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Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia virus and feline immunodeficiency virus with chronic feline gingivostomatitis

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Gingivostomatitis (GS) is a significant condition in cats because of oral discomfort and associated periodontal disease. Several infectious agents have been associated with the presence of GS, but a causal relationship is unclear. The cats in this study were housed together, had a history of flea exposure, and were vaccinated with a modified live FVRCP product. There were nine cats with active GS and 36 unaffected cats at the time of sample collection. Serum was tested for feline leukemia virus (FeLV) antigen and antibodies against feline immunodeficiency virus, feline calicivirus (FCV), feline herpesvirus 1 (FHV-1), and *Bartonella* species (enzyme-linked immunosorbent assay and Western blot immunoassay). PCR assays for *Bartonella* species and FHV-1 and a reverse transcriptase PCR assay for FCV were performed on blood and throat swabs. All cats were negative for FeLV. Assay results failed to correlate to the presence of GS in the group of cats studied.

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Gingivostomatitis (GS) is often recognized in feline patients and is clinically characterized by erythematous and swollen gums, halitosis and the potential for significant oral discomfort resulting in dysphagia, anorexia, and weight loss. In addition to being painful to the animal, the chronic inflammation of the gingiva can lead to progressive periodontal disease (Lyon 2005). The diagnosis is based on histopathology and characterization of reactive cells in the tissue, which most frequently consists of infiltrations of plasma cells primarily, with lesser numbers of lymphocytes, neutrophils, and macrophages (Lyon 2005). The syndrome is likely to be multifactorial and it is theorized to involve an exaggerated immune response to either infectious or non-infectious antigens. Genetic predisposition, nutritional factors, environment stresses, and domestication have also been proposed as playing a part in the syndrome (Lyon 2005).

Infectious agents suspected to be associated with GS in cats include feline immunodeficiency virus (FIV) (Knowles et al 1989, Tenorio et al 1991, Waters et al 1993), feline leukemia virus (FeLV) (Knowles et al 1989, Tenorio et al 1991), feline calicivirus (FCV) (Knowles et al 1989, 1991, Tenorio et al 1991, Waters et al 1993, Addie et al 2003, Lommer and Verstraete 2003), feline herpesvirus 1 (FHV-1) (Hargis and Ginn 1999, Lommer and Verstraete 2003), *Bartonella* species (Ueno et al 1996, Glaus et al 1997, Dowers and Lappin 2005), and a variety of other bacteria. However, all of these agents can be harbored by both healthy and clinically ill cats, so a causal relationship is difficult to prove in individual cats. It is also difficult to compare the results of previous studies because the agents tested for varied amongst the studies and the diagnostic

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methods were not standardized. Further information concerning the role infectious agents play in this syndrome is needed to help design improved diagnostic and therapeutic plans. The purpose of this study was to perform a standardized infectious disease diagnostic workup in a group of communally housed, mixed source, client-owned cats with and without GS in an attempt to determine infectious disease associations and to determine the optimal diagnostic workup.

Materials and methods

Feline colony

The cats in this study were in southern California and had been allowed to commingle for 3–15 years (mean = 8.2 years). The cats were owned by a veterinary technician that acquired them from a shelter (nine cats), as strays (nine cats), by capture (13 cats; considered feral), and from previous owners (eight cats). All of the cats had a history of exposure to fleas 1.5 years previously, but had no evidence of current infestation. Each of the cats had been vaccinated previously with a FHV-1, FCV, and panleukopenia containing vaccine (Feline UltraNasal FVRCP Vaccine; Heska Corporation, Loveland, Colorado) approximately 1 year prior to sample collection in this study. None of the cats were administered antimicrobial drugs within the 2 weeks before sampling.

Sample handling

At the time of sample collection, it was determined by one of the authors (TE) whether the cats were unaffected or currently had GS. GS was determined by presence of significant swollen, erythematous oral lesions including gingivitis, faucitis and stomatitis. Cats that were determined to be unaffected had no significant erythematous oral lesions. Blood (3 ml) was collected and placed into an EDTA tube (1.5 ml draw) and a clot tube for serum separation. A dry cotton swab was rolled over the lesion (GS cats) or caudal pharynx (normal cats) and was then placed in 1.0 ml of sterile 0.01 M phosphate buffered saline and allowed to equilibrate at room temperature for 2–3 h. The EDTA tube, serum, and swabs were then stored at −20°C until shipped to Colorado State University by overnight express on dry ice where they were stored at −70°C until assayed.

Assays

Serum was tested for FeLV antigen (SNAP Combo FeLV/FIV; Idexx Laboratories, Portland, ME) and antibodies against FIV (SNAP Combo FeLV/FIV; Idexx Laboratories, Portland, ME), FCV (Lappin et al 2002), FHV-1 (Lappin et al 2002), and *Bartonella* species (Lappin et al., 2006a). *Bartonella* species antibody responses were determined by both enzyme-linked immunosorbent assay (ELISA) and Western blot immunoassay.

After thawing the samples at room temperature, DNA and RNA were extracted from blood and swabs using a commercially available kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For swabs, 50 μg salmon sperm DNA (Invitrogen Corporation, Carlsbad, CA) was added per milliliter of manufacturer's lysis buffer. The swab was pressed against the side of the tube to expel all possible liquid and discarded. The entire sample was vortexed, transferred to a 1.5 ml of RNase and DNase free microcentrifuge tube and centrifuged for 5 min at 500 × *g*. Supernatant was decanted and the remaining pellet was resuspended in 400 μl of sterile 0.01 M PBS. The remainder of the manufacturer's protocol was followed as written. Each DNA extract was assessed for the presence of DNA by spectrophotometry. PCR assays used to amplify DNA of *Bartonella* species (Jensen et al 2000) and FHV-1 (Burgesser et al 1999) were performed as previously described on all DNA extracts. A reverse-transcription PCR (RT-PCR) assay used to amplify RNA of FCV was performed as previously described on all RNA extracts (Sykes et al 1998).

Statistical evaluation

To assess for associations between *Bartonella* species, FHV-1 and FCV and GS, numbers of positive test results in cats with and without GS were compared by two-tailed Fisher's exact test with significance defined as $P < 0.05$. To determine if any of the test results could be used to predict GS in cats, positive predictive value and negative predictive value (PPV and NPV) were determined for each assay by comparing to the current presence or absence of GS.

Because it is unknown whether the *Bartonella* species ELISA or Western blot immunoassay is the gold standard, sensitivity and specificity between the two assays could not be calculated. Thus, results of the ELISA and Western blot (WB) immunoassay were compared by

calculating percentage agreement using the following formula.

$$[(\text{ELISA}+,\text{WB}+)+(\text{ELISA}-,\text{WB}-)]/ \times [(\text{ELISA}+,\text{WB}+)+(\text{ELISA}-,\text{WB}-)+(\text{ELISA}+,\text{WB}-)+(\text{ELISA}-,\text{WB}+)] \times 100.$$

To determine whether *Bartonella* species antibodies detected by ELISA or Western blot (WB) correlated to the presence or absence of bacteremia, the PPV and NPV of the antibody tests were calculated using blood PCR results as a gold standard.

Results

Overall, samples were available from 45 cats; 36 unaffected cats and nine with GS. Of the unaffected cats, estimated ages were <1 year (zero cats), 1–3 years (zero cats), 4–6 years (six cats), 7–9 years (10 cats), 10–12 years (11 cats), >13 years (seven cats) and the age was unknown for two cats. Of the cats without GS, estimated ages were <1 year (zero cats), 1–3 years (zero cats), 4–6 years (one cat), 7–9 years (four cats), 10–12 years (four cats), >13 years (zero cats) and the age was unknown for zero cats. Seventeen males and 19 females were clinically unaffected and five males and four females exhibited GS. All of the cats were negative for FeLV antigen. Five cats were positive for FIV antibodies. Overall, prevalence rates for *Bartonella* species antibodies by ELISA, *Bartonella* species antibodies by Western blot, FHV-1 antibodies, FCV antibodies, and FIV antibodies were 44%, 57.8%, 95.6%, 100%, and 10.9%, respectively. The distribution of the serological test between groups is presented in Table 1. None of the positive test results was associated with the presence of GS. However, *Bartonella* species Western blot results were negatively associated with GS ($P = 0.0243$; odds ratio = 0.1429). None of the PPVs of the serological tests was >40% (Table 2).

When the ELISA and Western blot immunoassay tests were compared, a 60% percentage agreement was found. The discordant results were ELISA positive, Western blot negative ($n = 6$) and ELISA negative, Western blot positive ($n = 12$). Overall, 26 cats were Western blot positive (two cats had GS) and numbers of *Bartonella* species immunodominant antigens recognized ranged from two to eight antigens per cat. The most commonly recognized antigens had apparent molecular masses of 82 kDa (19 cats), 61.9 kDa (14 cats); 48 kDa (13 cats), and 57 kDa (11 cats).

Overall, prevalence rates for *Bartonella* species DNA in blood, *Bartonella* species DNA on oral swabs, FHV-1 DNA on oral swabs, and FCV RNA on oral swabs were 2.2%, 11.1%, 6.7%, and 6.7%, respectively. The distribution of PCR and RT-PCR test results between groups is presented in Table 3. None of the positive test results was associated with the presence of GS. The PPVs for *Bartonella* species DNA, FHV-1 DNA, and FCV RNA on oral swabs were 20%, 0%, and 0%, respectively (Table 4). While the PPV of the *Bartonella* species DNA in blood was 100%, only one positive sample was detected. Of the five cats with *Bartonella* species DNA amplified from oral swabs, only one had GS and none had *Bartonella* species DNA amplified from blood.

Positive *Bartonella* species culture or PCR assay on blood can be used to determine bacteremia. When compared to *Bartonella* species PCR assay results from blood, the PPV and NPV of the *Bartonella* species ELISA results for predicting bacteremia were 0% and 96%, respectively. When compared to *Bartonella* species PCR assay results from blood, the PPV and NPV of the *Bartonella* species WB results for predicting bacteremia were 0% and 95%, respectively.

Table 1. Distribution of serum antibody test results in group housed cats with ($n = 9$) and without ($n = 36$) stomatitis

Group	*Bartonella* ELISA		*Bartonella* Western blot		FHV-1 ELISA		FCV ELISA		FIV ELISA	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Stomatitis	4 (44.4%)	5 (55.6%)	2 (22.2%)	7 (77.8%)	9 (100%)	0 (0%)	9 (100%)	0 (0%)	2 (22.2%)	7 (77.8%)
Unaffected	16(44.4%)	20 (55.6%)	24 (66.7%)	12 (33.3%)	34 (94.4%)	2 (5.6%)	36 (100%)	0 (0%)	3 (8.3%)	33 (91.7%)
Fisher's[a]	$P = 1$		$P = 0.0243$		$P = 1$		$P = 1$		$P = 0.258$	

[a]Two-tailed Fisher's exact test; Pos = positive; Neg = negative.

Table 2. Predictive values of *Bartonella* species, FHV-1, FCV and FIV antibody tests for use with cats with stomatitis

	Bartonella ELISA	*Bartonella* Western blot	FHV-1 ELISA	FCV ELISA	FIV ELISA
PPV (%)	20.0	7.7	20.9	20.0	40
NPV (%)	80	63.2	100	0	82.5

PPV = positive predictive value; NPV = negative predictive value

Discussion

Bartonella species, FHV-1, FCV, FeLV, and FIV can be carried chronically by cats and have been proposed as causes of GS. The cats in this study were housed together, were vaccinated with a modified live FVRCP vaccine intranasally, and had a history of exposure to fleas. This history combined with the serologic test results and PCR assay results suggest that each of the cats had been exposed to at least one of these agents. However, evidence of infection with *Bartonella* species, FHV-1, FCV, FeLV or FIV was not overrepresented in the cats with GS and so the data fail to support the hypotheses that these organisms were a cause of GS in this population of cats. However, there are a number of factors or limitations that may have influenced the results of this study including sampling, shipping and storage factors, clearance or latency of the organisms, presence of subsets of GS, and potential variations in organism virulence.

Results of this study support previous research in the literature which asserts that because of the high prevalence rates of *Bartonella* species antibodies in normal cats from natural exposure, a positive antibody assay cannot be used to accurately predict clinical illness from this agent in individual cats (Breitschwerdt et al 2005, Pearce et al 2006). In an attempt to further evaluate clinical utility of antibody assay results, antigen recognition patterns were examined by Western blot. *Bartonella* species antigen recognition patterns in the two cats in this study with GS were similar to those of normal cats and so the Western blot immunoassay did not appear to provide additional predictive value. These results are similar to another study on cats with and without fever (Lappin et al., 2006a). Finally, the results of this study are also similar to those of others that show that detection of *Bartonella* species antibodies by any methodology does not accurately predict presence of *Bartonella* species bacteremia (Chomel et al 1995, Fabbi et al 2004, Guptill et al 2004, Lappin et al., 2006a).

While most cats with experimental *Bartonella* species infection have been clinically normal, fever, loss of appetite, transient anemia, injection site reactions, lymphadenopathy, and neurological signs have been detected in some cats (Regnery et al 1996, Abbot et al 1997, Guptill et al 1997, Kordick and Breitschwerdt 1997, Guptill et al 1998, 1999, Kordick et al 1999, O'Reilly et al 1999, Mikolajczyk and O'Reilly 2000, Powell et al 2002, Yamamoto et al 2002a, 2002b, 2003). In addition, some experimentally inoculated cats develop histopathological lesions including lymph node hyperplasia, abscesses, and lymphocytic inflammation of various tissues (Guptill et al 1997, Kordick et al 1999). However, no mention of gingival abnormalities was made in these studies. *Bartonella* species infection of naturally exposed cats has been associated with endocarditis (Chomel et al 2003) and ocular disease (Lappin and Black 1999, Lappin et al 2000, Ketring et al 2004). To date, the only studies suggesting that *Bartonella* species infection causes GS in cats were small serological studies from Europe and Japan (Ueno et al 1996, Glaus et al 1997). However, another ongoing study comparing results of *Bartonella* species PCR on blood and serum *Bartonella* species IgG ELISA results on cats with and without GS from which samples were

Table 3. Distribution of PCR and RT-PCR assay results in group-housed cats with ($n = 9$) and without ($n = 36$) stomatitis

Group	*Bartonella* PCR-blood		*Bartonella* PCR-swab		FHV-1 PCR-swab		FCV RT-PCR	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Stomatitis	1 (11.1%)	8 (89.9%)	1 (11.1%)	8 (89.9%)	0 (0%)	9 (100%)	0	9 (100%)
Unaffected	0 (0%)	36 (100%)	4 (11.1%)	32 (89.9%)	3 (8.3%)	33 (91.7%)	3 (8.3%)	33 (91.7%)
Fisher's[a]	$P = 0.2$		$P = 1$		$P = 1$		$P = 1$	

[a]Two-tailed Fisher's exact test; Pos = positive; Neg = negative.

Table 4. PPV and NPV for PCR tests for use with cats with stomatitis

	Bartonella PCR-blood	*Bartonella* PCR-swab	FHV-1 PCR-swab	FCV RT-PCR
PPV (%)	100	20.0	0	0
NPV (%)	81.8	80.0	78.6	78.6

collected the same day in the same clinic has also failed to show an association between *Bartonella* species and GS (Dowers and Lappin 2005).

The widespread use of FHV-1 and FCV containing vaccines and exposure to these common feline viruses has resulted in high prevalence rates of FHV-1 (Maggs et al 1999) and FCV (Lappin et al 2002, Mouzin et al 2004) antibodies in normal cats, lessening the predictive value of these assays. However, previous studies have shown increased FHV-1 (Lommer and Verstraete 2003) and FCV (Knowles et al 1989, Lommer and Verstraete 2003) carriage rates in cats with GS compared to normal cats by use of viral isolation. In addition, one cat with chronic stomatitis was PCR positive for FHV-1, 14 months after an upper respiratory infection (Hargis and Ginn 1999). These results suggested that detection of FHV-1, FCV, or *Bartonella* species in the oral cavity may be more predictive than the results of antibody assays.

To our knowledge, the study described here was the first to attempt to correlate presence of microbial DNA or RNA amplified from oral swabs collected from cats with and without GS housed in the same environment. Of the five *Bartonella* species positive samples, only one came from a cat with GS; FHV-1 and FCV were not amplified from any of the cats with GS. We believe false negative *Bartonella* species and FHV-1 PCR assays caused by poor sampling were uncommon because DNA was detected by spectrophotometry in all samples. The viral capsid of FCV protects against RNAase degradation and so we also believe false negative FCV RT-PCR assay results were unlikely. However, as mentioned previously, there are a number of factors that may have contributed to these findings.

It is possible that the organisms are not equally distributed through the oral cavity and could have been missed by our sampling technique. It is also possible that organisms were present in deeper tissues of the cats with GS but not the superficial samples collected by oral swab. However, *Bartonella* species DNA has rarely been detected in tissues of cats with GS in an ongoing prospective study in our laboratory (Dowers and Lappin 2005). It is also possible shipping and storage affected assay results. Another consideration is that chronic GS may be caused by a hypersensitivity reaction against the microbes, leading to clearance of the agents and false negative results in PCR, RT-PCR, or virus isolation. Sampling cats early in the syndrome may be more likely to show an association. Both FCV and FHV-1 have latent phases and so test results may only be positive intermittently (Hargis and Ginn 1999). It is possible that *Bartonella* species, FHV-1, and FCV are only involved with subsets of cats or GS and future studies of this type should attempt to classify the lesions by severity and location within the mouth. Lastly, different strains of *Bartonella* species (Mikolajczyk and O'Reilly 2000), FHV-1 (Hargis and Ginn 1999, Hamano et al 2003), and FCV (Geissler et al 1997, Poulet et al 2000, Hurley et al 2004) have different degrees of virulence which may relate to the development of GS.

To our knowledge, this is the first study to compare the results of *Bartonella* species PCR assay on blood and oral swab samples collected from naturally infected cats. While the cats had all been exposed to fleas previously, the owner had consistently used flea control products for the last 1.5 years and felt the cats had been flea free during that time. Thus, it is likely the *Bartonella* species infected cats in this study maintained their infections for months, confirming the findings of others using experimentally infected cats (Kordick et al 1999). *Bartonella* species bacteremia was only documented in one of 45 cats (2.2%), none of the five cats with *Bartonella* species DNA amplified from oral swabs were currently bacteremic, and only one cat with *Bartonella* species DNA amplified from an oral swab had stomatitis. The results show that use of blood or oral swab PCR assay results is unlikely to correlate with the presence of GS. In addition, the results also confirm that *Bartonella* species DNA can be present intermittently in blood and so detection of a single negative PCR assay result cannot be used to accurately assess infection status of cats (Brunt et al 2006). *Bartonella henselae* lives in flea dirt for 3–9 days after being passed by fleas (Higgins et al 1996, Finkelstein et al 2002) and *B henselae* or *Bartonella clarridgeaie* DNA was amplified from 59.7% of fleas collected from naturally exposed cats (Lappin et al 2006b). Thus, amplification of *Bartonella* species DNA from oral cavity swabs could result from grooming behavior and ingestion of *Bartonella* species in flea dirt.

However, the cats in this study had not been exposed to fleas in the last 1.5 years and so the positive *Bartonella* species PCR assay results may document the presence of infected red blood cells in the mouth even without gross evidence of GS. These findings support the recommendations that flea control should be maintained at all times and that bites and scratches should be avoided (Brunt et al 2006).

In conclusion, a correlation between the presence of GS and FCV, FHV, FIV, FeLV or *Bartonella* species in this colony of cases was not identified and so the results did not support disease causation by any of the five agents. Diagnostic test results for these infectious agents should be interpreted carefully as none appear to correlate to the presence or absence of disease.

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