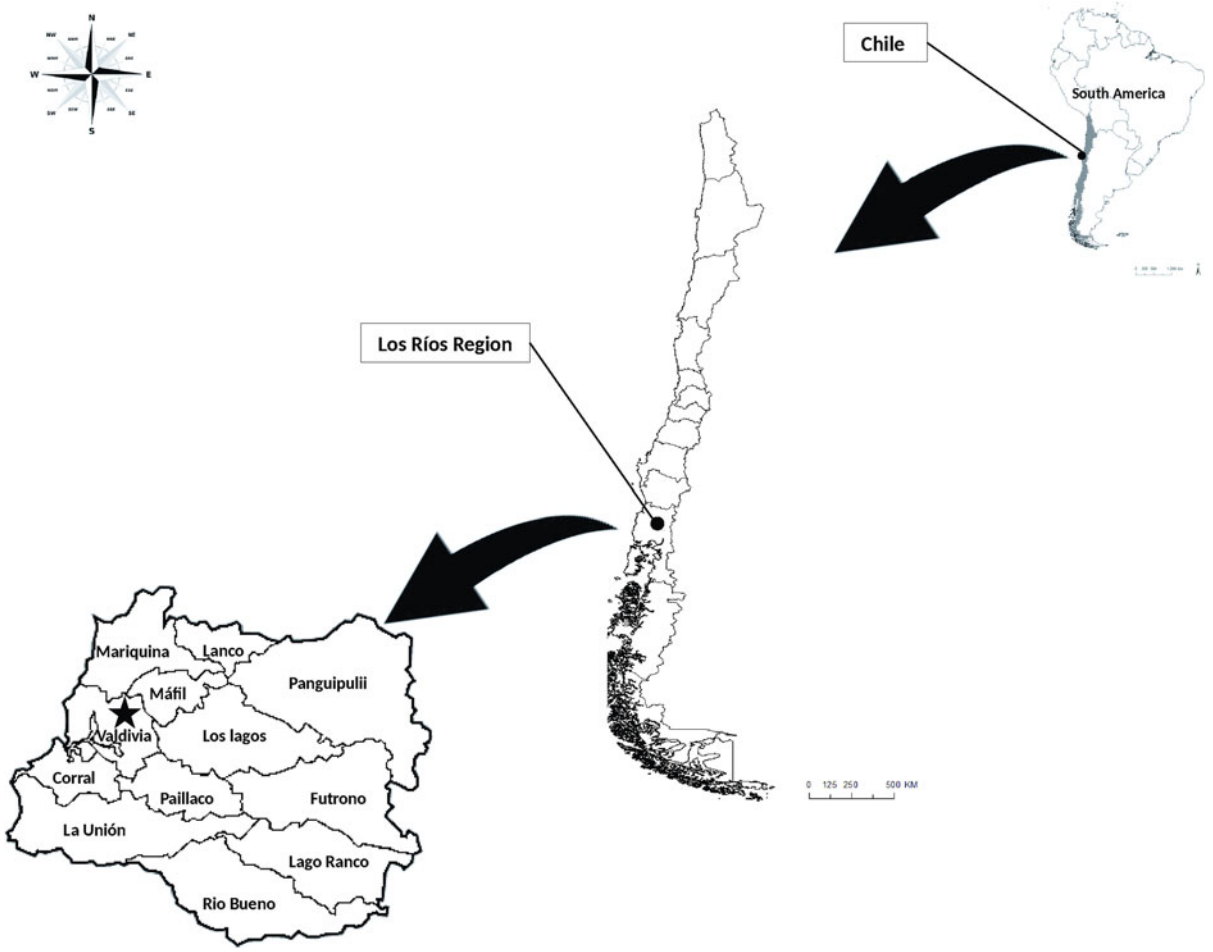

Fig. 1. Map of Chile, showing Valdivia City located in the Los Ríos Region, where samples from cats were taken (MapInfo Professional 7·5 SCP).

of PCR inhibitors. The reaction mixture was composed of 12·5 μL of Maxima®SYBR Green/Rox Master Mix (Thermo Scientific©, USA), 300 nM of the forward and reverse primers and 5 μL of DNA template, brought to a total volume of 25 μL with nuclease-free water (Thermo Scientific©, USA). The amplification conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in a Stratagene Mx3000P™ (Agilent Technologies).

qPCR for Bartonella spp.

28S rDNA cPCR-positive samples were subsequently submitted to a previously described qPCR for *Bartonella* spp. targeting *nuoG* gene (André *et al*. 2016). Amplification reactions were performed in duplicate using 10 μL of PCR mixtures containing 5 μL of Go Taq® Probe qPCR Master Mix, dTTP (Promega, Madison, WI, USA), 1,2 μM of each primer [F-Bart (5′-CAATCTTCTTTTG CTTCACC-3′) and R-Bart (5′- TCAGGGCTTT ATGTGAATAC-3′), hydrolysis probe [TexasRed-5′-TTYGTCATTTGAACACG-3′(BHQ2a-Q)3′] and 1 μL of the DNA sample. PCR amplifications were conducted in Low-Profile Multiplate™ Unskirted PCR Plates (BioRad©, Hercules, CA, USA) using a CFX96 Thermal Cycler (BioRad©). The amplification conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 10 min and 52·8 °C for 30 s. The qPCR was performed following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (Bustin *et al*. 2009). Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/\text{slope}}$). Copy numbers were estimated using 10-fold serial dilutions of pIDTSMART plasmids (Integrated DNA Technologies, Coralville, IA, USA) encoding the *nuoG B. henselae* sequence (insert containing 83 bp). The number of plasmid copies was determined according to the formula [Xg μL^{-1} DNA/ (plasmid length in bp × 660)] × 6·022 × 10^{23} × plasmid copies μL^{-1}. *Bartonella henselae* DNA obtained from a naturally infected cat (Miceli *et al*. 2013) was used as a positive control. All PCR runs were performed with

nuclease-free water (Thermo Scientific©, USA) as a negative control. Replicates showing a *Cq* difference higher than 0·5 were retested.

cPCR for Bartonella *spp.*

For further molecular characterization and species differentiation, *nuoG Bartonella* qPCR-positive samples were tested using a previously described (Billeter *et al*. 2011) cPCR targeting a 767-bp fragment of the citrate synthase gene (*gltA*), using primers CS443f (5′- GCTATGTCTGCATTC TATCA-3′) and CS1210r (5′-GATCYT CAATC ATTTCTTTCCA-3′). Each DNA sample (5 μL) was used as a template in 25 μL reaction mixtures containing 10× PCR buffer, 1·5 mM $MgCl_2$, 0·2 mM deoxynucleotide triphosphate (dNTPs) mixture, 0·625 U Platinum Taq DNA Polymerase (Invitrogen©, Carlsbad, CA, USA), and 0·5 μM of each primer. cPCR amplification reactions were performed using a T100 BioRad termocycler (BioRad©) with the following cycling conditions: 94 °C for 2 min; 45 cycles of 94 °C for 30 s, 48 °C for 1 min and 72 °C for 1 min; and one cycle of 72 °C for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide. To prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms. Gels were visualized under ultraviolet light using the Image Lab Software version 4.1 (BioRad©). The reaction products were purified using the Silica Bead DNA gel extraction kit (Fermentas©, São Paulo, SP, Brazil).

Sequencing and Phylogenetic analysis

Only *gltA*-cPCR-positive samples presenting strong band intensity were submitted for sequencing. Sanger sequencing was performed on purified amplified DNA fragments from positive samples in an automatic sequencer (ABI Prism 310 genetic analyser; Applied Biosystems©/Perkin-Elmer) for species identification and subsequent phylogenetic analysis. Consensus sequences were obtained through analysis of the sequenced products, from both the forward and the reverse oligonucleotides, using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Primer sequences were trimmed from the consensus sequences prior to Blastn analysis. Comparisons with sequences in GenBank were performed using the basic local alignment search tool (BLASTn). The sequences were aligned with sequences published in GenBank using Clustal/W and manually adjusted in Bioedit v. 7.0.5.3 (Carlsbad). Phylogenetic inference based on maximum-likelihood criterion (ML) was inferred with RAxML-HPC BlackBox 7.6.3 (Statamakis *et al*. 2008) through the CIPRES Science Gateway (Miller *et al*. 2010) estimating the proportion of invariable sites by an evolutive model GAMMA GTR + I.

Nucleotide diversity

The alignment sequences of the *gltA* gene, amplified in the present study, were used to calculate the nucleotide diversity (π), polymorphic level [haplotype diversity (*Hd*)], number of variable sites (*vs*) and the average number of nucleotide differences (*K*) using the DnaSP v5.10 (Librado and Rozas, 2009).

Statistical analysis

To determine *Bartonella* spp. prevalence, qPCR-positive cats were divided by the total number of cats sampled and multiplied by 100. The observed prevalence rates were expressed in percentages and the 95% IC was calculated. Descriptive statistics were obtained for haematological parameters and the cats were divided into two groups according to their *Bartonella* spp. status based on the qPCR results: *Bartonella* spp. negative or *Bartonella* spp. positive. The normal distribution of data was evaluated by a Shapiro–Wilk's test. The non-normally distributed data were analysed using the Kruskal–Wallis test to determine if there were any significant differences between the haematological variables of the *Bartonella* spp. status groups. A *P*-value $\leqslant 0{\cdot}05$ was considered statistically significant. Data were analysed using RStudio version 0.99.903 and were available for all 370 cats, except for platelet counts, which were available only for 171 cats.

RESULTS

Bartonella *spp. qPCR results*

All 370 DNA samples [median and standard deviation (S.D.) of DNA concentration = $26{\cdot}5 \pm 12{\cdot}3$ ng μL^{-1}; mean and S.D. 260/280 ratio = $1{\cdot}79 \pm 0{\cdot}07$] were positive for the feline 28S rDNA endogenous gene. Molecular prevalence of *Bartonella* DNA in cats by qPCR (mean and S.D. efficiency of reactions: $96{\cdot}1 \pm 0{\cdot}83\%$, $r^2 = 0{\cdot}998 \pm 0{\cdot}00046$) was 18·1% (67/370) (95% CI 14·422·5%). Thirty-eight samples had a consistent *Cq* (mean and S.D. $30{\cdot}21 \pm 2{\cdot}93$) and quantification (mean $1{\cdot}32 \times 10^3$; minimun–maximun $2{\cdot}13 \times 10^0$–$3{\cdot}19 \times 10^4$ *nuoG*-copies μL^{-1}) (Table 1). Twenty-nine cats had inconsistent *Bartonella*-qPCR quantification assays, due to that, their *Cq* and quantification results were not registered in the present work.

cPCR results and phylogenetic analysis

Of 67 *nuoG*-qPCR-positive samples, 49 (73%) were *gltA*-cPCR-positive and 29 were sequenced.

Table 1. *Bartonella* spp.-positive cat blood samples with their respective *Cq* (cycle of quantification) and quantification (nuoG-copies μL^{-1}) mean values obtained by qPCR assays, and the *Bartonella* species identified by BLASTn analysis

				Closest BLAST identity (%)		
Bartonella spp. nuoG qPCR-positive samples	*Cq* mean values	Quantification (nuoG-copies μL^{-1})	gltA cPCR	Organism	Identity	Accession number
A6B	NA	NA	Positive NS	–	–	–
A1C	NA	NA	Negative	–	–	–
A3C	28·18	$6{\cdot}22 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
A5C	30·85	$2{\cdot}22 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
A7C	NA	NA	Negative	–	–	–
A1E	NA	NA	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
A8E	NA	NA	Positive NS	–	–	–
A10E	29·72	$2{\cdot}47 \times 10^{3}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
A10F	NA	NA	Negative	–	–	–
A10G	NA	NA	Positive	*Bartonella henselae*	(100%)	HG965802.1
A5I	NA	NA	Negative	–	–	–
B10A	26·70	$4{\cdot}08 \times 10^{3}$	Positive	*Bartonella clarridgeiae*	(100%)	KJ170236.1
B4C	30·24	$4{\cdot}34 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
B5C	27·72	$2{\cdot}24 \times 10^{3}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
B6C	NA	$4{\cdot}42 \times 10^{2}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
B7C	NA	NA	Positive	*Bartonella khoelerae*	(100%)	AF176091.1
B2D	26·66	$4{\cdot}85 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
B4D	30·24	$8{\cdot}0 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
B1E	31·25	$5{\cdot}46 \times 10^{0}$	Positive	*Bartonella henselae*	(99%)	HG965802.1
B7E	29·47	$7{\cdot}35 \times 10^{1}$	Positive	*Bartonella henselae*	(99%)	HG965802.1
B1F	31·20	$5{\cdot}78 \times 10^{0}$	Positive	*Bartonella henselae*	(99%)	HG965802.1
B2F	30·10	$2{\cdot}11 \times 10^{2}$	Positive NS	–	–	–
B3F	21·80	$3{\cdot}19 \times 10^{4}$	Positive NS	–	–	–
B4F	32·12	$3{\cdot}15 \times 10^{1}$	Positive NS	–	–	–
B5F	31·59	$4{\cdot}49 \times 10^{1}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
B6F	27·22	$8{\cdot}14 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
B7F	32·23	$2{\cdot}19 \times 10^{1}$	Positive	*Bartonella henselae*	(100%)	HG965802.1
B9F	NA	NA	Negative	–	–	–
B2G	34·88	$2{\cdot}13 \times 10^{0}$	Positive NS	–	–	–
B3G	NA	NA	Positive	*Bartonella henselae*	(100%)	HG965802.1
B4G	NA	NA	Negative	–	–	–
B5G	NA	NA	Positive	*Bartonella henselae*	(99%)	HG965802.1
B5H	27·61	$1{\cdot}14 \times 10^{3}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
B8H	31·37	$9{\cdot}56 \times 10^{1}$	Positive	*Bartonella henselae*	(100%)	HG969191.1
B9H	35·85	$5{\cdot}05 \times 10^{0}$	Negative	–	–	–
B10H	29·12	$4{\cdot}24 \times 10^{2}$	Positive	*Bartonella henselae*	(100%)	HG969191.1
B2I	NA	NA	Positive NS	–	–	–
C6A	33·16	$2{\cdot}93 \times 10^{1}$	Positive NS	–	–	–
C9A	NA	NA	Positive NS	–	–	–
C6B	NA	NA	Negative	–	–	–
C7B	NA	NA	Negative	–	–	–
C8B	34·06	$2{\cdot}54 \times 10^{1}$	Positive NS	–	–	–
C2C	29·12	$2{\cdot}51 \times 10^{2}$	Negative	–	–	–
C3C	25·65	$2{\cdot}57 \times 10^{3}$	Negative	–	–	–
C2D	29·33	$3{\cdot}95 \times 10^{1}$	Positive	*Bartonella henselae*	(99%)	HG965802.1
C1E	NA	NA	Positive NS	–	–	–
C2E	NA	NA	Positive NS	–	–	–
C3E	NA	NA	Positive	*Bartonella henselae*	(99%)	HG965802.1
C4E	NA	NA	Positive NS	–	–	–
C5E	32·28	$4{\cdot}52 \times 10^{0}$	Positive NS	–	–	–
C9F	NA	NA	Positive	*Bartonella henselae*	(100%)	HG965802.1
C2G	NA	NA	Negative	–	–	–

Table 1. (*Cont.*)

Bartonella spp. nuoG qPCR-positive samples	*Cq* mean values	Quantification (nuoG-copies μL^{-1})	gltA cPCR	Closest BLAST identity (%)		
				Organism	Identity	Accession number
C7G	NA	NA	Negative	–	–	–
C8G	29·51	$2{\cdot}88 \times 10^{2}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
C5H	NA	NA	Positive NS	–	–	–
C1J	32·04	$1{\cdot}61 \times 10^{1}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
C2J	26·32	$8{\cdot}25 \times 10^{2}$	Positive	–	–	–
D9A	26·91	$5{\cdot}44 \times 10^{2}$	Positive	*Bartonella clarridgeiae*	(100%)	FN645454.1
D6B	32·27	$5{\cdot}67 \times 10^{0}$	Negative	–	–	–
D3D	33·05	$3{\cdot}51 \times 10^{0}$	Negative	–	–	–
D6D	30·72	$2{\cdot}47 \times 10^{2}$	Positive NS	–	–	–
D8D	35·08	$1{\cdot}66 \times 10^{1}$	Negative	–	–	–
D8E	32·11	$1{\cdot}04 \times 10^{2}$	Positive NS	–	–	–
D1H	NA	NA	Negative	–	–	–
D2H	NA	NA	Positive NS	–	–	–
D4H	NA	NA	Negative	–	–	–
D6H	30·19	$1{\cdot}13 \times 10^{2}$	Positive NS	–	–	–

cPCR, conventional PCR; NS, cPCR-positive sample but with a weak band intensity, which precluded sequencing; NA, data not available due to inconsistent results.

Twenty *gltA*-cPCR-positive samples presented weak band intensity, precluding sequencing. BLAST and phylogenetic analyses supported the identification of 62·0% (18/29) as *B. henselae*, 34·4% as *B. clarridgeiae* (10/29) and 3·4% (1/29) as *B. koehlerae* (Table 1).

Analysis of 14 sequenced products based on the *gltA* gene (GenBank accession numbers KX024499, KX024500, KX024503, KX024505, KX024509–KX024513, KX024515–KX024518, KX024520–KX024524) showed 99–100% identicalness with *B. henselae* (GenBank accession numbers HG965802; KJ170236). These fragments were positioned close to other *B. henselae* isolates, supported by high bootstrap values (96) in maximum-likelihood phylogeneitc analysis. Additionally, the analysis of six sequenced products based on the *gltA* region (GenBank accession numbers KX024501, KX024502, KX024504, KX024506, KX024507, KX024514, KX024519, KX024525–KX024527) showed 100% sequence identity with *B. clarridgeiae* (GenBank accession number FN645454 and KJ170236), and clustering with other *B. clarridgeiae* isolates. Finally, the analysis of one sequenced product based on the *gltA* region (GenBank accession number KX024508) showed 100% identicalness with *B. koehlerae* (GenBank accession number AF176091) and was positioned close to the American isolate (Fig. 2).

Nucleotide diversity

Overall, the *Bartonella* species identified in the present study had a low genetic diversity. Of the 18 *B. henselae gltA* sequences analysed, only three different haplotypes were identified. The haplotype number #Bh1 with ten sequences was the most abundant haplotype, haplotypes # Bh2 and # Bh3 contained two and six sequences, respectively. Additionally, only two haplotypes were identified in the *B. clarridgeiae* sequences. While nine sequences formed the haplotype number #Bc1, only one sequence formed the haplotype number #Bc2. Both *Bartonella* species had a low number of variable sites, haplotypes and nucleotide diversity (Table 2).

Haematological analysis

All variables were non-normally distributed. The only haematological parameter that was significantly different was the MCV ($P < 0{\cdot}0001$), which was significantly lower in *Bartonella*-positive cats (mean = 41·5 fL) than in *Bartonella*-negative ones (mean = 44 fL). There were no other significant differences between *Bartonella*-positive and -negative cats.

DISCUSSION

Cats are the major hosts of *B. henselae, B. clarridgeiae* and *B. koehlerae* (Mogollon-Pasapera *et al.* 2009). Since the pathogens can be transferred to humans through scratches or bites, public monitoring of its prevalence in cats is important (Breitschwerdt, 2008). This is the first study to investigate *Bartonella* spp. molecular prevalence in

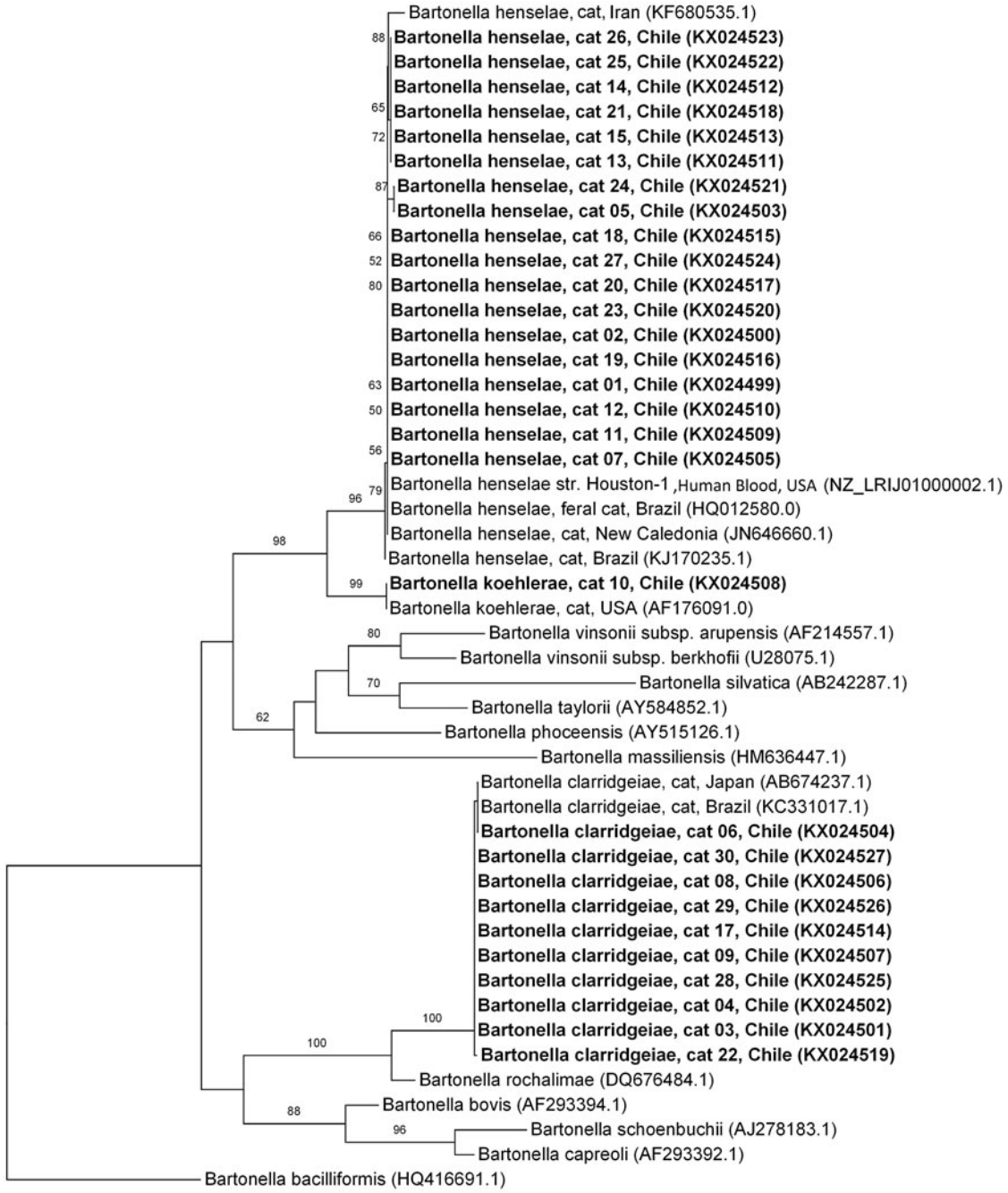

Fig. 2. Phylogenetic relationships within the *Bartonella* genus based on a 680 bp fragment of the gltA gene. The tree was inferred by using the ML method and evolutive model GAMMA GTR + I. The sequences detected in the present study are bold highlighted. The numbers at the nodes correspond to bootstrap values higher than 50% obtained with 1000 replicates. *Bartonella bacilliformis* was used as an outgroup.

Table 2. Polymorphism and genetic diversity of gltA *Bartonella* sequences detected in cats from Valdivia, Chile

Species	(bp)	*N*	VS	GC (%)	*h*	*hd* (mean ± S.D.)	π (mean ± S.D.)	*K*
Bartonella henselae	674	18	3	37·1	3	0·601 ± 0·080	0·001 ± 0·0003	0·888
Bartonella clarridgeiae	674	10	1	38·2	2	0·200 ± 0·154	0·0003 ± 0·0002	0·200

N, number of sequences analysed; VS, number of variable sites; GC, G + C content; *h*, number of haplotypes; *hd*, haplotypes diversity; S.D., standard deviation; π, nucleotide diversity (per site = PI); *K*, average number of nucleotide difference.

a population of domestic cats from southern Chile. Previous reports in Chile described high seroprevalence of *B. henselae* in Valdivia (Zaror *et al*. 2002; Ferrés *et al*. 2006*a*), Santiago and Coquimbo cities (Ferrés *et al*. 2005). Identification of *B. henselae* in solid cultures from cat (25/60) blood samples was performed in central Chile (Santiago City) (Ferrés *et al*. 2005).

Only few studies in South America have evaluated the molecular occurrence of *Bartonella* spp. in cats, mostly carried in Brazil (Staggemeier *et al*. 2010; Crissiuma *et al*. 2011; Braga *et al*. 2012; de Bortoli *et al*. 2012; Miceli *et al*. 2013). Cats from Valdivia, Southern Chile, showed a similar prevalence (18·1%) to the one described in Buenos Aires, Argentina (17·8%) (Cicuttin *et al*. 2014) and Southern Brazil (17·0%) (Staggemeier *et al*. 2010), lower than that observed in cats from Galapagos Island, Ecuador (44·0%) (Levy *et al*. 2008) and higher than central-western (2·2%) (Miceli *et al*. 2013), northeastern (4·5%) (Braga *et al*. 2012) and southeastern (4·3%) (de Bortoli *et al*. 2012) Brazil. However, comparison is difficult, because of the low number of cats and inclusion criteria used in some studies. In general, a higher prevalence was observed in stray cats (as high as 61·1%) (Boulouis *et al*. 2005; Gutiérrez *et al*. 2013), young adult cats living in shelters (36%) (Fleischman *et al*. 2015) and cats from spaying/neutering program (42·5%) (Crissiuma *et al*. 2011). Differences in prevalence may reflect variations in the groups of studied cats, or by geographical variations, such as climate and bloodsucking arthropod distribution, where the presence of fleas (Boulouis *et al*. 2005) is a risk factor for *Bartonella* infection. Furthermore, a direct comparison between studies is difficult because of the differences in cPCR and qPCR diagnostic assays (André *et al*. 2016).

As described worldwide (Chomel and Kasten, 2010), *B. henselae* was the most prevalent species in Southern Chile, followed by *B. clarridgeiae*. The latter was less frequently isolated from domestic cats than *B. henselae*, as it appears to be difficult to isolate and is unevenly distributed in cat populations (Chomel *et al*. 2004). Only one sampled cat was positive for *B. koehlerae*, which has rarely been detected in domestic cats worldwide (Avidor *et al*. 2004; Chomel and Kasten, 2010; Fleischman *et al*. 2015). To the best of our knowledge, *B. koehlerae* is detected for the first time in cats from South America.

The low genetic diversity of *Bartonella* species identified in cats from Southern Chile is in accordance with the high intra species similarity between *gltA* gene sequences of various *Bartonella* spp. (99·80–100%) (Birtles and Raoult, 1996). Since <1·00% genomic variety exists between various strains of *B. henselae*, a low diversity is a common finding (Guy *et al*. 2012). Due to the low genetic diversity, each *Bartonella* species from Chile clustered together and with other *Bartonella* spp. described in cats from Brazil, Iran and USA (Droz *et al*. 1999; André *et al*. 2014; Fard *et al*. 2016).

Bartonella henselae isolates clustered with both Houston-1 and Marseille strains, presenting a high similarity with these. Since Multi Locus Sequence Typing was not performed in the present study, it was not possible to determine which strains are circulating in Chilean cats. Most human cases of CSD are caused by *B. henselae* type Houston-1, suggesting that type Houston-1 strains could be more virulent to humans (Boulouis *et al*. 2005) than Marseille (type II), which is more frequently identified in cats (Chomel *et al*. 2004; Boulouis *et al*. 2005). *Bartonella henselae*-type Marseille is the dominant type in cat populations from Western Europe (France, Germany, Italy, The Netherlands and UK) and Australia (Boulouis *et al*. 2005), whereas type Houston-1 is more frequently reported in human cases in the same regions (Arvand *et al*. 2007). Houston-1 is more frequent in cats from Asia (Japan and the Philippines) (Boulouis *et al*. 2005). In North America (USA), type II is more prevalent in cats on the West Coast (California) (Chomel *et al*. 1995; Chang *et al*. 2002; Fleischman *et al*. 2015) but a 50–50% (types I and II) was described on the East Coast (North Carolina and Florida) (Guptil *et al*. 2004). *Bartonella henselae* isolates obtained from cats in Guatemala (Bai *et al*. 2015) and Argentina (Cicuttin *et al*. 2014) were Houston type I group, suggesting that it could be the major genotype in Central and South America. Nevertheless, more studies on *B. henselae* diversity in other countries, including Chile, are needed to prove this hypothesis.

The mean number of *Bartonella* spp. *nuoG*-copies μL^{-1} in cats from southern Chile was lower than that described in naturally infected cats from Brazil, using the same qPCR protocol (André *et al*. 2016). Indeed, low initial DNA copies in some blood samples from Chilean cats could produce inconsistent quantification results in the *Bartonella*-qPCR assay, represented by the Monte Carlo effect (Bustin *et al*. 2009). The low number of *nuoG-Bartonella* copies in blood samples from cats in our study may be explained by the characteristics of *Bartonella* spp. infection. After infecting their hosts, *Bartonella* may cause a persistent bacteraemia in cats, which is undetectable (Breitschwerdt *et al*. 2010*a*). Long-term intraerythrocytic bacteraemia in reservoir mammals is frequently described and represents a common strategy of *Bartonella* for achieving infection without producing organ damage, generating only chronic, asymptomatic, infection (Chomel *et al*. 2009).

A lower MCV, within the reference values, was the only haematological finding in *Bartonella*-positive cats and was not considered clinically relevant.

As observed in Chilean cats, haematological abnormalities are rarely described in naturally infected cats that seem to be healthy carriers of the bacterium (Boulouis *et al*. 2005; Chomel *et al*. 2009). In neotropical felids haematological abnormalities were not associated with *Bartonella* spp. natural infection (Guimaraes *et al*. 2010). On the contrary, eosinophilia (Kordick *et al*. 1999) and neutrophilia (Guptill *et al*. 1997) were observed in experimentally infected cats. It is important to state that in experimentally infected cats, usually the inoculum dose was very high (Guptill *et al*. 1997). Furthermore, strain variability among *B. henselae* isolates may contribute to enhanced pathogenicity in experimentally infected cats (O'Reilly *et al*. 1999).

Circulation of the three *Bartonella* species in Valdivia cats strengthens the importance of the feline population as a source of zoonotic agents and represents a potential infection risk to humans. While most cats are asymptomatic after becoming infected with *B. henselae*, they serve as reservoirs of the agent and may transmit the infection to humans (Breitschwerdt *et al*. 2010*a*). Data on CSD prevalence in Chile reports a 10·3% infection rate with *B. henselae* in children from Central Chile (Ferrés *et al*. 2006*b*). Also, asymptomatic cat-owners from southern Chile showed serological exposure (18%) to *B. henselae* (Zaror *et al*. 2002) and a high seroprevalence (60%) was observed in humans with an occupational risk in the Bío Bío region, Chile (Troncoso *et al*. 2016). The presence of *B. clarridgeiae* and *B. koehlerae* in cats suggests the need to consider these agents when testing clinical samples from suspected human cases in Chile, along with *B. henselae*.

Concluding remarks

The overall prevalence of *Bartonella* spp. in domestic cats from Valdivia, Southern Chile, is in accordance with that previously described in South America. *Bartonella*-positive cats had low DNA bacterial loads and their haematological parameters varied minimally. Low genetic diversity was reported among the *B. henselae* and *B. clarridgeiae* haplotypes in the present study. Three *Bartonella* species circulate in the studied cat population of Valdivia. *Bartonella clarridgeiae* is reported for the first time in cats from Chile and *B. koehlerae* in cats from South America.

ACKNOWLEDGEMENTS

We thank the veterinary team of the UACh Veterinary Hospital for their helpful contributions in collecting samples. We also wish to thank the team of the Inmunoparasitology Laboratory, Faculdade de Ciencias Agárias e Veterinarias of the Universidade Estadual Paulista, Jaboticabal, Brazil, for their technical support.

FINANCIAL SUPPORT

This work was supported by Beca Santander Iberoamérica Jovenes Investigadores, 2015, Chile.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

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