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Short Communication

Molecular detection and identification of *Bartonella* in the cat flea *Ctenocephalides felis felis* collected from companion animals in a border area in northeastern Argentina

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ABSTRACT

Molecular methods were used to detect and identify *Bartonella* species in the cat fleas *Ctenocephalides felis felis* from Puerto Iguazú, a border area in northeastern Argentina. The fleas were collected from 12 household animals, 9 dogs (*Canis lupus familiaris*) and 3 cats (*Felis silvestris catus*) during July 2016. Out of 15*C. f. felis* analyzed for PCR, only one flea collected from a cat was positive (6.66%) in screened for *Bartonella* spp. based on the *gltA* gene. *Bartonella clarridgeiae* was identified in the genetic analyses, this specimen clustered monophyletically with others *B. clarridgeiae* isolated from different geographical origins (1.0 PP), even, all shared the same haplotype. The results obtained provide evidence of the presence of *B. clarridgeiae* in cat fleas from Argentina suggesting the probable presence of related flea-borne diseases in the region and the role of cat fleas in the transmission of *Bartonella* among mammals including humans.

1. Introduction

Bartonella species are facultative intracellular Gram-negative bacteria that infect erythrocytes and endothelial cells of mammals. These bacteria present a complex cycle that includes the participation of mammals as hosts and arthropods as vectors with about 45 species and numerous genotypes described, of which about 13 species have been potentially associated with human diseases (Moreno Salas et al., 2019). *Bartonella* species produce emerging diseases in humans and animals known as bartonellosis, which are generally self-limited and characterized by high fever, headaches, rashes, muscle pain, regional lymphadenopathy, endocarditis and neurological manifestations among other symptoms (Chomel et al., 2004). Studies on *Bartonella* from Argentina are scarce, and most of the reports are from mammals (Cicuttin et al., 2014), with a few studies involving fleas (Oscherov et al., 2011; Cicuttin et al., 2019; Millán et al., 2019).

Considering the implication of the species of *Bartonella* and the cat flea *Ctenocephalides felis felis* (Bouché, 1835) (Pulicidae) in public health, the aim of the present study is to analyze the presence of these bacteria in fleas parasitic of household dogs and cats in the city of Puerto Iguazú, a triple border area at northeastern Argentina. The results obtained might contribute to the knowledge about the possible role that fleas have in the epidemiology of bacterial microorganisms with zoonotic importance.

2. Materials and methods

Samplings were carried out during July 2016 at the triple border Argentinean city of Puerto Iguazú (NE Argentina-Brazil-Paraguay). The fleas were collected from 9 dogs (*Canis lupus familiaris)* and 3 cats (*Felis silvestris catus*), according to the general guide of the OIE (World Organization for Animal Health). The household animals were randomly selected but taking into account that they were not recently brought from neighboring countries and that they did not co-inhabit with other animals of those countries, to avoid, in case of being positive for *Bartonella*, that the bacteria came from other regions outside from Argentina. The animals were immobilized on mattresses in the company of the owner, and in the case of dogs a protective muzzle was used. Each animal was examined manually, covering the entire body for 6 min in accordance with the general guidelines of the OIE. Fleas

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collected from each host were deposited in an individual plastic tube with 96% alcohol, and stored in freezer at −20 °C for further analyses. Fleas were identified under the binocular stereoscopic microscope based on morphological characteristics of diagnostic value present in the keys and descriptions presented by Johnson (1957).

Fifteen fleas chosen at random (at least one of a different cat or dog examined) and identified as *C. f. felis* were analyzed for the presence of *Bartonella*. The DNA was extracted from each individual crushed flea using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). A fragment of the citrate synthase gene (gltA) of *Bartonella* spp. of approximately 500 bp was amplified (Gil et al., 2010). *Bartonella henselae* was used as a positive control and nuclease-free water was used as a negative control. The PCR products were purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, U.S.A.) and sequenced with a 3500 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, U.S.A.) in the Neurovirus Service of the National Institute of Infectious Diseases (ANLIS Dr. Carlos G. Malbrán, Buenos Aires, Argentina).

The citrate synthase (*gltA*) sequence obtained was manually edited using MEGA v.7 (Kumar et al., 2016). This sequence was compared to other sequences from the GenBank database using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of *B. clarridgeiae* (accession number: KX001761, KY913634, KY913635, KY913636, FN645454) in addition other sequences of *Bartonella* spp. available in GenBank with accession numbers: *B. rochalimae* (JF429602; JF429590), *B. henselae* (KY913627; KY913624; KY913626; MF364384; MH019305; HQ012580), *B. tribocorum* (MG027995; MG027996), *B. koehlerae* (AF176091; KY913638), *B. quintana* (DQ383817; Z70014), *B. vinsonii* subsp. *vinsonii* (Z70015), *B. vinsonii* subsp. *berkonoffii* (AF143445) and *Bartonella* sp. (MH710576; MH710577; MH710578) were aligned using the software MUSCLE in Geneious® software (www.geneious.com/download). The DnaSP v.5 software (Librado and Rozas, 2009) was used to compare the haplotype of *B. clarridgeiae* obtained in Argentina with others *B. clarridgeiae* sequences from different geographical origins available from GenBank and the similarity and identity was calculated using the MatGat software (Campanella et al., 2003). Phylogenetic inference was analyzed using sequences of *Bartonella* spp. available in GenBank in addition to our sequences of *B. clarridgeiae* from Argentina (Table 1). The best-fit model of evolution was estimated using the Bayesian Information Criterion (BIC) as implemented in JModeltest v.2.1.7 software (www.darwin.uvigo.es/our-software; Darriba and Posada, 2012). The Bayesian inference (BI) was analyzed using MrBayes v.3.2 software (Ronquist et al., 2012), four Metropolis-coupled Markov Chain Monte Carlo (MCMC) were run for 220,000 generations to allow adequate time for convergence (≤ 0.005778). The first 25% of sampled trees were considered as burn in. The tree was visualized with FigTree v.1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Sequence of *B. bacilliformis* (access number: CP014012) was used as out-group.

3. Results

Out of the 15 specimens analyzed, only one flea collected from a cat tested positivity in PCR assays for *Bartonella* spp. based on the *gltA* gene. A 463 bp fragment was analyzed, the results of the BLAST identified the sequence as *B. clarridgeiae* (Nucleotide sequence data reported in this paper are available in the GenBank database under the accession number: MN101209), sharing indeed haplotype (100% identity and similarity) with *B. clarridgeiae* from Chile (KY913634-KY913636), Switzerland (FN645454) and Thailand (KX001761). Similarly, others two sequences present same haplotype, although the fragment available in GenBank was smaller (China: EU770616 and England: MG384320), for this reason, were not included in phylogenetic analyses. The BI analysis was constructed using the HKY + G model as the most appropriate for the data (-InL = 1787.4206; BIC = 3894.0031; Delta BIC = 0) with gamma of 0.3330. Two major clades (1.0 PP) were identified a) the first supported by 1.0 PP which is composed by *B. clarridgeiae* and *B. rochalimae* and b) second supported by 0.9 PP which composed by two subclades 1) *B. henselae, B. koehlerae, B. quintana*, 2) *Bartonella* sp., *B. vinsonii* and *B. tribocorum* (Fig. 1).

4. Discussion

The results obtained provide the first evidence of the presence of *B. clarridgeiae* in *C. f. felis* from Argentina. Given the epidemiological importance of flea-borne diseases, more studies are needed to determine if *C. f. felis* participates in the *B. clarridgeiae* transmission cycle. Previously, *B. clarridgeiae* was detected from 5.9% of cats examined from Buenos Aires, Argentina, while all dogs were negative by PCR and Reverse Line Blot hybridization assays (Cicuttin et al., 2014). Reports of *Bartonella* from Argentina also include *B. henselae* and *B. quintana* in humans, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* in canines and *B. henselae* in felines (Cicuttin et al., 2019). Concerning fleas, unidentified species of *Bartonella* at level species were reported from *C. f. felis* from Corrientes Province (Oscherov et al., 2011), as well as from *N. crackensis* associated with sigmodontine rodents from Argentinean Patagonia (Cicuttin et al., 2019). Moreover, *Pulex irritans* fleas collected from foxes from Argentinean Patagonia were mentioned associated with *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae* (Millán et al., 2019). However, among the above mentioned reports from both mammals and fleas, only a few genotypes are available at the GenBank (Cicuttin et al., 2019; Millán et al., 2019). Our analysis identified the same *B. clarridgeiae* haplotype circulating in several countries of the world, such as Chile, Switzerlan, Thailand, China and England suggesting null genetic polymorphism in *gltA* gene.

The identification of *B. clarridgeiae* in the cat fleas from cats reported herein is consistent with the literature. Domestic cats are considered to be the natural reservoir for *B. clarridgeiae*, a causative agent of cat scratch disease (CSD), and dogs may also be infected with these pathogens (Chomel et al., 2004). Indeed, *C. f. felis* is the main vector of *B. clarridgeiae*, *B. henselae* and *B. koehlerae*. The evidence of exposure to *Bartonella* spp. has been reported in cats from Southern Brazil using genus-specific polymerase chain reaction (PCR) (Staggemeier et al., 2010). In the city of Eldorado, 100 km away from Puerto Iguazú, a case of encephalitis has been reported in a young girl who lived in close relationship with cats infested with fleas and ticks. The PCR for *Bartonella* spp. in serum and the LCR was positive in this young girl, and the diagnostic was interpreted as *Bartonella* spp. encephalitis (Cornet et al., 2016). *Bartonella* species prevalence in humans remains unknown and is probably underestimated from Argentina, because the symptoms of this pathogen are common with other illness.

Ctenocephalides felis felis has been reported from a variety of wild and synanthropic animals in Argentina, such as rats and opossums (Lareschi et al., 2016). For this reason, the presence of this flea is a factor to take into account to assess flea-borne pathogens transmission risks, likewise to include *Bartonella* in the diagnosis of emerging pathologies, according to the epidemiology and clinical presentation. The studies on flea-borne diseases are important for monitoring animal health status and public health risks (Valente et al., 2019). The social practices concerning the transit and management of household animals, accompanied by rapid and disorganized urbanization, including deficiencies in environmental sanitation and poor housing, show the need to generate more studies in order to determine the role of fleas in the epidemiology of bacterial microorganisms with zoonotic importance.

Despite the low number of fleas analyzed, the identification of *B. clarridgeiae* is important as it consitutes the first report for these arthropods of Argentina. In addition, this finding is significant since the sampled household animals are local without antecedents of recent transit or cohabitation with non-local animals, in order to evaluate a local established scenario. Nevertheless, the studied area belongs to the three-country border of Argentina-Brazil-Paraguay with intense transit of humans and their companion animals, besides to be surrounded by

Table 1
Origin of *Bartonella* spp. samples included in analyses.

Bartonella specie	Host/tissue	GenBank *gltA* sequence accession no.	Collection site	Reference
Bartonella clarridgeiae	Crushed Flea	Submitted	Puerto Iguazú, Argentina	This study
Bartonella clarridgeiae (Lawson and Collins)	Crushed Flea	KY913634-KY913636	Chile	Müller, A., Rodríguez, E., Walker, R., Bittencourt, P., Pérez-Macchi, S., Gonçalves, L. R., André, M.R., 2018. Occurrence and genetic diversity of *Bartonella* spp. (Rhizobiales: Bartonellaceae) and *Rickettsia* spp. (Rickettsiales: Rickettsiaceae) in Cat Fleas (Siphonaptera: Pulicidae) From Chile. J Med Entomol, 55(6), 1627–1632. doi:https://doi.org/10.1093/jme/tjy12
Bartonella clarridgeiae	Cat/blood	KX001761	Thailand	Srisanyong, W., Takhampunya, R., Boonmars, T., Kerdsin, A., Suksawat, F., 2016. Prevalence of *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella vinsonii* subsp. *berkhoffii* in pet cats from four provincial communities in Thailand. *Thai J Vet Med*, *46*(4), 663–670.
Bartonella clarridgeiae	Cat/blood	FN645454	Switzerlan	Engel, P., Salzburger, W., Liesch, M., Chang, C.C., Maruyama, S., Lanz, C., Dehio, C., 2011. Parallel evolution of a type IV secretion system in radiating lineages of the host-restricted bacterial pathogen *Bartonella*. *PLoS Genetics*, *7*(2). doi:https://doi.org/10.1371/journal.pgen.1001296
Bartonella clarridgeiae	Cat/blood	EU770616	China	Li, D., Liu, Q., Song, X., Zhang, J., Xu, C., Yang, X., 2009. Biological and molecular characteristics of a cat-borne *Bartonella clarridgeiae*. Wei Sheng Wu Xue Bao = Acta Microbiologica Sinica, 49(4), 429–437.
Bartonella clarridgeiae	Human	MG384320	England	Unpublished
Bartonella rochalimae (Eremeeva)	Rat/spleen	JF429590/JF429602	USA	Gundi, V.A.K.B., Billeter, S.A., Rood, M.P., Kosoy, M.Y., 2012. *Bartonella* spp. in rats and Zoonoses, Los Angeles, California, USA. Emerg Infect Dis, 18(4), 631–633. doi:https://doi.org/10.3201/eid1804.110816
Bartonella koehlerae (Chomel)	Crushed Flea	KY913638	Chile	Muller et al. 2018
Bartonella koehlerae	Cat/blood	AF176091	USA	Droz, S., Chi, B., Horn, E., Steigerwalt, A.G., Whitney, A.M., Brenner, D.J., 1999. *Bartonella koehlerae* sp. nov., isolated from cats. J Clin Microbiol, *37*(4), 1117–1122.
Bartonella henselae(Brenner)	Cat/blood	MH019305	Brazil	Da Silva, B.T.G., de Souza, A.M., Campos, S.D.E., de Lemos, E.R.S., Favacho, A.R. de M., Almosny, N.R.P., 2018. Presence of *Bartonella* spp. In domestic cats from a state park in rio de Janeiro, Brazil. Rev. Inst Med Trop Sao Paulo. doi:https://doi.org/10.1590/S1678-9946201860014
Bartonella henselae	Crushed Flea	KY913624/KY913626-KY913627	Chile	Muller et al. 2018
Bartonella henselae	Cat/blood	HQ012580	Brazil	De Oliveira Braga, M. do S.C., de Paiva Diniz Diniz, P.P.V., André, M.R., de Bortoli, C.P., Machado, R.Z., 2012. Molecular characterisation of Bartonella species in cass from São Luís, state of Maranhão, north-eastern Brazil. Mem Inst Oswaldo Cruz, 107(6), 772–777. doi:https://doi.org/10.1590/S0074-02762012000600011
Bartonella henselae	Crushed Flea	MF374384	Austria	Duscher, G.G., Hodzić, A., Potkonjak, A., Leschnik, M.W., Spergser, J., 2018. *Bartonella henselae* and *Rickettsia felis* Detected in Cat Fleas (*Ctenocephalides felis*) Derived from Eastern Austrian Cats. *Vector-Borne and Zoonotic Diseases*, *18*(5), 282–284. doi:https://doi.org/10.1089/vbz.2017.2215
Bartonella quintana(Fuller)	Dogs/blood	DQ383817	New Zealand	Kelly, P., Rolain, J.M., Maggi, R., Sontakke, S., Keene, B., Hunter, S., … Raoult, D., 2006. *Bartonella quintana* endocarditis in dogs. *Emerging Infectious Diseases*, *12*(12), 1869–1872. doi:https://doi.org/10.3201/eid1212.060724
Bartonella quintana	Human	Z70014	France	Birtles, R.J., Raoult, D., 1996. Comparison of partial citrate synthase gene (gltA) sequences for phylogenetic analysis of *Bartonella* species. IJSB, *46*(4), 891–897. doi:https://doi.org/10.1099/00207713-46-4-891
Bartonella sp.	Crushed Flea	MH710576-MH710578	Patagonian, Argentina	Cicuttin et al. 2019
Bartonella vinsonii subsp. *vinsonii* (Baker)	Human	Z70015	France	Birtles, R. J., & Raoult, D. 1996
Bartonella vinsonii subsp. *berkhoffii* (Kordick)	Human	AF143445	France	Roux, V., Eykyn, S.J., Wyllie, S., Raoult, D., 2000. *Bartonella vinsonii* subsp. *berkhoffii* as an agent of afebrile blood culture-negative endocarditis in a human. *J Clin Microbiol*, *38*(4), 1698–1700.
Bartonella tribocorum (Heller)	Rat/spleen, heart	MG027995-MG027996	USA	Peterson, A.C., Ghersi, B.M., Alda, F., Firth, C., Frye, M.J., Bai, Y., … Blum, M.J., 2017. Rodent-Borne *Bartonella* Infection Varies According to Host Species Within and Among Cities. *EcoHealth*, *14*(4), 771–782. doi:https://doi.org/10.1007/s10393-017-1291-4
Bartonella bacilliformis	Human	CP014012	USA	Unpublished

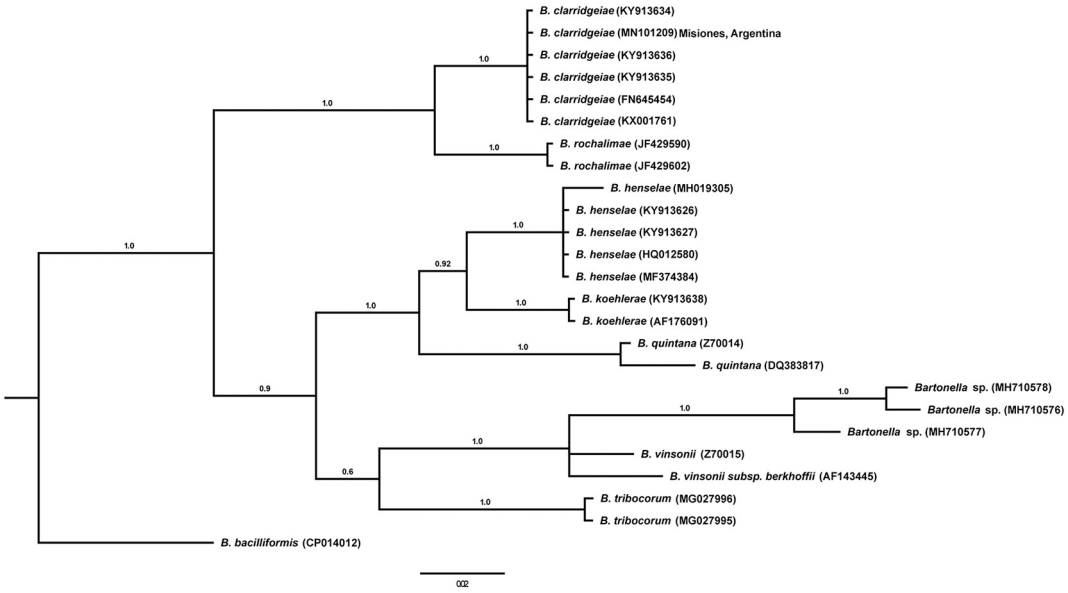

Fig. 1. Phylogenetic tree of *Bartonella clarridgeiae* constructed by Bayesian Inference method, using HKY + G model. Posterior probabilities values are shown on the branches. *Bartonella bacilliformis* was used as outgroup. The scale bar represents the expected number of nucleotide substitutions per site.

national parks with preserved wild fauna, and so forest-periurban ecotones. Therefore, these results bring the alert to improve surveillance and medical monitoring on bartonellosis in humans and animals from the region, but also to encourage the flea-borne *Bartonella* studies in the area in order to understand its dynamic and dispersion both through country borders and between household and wild animals.

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Declaration of Competing Interest

None.

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