Journal of Veterinary Internal Medicine

ACVIM American College of Veterinary Internal Medicine

Open Access

Brief Communication

J Vet Intern Med 2018;32:274–279

Serum Cytokine Profiling in Cats with Acute Idiopathic Cystitis

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Background: Feline idiopathic cystitis (FIC) is a common lower urinary tract disorder of domestic cats that resembles interstitial cystitis/painful bladder syndrome (IC/PBS) in humans. Diagnosis of FIC is based on clinical signs and exclusion of other disorders because of a lack of specific pathologic findings or other objective biomarkers. Cytokines are potential noninvasive biomarkers to define the presence, severity, and progression of disease, and response to treatment.

Objectives: The objective of this pilot study was to determine concentrations of selected cytokines in serum from healthy cats and cats with acute FIC.

Animals: Serum samples from 13 healthy cats and from 12 cats with nonobstructive acute FIC were utilized.

Methods: Multiplex analysis of 19 cytokines (CCL2, CCL5, CXCL1, CXCL12, CXCL8, Flt3L, GM-CSF, IFN-γ, IL-12 (p40), IL-13, IL-18, IL-1β, IL-2, IL-4, IL-6, PDGF-BB, SCF, sFas, and TNF-α) was performed with a commercially available feline-specific multiplex bead-based assay.

Results: Mean serum concentrations of IL-12 (p40; $P < 0.0001$), CXCL12 ($P = 0.002$), IL-18 ($P = 0.032$), and Flt3L ($P = 0.0024$) were significantly increased in FIC cats compared to healthy cats. GM-CSF, IL-1b, IL-2, and PDGF-BB were undetectable or detected in an insufficient number of cats to allow meaningful comparisons.

Conclusions and Clinical Importance: We have identified increased serum concentrations of pro-inflammatory cytokines and chemokines CXCL12, IL-12, IL-18, and Flt3L in FIC-affected cats. These findings suggest potential candidates for noninvasive biomarkers for diagnosis, staging, and therapeutic outcome monitoring of affected cats and provide additional insight into the etiopathogenesis of FIC.

Key words: Feline; Interstitial; Multiplex assay; Urine.

Feline idiopathic cystitis (FIC) is the most common lower urinary tract disorder in domestic cats and is characterized by clinical signs of dysuria, pollakiuria, periuria, stranguria, hematuria, and, in males, urinary tract obstruction.[1,2] The clinical and morphologic features of FIC are strikingly similar to those of an idiopathic cystopathy of people called interstitial cystitis/painful bladder syndrome (IC/PBS).[1,2] As of yet, no consistently reliable diagnostic markers establish a diagnosis of FIC or IC/PBS and differentiate among various clinical and pathologic disease phenotypes.

Over the past several decades, a large number of potential serum and urine proteins that may be utilized as biomarkers have been identified and evaluated in people with IC/PBS.[3,4] Similarly in cats with FIC, several urine proteins have been found to be increased (C4a, NF-kB p65, p38 MAPK, fibronectin, galectin-7, thioredoxin)[5–8] or decreased (GP-51, I-FAB, trefoil factor 2)[7–9] in urine of affected cats compared to healthy controls. In contrast, studies investigating serum biomarkers in cats with acute or chronic FIC have not been performed to date. Because FIC may be associated with factors that can induce systemic immune activation and pro-inflammatory cytokine release (eg, bladder inflammation, comorbid nonurinary

Abbreviations:

CCL2	C-C motif chemokine ligand 2 (MCP-1)
CCL5	C-C motif chemokine ligand 5 (RANTES)
CXCL12	C-X-C motif chemokine ligand 12 (SDF-1)
CXCL1	C-X-C motif chemokine ligand 1 (KC)
CXCL8	C-X-C motif chemokine ligand 8 (IL-8—interleukin 8)
FeLV	feline leukemia virus
FIC	feline idiopathic cystitis
FIV	feline immunodeficiency virus
Flt3L	fms-related tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
IC/PBS	interstitial cystitis/painful bladder syndrome
IFN-γ	interferon gamma
IL-12 (p40)	interleukin 12 subunit p40
IL-13	interleukin 13
IL-18	interleukin 18
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-4	interleukin 4
IL-6	interleukin 6
ND	not detected
NSAIDs	nonsteroidal anti-inflammatory drugs
PDGF-BB	platelet-derived growth factor subunit B
SCF	stem cell factor
sFas	soluble Fas cell surface death receptor
TNF-α	tumor necrosis factor alpha

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Work presented in this manuscript was performed at Michigan State University College of Veterinary Medicine.

This study was presented at 2016 ACVIM Forum in Denver, CO, and it is a part of PhD dissertation of Dr. Maciej Parys.

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Submitted July 12, 2017; Revised October 16, 2017; Accepted November 28, 2017.

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DOI: 10.1111/jvim.15032

inflammatory conditions, and activation of sympathetic nervous system),[10–14] characterization of serum cytokine and chemokine responses could offer additional insight into the pathogenesis of FIC and identify potential noninvasive biomarkers for diagnosis, prognosis, and prediction of therapeutic responses. In 1 study of 22 nonobstructed cats with chronic FIC, serum concentrations of tryptophan and its metabolite kynurenine were higher in FIC cats compared to healthy controls, but differences did not reach statistical significance.[15]

Inflammation appears to be involved in the pathogenesis of some forms of FIC.[12,13,16] Cytokines and chemokines are factors important for migration, maturation, and proliferation of immune cells and play key roles in host inflammatory and immune responses. In humans with IC/PBS, cytokines and chemokines have been intensively studied as potential serum or urine biomarkers of the disorder.[11–13,16] Recently, a feline-specific bead-based multiplex assay has become available for simultaneous detection of immunologically active proteins in cats. Studies investigating the analytical performance of the multiplex assay suggested that at higher blood analyte concentrations, recovery was linear and allowed for reliable cytokine detection in serum or heparinized plasma samples.[17] The objective of our study was to investigate expression of 19 cytokines and chemokines in serum of cats with untreated nonobstructive acute FIC and in unaffected healthy control cats.

Materials and Methods

Study Population

In this retrospective pilot study, specimens included archived frozen serum samples collected from 12 untreated cats with a clinical diagnosis of nonobstructive acute FIC. All animals were evaluated at Michigan State University Veterinary Medical Center. Control samples were obtained from 13 clinically healthy client-owned, blood donor or research colony cats. Acute FIC was diagnosed based on clinical signs of dysuria, periuria, hematuria, or stranguria, where other disease processes with similar clinical presentation had been excluded. All cats were evaluated with a standardized medical history, a complete physical examination, a complete urinalysis, a urine culture, survey abdominal radiographs, and an abdominal ultrasound examination to exclude other comorbidities. Additionally, all FIC cats had serum biochemistry profiles, CBC, and FeLV/FIV testing performed. Cats with acute FIC were excluded from the study if they had another detectable disease process, had concurrent urethral obstruction, or had been treated with antimicrobials, antihistamines, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), anticholinergics, antidepressants, urinary acidifiers, glycosaminoglycans, diuretics, dimethyl sulfoxide, cyclophosphamide, or any other medication used to treat IC/PBS.

Peripheral blood samples were obtained by venipuncture. Collected samples were centrifuged at 2,000 *g* for 10 minutes. Serum samples subsequently were cryopreserved at −80°C and remained frozen until analysis.

All samples utilized in this study were obtained under informed consent from the owners and with the approval of the Michigan State University Institutional Animal Care and Use Committee.

Multiplex Cytokine Assays

A commercial feline-specific multiplex bead-based assay[a] was utilized allowing for simultaneous measurement of 19 cytokines: sFas, TNF-α, CXCL12, SCF, CCL5, PDGF-BB, CCL2, CXCL1, IL-18, IL-13, IL-12 (p40), CXCL8, IL-6, IL-4, IL-2, IL-1β, IFN-γ, GM-CSF, and Flt3L.[17] Serum samples were assayed according to manufacturer's recommendations. Sample analyte concentrations were measured using a multiplexing platform,[b] and data were analyzed using a commercial analytical software package.[c] All assays were run in duplicate.

Statistical Analysis

Statistical analysis was performed using commercially available statistical software.[d] D'Agostino and Pearson omnibus test was used to evaluate normality of data. Comparisons of cytokine concentrations between groups with normal distribution were performed by Student *t*-test, otherwise the nonparametric Mann-Whitney *U* test was performed.

Results

Animals

Samples from 12 FIC-affected cats (4 females and 8 males) and 13 healthy control cats (5 females and 8 males) were utilized in the study. Average age of affected cats was 4 years, 11 months (SD ± 1 year, 10 months; range, 1 year, 5 months to 7 years), and 5 years, and 2 months (SD ± 1 year, 10 months; range, 1–7 years, 2 months) for control cats. All FIC cats had clinical signs consistent with acute cystitis, with clinical signs lasting an average of 3 days (SD ± 2.21 days; range, 1–8 days) before presentation. The mean urine specific gravity of FIC cats was significantly lower than that of the control cats ($P = 0.0081$, *t*-test; FIC: 1.048 ± 0.012 versus control 1.060 ± 0.009). There were no significant differences in urinary pH between the groups (FIC: 6.6 ± 0.53 versus control: 6.72 ± 0.58). Struvite crystals were present in 3 affected and 3 control cats. Bacterial culture was negative in all animals. Microscopic hematuria was present in 9 FIC cats and 2 controls. Pyuria was not observed in any affected or control cat.

Some FIC cats had mild abnormalities in their blood chemistry and CBC results including hyperglycemia (3/12), increased CK activity (10/12), hypo- (1/12) or hyper-natremia (1/12), hypochloremia (1/12), hypoglobulinemia (1/12), hypocholesterolemia (2/12), hypoferritinemia (2/12), increased TCO_2 (1/12), increased AST activity (2/12), increased ALT activity (1/12), hypophosphatemia (1/12), decreased hematocrit and hemoglobin (2/12) as well as decreased RBC counts (1/12). All other results were within reference ranges.

Serum Cytokine/Chemokine Concentration

Twelve FIC and 13 control serum samples were available for evaluation at the time of experiments. Four of 19 analytes were undetectable or detectable in an insufficient number of samples to make a meaningful comparison and were excluded from analyses. These analytes were GM-CSF, IL-1b, IL-2, and PDGF-BB (Table 1). Concentrations of 4 cytokines were significantly increased in serum of FIC cats compared to controls: IL-12 (p40) ($P < 0.0001$ Mann-Whitney test; FIC:

median, 1,069; range, 551.3–2,042 pg/mL versus control: 1,221.8, 0–1,503 pg/mL), CXCL12 (SDF1) (P = 0.002, Mann-Whitney test; FIC: 2,349; 0–3,523 pg/mL versus control 0; 0–3,571 pg/mL), IL-18 (P = 0.032, Mann-Whitney test; FIC: 379.8; 0–1,179 pg/mL versus control: 0; 0–978.7 pg/mL), and Flt3L (P = 0.0024, Mann-Whitney; FIC: 365.6; 219.7–563.8 pg/mL versus control: 177; 112.7–1,215 pg/mL; Table 1, Fig 1).

Discussion

To our knowledge, ours is the first study investigating serum concentrations of cytokines and chemokines in cats with acute FIC. We have identified 4 cytokines that were significantly higher in serum of FIC-affected cats compared to healthy controls (IL-12 [p40], CXCL12, IL-18, Flt3L). Studies investigating the expression of these 4 cytokines in humans with IC/PBS have not been reported. Although we did not detect significant differences in other serum cytokines in FIC cats compared to controls, increased serum or plasma concentrations of IL-1β, IL-6, CXCL8 (IL-8), and TNF-α have been reported in people with IC/PBS compared to healthy controls.[18,19] Factors responsible for differences in cytokine profiles between acute FIC and IC/PBS are unknown, but may be related to differences in chronicity of disease, pathophysiologic mechanisms, and analytical performance of cytokine assays. No studies have investigated the expression of IL-2, IL-4, IL-12, IL-13, IL-18, sFas, Flt3L, GM-CSF, IFN-γ, CXCL1 (KC), CXCL12 (SDF1), CCL2 (MCP-), CCL5 (RANTES), PDGF-BB, and SCF in serum of people with IC/PBS. Although comparative data on each cytokine is not available for comparison of human IC/PBS and FIC serum cytokine profiles, increased levels of circulating pro-inflammatory cytokines are present in both disorders.

Table 1. Median and range of concentrations of 19 cytokine/chemokine measured in serum from 12 cats with acute FIC and 13 healthy control cats. In bold are cytokines significantly different between 2 groups.

	Serum Concentration in pg/mL (Median, Range)		
Cytokine	FIC (n = 12)	Healthy (n = 13)	P Value
sFas	0 (0–161.3)	0 (0–127.4)	0.38
Flt3L	365.6 (219.7–563.8)	177 (112.7–1,215)	0.0024
GM-CSF	ND	ND	
INF-γ	371.3 (0–1,358)	692 (0–19,626)	0.29
IL-1β	ND	ND	
IL-2	ND	ND	
PDGF-BB	ND	ND	
IL-12 (p40)	1,069 (551.3–2,042)	121.8 (0–1,503)	<0.0001
IL-13	27.02 (0–164.1)	45.05 (0–217.1)	0.66
IL-4	416.6 (89.35–1,734)	1,204 (72.35–3,598)	0.14
IL-6	0 (0–929.7)	260.6 (0–1,929)	0.33
CXCL8 (IL-8)	24.43 (0–148.4)	82.46 (0–673)	0.17
CXCL1 (KC)	48.04 (0–468.7)	13.18 (0–110.7)	0.11
CXCL12 (SDF1)	2,349 (0–3,523)	0 (0–3,571)	0.002
RANTES	113.8 (51.9–361.9)	96.4 (12.7–683.5)	0.27
SCF	219 (0–493.6)	223.9 (1.39–1,525)	0.73
MCP-1	0 (0–8,249)	4,214 (0–35,065)	0.18
TNF-α	0 (0–817)	60.4 (0–7,495)	0.4342
IL-18	379.8 (0–1,179)	0 (0–978.7)	0.032

CCL2, C-C motif chemokine ligand 2 (MCP-1); CCL5, C-C motif chemokine ligand 5 (RANTES); CXCL1, C-X-C motif chemokine ligand 1 (KC); CXCL12, C-X-C motif chemokine ligand 12 (SDF-1); CXCL8, C-X-C motif chemokine ligand 8 (IL-8, interleukin 8); Flt3L, fms-related tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IL-12 (p40), interleukin 12 subunit p40; IL-13, interleukin 13; IL-18, interleukin 18; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; ND, not detected; PDGF-BB, platelet-derived growth factor subunit B; SCF, stem cell factor; sFas, soluble Fas cell surface death receptor; TNF-α, tumor necrosis factor alpha.

Interleukin 12 is a pro-inflammatory cytokine composed of 2 subunits: p35 and p40.[20] Antigen-presenting cells are the main source of IL-12 in the body, and IL-12 is an important mediator of Th1 responses and induction of INF-γ production.[20–22] Limited information is available on the role of IL-12 in humans with IC/PBS. This cytokine was not significantly increased in bladder biopsies of human patients.[23] However, urine IL-12 was increased in human patients with overactive bladder syndrome.[24] Increased RNA expression of IL-12 (p40) in bladder tissues also was identified in mice with experimental autoimmune cystitis.[25] Interestingly, human patients receiving intravesicular instillation of IL-12 for treatment of superficial bladder transitional cell carcinoma had adverse effects that were similar to IC/PBS such as pain, dysuria, or increased frequency.[26] Identification of presence of IL-12 in urine of cats with FIC warrants further investigation and will depend on validation and optimization of assays for detection of cytokines in feline urine.

Interleukin 18 is another Th1 pro-inflammatory cytokine, and significantly increased serum concentrations were observed in our cohort of FIC-affected patients. Interleukin 18 was observed to be significantly increased in bladder tissues of IC/PBS-affected human patients.[23] Furthermore, decreasing urine concentrations of this cytokine in patients with IC/PBS have correlated with clinical improvement after hydrodistention therapy.[23] It is well established that IL-18 and IL-12 work in synergy to induce INF-γ expression in T-cells, NK cells, and dendritic cells.[22,27] Although in our study we did not identify increased concentrations of INF-γ in FIC patients, previous studies have identified a potential serum biomarker, kynurenine,[15] which is produced by an INF-γ-inducible gene IDO1.[28–30] Importantly, this gene is known to be INF-γ inducible in cats as well,[30] so one can speculate that INF-γ may be more locally produced in FIC cats in early stage of the disease, and furthermore, our findings of increased serum concentrations of IL-12 and IL-18 suggest they may work in conjunction with INF-γ in FIC.

CXCL12 (also known as SDF1) is a chemokine, which induces migration of various cells such as T-cells, B-cells, macrophages, and dendritic cells which express

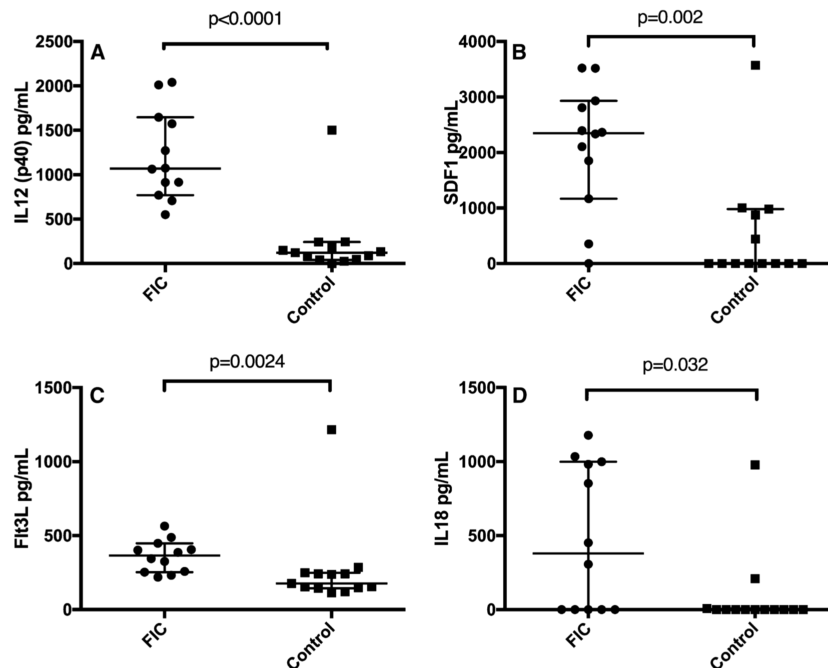

Fig 1. Concentrations of IL-12(p40) (**A**), SDF1 (**B**), Flt3L (**C**), and IL-18 (**D**) in serum of 12 acute FIC cats were significantly higher than in 13 healthy control cats. Graph presents median with 95% confidence interval.

the CXCL12 receptor CXCR4.[31,32] We observed significantly increased concentrations of CXCL12 in serum of cats with acute FIC. Studies investigating the role of this chemokine in cystopathies of humans or cats have not been reported. However, increased concentrations of bladder tissue CXCL12 were documented in a cyclophosphamide-induced bladder inflammation rat model.[33] Pain is one of the elements of both FIC and IC/PBS, and the chemokine CXCL12 has been shown to directly affect nociceptive neurons and induce pain.[34]

Another significantly increased cytokine in the serum of FIC cats was Flt3L. The receptor for this ligand (Flt3) is expressed in hematopoietic progenitor cells and is crucial for development of dendritic cells and enhances production of lymphoid progenitors.[35–37] Flt3L has been implicated as a pro-inflammatory factor in the pathogenesis of a variety of inflammatory diseases.[38–40] It also has been shown to have a protective, anti-inflammatory role in some animal models.[39] One proposed mechanism for this action of Flt3L has been the induction of T regulatory cells.[41] The reason for increased Flt3L in our cohort of FIC-affected cats is unknown. To our knowledge, studies investigating the role Flt3L in humans with IC/PBS have not been reported. One could speculate that the reason for increased serum Flt3L concentration is in response to infection, whereas previously it has been noted that Flt3L is transiently induced upon murine cytomegalovirus infection or exposure to toll-like receptor agonists.[42] The potential role of viral infection in the pathogenesis of FIC has been previously investigated.[43] Caliciviruses,[44] feline foamy viruses,[45,46] and gamma herpesviruses[47] have been proposed to be potential causative agents for FIC, but their role in pathogenesis is unclear and needs further investigation.[43,48] Flt3L also can be produced by a wide variety of cells in the body including both immune cells as well as nonimmune cells such as fibroblasts or endothelial cells,[49] and NK cells are the major source of Flt3L in the body.[50]

We have identified several potential biomarkers of acute FIC in serum of affected cats. However, our study has limitations including relatively small sample size, lack of samples from cats with chronic FIC and lack of histologic phenotyping by light microscopy, and the retrospective nature of the study. In addition, future studies incorporating additional control populations of cats with non-FIC lower urinary tract diseases (eg, urolithiasis, bacterial urinary tract infection) as well as nonbladder diseases to assess the specificity of changes in cytokine and chemokine concentrations are needed. The limited number of patients decreased the statistical power to detect more subtle differences in cytokine concentration and increased the risk of type II statistical error in our data set, which raises the question of whether the significant differences in cytokines observed in the present study would be similar in a larger cohort that included chronic cases. This limitation emphasizes the need for future studies analyzing cytokines in cohorts of patients with chronic FIC, because these cats would especially benefit from sensitive and specific biomarkers to measure treatment responses. Cases of FIC in general and of chronic FIC in particular would benefit from thorough phenotyping including cystoscopy and bladder biopsies to be able to fully assess the diagnostic utility of cytokines in these cases.

Another potential limitation of our study may be relatively low recovery of the multiplex assay and its ability to identify more subtle differences in analytes with lower

serum concentrations.[17] Whereas concentrations of all of the cytokines found to be significantly increased in serum of FIC cats (IL-12 p40, SDF1, Flt3L, and IL-18) were relatively high and well above the lower limits of detection, it is conceivable that concentrations of other cytokines could have been significantly different between groups, but remained undetected because of poor analyte detection at low concentrations.

Another potential limitation is that in some of the affected animals, slight increases in CK, AST, and ALT enzyme activities were detected. Although the changes were minimal and most likely not clinically relevant, we cannot completely exclude that these findings may be secondary to another ongoing disease process, which could cause increases in serum cytokine concentrations. The increase in CK activity was identified in the majority of cats, but none of the animals was symptomatic for myositis, and the increases probably were related to muscle damage secondary to restraint, intramuscular injection of sedatives or venipuncture. None of the animals developed any signs of muscular disease during months of close monitoring during the clinical trial. Increases in ALT (1/12) and AST (2/12) activities were minimal and slightly above the reference range. None of the cats had any changes identified on abdominal ultrasound examination that were suggestive of ongoing hepatic disease, such as cholangiohepatitis. The increases could have been a result of in vitro hemolysis after blood sampling.

Simultaneous determinations of serum and urine concentrations of cytokines and chemokines in affected and healthy cats would have been of value in differentiating local versus systemic responses. However, the optimal methods for preparation and storage of feline urine specimens for detection of urine cytokines have not been determined. In addition, studies investigating the analytical performance of the feline-specific multiplex assay for detection of cytokines and chemokines in cat urine have not been reported, but are being investigated in our laboratory.

Ours is the first study investigating serum cytokine concentrations in cats with FIC. We have identified several cytokines that are increased in the serum cats with FIC, which points to a pro-inflammatory milieu. Our study creates a foundation for future studies investigating the role of cytokines in FIC and their utilization as noninvasive biomarkers for diagnosis, differentiating disease phenotypes, and monitoring therapeutic outcomes in different forms of the disease. Future studies are needed to optimize sample preparation and storage, and to evaluate the specificity, sensitivity and clinical utility of these serum and urine cytokines as noninvasive biomarkers for FIC.

Footnotes

[a] Milliplex MAP Feline Cytokine/Chemokine Magnetic Bead Panel; Millipore, Billerica, MA

[b] Luminex 100; Luminex Corporation, Madison, WI

[c] Bio-Plex Manager 4.1.1; Bio-Rad, Hercules, CA

[d] GraphPad Prism 7.0; GraphPad, La Jolla, CA

Acknowledgments

Grant support: This study was supported by a grant from the Michigan State University Center for Feline Health and Well-being.

Authors thank Dr. Andrea Amalfitano and Dr. Yasser Aldhamen for their technical assistance.

Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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