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Coexistence of *Bartonella henselae* and *B. clarridgeiae* in populations of cats and their fleas in Guatemala

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Abstract

Cats and their fleas collected in Guatemala were investigated for the presence of *Bartonella* infections. *Bartonella* bacteria were cultured from 8.2% (13/159) of cats, and all cultures were identified as *B. henselae*. Molecular analysis allowed detection of *Bartonella* DNA in 33.8% (48/142) of cats and in 22.4% (34/152) of cat fleas using *gltA*, *nuoG*, and 16S–23S internal transcribed spacer targets. Two *Bartonella* species, *B. henselae* and *B. clarridgeiae*, were identified in cats and cat fleas by molecular analysis, with *B. henselae* being more common than *B. clarridgeiae* in the cats (68.1%; 32/47 vs 31.9%; 15/47). The *nuoG* was found to be less sensitive for detecting *B. clarridgeiae* compared with other molecular targets and could detect only two of the 15 *B. clarridgeiae*-infected cats. No significant differences were observed for prevalence between male and female cats and between different age groups. No evident association was observed between the presence of *Bartonella* species in cats and in their fleas.

Keywords

Cats; cat fleas; *Bartonella* ; *B. henselae* ; *B. clarridgeiae* ; Guatemala

INTRODUCTION

At least three *Bartonella* species, *B. henselae, B. clarridgeiae*, and *B. koehlerae*, are associated with cats. With a worldwide distribution, *B. henselae* is the most common of the three species with a considerable variation in prevalence observed across different regions (Chomel et al. 1995, 2002, Bergmans et al. 1996, Branley et al. 1996, Maruyama et al. 2001). *B. clarridgeiae* is also reported throughout most temperate regions of the world (Heller et al. 1997, Chomel et al. 1999, Marston et al. 1999, Maruyama et al. 2000), while *B. koehlerae* is less common compared with the other two species (Droz et al. 1999). *Bartonella* infections are more likely in younger cats (<1 year old) (Chomel et al. 1995). Two main

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genotypes of *B. henselae* (Houston I and Marseille) have been identified based on 16S rRNA gene sequences (Bergmans et al. 1996, La Scola et al. 2002). The respective prevalence of these two genotypes varies considerably among cat populations from different geographical areas. *B. henselae* Marseille is the dominant type in cats from western U.S.A., Australia, and most of Europe, whereas Houston I represents the majority of *B. henselae* isolates in cats from the eastern U.S.A. and East Asia (Boulouis et al. 2005). Epidemiological evidence and experimental studies have shown that the cat flea (*Ctenocephalides felis*) plays a major role in the transmission of *B. henselae* and *B. clarridgeiae* among cats (Chomel et al. 1996). Cats infected with *B. henselae* and other *Bartonella* species are typically asymptomatic with a persistent bacteremia lasting from several months to years (Koehler et al. 1994, Abbott et al. 1997). *B. henselae* is responsible for various human infectious diseases, including vasoproliferative illness (bacillary angiomatosis), hepatosplenic granulomatosis, peliosis hepatitis, fever, central nervous disorders, and, most commonly, cat scratch disease (CSD) (Welch et al. 1992, Branley et al. 1996). Recently, *B. henselae* has been identified as the causative agent of infective endocarditis in Thailand (Pachirat et al. 2011, Watt et al. 2014). *B. clarridgeiae* also has been reported as a causative agent of cat scratch disease (Kordick et al. 1997, Margileth and Baehren 1998), as well as other diseases (Sander et al. 2000).

In Guatemala, *Bartonella* infections are prevalent in cattle and bats (Bai et al. 2011, 2013). However, cats and their fleas have not been assessed for *Bartonella* infections in this country. Considering the ubiquity of cats, their association with humans, and the distribution of *Bartonella* species, it is important to estimate the status of *Bartonella* infections in local populations of cats and cat fleas in Guatemala. This in turn can provide information for estimating the risk of acquiring cat-originated *Bartonella* species by people. The present study aimed to identify *Bartonella* species using both blood culture and molecular detection in cats and their fleas, and determine its prevalence.

MATERIALS AND METHODS

Sample collection

Cats from pet clinics or neutering and spaying campaigns conducted in seven sites within Guatemala were recruited to the study during January, 2013 and August, 2013. Cat fleas were collected in 70% alcohol; cats were recorded for gender, age, weight, clinical symptoms, and flea infestation status. Collected blood was stored at −70° C until processing.

Isolation of *Bartonella* bacteria from cat blood

Cat blood was thawed at 4° C and re-suspended 1:4 in brain heart infusion broth supplemented with 5% amphotericin B (1μg/ml) for the purpose of reducing fungal contaminants. Then 100 μl diluted blood (25 μl whole blood) was plated on heart infusion agar containing 10% rabbit blood and incubated in an aerobic atmosphere with 5% carbon dioxide at 35° C for up to four weeks. Bacterial growth was monitored at the end of each week. Bacterial colonies were presumptively identified as *Bartonella* based on colony morphology. Subcultures of *Bartonella* colonies from the original agar plate were streaked

onto secondary agar plates and incubated at the same conditions until sufficient growth was observed. Pure cultures were harvested in 10% glycerol.

Confirmation and multi-locus sequence typing (MLST) of *Bartonella* isolates

Crude genomic DNA was prepared by heating a heavy suspension of pure culture for 10 min at 95° C followed by centrifugation of the lysed cells for 1 min at 3,000 rpm. The supernatant was then transferred to a clean centrifuge tube to be used as the template DNA. All isolates obtained from the blood were first verified as *Bartonella* species by amplifying and sequencing a specific region in the *gltA*, and then further characterized by six additional targets, including *ftsZ*, *nuoG*, *ribC*, *rpoB*, *ssrA*, and 16S–23S internal transcribed spacer (ITS), using primers that have been previously applied (Bai et al. 2013). All positive PCR products were purified using Qiagen QIAquick PCR Purification Kit (Qiagen, MD) and sequenced in both directions using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The obtained sequences were aligned by each locus and compared among the isolates and with other known *Bartonella* species using the Lasergene software package (DNASTAR, Madison, WI). Based on the allelic profile, each unique combination for the isolates was designated as a sequence type (ST) and sequences for the seven loci were concatenated.

Molecular detection and identification of *Bartonella* species in cat blood and cat fleas

Cat blood DNA was extracted using the Qiagen QIAamp kit following the blood protocol. To determine what targets to apply, a pilot study on 48 cat samples from the present study was first conducted using nested *gltA* and the other PCR targets applied to characterization of the *Bartonella* isolates. The pilot study indicated that nested *gltA*, conventional *nuoG*, and ITS PCRs were more sensitive than the other targets (data not shown). The nested *gltA* was performed using the primer for the isolates characterization as the outer primer, and then Bhcs.781p and Bhcs.1137n (Norman et al. 1995) as the inner primers. For flea DNA preparation, individual fleas were first triturated using a bead beater protocol (Halos et al. 2004) and then processed following the Qiagen tissue protocol. Flea DNA was tested for ITS and *gltA* (using the same primers as used for isolates characterization). All positive cat blood and fleas were subject to sequencing as described above for *Bartonella* species identification.

RESULTS

Cats and fleas

In total, blood was collected from 160 cats, consisting of 84 females and 64 males (12 cats were missing gender information). Cat ages varied from one month to seven years old, with 65 cats of <1 year old, 66 cats of 1 to 4 years old, five cats >4 years old (24 cats had no age information). Flea infestation was observed in 71 cats, from which 152 fleas were collected with ranges of 1 to 12 fleas per cat. All fleas were subsequently identified as cat fleas (*Ctenocephalides felis*). Seventy-seven cats were free from flea infestations, and 12 cats had no flea infestation information.

Bartonella culturing and MLST characterization of the isolates

Of the 160 blood samples, 159 were cultured for *Bartonella*. *Bartonella*-like bacteria were observed on agar inoculated with 13 (8.2%) samples after one to two weeks post-inoculation. Bacteremia levels varied from 40 to 1,480 CFU per milliliter of blood. PCR amplification of *gltA* confirmed all 13 isolates as *Bartonella* species. The *gltA* sequences showed that all of these isolates belonged to *B. henselae* Houston type I. The sequences were close to each other (99.7% similarity) and were identical by the *gltA* PCR assay to two previously identified variants [GenBank: AJ439406 and NC005956]. Characterization of the isolates with the other six targets (*ftsZ*, *nuoG*, *ribC*, *rpoB*, *ssrA*, and ITS) further confirmed that all of these isolates are *B. henselae* Houston type I, with identification of four *ftsZ* variants, three *nuoG* variants, two *ribC* variants, four *rpoB* variants, and four ITS variants. All isolates were invariant by *ssrA* and identical to a previously described variant [GenBank:JN029785]. The isolates were of five sequence types based on the MLST allelic profile (Table 1), with divergence of 0.1 to 0.4% among all STs. Novel variants of each target were submitted to GenBank with the following GenBank accession numbers: KP822810 to KP822812 (*ftsZ*), KP822813 (*nuoG*), KP822814 to KP822815 (*ribC*), KP822816 to KP822819 (*rpoB*), and KP822820 to KP822821 (ITS).

Molecular detection and identification of *Bartonella* species in cat blood

Molecular detection using nested *gltA*, *nuoG*, and ITS was applied to 142 of the 160 blood samples based on sample availability for *Bartonella* infection. A total of 48 (33.8%) were positive for *Bartonella* DNA for at least one of the three tested targets, showing a significant higher detection rate when compared to culturing (χ^2=24.3, p<0.001). Of the 48 positive samples, 27 were positive for all three targets; 15 samples were positive for either two of the three targets; and six samples were positive for a single target. By target, *Bartonella* species was detected in 44 (31.0%), 44 (31.0%), and 29 (20.4%) by nested *gltA*, ITS and *nuoG*, respectively. Of the 13 culture positive samples, blood DNA was available for ten samples. All three targets were positive for *Bartonella* species in nine of the ten samples, but none of the targets was amplified in one sample which presented a bacteremia of 40 CFU. For all positive samples, there is no statistical difference with respect to either gender or age (p > 0.05).

Sequences were obtained for 47 of the 48 *Bartonella*-positive samples by one or more targets. Two *Bartonella* species, *B. henselae* and *B. clarridgeiae*, were identified among the sequences, with 32 (68.1%) of them as *B. henselae* and 15 (31.9%) as *B. clarridgeiae*. For the 32 *B. henselae* infected samples, 21 were confirmed by all three targets, seven were confirmed by two targets (including two by ITS and nested *gltA*, three by *nuoG* and ITS, and two by *nuoG* and nested *gltA*), and four were confirmed by a single target (including two by nested *gltA* and two by ITS). By target alone, *B. henselae* was confirmed in 28, 27, and 26 samples by ITS, nested *gltA*, and *nuoG*, respectively. Genetic variants identified in cat blood were the same as those in cultures by each of the three targets. For the 15 *B. clarridgeiae*-infected samples, two samples were confirmed by all three targets, 11 were confirmed by both ITS and nested *gltA*, and two were confirmed only by one target, either nested *gltA* or ITS. In fact, the two samples confirmed by all three targets were the only samples that were amplified by *nuoG* among the 15 *B. clarridgeiae* samples. The sequences

of all *B. clarridgeiae* positive samples were invariant for each target, and all were previously described with GenBank accession numbers as KC331017, KC331014, and FN645454 for *gltA*, ITS, and *nuoG*, respectively.

Molecular detection and identification of *Bartonella* species in cat fleas

Molecular detection of *Bartonella* infection using *gltA* and ITS was applied to the 152 fleas collected from 71 cats. Thirty-four fleas (22.4%) collected from 19 cats were positive for *Bartonella* by at least one of the tested targets. Among the positive fleas, 24 fleas were positive by both *gltA* and ITS, nine fleas were positive by ITS, and one flea was positive by *gltA* alone.

Sequences were obtained from 32 fleas, either *gltA* or ITS or both. Sequencing analysis demonstrated the fleas were infected with the same two *Bartonella* species, *B. henselae* and *B. clarridgeiae*, as found in cats, with 18 fleas (from seven cats) infected with *B. henselae* and 14 fleas (from 11 cats) infected with *B. clarridgeiae*. Of the 18 fleas with *B. henselae*, 11 fleas were confirmed by both *gltA* and ITS, six by ITS, and one by *gltA*. The sequences for *B. henselae* were of the same two variants for *gltA* and three of the four ITS variants, which were identified in cats. Of the 14 fleas with *B. clarridgeiae*, nine fleas were confirmed by both *gltA* and ITS; the other five fleas were confirmed by ITS alone. All ITS sequences and *gltA* sequences for *B. clarridgeiae* were identical to those identified in cats.

Relationships of *Bartonella* infection between cats and flea infestations

Flea infestation information was recorded in 132 cats, with 65 cats infested and 67 cats not infested. *Bartonella* was detected in 40% (26/65) of cats infested with fleas, and in 30.0% (20/67) of cats not infested with fleas. *Bartonella* infection in cats did not show any significant correlation to flea infestation status (χ^2=0.98, p=0.32).

Of the 48 cats that had *Bartonella* infection in the study, 22 of them were flea-infested; the other 26 were free from fleas. Fleas from 8 of the 22 flea-infested cats were *Bartonella-positive*, but the rest (14 cats) were *Bartonella*-negative. Of the 19 cats that had positive fleas (see previous section), blood specimens were available for 13 cats and for *Bartonella* testing. Eight of them were positive and the rest of the cats were negative. Six cats were infected with the same *Bartonella* species as their fleas, with *B. henselae* in four cats and their fleas, and *B. clarridgeiae* in two cats and their fleas; two cats were infected with *B. henselae* but their fleas were infected with *B. clarridgeiae*.

DISCUSSION

Using both culturing and molecular detection by PCR directly in blood, we report the presence of *Bartonella* infections in cats and their fleas from Guatemala. Similar to reports from other regions (Chomel et al. 1995, 1999, 2002, Bergmans et al. 1996, Branley et al. 1996, Heller et al. 1997, Marston et al. 1999, Maruyama et al. 2000, 2001), *Bartonella* infections were prevalent in cats in this country. Nevertheless, the prevalence estimated by molecular detection (33.8%) was significantly higher than by culturing (8.2%). It is not surprising that a molecular approach is more sensitive than culturing, but the molecular approach does not provide evidence of viable bacteria in animal samples. In all 13 culture-

positive cats, bacteremia levels were quite low (40 to 1,480 CFU per milliliter). The observation of low concentrations of *Bartonella* bacteria in cat blood can explain the overall low success of culture. Alternatively, the growth requirement for the bacteria may not be met by the media. Prevalence of infection between male and female cats, as well as in different age groups, showed no significant differences between the groups compared.

Two *Bartonella* species, *B. henselae* and *B. clarridgeiae*, were identified in the cats and their fleas, with *B. henselae* more common than *B. clarridgeiae*. Interestingly, all cultures obtained from cats exclusively were of *B. henselae*. It is unknown why no *B. clarridgeiae* was cultured from any cats. Possibly a very low bacteremia level caused by this particular species limited its detection by culturing. However, *B. clarridgeiae* may possess some special biological characteristics or requirements that affect the growth of the bacterium on the agar that prevented culture. Results from a recent study by Zhu et al. (2014) suggests that *Bartonella* species forming a phylogenetic group (lineage-3), to which *B. clarridgeiae* belongs, lack the *gpsA* and other metabolically related genes that are important in the phospholipid pathway. Other studies reported that the *Bartonella* bacteria in lineage-3 are difficult to isolate and culture in artificial medium (e.g., blood agar, BACTEC) compared to *Bartonella* of other lineages (Podsiadly et al. 2007) but are readily detected by PCR (Zhu et al. 2014). Noticeably, PCR using *nuoG*, ITS, and nested *gltA* showed sensitivities to these three targets and were comparable in detecting *B. henselae* in cats; however, *nuoG* was less sensitive in detecting *B. clarridgeiae* compared to the other two targets.

Using the MLST platform, we further demonstrated that all *B. henselae* isolates obtained in the cats belonged to the Houston type I group, suggesting that it is the major genotype in Guatemala. As the sample size, as well as the investigated area, is relatively small, further studies are required to support this assumption. Although all identified genotypes belong to the same type, our MLST analysis allowed us to distinguish five sequence types among the isolates. The genetic differences demonstrated by MLST may help to identify a link between human cases and their cat sources.

Although cat fleas were frequently infected with both *B. henselae* and *B. clarridgeiae*, we could not demonstrate an association between occurrences of *Bartonella* in fleas and their cat hosts. A *Bartonella*-infected cat may or may not be infested with fleas and, if infested, the fleas could be either positive or negative for *Bartonella* infection. On the other hand, fleas collected from positive cats were not always positive. Similar observations were reported in some other studies (Morway et al. 2008, Tsai et al. 2011, Gutiérrez et al. 2014). These observations are challenging considering the well-documented role of fleas in transmitting *Bartonella* bacteria among cats (Chomel et al. 1996). It is possible that *Bartonella* infections may persist in both cats and fleas as observed in rodents (Kosoy et al. 2004, Bai et al. 2011). After infecting their hosts, *Bartonella* bacteria may cause a persistent bacteremia in cats at an undetectable level. The bacteremia level may cyclically fluctuate, occasionally reaching detectable levels of bacteremia. In such a scenario, it would be hard to notice any evident correlation of the infection in cats and their fleas. Also, we cannot exclude alternative modes of transmission, such as cat bites and scratches, which might contribute to the lack of correlation between prevalence of *Bartonella* infection and infestation of fleas in cats. *Bartonella henselae* is responsible for most CSD cases in

America and across the world. It also causes other clinical manifestations. While most cats are asymptomatic after becoming infected with *B. henselae*, they serve as reservoirs of the agent and transmit the infection to humans. Data on prevalence of CSD in Guatemala are limited, but people commonly come into contact with cats and are potentially at risk for cat-borne diseases, including CSD. The presence of *B. clarridgeiae* in cats and cat fleas suggests the need to include this agent when testing clinical samples from human cases suspected for CSD along with *B. henselae*.

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Table 1.

Allelic profiles and sequence types (ST) for the 13 *Bartonella* isolates obtained from cats in Guatemala.

Isolate	ftsZ	gltA	nuoG	ribC	rpoB	ssrA	ITS	ST
B40683	1	1	2	2	2	1	1	ST1
B40684	1	1	2	2	2	1	1	ST1
B40885	1	1	2	2	2	1	1	ST1
B40915	1	1	2	2	2	1	1	ST1
B40916	1	1	2	2	2	1	1	ST1
B40888	2	2	3	1	3	1	2	ST2
B40887	2	2	3	1	3	1	2	ST2
B40575	3	1	2	2	1	1	3	ST3
B40917	3	1	2	2	1	1	3	ST3
B40918	3	1	2	2	1	1	3	ST3
B40914	4	2	1	2	4	1	1	ST4
B40577	4	2	1	2	4	1	4	ST5
B40919	4	2	1	2	4	1	4	ST5