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Bartonella species antibodies and DNA in cerebral spinal fluid of cats with central nervous system disease

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Bartonella species infection is associated with central nervous system (CNS) disease in some humans and cats but the diagnosis is difficult to confirm with blood or serum test results. In this retrospective study of 100 client-owned cats, serum and cerebral spinal fluid (CSF) were assayed for *Bartonella* species IgG antibodies and CSF was assayed for *Bartonella* species DNA. *Bartonella* species IgG antibodies were detected in serum of 36 cats, *Bartonella* species *C*-values > 1 (suggesting antibody production by the CNS) were detected in CSF of 11 cats, and *B henselae* DNA was amplified from the CSF of 10 cats. While the clinical significance of these findings cannot be assessed without a control group, the development of neurological signs in some cats inoculated with *B henselae* and the results of this study warrant prospective evaluation of the association of *Bartonella* species with feline CNS disease.

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*B*artonella species are aerobic, Gram-negative pleomorphic bacteria responsible for a number of diseases in cats and humans (Carithers 1985, Carithers and Margileth 1991). In the United States, cats are most commonly infected with *B henselae* or *B clarridgeiae* (Lappin et al 2005, Brunt et al 2006). Common manifestations of disease in humans associated with *B henselae* infection include regional lymphadenopathy with mild clinical signs of fever, lethargy and occasionally abscessed lymph nodes (cat scratch disease). Less commonly, neurological manifestations such as seizure, coma, behavioral changes (aggression), peripheral neuropathy, and encephalopathy occur (Carithers and Margileth 1991, Schwartzman et al 1994, 1995, Glaser et al 2003).

Like humans, most cats infected with *Bartonella* species are clinically normal (Brunt et al 2006). However, *Bartonella* species infections have been associated with a wide variety of clinical signs in cats. Fever, lymphadenopathy, anorexia, depression, lethargy, uveitis, gingivitis and neurological manifestations are most commonly reported (Kordick and Breitschwerdt 1997, O'Reilly et al 1999, Yamamoto et al 2002). Studies have shown that *B henselae* can replicate in microglial cells grown from fetal brain tissues (Munana et al 2001) and the organisms have been grown from brain tissue of experimentally inoculated cats (Guptill et al 1997, Kordick et al 1999). In addition, neurological dysfunction, including behavior change, focal seizures, nystagmus and generalized tremors have been reported in experimentally inoculated cats (Guptill et al 1997, O'Reilly et al 1999, Mikolajczyk and O'Reilly 2000, Chomel et al 2003). Thus, *Bartonella* species infection may be an important cause of neurological disease in client-owned cats. A previous study investigated whether neurological bartonellosis was common in client-owned cats by comparing *Bartonella* species specific antibodies in serum of cats with and without clinical signs of neurological dysfunction (Pearce et al 2006). While many of the cats were seropositive, there were no statistical differences in *Bartonella*

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species prevalence rates between cats with neurological diseases (49.7%), cats with other non-neurological diseases (63.8%), and healthy cats (70.1%). As seroprevalence rates in normal cats are also very high, serological test results cannot be used alone to make disease associations with *Bartonella* species infections in individual cats (Nutter et al 2004, Pearce et al 2006). Similar problems have been noted in cats with ocular disease (Fontenelle et al 2008).

In cats with endogenous uveitis, other means of assessing ocular *Bartonella* species infection have included determination of *Bartonella* species aqueous humor antibody coefficients (*C*-values) to suggest local antibody production by ocular tissues (Lappin and Black 1999, Lappin et al 2000), and polymerase chain reaction (PCR) amplification of *Bartonella* species DNA from aqueous humor (Lappin et al 2000). Detection of *Bartonella* species antibodies in cerebrospinal fluid (CSF) has been used to aid diagnosis of central nervous system (CNS) bartonellosis in humans (Schwartzman et al 1994). However, to our knowledge these techniques have not been applied to cats with CNS disease. The purpose of this study was to determine whether *Bartonella* species DNA or *Bartonella* species antibodies can be detected in the CSF of client-owned cats with neurological disease.

Materials and methods

Case selection

The serum and CSF samples (100 pairs) used in this study had previously been submitted by veterinarians in the United States for performance of *Toxoplasma gondii*-specific IgM and IgG antibody assays and calculation of *T gondii* antibody production indices (*C*-values) in the CSF (Specialized Infectious Disease Laboratory, Colorado State University, Fort Collins, CO). The samples were selected sequentially from those submitted from 1992 to 1998 and were chosen solely based on sample availability for performance of additional tests.

The only source of information concerning history, signalment, and physical examination findings was the submission sheet used at the time of the initial testing, so information in some categories was incomplete. The age, sex, and state of origin were available for most cases. The state of origin was used to classify each cat as low risk of exposure to fleas (Alaska, Arizona, Colorado, Idaho, Montana, Nevada, New Mexico, Utah, Wyoming) or high risk (all other states) using a previous study that was based on climatic factors as more specific information was not available (Jameson et al 1995). All cats were client-owned and while information was incomplete, we assumed that all cats had some clinical signs consistent with CNS disease as most veterinarians do not submit CSF from normal cats for infectious disease testing. Because not all cases were submitted by veterinary neurologists and because neurological examination findings were incomplete, the cases could not be classified into categories more detailed than seizures or other neurological signs.

Antibody assays

The serum and CSF samples were frozen at −20 or −80°C until utilized in this study. After thawing at room temperature, an aliquot of each serum and CSF sample was assayed for *Bartonella* species IgG (heavy-chain specific) using an adaptation of a previously described enzyme-linked immunosorbent assay (ELISA) (Lappin et al 2000). For this study, the CSF samples (various volumes of 50−300 µl) were transferred into an RNase/DNase free tube and centrifuged at 13,200 rpm for 10 min. After centrifuging, the supernatant was pipetted back into its original tube to be assayed for antibodies and the pellet was used for DNA extraction and the *Bartonella* species PCR assay.

Serum samples were assayed for *Bartonella* species IgG at 1:10 and 1:64 in triplicate wells; CSF samples were assayed at 1:10 in triplicate wells. Positive control wells (serum from experimentally infected cats), negative serum control wells (serum from specific pathogen-free [SPF] cats), enzyme control wells, and substrate control wells were included on each micro-ELISA plate. The mean absorbance values for each serum or CSF sample were converted to %ELISA units by use of the following formula: (test sample mean absorbance minus the negative control sample mean absorbance)/(positive control sample mean absorbance minus the negative control sample mean absorbance) multiplied by 100. An individual sample was considered positive for *Bartonella* species IgG antibodies if the %ELISA value was greater than the mean %ELISA value plus 3SD of the pre-inoculation samples for a group of 26 SPF cats (10 kittens at 8 weeks of age and 16 cats at 3 years of age). The *Bartonella* species serum antibody titer was estimated by comparing results from the study samples at the 1:64 dilution to a standard curve generated

from positive and negative control sera assayed on each plate using a cut off value of 1:64.

Levels of total and specific antibody in CSF are generally much lower than serum and so results of the 1:10 dilution were used in the calculation of *Bartonella* species *C*-values (Lappin et al 2000). CSF samples that were positive for *Bartonella* species antibodies were assayed for calicivirus IgG using an adaptation of a previously described ELISA (Hill et al 1995). The *Bartonella* species IgG *C*-value was then calculated as follows:

$$\frac{\text{Bartonella species IgG \%ELISA units in CSF} \times \text{calicivirus IgG \%ELISA units in serum}}{\text{Bartonella species IgG \%ELISA units in serum} \times \text{calicivirus IgG \%ELISA units in CSF}}$$

A *C*-value > 1 was considered suggestive of local production of *Bartonella* species antibody by the CNS.

PCR assays

The pellet from each CSF sample was reconstituted with 200 µl of sterile phosphate buffered saline solution and then total DNA was extracted using a commercially available kit (Qiamp DNA Blood Kit, Qiagen Sciences, MD). After DNA extraction, 5 µl was assessed in a PCR assay capable of amplifying multiple *Bartonella* species (Jensen et al 2000). The different *Bartonella* species are identified by characteristic band sizes. In addition, each sample was assayed for DNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a PCR assay to assess for PCR inhibitors or DNA extraction failure (Kipar et al. 2001). Frequently, CSF samples are of low cellularity and so samples that were negative for GAPDH DNA were assessed by spectrophotometry to assure DNA was present.

Statistical analysis

Bartonella species prevalence rates based on different individual tests and combinations of tests were calculated. *Bartonella* species prevalence rates obtained from cats with seizures or other neurological signs and within each sex were compared by two-tailed Fisher's exact test with significance defined as $P < 0.05$.

Results

Of the 100 cats, the state of origin was known for 94 cats; North Carolina (14 cats), Washington (14 cats), Florida (nine cats), Colorado (nine cats), Texas (eight cats), Indiana (eight cats), California (six cats), Minnesota (five cats), Louisiana (three cats), Ohio (three cats), Wisconsin (two cats), New York (two cats), Illinois (two cats), Michigan (one cat), Kansas (one cat), Nebraska (one cat), Oklahoma (one cat), New Mexico (one cat), Massachusetts (one cat), Missouri (one cat), Virginia (one cat), and Mississippi (one cat). The sex was recorded for 94 cats; 57 cats (60.6%) were male and 37 cats (39.4%) were female. Sufficient clinical information was available on the submission sheet to classify 23 cats with seizures and 34 cats with other neurological dysfunction (Table 1). Clinical signs included in the 34 cats classified as other neurological dysfunction included paresis (11 cats), vestibular dysfunction (seven cats), blindness (four cats), neuromuscular weakness (two cats), circling (one cat) and non-specific neurological signs (nine cats).

Overall, 36% of the cats were positive in one or more *Bartonella* species tests (Table 1). The sex was known for 30 of 36 *Bartonella* species positive cats; 21 cats (70%) were males and nine cats (30%) were females. The difference in *Bartonella* species prevalence rates between male and female cats was not significantly different ($P = 0.26$). Of the *Bartonella* species positive cats for which the state of origin was known, 32 of 33 cats (97%) were from states classified as high flea risk. Estimated serum *Bartonella* species IgG titers (25 cats) ranged from 1:64 to 1:8192 and were detected in the serum of significantly more cats with seizures (47.8%) than cats with other neurological signs (12.1%). *Bartonella* species IgG was detected in the CSF of 11 cats; all of these cats had IgG *C*-value > 1 (range 1.14−85.7; mean = 10.39). Each of the 11 cats were positive for *Bartonella* species IgG in CSF and serum at the 1:10 dilution and six of these cats were concurrently positive for *Bartonella* species IgG serum antibodies at the 1:64 dilution. All 10 PCR assay positive CSF samples were *B henselae* based on band size (Jensen et al 2000). Of these 10 PCR assay positive cats, two cats had *Bartonella* species *C*-values > 1 and four cats had *Bartonella* species antibodies in serum assayed at the 1:64 dilution.

Table 1. Distribution of positive *Bartonella* species test results in cats with clinical signs of CNS disease

Test	All cats ($n = 100$)*	Seizures ($n = 23$)	Other signs ($n = 33$)	P-value†
Bartonella IgG (serum)	25 (25.0%)	11 (47.8%)	4 (12.1%)	0.005
Bartonella IgG CSF C-value > 1‡	11 (11.0%)	3 (13.0%)	1 (3.0%)	0.295
Bartonella CSF PCR assay	10 (10.0%)	1 (4.3%)	4 (12.1%)	0.394
Any *Bartonella* species positive result	36 (36.0%)	13 (56.5%)	10 (30.3%)	0.06

‡All *Bartonella* species IgG C-values were >1 and ranged from range 1.14 to 85.7 (mean = 10.39).
*The neurological signs were not listed for some cats.
†Prevalence rates between cats with seizures and other neurological signs were compared by the two-tailed Fisher's exact test with significance defined as $P < 0.05$.

Discussion

Overall, 36% of the 100 cats in this study had evidence of previous or current infection by a *Bartonella* species which is similar to the prevalence rate in other studies (Brunt et al 2006). The majority of cats came from states with high flea risk. There was no significant difference in *Bartonella* species prevalence rates between males and females.

In these 100 cats, *Bartonella* species IgG was detected in the CSF of 11 cats, each of which had a *Bartonella* IgG C-value > 1. In addition, *B henselae* DNA was amplified from CSF of 10 cats. These results support the hypothesis that *Bartonella* species infects the CNS of some client-owned cats with neurological disease. As this study was performed retrospectively, some of the samples were stored for up to 14 years and had been frozen and thawed which may have lead to false negative results in the *Bartonella* species assays and so it is possible that prevalence rates were even higher. Future studies of this type should use fresh samples if possible.

It is known that *Bartonella* species DNA can be amplified from brain tissues of healthy experimentally inoculated cats, so positive test results in PCR assays performed on CSF may not correlate with the presence of neurological disease (Kordick et al 1999). In addition, as *Bartonella* species reside within erythrocytes (Kordick and Breitschwerdt 1995) and cats are commonly bacteremic (Shaw et al 2001, Chomel et al 2003, Lappin et al 2006) positive PCR assay results on CSF may only indicate blood contamination from inflammation or the CSF collection procedure. Results of CSF analyses from the cats in this study were not available and so the possibility or magnitude of CSF blood contamination could not be assessed. In future prospective studies, results of *Bartonella* species PCR assays should be correlated with CSF analysis of both diseased and normal cats.

When the blood brain barrier is compromised, serum antibodies may leak into CSF and so detecting *Bartonella* species in CSF alone does not prove local antibody production by the CNS. To calculate a local antibody production index (C-value), the CSF to serum ratio of the specific antibody of interest is compared to the serum to CSF ratio of a constant that is common in the serum but generally only present in the CSF from leakage across a damaged blood brain barrier (Andiman 1991, Munana et al 1995). Calicivirus antibodies are extremely common in the serum of cats from natural exposure or vaccination but calicivirus infection of the CNS of cats is not common (Hill et al 1995). Similar techniques have been used for evaluating for *Bartonella* species antibody production by the eyes (Lappin and Black 1999, Lappin et al 2000).

Of the 11 cats with *Bartonella* species IgG C-values > 1, eight had C-values > 2. The higher the C-value, the more likely that antibody was locally produced. However, results of this study cannot be used to determine whether detection of *Bartonella* species IgG C-values > 1 in CSF proves CNS disease induced by the infection because a healthy control group was not available. Following experimental inoculation of cats, *B henselae* infects endothelial cells and erythrocytes and it has been documented in the brain tissues of experimentally inoculated cats (Kordick et al 1999, Munana et al 2001). During widespread infection, it is possible that immune cells in localized tissues like the CNS could mount an immune response against the organism. For example, it is known that *Bartonella* IgG C-values > 1 are detected at least transiently in the aqueous humor of experimentally inoculated cats without uveitis (Lappin et al 2000). In that study, *Bartonella* IgM C-values were also

measured and values >1 were only detected in cats with uveitis, not experimentally inoculated healthy cats. In this proof of concept study, we only measured *Bartonella* species IgG. To further evaluate the predictive values of *Bartonella* species *C*-values using CSF, *Bartonella* species IgM and IgG responses in CSF should be measured temporally in experimentally inoculated cats and prevalence rates should be determined from clinically normal, naturally exposed cats with a more detailed clinical history, including response to therapy.

Of the 10 cats with *Bartonella* species DNA amplified from CSF, all were infected with *B henselae*. In a recent study, prevalence rates of *B henselae* and *B clarridgeiae* in blood of 92 normal cats in the United States were 34.8% and 20.7%, respectively (Lappin et al 2006), and so it is likely that at least some cats in the current study were exposed to *B clarridgeiae*. Thus, the fact that only *B henselae* was amplified from the CSF of the cats with neurological disease described here may suggest that *B henselae* is more likely than *B clarridgeiae* to induce CNS disease. Unfortunately, blood was not available to determine *B henselae* and *B clarridgeiae* prevalence rates and so it is also just possible that the cats described here were only infected by *B henselae*. Whether one or more *Bartonella* species are associated with neurological disease should be evaluated in a future prospective study by comparing blood and CSF results from the same clinically ill cats and including a control group of normal cats.

Of the 10 cats with *B henselae* DNA amplified from CSF, four cats had *Bartonella* species IgG titers in serum and two cats had *Bartonella* species *C*-values > 1 in CSF. Of the 11 cats with *Bartonella* species *C*-values > 1, internal controls included on all *Bartonella* species PCR assays performed as expected and the samples when used previously were handled appropriately and so there is little reason to expect false positive PCR assay results from assay contamination. The failure to detect *Bartonella* species antibodies in some PCR assay positive cats has several potential explanations including the possibilities that the infection was acute and the cats were PCR assay positive prior to development of a detectable humoral immune response or the individual cats were not capable of mounting a humoral response. It is also possible the antibody test results were falsely negative because of being frozen and thawed previously. The failure to detect *Bartonella* species DNA in the other cats with a *Bartonella* species *C*-value > 1 suggests that the organism had been cleared to levels that were undetectable with the assays used or the assay was otherwise falsely negative (Jensen et al 2000). Overall, these results suggest that if CSF is to be used for evaluation for *Bartonella* species infection in individual cats, both PCR assays and antibody assays should be used. In people and cats with infectious diseases associated ocular inflammation, the combination of antibody production indices with nucleic acid amplification techniques is considered superior to either technique alone (De Groot-Mijnes et al 2006).

In the study described here, *Bartonella* species antibodies were detected in serum of more cats with seizures than in cats with other neurological manifestations. In another study, median *B henselae* serologic titers of cats with seizures were significantly greater than cats with other neurologic disease (Pearce et al 2006). The results of the studies suggest that *Bartonella* species infection may be associated with seizure disorders in client-owned cats. However, clinical bartonellosis cannot be diagnosed on serological test results alone. For example, in the previous study, the prevalence of *Bartonella* species antibodies in the serum of cats with neurological dysfunction was less than in a group of clinically ill cats without neurologic signs and a group of healthy cats (Pearce et al 2006). This underscores the need for future studies that combine both CSF antibody and PCR testing, as serology alone is a poor indicator of clinically significant disease (Kordick et al 1999, Guptill et al 2003, Pearce et al 2006).

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