Prevalence of *Rickettsia* and *Bartonella* species in Spanish cats and their fleas

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ABSTRACT: The aim of this study was to determine the prevalence of *Bartonella henselae*, *Rickettsia felis,* and *Rickettsia typhi* in fleas and companion cats (serum and claws) and to assess their presence as a function of host, host habitat, and level of parasitism. Eighty-nine serum and claw samples and 90 flea pools were collected. Cat sera were assayed by IFA for *Bartonella henselae* and *Rickettssia* species IgG antibodies. Conventional PCRs were performed on DNA extracted from nails and fleas collected from cats. A large portion (55.8%) of the feline population sampled was exposed to at least one of the three tested vector-borne pathogens. Seroreactivity to *B. henselae* was found in 50% of the feline studied population, and to *R. felis* in 16.3%. *R. typhi* antibodies were not found in any cat. No *Bartonella* sp. DNA was amplified from the claws. Flea samples from 41 cats (46%) showed molecular evidence for at least one pathogen; our study demonstrated a prevalence rate of 43.3 % of *Rickettsia* sp and 4.4% of *Bartonella* sp. in the studied flea population. None of the risk factors studied (cat's features, host habitat, and level of parasitation) was associated with either the serology or the PCR results for *Bartonella* sp. and *Rickettsia sp.*. Flea-associated infectious agents are common in cats and fleas and support the recommendation that stringent flea control should be maintained on cats. ***Journal of Vector Ecology* 40 (2): 233-239. 2015**.

Keyword Index: Cats, fleas, *Bartonella henselae*, *Rickettsia felis, Rickettsia typhi.*

INTRODUCTION

Infections caused by species of *Bartonella* sp. and *Rickettsia* produce a broad group of zoonoses with a worldwide distribution and high prevalences in areas where conditions are most favorable for arthropod vectors (Chomel et al. 2006) Murine typhus is a worldwide zoonosis caused by *Rickettsia typhi*. Two cycles are involved in *R. typhi* transmission: a classical cycle that involves rats as a reservoir and *Xenopsylla cheopis* as a vector, and a peridomestic cycle, involving cats, dogs, peridomestic rodents, and their fleas, *Ctenocephalides felis*) (Gillespie et al. 2009). In Spain, some epidemiological studies have shown a seroprevalence of *R. typhi* of up to 18% in human populations (Santibáñez et al. 2009). Although the maintenance of *R. typhi* in the peridomestic cycle is of potential public health importance and a major health risk, the extent of exposure to this pathogen remains incompletely described in cats and their fleas. In Spain, only one study conducted in northeast Spain provides information related to *R. typhi* infection in cats and their fleas (Nogueras et al. 2013).

Rickettsia felis, also known as cat flea typhus, is an emerging clinical disease of humans worldwide, including Spain. *C. felis* is recognized as both a reservoir host and a main vector for *R. felis* (Reif and Macaluso 2009). *R. felis* has been found in cat fleas on all continents with different prevalences (Shaw et al. 2004, Hawley et al. 2007, Gilles et al. 2008, Giudice et al. 2014). In Spain, despite *R. felis* having also been detected in fleas, studies only processed fleas from dogs, facilities, and wild animals and usually involved few cats (Blanco et al. 2006, Márquez et al. 2006, Lledó et al. 2010, Nogueras et al. 2011). *R. felis* antibodies have been detected in serum of some cats, suggesting exposure may be relatively common (Case et al. 2006, Bayliss et al. 2009). However, in Spain, as far as we know, there are no data regarding cats.

Bartonella henselae is the main causal agent of cat scratch disease (CSD), and several other syndromes in humans (Chomel et al. 2004). In Spain, a seroprevalence of up to 24.7% has been reported (García-García et al. 2005). Domestic cats are the natural reservoir of *B. henselae* and play an important role as a source for human infection. *B. henselae* has a worldwide distribution in domestic cats (Boulouis et al. 2005). Exposure to *B. henselae* in cats from Spain has been evaluated in previous reports but sampling was restricted to only two areas (Pons et al. 2005, Solano-Gallego et al. 2006, Ayllón et al. 2012). *C. felis* is the main vector of *B. henselae* and their presence is essential for the maintenance of the infection within the cat population (Chomel and Kasten 2010). Although *Bartonella* sp. presence has been assessed in Spain in previous studies, fleas were sampled from small cat populations restricted to specific areas (Blanco et al. 2006, Márquez et al. 2009). Transmission of *B. henselae* by cat fleas occurs mainly through infected flea feces (Chomel and Kasten 2010). *B. henselae* can multiply in the digestive system of the cat flea and survive several days in the flea feces (Finkelstein et al. 2002). Therefore, the main source of infection appears to be the flea feces that are inoculated by contaminated cat claws into people, cats, or other animals when bitten or scratched. However, limited scientific data are available to support the role of cat claws in the transmission of *B. henselae* between cats, or to humans and there is little information available

concerning numbers of naturally exposed cats that have these organisms in their claws.

Cats and their arthropod parasites can be important sources of zoonotic diseases in humans and the prevalence rates can differ among cat populations and geographical locations. Determination of prevalence rates for *Bartonella* and *Rickettsia* species in cats and fleas would be useful for predicting the likelihood of infection in cats as well as for assessing potential zoonotic risks. In Spain, the information is scarce and comes from studies conducted in very specific areas. To provide further information concerning these organisms among cats living in Spain, the aim of this study was to determine the prevalence of *Bartonella* spp. and *Rickettsia* spp. in fleas and companion cats (serum and claws) living in different regions of the country and to assess their presence as a function of host, host habitat, and level of parasitism.

Figure 1. Map of Spain showing the location of regions (in grey) where cats were sampled (1. Andalucía, 2. Baleares, 3. Canarias, 4. Cataluña, 5. Galicia, 6. Madrid, 7. Murcia, 8. País Vasco, 9. Comunidad Valenciana).

MATERIALS AND METHODS

Cats and fleas

The survey was carried out on 89 cats belonging to 24 veterinary practices in nine regions in Spain (Figure 1). The veterinary practices collaborated voluntarily in the survey and only cats parasitized by fleas were used. The number of practices ranged from one to five per region and the number of cats ranged from one to eight per practice. The study took place from April, 2010 to August, 2011. Data recorded from each cat included age, sex, hair type, and breed. In addition to cat features, the following other habitat variables were recorded for each cat: 1. Location of the household - urban or rural/suburban. 2. Activity - partially or totally outdoors (access to a garden, street, or other outside environment) or entirely indoors. 3. Multi-pet households - whether residence was shared with other pets. 4. Contact with other pets - whether cats had contact with other pets in the neighborhood. 5. Period of the year - whether the cat was sampled in the warm period (May-October) or the cold period (November-April). 6. Information related to previous treatments - product and frequency of use. After the owner had been informed and consent obtained, fleas, blood samples, and nails (4-5) were collected from each cat.

The flea index was calculated as the number of fleas per host on day 0. The examining veterinarian performed a total flea count according to a defined procedure (Marchiondo et al. 2007). From one to eight fleas from each cat were collected for analysis. The collected serum, cat nails, and flea samples were stored at -20° C in the veterinary hospitals, from where they were air freighted on dry ice and transferred to the Department of Animal Pathology, (Zaragoza University, Spain) and stored at -70° C until assay. The collected fleas were identified to species using the key of Beaucournu and Launay (1990).

R. typhi, R. felis, and *Bartonella henselae* serology

Serum samples were thawed at room temperature and assayed by indirect inmunofluorescence assay (IFA) for *Bartonella henselae* and *Rickettsia* species IgG antibodies. *Rickettsia typhi* IgG and *Rickettsia felis* IgG antibodies in serum were detected by IFA using an in vitro assay (MegaScreen®FLUORICKETTSIA typhi and MegaScreen®FLUORICKETTSIA felis, respectively) with a 1:16 threshold. Following the kit instructions, titers <1:16 were considered negative, while titers ≥1:16 were considered positive for both assays. Positive and negative controls were provided by the kit. *Bartonella henselae* IgG antibodies in serum were detected by IFA using an *in vitro* assay (MegaScreen®FLUOBARTONELLA henselae) with a 1:50 threshold. Following the kit's instructions, titers <1:50 were considered negative and titers ≥1:50 were considered positive. Positive and negative controls were provided by the kit.

Table 1. Primers used in the study.

Organism	Primer	Sequence (5'-3')
Bartonella spp.[a]	Bart/16-23F	bio-TTGATAAGCGTGAGGTCGGAGG
	Bart/16-23R	bio-CAAAGCAGGTGCTCTCCCAG
Rickettsia spp.[b]	RCK/23-5-F	CTCAAAACTAGACTTCCCYATTAG
	RCK/23-5-R	TCGGGATGGGATCGTGTGTTTC

[a]García-Esteban et al. 2008.
[b]Jado et al. 2006.

DNA extraction and PCR assays (in pool fleas and cat nails)

Flea samples were processed once they were thawed. DNA was extracted from samples of one to five fleas depending on the number of fleas collected from each animal. Samples containing more than one flea were pooled. A total of 90 pools from 468 cat fleas (89 pools of *Ct. felis* and one of *E. gallinacea*) from 89 cats were used for analysis. Fleas in each pool were cut with individual scalpels and macerated using individual pestles and mortars, and every effort was taken to minimize the generation of aerosols that could result in sample cross-contamination. DNA from cat claws (as potential *Bartonella* spp. DNA carriers) and fleas (as potential *Bartonella* spp. and *Rickettsia* spp. DNA carriers) was extracted with a High Pure PCR Template Preparation Kit (Roche). PCR assays used to amplify DNA on all DNA extracts were performed as previously described, whether for *Bartonella* (García-Esteban et al. 2008) by targeting the 16S-23S internal transcribed spacer or *Rickettsia* species by targeting the 23S-5S internal transcribed spacer (Jado et al. 2006) with a modified forward primer for *Rickettsia*, following the instructions (Table 1). A Bio-Rad MJ Mini thermal cycler was used. The expected amplicons comprised 438 bp and 434 bp, respectively. One negative water control and one positive control (*B. henselae* and *R. typhi*) were included in every reaction. The *Bartonella/Rickettsia* genus PCR assay gives a specific band size for each genus, so positive samples were not characterized by genetic sequencing. Results were considered positive only if a band of the expected *Bartonella/Rickettsia* genus size was detected. The determination of sensitivity of the technique was performed by serial ten-fold dilutions of these DNAs (positive control). The recorded sensitivity to *Bartonella* sp and *Rickettsia* sp. was 10^{-2} and 10^{-4}, respectively. A previous PCR was performed using a mixture of DNA extracted from each claw or flea pool with the previously referred detectable minimum concentration of target DNA from each control to assess the absence of inhibition.

Statistical analysis

Associations of serological prevalence or DNA amplification with cat features, habitat variables, flea index, and region were explored for each pathogen by fitting logistic models in which the presence of antibodies or amplified DNA were the dependent variables and the remaining variables the predictor ones. Moreover, in order to explore the relationship between serological prevalence and DNA amplification within each pathogen, the presence of antibodies was also included as a predictor in the initial model when presence of amplified DNA was the dependent variable and vice-versa. Initial models were refined by a backward-stepwise algorithm to obtain the refined final models. Wald $P = 0.05$ was used as the threshold value to enter or remove variables during step-wise procedures.

RESULTS

Habitat and cat features

A total of 89 cats were sampled. Of the 89 cats, 47 were males and 42 were females. Of the 83 cats whose age was known, 16 (19.3%) were 1-12 months-old, 28 (33.7%) were between one and two years-old, 20 (24.1%) were between two and five years-old, 10 (12%) were between six and nine years-old and nine (10.8%) were between ten and thirteen years-old. Most cats were European (57 cats), followed by hybrid breeds (15), Siamese (nine) and Persian (eight). Seventy-one cats had short hair, ten medium length and eight were long-haired. Of the 89 cats, 42 (47.2%) lived in a rural/suburban location, 12 (13.5%) had entirely indoor activity, ten (11.2%) shared an abode with other pets, and 51 (57.3%) had contact with other pets. Sixty-three cats (70.8%) were surveyed in the warm period (May-October) and twenty-six (29.2%) in the cold period (November-April). Out of the 41 owners, 46.3% who gave information about the frequency of treatment against fleas treated every one to three months, 7.3% treated every three to six months, 19.5% treated only in summer time and, finally, 26.8% treated when the owner deemed it appropriate.

Flea identification

A total of 458 fleas were collected from 89 cats. *Ct. felis* was the most abundant (448 fleas, 98.9% of the total), while *E. gallinacea* was only recorded in one cat (five fleas, 1.1% of the total). Five fleas were not identified.

Serology and PCR results

Serology and PCR results are show in Table 2. A total of 86 serum samples were collected from 89 cats. Forty-eight (55.8%) out of the 86 cat serum samples were seropositive for at least one pathogen and nine (10.5%) were seropositive for both pathogens. The prevalence of *Bartonella henselae* IgG antibodies in serum was 50%. The number of seropositive cats in each hospital ranged from none (seven clinics) to 100% (three clinics). The prevalence of *R. felis* IgG antibodies in serum reached 16.3%. The number of seropositive cats in each hospital ranged from none (in 15 clinics) to 100% (in one clinic). None of the 86 cat sera showed IgG antibodies against *R. typhi*. Out of 24 hospitals sampled, (75%) had seropositive samples for at least one pathogen and eight (33.3%) had positive samples for both pathogens. Seropositive hospitals were located in all regions studied (*Rickettsia* spp. in five regions; *Bartonella* spp. in the nine regions studied).

DNA of at least one organism was amplified from 41 (45.5%) out of the 90 flea pool sets, (one flea set per cat with *Ct. felis* and one flea set with *E. gallinacea* from one cat), and two (2.2%) were infected with two of the studied pathogens. *Rickettsia* spp. DNA was amplified in 39 (43.3%) flea pools. The number of positive flea pools in each hospital ranged from none (in eight clinics) to 80% (in three clinics). Four flea pool sets (4.4%) were positive for *Bartonella* spp. The number of positive flea pools in each hospital ranged of none (in 20 clinics) to 25% (in one clinic). DNA was amplified from *Ct. felis* pools but not from *E. gallinacea*. Sixteen out of the 24 sampled hospitals had positive flea pool sets for at least one pathogen and four (16.6%) had positive samples for both pathogens. Positive flea pools were located in all the regions studied (*Bartonella* spp. in four regions and *Rickettsia* spp. in

Table 2. Distribution of seroprevalence and DNA results of *Bartonella* spp. and *Rickettsia* spp. in feline and cat fleas samples from different regions of Spain.

	No. (%) positive sera			No. (%) positive PCR		
	R. felis	***R. typhi***	***B. henselae***	***Rickettsia* spp. (fleas)**	***Bartonella* spp. (fleas)**	***Bartonella* spp. (nails)**
Andalucía Clinics: n=5 Cats: n=19	0 (0%)	0 (0%)	9 (47.4%)	7 (36.8%)	1 (5.3%)	0 (0%)
Baleares Clinics: n=2 Cats: n=9	2 (22.2 %)	0 (0%)	4 (44.4%)	5 (55.5%)	0 (0%)	0 (0%)
Canarias Clinics: n=5 Cats: n=22	4 (18.2%)	0 (0%)	13 (59.1%)	8 (36.4%)	1 (4.5%)	0 (0%)
Cataluña Clinic: n=3 Cats: n=10	3 (30%)	0 (0%)	2 (20%)	4 (40%)	0 (0%)	0 (0%)
Galicia Clinics: n=3 Cats: n=12	4 (33.3%)	0 (0%)	8 (66.6%)	7 (58.3%)	1 (8.3%)	0 (0%)
Madrid Clinics: n=3 Cats: n=6	0 (0%)	0 (0%)	1 (16.7%)	2 (33.3%)	0 (0%)	0 (0%)
Murcia Clinics: n=1 Cats: n=2	1 (50%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	0 (0%)
País Vasco Clinics: n=1 Cats: n=5	0 (0%)	0 (0%)	4 (80%)	2 (40%)	0 (0%)	0 (0%)
Comunidad Valenciana Clinics: n=1 Cats: n=4	0 (0%)	0 (0%)	1 (25%)	3 (75%)	1 (25%)	0 (0%)
TOTAL 9 Regions 24 Clinics 89 Cats	**14(16.3%)**	**0 (0%)**	**43 (50%)**	**39 (43.3%)**	**4 (4.4%)**	**0(0%)**

the nine regions studied). For *Bartonella* spp. analysis, a total of 86 nail samples were collected from 89 cats. *Bartonella* spp. DNA was not amplified from any cat nail samples. Two (4.6%) out of the 43 *B. henselae*-seropositive cats had *Bartonella*-positive fleas. One cat had seronegative *B. henselae*, but *Bartonella*-positive fleas. Five (35.7%) out of the 14 *R. felis*-seropositive cats had *Rickettsia*-positive fleas. Thirty-two cats had seronegative *R. felis* but *Rickettsia*-positive fleas.

Factors associated with *Bartonella* spp. and *Rickettsia* spp. infections

None of the predictor variables was retained in any of the final logistic models, therefore no relationship was detected between factors studied (level of parasitism or flea index, cat features, host habitat, and region) and the serology and PCR results for *Bartonella* and *Rickettsia*. Moreover, no correspondence between presence of antibodies and DNA amplification was found at host level.

DISCUSSION

A large portion (55.8%) of the cat population sampled could potentially be exposed to at *B. henselae or R. felis* (50% and 16.3%, respectively), whereas no evidence for *R. typhi* was found. The *B. henselae* prevalence rate (50%) in the serum of cats found in this study suggests the exposure to the pathogen in the main reservoir of this microorganism. Our results are similar to those obtained in seroprevalence studies carried out in the U.S.A. (Lappin and Hawley 2009, Sykes et al. 2010) and Holland (Bergmans et al. 1997), while higher than those reported previously in Spain (29.6% and 23.8%) (Pons et al. 2005, Ayllón et al. 2012), Scotland (15.3%)

(Bennet et al. 2011), U.S.A. (14.7%, 17%) (Case et al. 2006, Sykes et al. 2010) and Argentina (17.8%) (Cicuttin et al. 2014) and lower (71.4%) than the ones reported by Solano-Gallego et al. (2006) in a report carried out in northeast Spain. *B. henselae* has a worldwide distribution in domestic cats but with varying antibody and infection prevalence rates according to geographical location, status (pet or stray), and the assays used (Boulouis et al. 2005). The organism is transmitted among cats by *C. felis,* so variables that influence flea epidemiology also have influence in the *Bartonella* spp. prevalence.

It is well established that there is an increased risk of *B. henselae* infection in cats parasitized by fleas (Chomel et al. 2006) and there is higher seroprevalence in older cats than in younger animals (Guptill 2010). In our study, all the cats were parasitized with fleas but no association was found with seropositivity, flea abundance, cat's features, or host habitat. The observed 4.4% prevalence of *Bartonella* spp. DNA in flea pools in this trial is similar to a previous study carried out in Spain (3.4%) (Blanco et al. 2006) and lower than those obtained from France (26.2%) (Rolain et al. 2003), United Kingdom (17%) (Shaw et al. 2004), Spain (13.5%) (Márquez et al. 2009) and U.S.A. (22.8%) (Lappin et al. 2006). In our work, 50% of the feline studied population was seropositive, but the presence of *Bartonella* DNA in flea samples was not similar. Discrepancy between IgG seroprevalence in cats and detectable pathogen DNA in fleas is not unusual. Bacteremia is common in infected cats for weeks, months, and even for more than a year (Abbott et al. 1997, Kordick et al. 1999) and likely facilitates vector transmission (Guptill 2010). However, measuring serum antibodies has a limited value for determining whether a cat has an active *Bartonella* infection, since the antibody levels have a time lag with respect to acute infection. Thus, bacterial burden can increase while the immune response begins, develops, and gets to reach proper effectiveness. This means that the quantification of the immune response can fail to determine the status of an infection, even more in case of acute infections with rapid development of bacterial burdens. Furthermore, no cut-off values allowing use of serologic testing to determine whether a cat is currently infected with *Bartonella* have been established (Yamamoto et al. 2003).

One possibility to explain the discrepancy between the high seroprevalence of cats and the low DNA detection in the fleas found on those cats might be that the burden of bacterial DNA in fleas was insufficient for its detection by the techniques used in this study (our detection threshold is above 10^{-2}). Another possibility is that these cats could have acquired the fleas from other uninfected cats, and thus there was not enough time for sufficient bacterial multiplication inside the flea following their recent feeding on infected cats. *Bartonella* spp. was not amplified from cat claws in this study. Our results may reflect the lack of current infection in fleas (only 4.4%), as nail samples were processed together with internal control to discard a potential inhibition. However, the failure to amplify *Bartonella* sp. from feline claws has been reported in other studies (Bennet et al. 2011) while in previous reports, *Bartonella* spp. DNA was, in fact, amplified from claws (Lappin and Hawley 2009)

Rickettsia typhi is distributed worldwide, and could be widely distributed in our country (Santibáñez et al. 2009). However, all the 86 sera from our cats were negative to *R. typhi* in the present survey. Why *R. typhi* was not detected in this study is unclear. Based on the results of the study described herein, it appears unlikely that domestic cats are an effective reservoir host for *R. typhi* and it is possible that other animals work as reservoir hosts for *R. typhi* (Eisen and Gage 2012). However, 15.8% prevalence in naturally infected cats was found in a recent study carried out in northeast Spain (Nogueras et al. 2013). This discrepancy between the two studies might be due to the different origin of the cats involved (veterinary clinics vs veterinary clinics, shelters, and the street) or the sample area. A high variability in prevalence (none vs 62.9%) was observed according to the area sampled (Nogueras et al. 2013). It is important to maintain a high level of surveillance for this infectious disease.

To our knowledge, this is the first report of *R. felis* seroprevalence in naturally infected cats in Spain. In our study, *R. felis* antibodies were detected in 14 out of 86 sera (16.3%), data similar to previous reports from the U.S.A. (Case et al. 2006, Bayliss et al. 2009). There was no significant relationship between seropositivity rates and any of the surveyed items. *R. felis* has been identified in flea vectors in different countries including Spain, with prevalences between 20 and 70% (Shaw et al. 2004, Hawley et al. 2007, Gilles et al. 2008, Giudice et al. 2014, Blanco et al. 2006, Márquez et al. 2006, Lledó et al. 2010, Nogueras et al. 2011). *Rickettsia* sp DNA was amplified from 43.3% of flea pools in this study. Unfortunately, we lack information regarding the species found in cat fleas to confirm that these prevalence data correspond to *R. felis*. Nevertheless, since fleas assessed in this study were collected directly from cats, and due to negative results for *R. typhi*, we could assume that this pathogen is *R. felis*. In any case, since 43.3% of flea pools collected from cats were positive for *Rickettsia* sp. DNA, this pathogen may mean a serious risk to human health in Spain.

As fleas are hematophagous, the detection of pathogens in fleas may reflect components of both host and vector infection. The discrepancy between the seroprevalence of cats and the frequency of DNA detection in the fleas found on those cats suggests that these cats could have acquired the fleas from other infected cats, and thus there was not enough time for sufficient bacterial multiplication in the new host. Moreover, detection of antibodies does not necessarily imply that an active infection is present since serological studies only document exposure to infection, and therefore it is important that they do not effectively correlate with PCR surveys. Research on colony cats exposed to fleas known to be carriers of *R. felis* indicated they became seropositive by the fourth month post-exposure (Wedincamp and Foil 2000). *R. felis* can be maintained within populations of fleas by vertical transmission and can maintain infections for at least 12 generations without feeding on an infected host (Wedincamp and Foil 2002). Therefore, arthropod vectors also act as reservoirs of *Rickettsia* (Parola et al. 2005).

In conclusion, although the sample was very small from

different locations, our results show that a large portion of the cat and flea populations sampled was exposed to at least one of the three tested vector-borne pathogens. This information is of potential public health importance and a major health risk. In this study, a large population of cat fleas were collected and analyzed, but it was not possible to obtain specific information about species found in cat fleas and additional studies will be required to determine the species of pathogens detected in flea pools. Finally, due to the potential for these agents to cause illness in cats or humans, these results support the recommendation that flea control for cats is highly recommended in the studied area to decrease the exposure of cats and people to pathogens harbored by fleas.

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