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BIOMARKERS OF GASTROINTESTINAL DISEASE IN CHEETAHS (*ACINONYX JUBATUS*)

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Abstract: Gastrointestinal disease is a common clinical problem in captive cheetahs (*Acinonyx jubatus*). It is reported that gastritis affects the vast majority of the captive population of cheetahs. Pancreatitis and acute and chronic enteritis have also been reported. These issues pose significant long-term health and welfare implications for cheetahs. Cobalamin, folate, methylmalonic acid (MMA), gastrin, feline pancreatic-specific lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI) immunoassays are important biomarkers of gastrointestinal disease in domestic cats. The goal of this study was to determine if these immunoassays validated in domestic cats could be used clinically in cheetahs, by establishing reference intervals (RI) for these biomarkers in cheetahs. A cohort of 40 clinically healthy cheetahs was selected from three zoological institutions on the basis of being free of clinical gastrointestinal disease and extra-gastrointestinal disease that could affect biomarkers, as well as having banked frozen serum. Cheetah biomarker RI, with domestic cat RI for comparison in parentheses, are as follows: cobalamin 470–618 pg/ml (290–1500 pg/ml), folate 2.2–15.7 ng/ml (9.7–21.6 ng/ml), MMA 365–450 nM/L (139–897 nM/L), fPLI 0.5–1.2 μg/L (0–4 μg/L), and gastrin 30–50 pg/ml (<10–39.5 pg/ml). This study shows that RI for gastrointestinal biomarkers can be notably different, even between species that are as closely related as the domestic cat and the cheetah. Additionally, it was found that the fTLI assay does not cross-immunoreact with cheetahs. In conclusion, this study emphasizes the importance of developing species-specific RI for biomarker assays and using caution when extrapolating RI from other species.

INTRODUCTION

Cheetahs (*Acinonyx jubatus*) are in the subfamily Felinae, and are separated from the subfamily Pantherinae due to their inability to roar and their ability to purr. They are in the *Acinonyx* genus, one of three genera in the Puma lineage of Felidae. Out of the eight felid lineages, the Puma lineage is the second most closely related to the domestic cat lineage, indicating that they are more related to domestic cats than other large exotic cats.[21] Cheetahs are classified as vulnerable by the International Union for Conservation of Nature (IUCN) Red List, with only an estimated 6,700 animals remaining in the wild.[11] Wild populations are threatened by habitat loss and fragmentation, conflict with livestock farmers, and illegal hunting and trade. To compound these threats, cheetahs have very low genetic diversity, low reproductive success, and a high rate of infant mortality compared with other animal species.[10,28]

Morbidity and mortality in North American captive cheetahs are predominantly due to chronic degenerative diseases rather than infectious diseases.[27] These include glomerulosclerosis, veno-occlusive disease, chronic lymphocytic-plasmacytic gastritis, and systemic amyloidosis.[26] Chronic stress is suspected to be an important contributing factor for these diseases, as baseline cortisol concentrations are reported to be significantly higher, and adrenal cortices larger, in comparison with wild cheetahs.[37,43] Because these diseases are rarely observed in free-ranging cheetahs, environmental factors appear to play an important role in the disease pathogenesis; additionally, an immune-mediated mechanism, rather than a genetic one, has been suspected.[6,27]

Gastrointestinal disease is a common clinical problem in cheetahs. Both pancreatitis and enteritis have been reported in captive cheetahs, including cases of astrovirus, coronavirus, and *Clostridium*.[2,5,14,26,44] Most commonly, however, it

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has been reported that gastritis affects over 99% of the captive population of cheetahs in North America and South Africa, which has been confirmed on histopathology.[26,27] A more recent review of the Cheetah Species Survival Plan (SSP) Pathology Database from 2010 to 2018 found that approximately 80% of captive cheetahs have histologic changes consistent with gastritis (Karen Terio, unpubl. data). The histologic criteria for gastritis in cheetahs have been well documented.[38] Commonly reported clinical signs include vomiting, diarrhea, chronic weight loss, and failure to thrive. This has significant long-term welfare implications for the species. *Helicobacter* spp. are suspected to contribute in the pathogenesis of chronic gastritis in captive cheetahs. However, free-ranging cheetahs have been reported to have a similarly heavy colonization rate with *Helicobacter* spp., but only 3% have gastritis.[6,27]

Cobalamin, folate, methylmalonic acid (MMA), gastrin, feline pancreatic-specific lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI) are important biomarkers of gastrointestinal disease in domestic cats; however, their routine use in the cheetah has not yet been evaluated.[33]

Folate, or vitamin B9, is a water-soluble vitamin. In dogs and cats with proximal small intestinal disease, folate malabsorption can occur, ultimately resulting in decreased serum folate concentrations.[33] Serum folate concentrations may also be affected by hepatic or renal disease due to reduced storage or increased excretion, respectively.[1,24] Conversely, increased serum folate concentrations can be observed with small intestinal dysbiosis, as many bacterial species synthesize folate. Folate is a cofactor for the synthesis of amino acids and vitamins, and also plays an important role in DNA replication, repair, and methylation.[20] Folate deficiency in cats can cause poor appetite and growth, as well as bone marrow changes.[8,20,46]

Cobalamin, or vitamin B12, is a water-soluble vitamin. Herbivores are able to absorb cobalamin synthesized by their physiological gastrointestinal microbiota; however, carnivores are dependent on dietary intake.[33] Small intestinal disease involving the ileum can lead to destruction of ileal cobalamin receptors, causing cobalamin malabsorption and ultimately a decrease in serum cobalamin concentrations.[33] In dogs and cats, exocrine pancreatic insufficiency (EPI) can lead to cobalamin deficiency secondary to a carrier protein deficiency (i.e. intrinsic factor) and a deficiency in pancreatic proteases that would normally digest the cobalamin-binding protein, R protein. Thus, cobalamin deficiency can be caused by severe and long-standing ileal disease, EPI, and also small intestinal dysbiosis.[33] Because cobalamin plays a role in DNA synthesis, it commonly affects rapidly dividing cells, such as cells of the gastrointestinal and hematopoietic systems.[20] The clinical consequences of cobalamin deficiency in dogs, cats, and humans include gastrointestinal disease, central neuropathies, peripheral neuropathies, immunodeficiencies, and myelopathies.[20]

MMA is used as a marker of cobalamin deficiency on a cellular level.[30] Formation of MMA is favored when cells are deficient in cobalamin. Because cobalamin-dependent reactions occur intracellularly, within the cytoplasm and mitochondria, MMA is a better indicator of true cellular cobalamin deficiency than simply measuring serum cobalamin concentrations. However, because MMA is excreted through the kidneys, values in animals with kidney disease should be interpreted cautiously.[4]

Gastrin is a peptide hormone produced by G cells present in the stomach and proximal duodenum.[19] Its function is to stimulate gastric acid production; however, it also has trophic effects on the gastric mucosa. Serum gastrin concentrations can be mildly increased in dogs with gastritis or inflammatory bowel disease, and in cats with chronic kidney disease; however, marked elevations are typically indicative of gastrinomas in dogs and cats.[9,15,18,19,23] Additionally, serum gastrin can also be significantly increased in dogs and cats after administration of proton pump inhibitors.[17,19]

Serum fPLI assays specifically measure the serum concentrations of pancreatic lipase, which if elevated, indicates the presence of acinar cell damage, most likely due to pancreatitis.[33] Pancreatitis can be acute or chronic. Acute pancreatitis is often associated with various systemic complications, while chronic pancreatitis can lead to an acute exacerbation, diabetes mellitus, or EPI. This assay in domestic cats has a sensitivity of 54–100% and a specificity of 67–100%.[12] It is important to note that false negative results are possible, especially in cats with mild or chronic pancreatitis.[45] This is because sensitivity varies based on the severity of disease and because leakage of pancreatic enzymes does not occur with histopathologic lesions associated with chronic pancreatitis, such as fibrosis and atrophy.[45]

Serum TLI measures trypsin and trypsinogen released into the bloodstream from pancreatic

acinar cells.[33] Exocrine pancreatic insufficiency leads to severely decreased serum TLI concentrations. In domestic cats, pancreatitis is the most common cause of EPI.[32] EPI leads to impairment of nutrient absorption, leading to soft, voluminous stools and weight loss, as well as vitamin deficiencies.[34] This assay in domestic cats has a specificity of 85–100% and an undetermined sensitivity.[35]

Despite the high occurrence of gastrointestinal disease in captive cheetahs, reference intervals (RI) for biomarkers of gastrointestinal disease have not previously been established. Therefore, this study aims to fill this important gap in order to help diagnose these conditions and thus, improve the health and welfare of captive cheetahs.

MATERIALS AND METHODS

Study design

This study used a retrospective design to establish RI for biomarkers of gastrointestinal disease in cheetahs. A cohort of cheetahs was selected on the basis of having banked serum stored in a –80°C freezer, as well as a good overall health status, as defined below. Serum samples from cheetahs owned by three different zoological institutions were used for this study. Samples were shipped on dry ice to the Texas A&M Gastrointestinal Laboratory to be analyzed. The minimum serum sample size required to run the panel containing fPLI, fTLI, cobalamin, and folate was 0.7 ml. For gastrin and MMA, an additional 0.5 ml of serum was required for each test. The 40 cheetahs ranged in age from 0.5 to 10.9 y and included 13 males and 27 females. All cheetahs were part of a preventative health program that included a full physical examination with bloodwork and diagnostics every 2–3 y, as well as routine vaccinations and preventative medications for heartworm disease and internal and external parasites.

The main criterion for inclusion into the study was a designation of good health. Species360 Zoological Information Management System (ZIMS) (Bloomington, MN 55425, USA), an online information database used by numerous zoos, contains both medical records and RI for zoo species. Each institution's ZIMS medical records were searched for the most recent immobilization that contained a full physical examination, CBC, biochemistry panel, fecal examination, and whole body radiographs, as well as a urinalysis and abdominal ultrasound, when available. For inclusion purposes, an animal was considered healthy if its physical examination and diagnostic tests showed no significant findings related to gastrointestinal disease or extra-gastrointestinal disease that would be expected to affect any of the biomarkers evaluated in this study. Cheetahs that had minor medical issues that were not expected to have an impact on the gastrointestinal tract were also included in this study. For example, animals with wounds, low-grade heart murmurs, and/or minor transient urinary or upper respiratory tract infections were included in this study. The examination and testing needed to be performed within 1 y from the date the serum was collected. Each animal had to be up-to-date on vaccinations, as recommended by the Cheetah SSP. Additionally, all animals had to be asymptomatic and untreated for any gastrointestinal disease within the past year, as well as free of any extra-gastrointestinal disease that might affect any of the biomarkers (i.e., kidney disease, hepatic disease, biliary disease). Some cheetahs had gastric biopsies performed opportunistically during routine examinations. Based on histopathology, the severity of gastritis was categorized as either mild, mild to moderate, moderate, moderate to severe, or severe. Cheetahs that had moderate gastritis or less were included in the study as long as they met all other inclusion criteria. Cheetahs with moderate to severe gastritis or above were excluded. Additionally, all animals had to be negative for heartworm antibody, *Toxoplasma gondii* antibody, FeLV antigen, FIV antibody, and feline enteric coronavirus PCR.

Analytical methods and assay interpretation

RI from domestic cats for the biomarkers measured in the cheetahs are presented in Table 1, as previously determined by the Texas A&M University Gastrointestinal Laboratory.[31,39–42]

Serum cobalamin, folate, and gastrin concentrations were measured using an automated chemiluminescent assay (Immulite 2000, Siemens Healthcare Diagnostics, Malvern, PA 19355, USA). Low serum concentrations of folate indicate proximal small intestinal disease, whereas increased concentrations indicate small intestinal dysbiosis. Decreased serum cobalamin concentrations indicate distal small intestinal disease, exocrine pancreatic insufficiency, or small intestinal dysbiosis. Increased fasted serum gastrin concentrations suggest the presence of a gastrinoma or treatment with a proton-pump inhibitor.[9,17]

Table 1. Reference intervals (RI) and descriptive statistics for biomarkers of gastrointestinal disease in cheetahs. RI for biomarkers in domestic cats are included for reference.[31,39–42]

Biomarker[a]	*N*	Median	Min	Max	Distribution	Method	RI	90% CI		Domestic cat RI
								Lower limit	Upper limit	
Cobalamin (pg/ml or ng/L)	40	540	229	1201	NG	R	470–618	467–474	614–626	290–1,500
Folate (ng/ml or μg/L)	40	9.1	3.3	16.3	G	P	2.2–15.7	0.8–3.6	14.1–17.1	9.7–21.6
MMA (nM/L)	40	405	200	1076	NG	R	365–450	363–368	454–448	139–897
fPLI (μg/L)	40	0.8	0.5	1.1	G	P	0.5–1.2	0.4–0.6	1.1–1.2	0–4
fTLI (μg/L)	40	2	0	6	NA	NA	NA	NA	NA	12–82
Gastrin (pg/ml or ng/L)	40	39	10	217	NG	R	30–50	30–31	49–51	<10–39.5

[a] MMA, methylmalonic acid; fPLI, feline pancreatic lipase immunoreactivity; fTLI, feline trypsin-like immunoreactivity; NG, non-Gaussian; G, Gaussian; NA, not applicable; R, robust; P, parametric.

Serum concentrations of MMA were determined using a stable-isotope dilution gas chromatography–mass spectrometry assay.[29] Elevated MMA concentrations indicate cobalamin deficiency on a cellular level.[30]

Serum concentrations of fPLI were measured using a commercial ELISA (Spec fPL; Idexx Laboratories, Westbrook, ME 04094, USA). fPLI values of >5.4 μg/L suggest pancreatitis in domestic cats.[40]

Serum concentrations of fTLI were measured using an in-house radioimmunoassay.[36] In domestic cats, fTLI values at or below 8 μg/L are diagnostic for EPI, and values 8–12 μg/L are equivocal.[35]

Statistical analysis

RI were established using JMP Pro 14.2.0 (SAS Institute Inc, Cary, NC 27513, USA) and Reference Value Advisor 2.1[16] (National Veterinary School, Toulouse 31000, France) statistical software packages, following the guidelines provided by the American Society for Veterinary Clinical Pathology (ASVCP).[13] Assumptions of normality were evaluated and verified via the Shapiro–Wilk test. Data was screened for outliers using quantile and Q–Q plots and confirmed by Tukey HSD.

Folate and fPLI data were normally distributed (Gaussian); therefore, 90% CI were established using untransformed parametric methods. Ninety percent CI for cobalamin, MMA, and gastrin were established following $\log_{10}$ data transformations, due to the data not being normally distributed (non-Gaussian). This was followed by bootstrapping using nonparametric methods. This was considered the robust method.

The potential effects of age (continuous) and sex (nominal) on all five biomarkers were assessed by analysis of variance (ANOVA) and student's *t*-test, respectively. Statistical significance was set at $P < 0.05$. Lastly, the correlation between cobalamin and MMA was assessed by ANOVA.

RESULTS

The RI established for five biomarkers of gastrointestinal disease in cheetahs (cobalamin, folate, MMA, fPLI, and gastrin) are presented in Table 1. A RI was unable to be established for fTLI. There was no correlation between any of the five biomarkers and sex. The relationship between decreasing folate and increasing age trended towards statistical significance ($r = -0.30$, $P = 0.0591$). The relationship between decreasing fPLI and increasing age was statistically significant ($r = -0.37$, $P = 0.0184$); however, a low fPLI is not clinically relevant. No other biomarkers correlated with age. There was no statistically significant correlation between cobalamin and MMA.

DISCUSSION

RI for biomarkers of gastrointestinal disease are well-established for domestic cats and dogs. However, they have not yet been established for cheetahs. Species360 ZIMS contains the most comprehensive data on RI for hematological and biochemical values of zoological species. However, it does not include RI for the gastrointestinal biomarkers evaluated here. The goal of this study was to determine if the cobalamin, folate, MMA, gastrin, fPLI, and fTLI immunoassays validated in domestic cats could be used clinically in cheetahs, by establishing RI for these biomarkers in cheetahs.

A meaningful RI for serum fTLI concentrations could not be established. This is because all

cheetahs had serum concentrations in the very low end of the working range of the assay, far lower than would be required to differentiate healthy cats from cats with EPI, which is the primary use of this measurement. These consistently low values indicate a lack of species cross-immunoreactivity for this assay. A recent study performed in tigers revealed that serum fTLI values in healthy tigers were also lower than in domestic cats.[25] However, some tigers with chronic clinical signs of gastrointestinal disease were found to have significantly lower serum fTLI concentrations than healthy tigers.[25] Therefore, in that study, the domestic cat fTLI assay was used to establish a RI for tigers. In the future, a cheetah-specific fTLI assay would need to be established in order to effectively diagnose EPI using this biomarker.

In general, when considering the biochemical relationship between serum cobalamin and serum MMA concentrations, serum MMA concentrations would be expected to be higher in cheetahs with low cobalamin.[30] However, this study did not include animals with suspected cobalamin deficiency, and thus a negative correlation between serum cobalamin and MMA concentrations was not expected in this set of patients. Interestingly, however, in dogs with clinical signs of gastrointestinal disease and a proven cobalamin deficiency, it was shown that not all dogs with hypocobalaminemia have a cellular deficiency of cobalamin, or an elevated serum MMA concentration.[4] Future studies should evaluate whether there is a correlation between these two biomarkers in clinically ill cheetahs.

Notably, six samples from cheetahs that did not meet inclusion criteria due to clinical symptoms of gastritis or moderate to severe gastritis on histopathology had serum gastrin concentrations greater than the upper limit of the RI, ranging from 59.3 to 438 ng/L. None of these animals had been administered treatment for gastritis, including proton-pump inhibitors, for at least 6 mo before the sampling date. A study in dogs revealed increased blood gastrin concentrations with acute and chronic gastritis compared with controls.[18] In that study, the highest gastrin concentrations were seen in dogs with chronic gastritis. However, gastrin has not been established as a clinically valid biomarker for gastric disease in any species to date. Because our study only evaluated clinically healthy cheetahs, we were unable to evaluate whether gastrin concentrations in cheetahs are affected by clinical gastritis.

There was a trend towards statistical significance of decreasing folate with increasing age. This suggests either a decrease in absorptive capacity for folate with increasing age, or an increased incidence in proximal small intestinal disease in older cheetahs. A similar correlation of serum folate concentration and age has previously been documented in older domestic cats and humans. In humans, this is a result of decreased folate conjugase enzymes that occurs with increasing age.[3,20]

A main limitation of this study was the sample size. Standard recommendations for the generation of RI call for datasets of at least 120 individual animals. However, ASVCP guidelines for sample sizes between 40 and 120 with no outliers have been published.[13] This study represents 10.5% of the cheetah population registered with the Association of Zoos and Aquariums (AZA) Cheetah SSP.[7] Thus, a much higher number of samples would not have been easily achievable. Another key limitation of this study was determining inclusion criteria when the majority of clinically normal captive cheetahs have gastritis on histopathology (Karen Terio, pers. comm.).[26,27] However, it is important to note that even in domestic cats, there is limited information on what is normal histomorphology of the gastrointestinal tract as cats age.[22] A recent study showed that intestinal biopsies of asymptomatic cats showed a variety of findings that are considered abnormal, including 6 of 20 with lymphoplasmacytic enteritis.[22] Because cheetahs in this study were considered healthy and asymptomatic for gastrointestinal disease, future studies are needed to validate the relationship between animals with clinical signs of gastrointestinal disease and abnormal biomarker values. Additionally, future studies should further investigate the correlation between histopathologic findings and clinical disease in cheetahs.

This study represents the first report in the literature on RI for biomarkers of gastrointestinal disease in cheetahs. RI for domestic cats have been established for all six biomarkers, but this study shows that RI can be significantly different, even between species that are as closely related as the domestic cat and the cheetah. In particular, this study showed that the fTLI assay does not cross-immunoreact with cheetahs. In conclusion, this study emphasizes the importance of developing species-specific RI for biomarker assays, and using caution when extrapolating RI from other

species, even when they are closely related genetically.

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