# Molecular detection of *Bartonella henselae, Bartonella clarridgeiae* and *Rickettsia felis* in cat and dog fleas in Tenerife, Canary Islands, Spain

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ABSTRACT: The cat flea *Ctenocephalides felis* is the main vector of *Bartonella henselae* and *Bartonella clarridgeiae,* the causative agents of cat-scratch disease (CSD) and the spotted-fever agent *Rickettsia felis*. In spite of their worldwide distribution, there are no data on the occurrence of CSD-causing *Bartonella* species or the prevalence of *Rickettsia* species in the Canary Islands, Spain. Therefore, the aim of our study was to screen cat and dog fleas for both pathogens. A total of 128 *C. felis* from cats and dogs were screened for *Bartonella* and *Rickettsia* by PCR. *Bartonella henselae* (2.3%) and *B. clarridgeiae* (3.9%) were found in fleas infesting cats, whereas *R. felis* was identified in both cat (36.6%) and dog (40.7%) fleas. Further, co-infections were observed. This work constitutes the first finding of CSD-causing *Bartonella* species and the first study on the prevalence of *R. felis* in fleas from domestic animals in the Canary Islands. These results indicate public health importance, as associated infections could be misdiagnosed in the Archipelago despite their clinical relevance. Establishing human and animal routine diagnosis procedures for these pathogens along with improving vector control in shelters is necessary in order to prevent the spread of the infections among animals. ***Journal of Vector Ecology* 45 (2): 233-240. 2020.**

*Keyword Index: Bartonella henselae*, *Bartonella clarridgeiae*, *Rickettsia felis*, *Ctenocephalides felis*, cat flea, Canary Islands.

## INTRODUCTION

Fleas are recognized vectors for several zoonotic pathogens of great importance for human health. The cat flea, *Ctenocephalides felis,* is the most common flea species infesting both cats and dogs worldwide. Numerous studies prove that *C. felis* is a competitive vector of several bacterial human pathogens, including *Bartonella* and *Rickettsia* species (Chomel et al. 2006, Reif and Macaluso 2009).

*Bartonella* is a Gram-negative, intracellular bacterium that has been described in a great variety of domestic and wild mammals worldwide (Kosoy et al. 2012). *Bartonella henselae* is the most common species described in both humans and cats, and also reported in other mammalian hosts such as dogs, rabbits, and guinea pigs (Mazur-Melewska et al. 2015). This species is considered the causative agent of cat-scratch disease (CSD) (Anderson and Neuman 1997), although *Bartonella clarridgeiae* and *Bartonella koehlerae* have been occasionally reported as the etiological agent of the disease (Kordick et al. 1997, Avidor et al. 2004). *Ctenocephalides felis* can occasionally transmit CSD-related *Bartonella* to humans by biting, but flea feces involve the main source of infection in humans from a cat scratch or bite, along with the saliva of an infected cat (Abbot et al. 1997). Nevertheless, asymptomatic infection of *Bartonella* has been reported in blood donors (Noden et al. 2014) that may constitute an unusual, but potential, source of infection, as well as by accidental needle stick or perinatal transmission (Breitschwerdt et al. 2010, Oliveira et al. 2010). In humans, the infection is characterized by a self-limited regional lymphadenitis but may also cause a great range of clinical manifestations, from fever of unknown origin to splenic or hepatic issues, encephalitis, endocarditis, or ocular disease (Maguina et al. 2009). Further, it may develop into potentially fatal disorders in immunocompromised patients. Naturally infected cats do not usually show any clinical manifestation, but cardiac signs such as endocarditis or myocarditis, or ocular issues may appear in some cases, especially when the cat is under two years old (Guptill et al. 2004). Infections in dogs present similar range and clinical manifestation as human infections (Friedenberg et al. 2015). Occurrence of vectors is critical for spreading of both *B. henselae* and *B. clarridgeiae*, as a transmission among animals was not observed in a flea-free environment (Mosbacher et al. 2011).

The genus *Rickettsia* contains gram-negative, obligate intracellular bacteria that have a worldwide distribution, although endemic areas are reported (Chikeka and Dumler 2015). This genus is divided into two major groups, the spotted fever group (SFG) and the typhus group (TG) (Bhengsri et al. 2016), both being transmitted to accidental hosts by arthropod vectors, mainly ticks, but fleas may be involved in some cases (Blanton and Walker 2017). *Rickettsia felis* is a flea-borne causal agent of spotted-fever in humans (Bouyer et al. 2001), and domestic cats and dogs are considered its reservoir hosts (Hii et al. 2011, Gracia et al. 2015). *Ctenocephalides felis* is both host and vector of the pathogen, although

recent studies involve other flea species as potential vectors (Rakotonanahary et al. 2017). Unlike *Bartonella* species, the transmission of *Rickettsia* to humans is directly by flea bites (Foil et al. 1998). Infection by *R. felis* in humans comprises a wide range of clinical manifestations, from fever, headache, or cough, to myalgia, hepatomegaly, abnormal blood and liver biochemistry, and kidney malfunction (Hernández-Cabrera et al. 2004, Pérez-Arellano et al. 2005). Infection in dogs seems to cause mild clinical manifestation, like self-limited diarrhea or gingival petechial hemorrhages, and appetite reduction (Ng-Nguyen et al. 2020), whereas larger and more severe manifestations may occur in cats with blood disorders and neurological symptoms, but the isolation of *R. felis* from animals with evidence of rickettsial infection has not been performed (Labruna and Walker 2014).

CSD-*Bartonella* species have been reported in humans, and domestic animals and their fleas in mainland Spain (Gil et al. 2013). However, there have been no recent studies of the presence of CSD-related *Bartonella* species in the Canary Islands (Spain) despite their clinical relevance. Otherwise, serological evidence of *R. felis* in cats (Gracia et al. 2015) and molecular detection in fleas of a dog owned by a *R. felis*-infected patient (Pérez-Arellano et al. 2005) have been reported in the Archipelago.

Therefore, the aim of the present study was to screen fleas from stray and sheltered cats and dogs for the presence of *Bartonella* and *Rickettsi*a species by PCR amplification and to identify bacterial and flea species involved in the transmission of the pathogens.

## MATERIALS AND METHODS

Flea samples were collected in two areas in the north part of Tenerife, the largest island in the Canary Islands (Spain), an archipelago located in NW Africa (13°23'-18°8´W and 27°37'- 29°24'N). In December, 2019, 54 fleas were collected from two adopted stray cat siblings of approximately three months old in a rural area of La Orotava (16º31'W and 28º22'N). In January and February, 2020, 74 additional fleas infesting ten stray cats and six dogs from urban areas were collected in the veterinary service of an animal shelter in La Laguna City (16º19'W and 28º29'N) during an animal neuter campaign. Thus, a total of 128 fleas from cats (101) and dogs (27) were included in the study.

Fleas were stored in absolute ethanol to be submitted to DNA extraction following López et al. (2015), where each flea was ground in a 1.5 ml tube containing 250 µl of lysis buffer (30 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.4% SDS) and 5 µl of proteinase K (20 ng/µl) and then incubated at 56° C overnight. Afterward, 250 µl of $NH_4Ac$ 4M was added and mixed thoroughly followed by 30 min incubation at room temperature. Afterwards, samples were centrifuged at 13,000 rpm for 10 min and supernatant was transferred to another tube and submitted to centrifugation with absolute ethanol and 70° C ethanol in two consecutive steps. DNA extraction procedure was checked using a spectrophotometer. A 767-bp region of the citrate synthase gene (*gltA*), including a 327-bp sequence used to distinguish *Bartonella* species (Norman et al. 1995), was targeted by PCR. In addition, amplification of a 381-bp fragment of the rickettsial *gltA* gene was carried out to determine the presence of SFG or TG species (Choi et al. 2005). In both cases, when amplification was observed, a second PCR protocol was performed in order to confirm the identity of the pathogens by analyzing a 346 bp-fragment of the NADH dehydrogenase gamma subunit (*nuoG*) for *Bartonella* (Colborn et al. 2010) and the fragment I of the outer-membrane protein gene (*ompB*) for *Rickettsia* (Roux and Raoult 2000).

PCR targeting a fragment of the cytochrome c oxidase subunit I (*cox1*) gene was performed following Lawrence et al. (2014), in order to molecularly identify species of the pathogen-positive fleas. PCR amplicons were purified using commercial kits following the manufacturer recommendations and then were sequenced in Macrogen (Spain) in both directions for the pathogens and in one single direction for fleas, using in this case the sequence obtained with reverse primer as it is recommended by authors (Lawrence et al. 2014).

The analysis of the sequences was carried out with software MEGA X (Molecular Evolutionary Genetic Analysis) (Kumar et al. 2018), using the multiple alignment program ClustalW included in MEGA X and minor corrections were made by hand. Sequences were compared with available published sequences in GenBank by the BLAST (blastn) program. Phylogenetic relationships based on the neighbor-joining method were carried out with the p-distance model (Nei and Kumar 2000). Bootstrap analysis was performed with 1,000 trials.

## RESULTS

Of the 128 fleas tested, 55 harbored at least one of the pathogens of interest (43%) (Table 1). Eight out of the 128 tested samples were positive for *Bartonella* (6.3%), including two fleas obtained from cats in La Orotava and six fleas collected from a cat from the shelter. All *Bartonella*-positive fleas were collected from cats (8/101), as no fleas collected from dogs were found positive for *Bartonella* species (0/27). Seven *gltA* amplicons were successfully sequenced. Of those, three sequences were identical to each other and to *B. henselae* type I (strain Houston-1) sequence of reference (accession number BX897699), whereas other four sequences were identical to *B. clarridgeiae* (accession number FN645454) with no observed differences among sequences. Phylogenetic analysis of the sequences based on the *gltA* genes has also demonstrated clustering of both groups with *B. henselae* and *B. clarridgeiae* (Figure 1). All identities were confirmed by analyzing the *nuoG*, except for one sample that showed 100% similarity to *B. clarridgeaie* by *gltA* analysis and to *B. henselae* type I by *nuoG*. *Bartonella gltA*-sequences obtained within this study were deposited in GenBank under accession numbers MT459991-97, and *nuoG* sequences under MT459998-460000.

Screening of the 128 fleas by PCR using the *gltA* gene target has revealed 48 fleas positive for *Rickettsia* (37.5%), including 37 positive fleas collected from cats (36.6%), where two positive fleas were collected in the shelter and 35 in La Orotava, and 11 positive fleas were collected from dogs

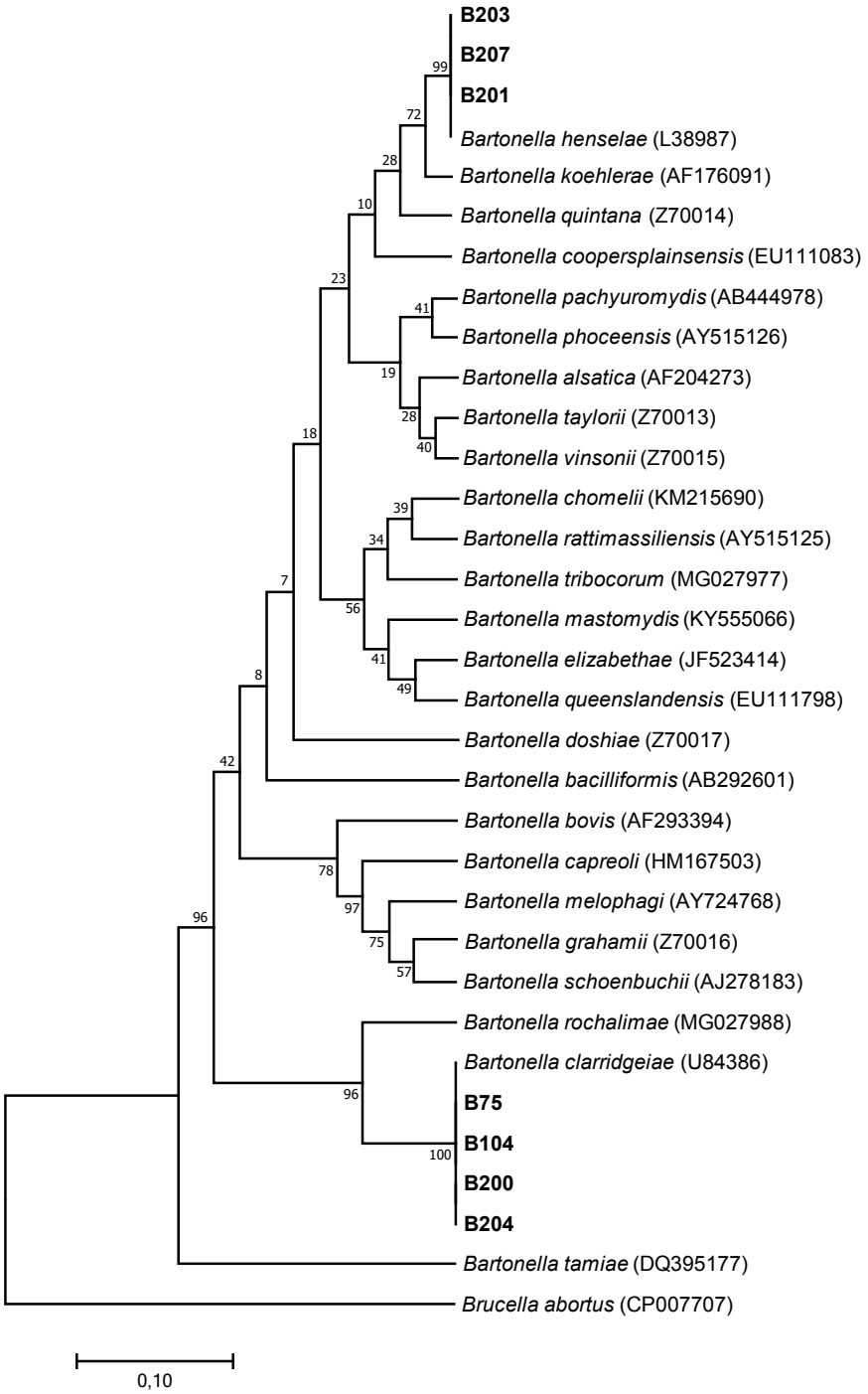


Figure 1. Phylogenetic analysis of the *Bartonella* sequences obtained in fleas infesting stray cats in Tenerife, based on the 327-bp fragment of the *gltA* gene. *Brucella abortus* (CP007707) was used as the outgroup. Sequences obtained within this work are shown in bold.

Table 1. Prevalence of *Bartonella* sp. and *Rickettsia* sp. in fleas infesting cats and dogs in Tenerife (Canary Islands, Spain). (+/n (P): positive samples/number of fleas analyzed (prevalence).

| Location | Host | *Bartonella* sp. (+/n (P)) | *Rickettsia* sp. (+/n (P)) |
|---|---|---|---|
| La Orotava | Cat | 2/54 (3.7%) | 31/54 (57.4%) |
| La Laguna (shelter) | Cat | 6/47 (12.8%) | 6/47 (12.8%) |
| | Dog | 0/27 (0%) | 11/27 (40.7%) |
| TOTAL | | 8/128 (6.3%) | 48/128 (37.5%) |

(40.7%) from the shelter. Among these positive samples, 38 could be successfully sequenced afterwards, showing all sequences with 100% identity to *R. felis* (accession number GQ329873). Rickettsial identity was confirmed by phylogenetic analysis of the *gltA* gene (Figure 2) and the *ompB* analysis, as similar identities were obtained (data not shown). All *gltA*-rickettsial sequences were deposited to the GenBank under accession numbers MT460001-41, as well as an *ompB* representative sequence since they were all identical (accession number MT460042). Co-infections of *Bartonella* and *Rickettsia* species were detected in two fleas from cats (1.6%), with one flea collected from a cat from the shelter harboring both *R. felis* and *B. henselae* and another flea from a cat from La Orotava harboring *R. felis* and *B. clarridgeiae*.

The molecular identification of 45 of the pathogen-positive fleas using the *cox1* gene showed that all these fleas were 100% identical to *C. felis* (accession number MG668603) and sequences were submitted to GenBank under accession numbers MT460043-88.

## DISCUSSION

The CSD has a worldwide distribution, making it the most common infection in humans caused by *Bartonella* species (Klotz et al. 2011), but there were no previous data about the presence of these species in the Canary Islands. Therefore, this work constitutes the first report of CSD-related *Bartonella* species in the Archipelago. Some zoonotic species of *Bartonella* are known to be circulating among rodents and their fleas in the Canary Islands (Abreu-Yanes et al. 2018). Arthropod vectors are the main source of transmission of *Bartonella* among rodents, although vertical transmission has been also demonstrated (Kosoy et al. 1998).

Although animals included in this study showed relatively high flea infestation, the prevalence of *Bartonella* in cat fleas seems lower when compared with studies in mainland Spain (Andalucía 11.5%, La Rioja 21.5%) (Márquez et al. 2009, Gil et al. 2013) and some other Mediterranean countries, such as Israel (64%) or Tunisia (33.4%) (Gutiérrez et al. 2015, Zouari et al. 2017). Additional investigations covering other islands of the Archipelago might change the prevalence rate. Although *Bartonella* species have been reported in dog fleas with low prevalence (Chomel et al. 2004), no dogs were found to be infested with *Bartonella*-positive fleas in our study. In a number of investigations, *B. clarridgeiae* was the most common species found in cat fleas with *B. henselae* being the second species most frequently identified (Mokhtar and Tay 2011). In our study, prevalence of both *Bartonella* species was quite similar, though limited sample size did not allow any statistical comparison.

Our findings are important for public health, since *B. henselae* is considered the main causative agent of CSD. Besides, some investigations indicate that strains of *B. henselae* belonging to type I are more related to human disease than type II (Bouchouicha et al. 2009). In addition, *B. clarridgeiae* has been also detected in patients with the disease, although it has not been isolated from blood or lymph node samples of the patients (Boulouis et al. 2005). Besides, co-infection of both *Bartonella* species in fleas has been reported (Mokhtar and Tay 2011) and it likely was also observed in this study based on the presence of different *gltA* and *nuoG* sequences in one flea. The detection of the coinfection with both species is probably associated with the lower sensitivity of *nuoG* to detect *B. clarridgeiae* when compared to *gltA* (Bai et al. 2015).

*Ctenocephalides felis* is the main vector in the transmission of *B. henselae* and *B. clarridgeiae,* although other species belonging to this genus, such as *Ctenocephalides canis* and *Ctenocephalides orientis*, may be involved in some cases (Zouari et al. 2017, Kernif et al. 2012). Occasionally, other flea species have been found to be harboring CSD-*Bartonella* species, i.e., *Pulex irritans* (Cáceres et al. 2013), although their vectorial capacity has not been proved. Our results support the association between *B. henselae* and *B. clarridgeiae* with *C. felis* in the Canaries, as *C. felis* is the only flea species infesting cats and dogs identified in this study.

Since infected cats usually remain asymptomatic, close contact with stray and wild cats may present a risk of acquiring *Bartonella* infection for humans and animals. In some cases, an accurate diagnosis of bartonellosis is complicated due to unspecific symptoms and the type of samples analyzed. Some studies suggest that infected cats could prove to be positive to *Bartonella* when analyzing the saliva but not based on the blood analysis, and vice versa (Lappin and Hawley 2009). Further, cases of asymptomatic *Bartonella*-positive blood donors have been reported (Noden et al. 2014).

The results of this study have for the first time demonstrated a high prevalence of pathogenic *R. felis* in fleas infesting stray cats and dogs in Tenerife. The overall rickettsial prevalence is similar to those detected in previous studies in the Archipelago (Gracia et al. 2015). However, this prevalence was only in fleas from the feline population and not from dogs, as there were only anecdotal data available. In

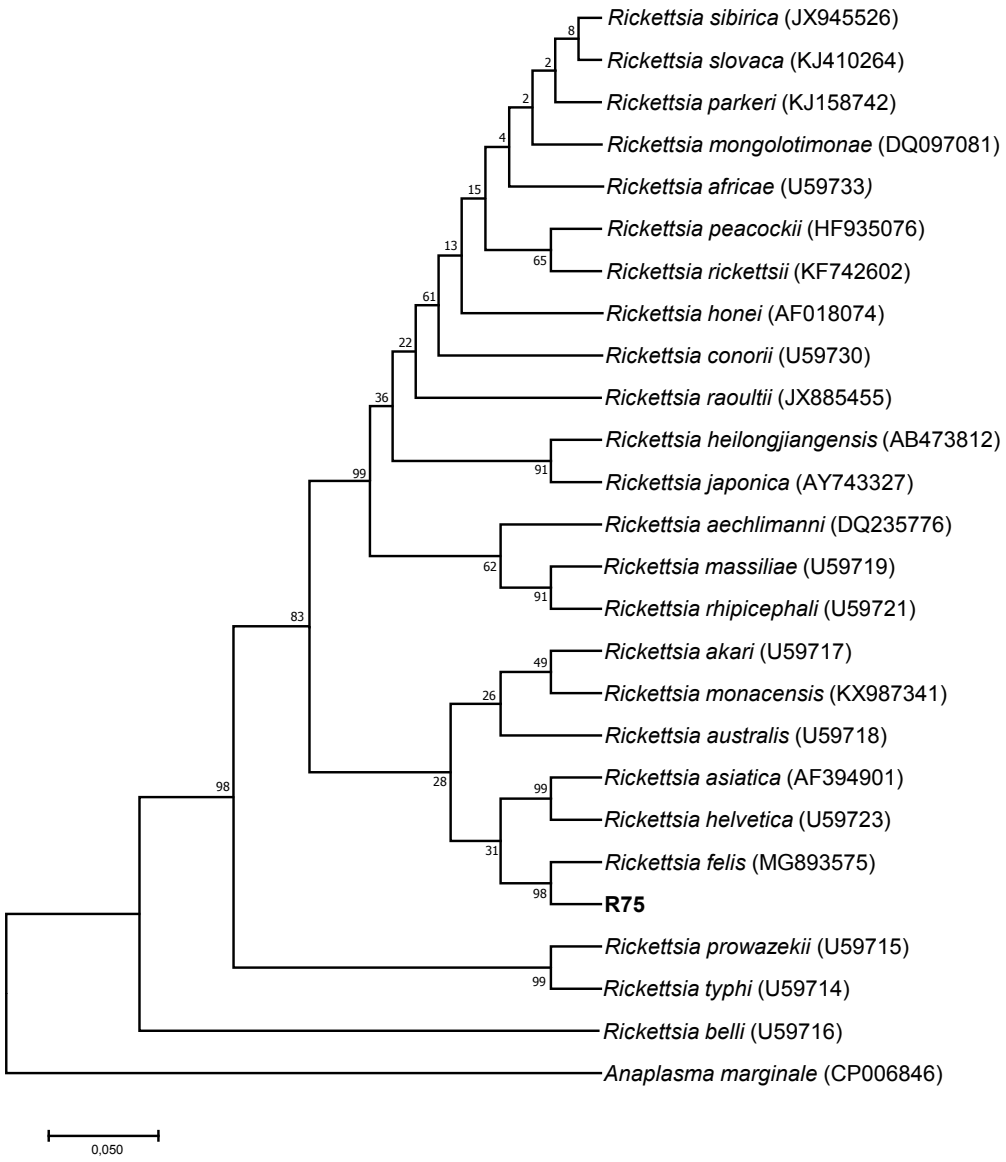


Figure 2. Phylogenetic analysis of the *Rickettsia* sequences obtained in fleas infesting stray cats and dogs in Tenerife by analyzing a 381-bp fragment of the *gltA* gene. *Anaplasma marginale* (CP006846) was used as the outgroup. Only one sequence obtained within this work is shown (in bold) as representative of all sequences as they are identical.

this study, prevalence of *R. felis* in dog fleas was 24.9%, with positive fleas found in five of six dogs analyzed. This rate is close to that previously reported (Gilles et al. 2008, Giudice et al. 2014). Thus, *C. felis* is both host and vector of *R. felis* (Reif and Macaluso 2009) and the main flea species infesting both cats and dogs (Rust 2005). Our results support the close relation between *C. felis* and *R. felis* as they both were the only flea and *Rickettsia* species identified in this study. Co-infections of *R. felis* with *B. henselae* and *B. clarridgeiae* were also observed in this work, similarly to the studies conducted in Lebanon and Malaysia (Mba et al. 2011, Kernif et al. 2012).

Due to the occurrence of a competent flea vector and relatively high prevalence, the potential risk of rickettsial infection for humans on the island is high, as well as a possibility of spreading the pathogen among animals. Infection in humans by *R. felis* may produce different clinical manifestations; the diagnoses of the infection in the Canary Islands was established based on serological, Western blot assay, or cross-adsorption tests (Pérez-Arellano et al. 2015), which are not of common use in healthcare.

This work involves a great interest for human and animal health due to the clinical relevance of the species found, as well as the high prevalence of *R. felis* and the potential occurrence of unspecific symptoms of *Bartonella* and *Rickettsia* infections that may lead to misdiagnoses. In this sense, our results show the need for including detection procedures for both bacteria in public healthcare for accurate and quick diagnoses. Further, as samples were obtained in two restricted locations in the north side of the island of Tenerife, studies on the presence of *Bartonella* and *Rickettsia* in pets and domestic animals along the Archipelago should be carried out, including vectors involved in the spreading of these pathogens. Likewise, since animals included in this study were stray and animals that may be adopted from both urban and rural areas, routine procedures of detection in shelters should be carried out, as well as effective vector control, in order to reduce the spread of the pathogens and avoid health problems in both pets and their owners.

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