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Results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsy specimens from clinically healthy client-owned cats

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Abstract

Background: Histopathology, immunohistochemistry, and molecular clonality testing are metrics frequently used to diagnose chronic enteropathy (CE) in cats. However, normal values for these metrics have been based mainly on samples from cats that were relatively young, specific pathogen-free, or both.

Objectives: To describe results of histopathology, immunohistochemistry, and clonality testing of endoscopically-derived biopsy specimens of the upper small intestinal tract from a cohort of clinically healthy client-owned cats.

Animals: Twenty clinically healthy client-owned cats ≥3 years of age.

Methods: Tissue specimens were collected from the stomach and duodenum and evaluated single blinded by a board-certified pathologist. In addition, samples were evaluated by routine immunohistochemistry and clonality testing. Cats were followed after the procedure for signs of CE.

Results: Integrated results from histopathology, immunohistochemistry, and clonality testing were interpreted as consistent with small cell lymphoma (SCL; n = 12), emerging SCL (n = 1), lymphocytic enteritis (n = 6), and pseudoclonality (n = 1). On follow-up, 3 cats eventually developed clinical signs of CE, of which 2 were euthanized 295 and 654 days post-endoscopy. The remaining 17 cats did not show clinical signs of CE after a median of 709 days (range, 219-869 days).

Conclusions and Clinical Importance: Intestinal biopsy specimens from clinically healthy client-owned cats commonly had abnormal findings on histopathology, immunohistochemistry, clonality testing, or some combination of these without apparent clinical relevance. Current diagnostic metrics for diagnosing CE in cats may need modification to be applicable to the general population of cats.

KEYWORDS

feline chronic enteropathy, inflammatory bowel disease, PARR, PCR for antigen receptor rearrangements, small cell lymphoma

Abbreviations: CE, chronic enteropathy; FFPE, formalin-fixed, paraffin-embedded; fPLI, pancreatic lipase immunoreactivity; fTLI, feline trypsin-like immunoreactivity; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; NK, natural killer; PARR, PCR for antigen receptor rearrangements; SCL, small cell lymphoma; SI, small intestinal; SPF, specific pathogen-free; TCR, T cell receptor; TRG, T cell receptor gene; WSAVA, World Small Animal Veterinary Association.

1 | INTRODUCTION

In 2008, the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group published histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from cats and dogs.[1] The histopathological standards include the description of normal and abnormal findings in the gastrointestinal tract of cats. Scoring sheets and pictorial templates of histopathological images were developed for the description and grading of morphological and inflammatory changes, thereby establishing a histopathologic standard of "normal" and "abnormal."[1] However, most of the studies used for developing the WSAVA criteria were performed on full-thickness biopsy specimens from specific pathogen-free (SPF) colony cats that predominantly were relatively young, with most cats being between 5 and 18 months of age.[2,3] This reference group differs from the population to which the standard criteria are actually applied. More specifically, cats with chronic enteropathy (CE) usually are middle-aged to older, with a variety of backgrounds and lifestyles.[4–9] Therefore, the question arises as to whether the standard criteria are applicable to the general population of cats presenting with possible CE, and whether the definition of "normal" by WSAVA standards represents the findings in normal cats in the corresponding age group.

Immunohistochemistry and molecular clonality testing frequently are used as additions to routine histopathologic assessment to differentiate inflammatory from neoplastic changes in cats with CE. Antigenic stimulation induces clonal expansion of memory lymphocytes as part of the physiologic immune response in inflammation.[10] This affects many different lymphocyte clones with a wide range of antigen receptor specificities and thus expansion and antigen receptor rearrangements become polyclonal.[11] Lymphocytic neoplasms comprise clones of a single or very few lymphocytic precursors, showing the same receptor rearrangement as their parent cell, resulting in monoclonal or oligoclonal receptor rearrangements.[11] Immunohistochemistry is used to determine whether a mixed population of T- and B-lymphocytes, indicative of inflammatory lesions, is present in the tissue or whether the lymphocytic population mainly consists of a single lymphocyte type, as seen in alimentary lymphomas in cats.

Clonality testing assesses the diversity of lymphocyte antigen receptor gene rearrangements, thereby differentiating clonal from polyclonal rearrangements. Polymerase chain reaction for antigen receptor rearrangements (PARR) is the most commonly used clonality assay for differentiating inflammatory bowel disease (IBD) from small cell lymphoma (SCL) in cats, because this test is widely available and can be performed on formalin-fixed and paraffin-embedded (FFPE) tissue.[12] However, similar to the WSAVA histology criteria, the assay has been developed on tissue from SPF colony cats.[12]

In this study, we characterize histopathological, immunohistochemical (for immunophenotyping), and clonality assay findings in endoscopically-derived biopsy specimens of the upper small intestinal (SI) tract from a group of clinically healthy client-owned cats with demographic characteristics resembling those of the cats to which these tests are applied. We hypothesized that a cohort of client-owned clinically healthy cats would show findings deemed to be normal according to the current standard tests for CE.

2 | MATERIALS AND METHODS

2.1 | Cats

This prospective study was conducted at the Veterinary Medical Teaching Hospital, Texas A&M University. The study protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee, and written owner consent was obtained for each cat before enrollment into the study. Twenty cats were included in the study.

2.2 | Inclusion and exclusion criteria

Clinically healthy, adult, client-owned cats ≥3 years of age, undergoing an elective procedure requiring general anesthesia were eligible for enrollment in the study. Study eligibility initially was determined by an owner questionnaire on general and gastrointestinal health. The questionnaire covered the following areas: attitude, activity, appetite, drinking, urination, chronic illnesses, weight loss, vomiting, diarrhea, and treatment with antibiotics, antacids, anti-inflammatory drugs, or corticosteroids. A physical examination was performed by a single board-certified internist (SM). Blood was collected from a peripheral vein or the jugular vein and the following tests were performed: CBC, serum biochemistry profile, serum total T4 concentration, and serum concentrations of cobalamin, folate, feline pancreatic lipase immunoreactivity (fPLI), and feline trypsin-like immunoreactivity (fTLI). Additionally, a serum feline immunodeficiency virus antibody test and feline leukemia virus antigen test were performed if the status of the cat was unknown (n = 4). Cats with gastrointestinal signs (eg, weight loss, hyporexia, vomiting > twice per month, and diarrhea) within 6 months before enrollment were excluded. In addition, cats with systemic diseases, chronic illnesses, or laboratory abnormalities that were deemed to be clinically relevant were excluded from the study. Cats with a serum cobalamin concentration <350 ng/L also were ineligible. Finally, cats that had received any antibiotics, antacids, anti-inflammatory drugs, or corticosteroids within the past 6 months were excluded from the study.

2.3 | Sample collection and processing

After a routine dental procedure under general anesthesia, all cats underwent gastroduodenoscopy. Six biopsy specimens each from the stomach and upper SI tract were collected for histopathologic examination, immunohistochemistry, and clonality testing. In 1 cat, anesthesia time and personnel allowed for an additional ileo-colonoscopy, whereas in 2 cats only duodenal biopsy specimens were collected because of a longer than usual setup time.

Histopathologic examination of hematoxylin and eosin (H&E)-stained endoscopic FFPE tissue sections was performed by a single

board-certified pathologist (MA) blinded to the clinical status of the cats (ie, the pathologist was unaware that the samples were from healthy control cats). Findings were reported descriptively, and numerically scored according to the WSAVA histopathologic scoring system.[1,13] Briefly, morphological features (eg, surface epithelial injury, crypt hyperplasia, crypt dilatation or distortion, and fibrosis or atrophy) and inflammatory changes (eg, lamina propria lymphocytes, plasma cells, eosinophils, neutrophils, and macrophages) were assessed histologically and assigned a score (normal = 0, mild = 1, moderate = 2, and marked = 3).[1]

Sections of FFPE tissue were sent to a single external laboratory for immunohistochemistry and clonality testing (the immunohistochemistry and molecular clonality analyses of biopsy specimens were performed at the Leukocyte Antigen Biology Laboratory, University of California Davis, SVM-PMI, 1 Shields Ave, 4206 VM3A, Davis, CA 95616, on a fee-for-service basis). Pathologists at the external laboratory were blinded to the health status of the cats. Biopsy sections were reassessed with H&E staining, and then immunohistochemistry and clonality testing were performed.

Immunohistochemistry was conducted using a stepwise approach. Staining for T-, B-, and natural killer (NK) cell markers (CD3, CD79a, granzyme B, respectively) were performed at the external pathologist's discretion and based on the results of the H&E staining (ie, size and distribution of mucosal lymphocytes).

Molecular clonality testing was conducted on at least 2 sections (each 25 μm) of FFPE tissue using PARR analysis. Total DNA content was measured before the PCR procedure to ensure that enough tissue was present for accurate PARR testing. Results from the H&E-based histopathology, immunohistochemistry, and molecular clonality analysis were integrated and reported by the external pathologist.

2.4 | Follow-up

All cats were followed up after endoscopy at various timepoints. An owner questionnaire on general and gastrointestinal health since endoscopy was used to assess the cat's health status. If abnormal findings were reported, owners were contacted and a more detailed history was obtained.

2.5 | Statistical analysis

The association between the results of laboratory tests, histopathology, and clonality assays was assessed using chi-squared tests or a Fisher's exact test, as appropriate. Statistical significance was set at $P < .05$. Statistical analyses were performed using a statistical software package (GraphPad Prism, GraphPad Software, Inc, San Diego, California).

3 | RESULTS

3.1 | Study population

Twenty cats were included in the study. Cats had a median age of 9.5 years (range, 3-18 years), median body weight of 5.0 kg (range, 2.6-10.8 kg), and median body condition score of 6 out of 9 (range, 5-9). There were 12 female spayed and 8 male neutered cats. Breeds included domestic shorthair (n = 12), domestic longhair (n = 3), Siamese (n = 2), Burmese (n = 1), Norwegian Forest Cat (n = 1), and Persian (n = 1).

According to the owner, 1 cat had a short episode of acute self-limiting diarrhea within the 6 months before the study. This cat had a minimally increased serum folate concentration (22.4 μg/L; reference interval, 9.7-21.6 μg/L). One cat had an increased fPLI concentration (15.6 μg/L; reference interval, ≤3.5 μg/L) without any current or prior associated clinical signs. Five cats had increased serum folate concentrations (25.3, 27.3, 33.8, 62.5, and 65.5 μg/L) without any current or prior associated clinical signs.

Gastric biopsy specimens were available from 18 cats, and upper intestinal tract biopsy specimens were available from all 20 cats.

Demographic characteristics, histopathological findings, and results of molecular clonality testing are shown in Table 1. Detailed results for individual cats are shown in Supporting Information Table S1.

3.2 | Histopathologic results

Sample number and quality were reported by the pathologist to be adequate for all cats. A detailed list of the WSAVA scores is shown in Supporting Information Table S2.

TABLE 1 Demographic data, histopathology, and molecular clonality testing

Number of cats	20
Demographic information	
Median age in years (range)	9.5 (3-18)
Median body weight in kg (range)	5.0 (2.6-10.8)
Median BCS (range)	6 (5-9)
Sex	12 FS, 8 MN
Breeds	12 DSH, 3 DLH, 2 Siamese, 1 Burmese, 1 Norwegian Forest Cat, 1 Persian
Results for histopathology	
Median gastric WSAVA score (range)	1.5 (0.5-3.5)
Median duodenal WSAVA score (range)	2.5 (1.5-5.5)
Results for molecular clonality assays on FFPE duodenal biopsy specimens (n = 20)	
Clonal rearrangements	8
Clonal rearrangements in a polyclonal background	5
Polyclonal rearrangements	6
Pseudoclonal rearrangements	1

Abbreviations: BCS, body condition score, 1-4: underweight, 5: ideal, 6-9 overweight; DLH, domestic longhair; DSH, domestic shorthair; FFPE, formalin-fixed, paraffin-embedded; FS, female spayed; MN, male neutered; WSAVA, World Small Animal Veterinary Association.

Histopathologic evaluation of H&E-stained gastric biopsy sections had abnormalities in all 18 available cats. Lymphocytic-plasmacytic gastritis was identified in all cats. This finding was reported to be minimal in 4, minimal to mild in 2, mild in 5, mild to moderate in 5, and moderate in 2 cats. One of the 2 cats with moderate lymphocytic-plasmacytic gastritis was reported to have focally extensive nodular lymphocytic and plasmacytic gastritis. Fibrosis was the most commonly reported morphologic abnormality, present in 17 cats (minimal in 3, minimal to mild in 2, mild in 11, and moderate in 1); lymphocytic nodule formation was present in 2 cats and 1 cat had occasional mucosal cyst formation.

Histopathologic evaluation of duodenal biopsy specimens showed some degree of lymphocytic-plasmacytic mucosal infiltration in all 20 cats (minimal to mild in 4, mild in 4, mild to moderate in 6, and moderate in 4). In 2 cats, a diagnosis of SCL was made based on histopathology. In addition to a diffuse infiltration of the lamina propria with monomorphic small lymphocytes, both cats had moderate epithelial infiltration with small lymphocytes. Morphologic changes were present in 19 cats. The most common architectural change observed in the duodenum was crypt hyperplasia in 18 cats (minimal in 3, minimal to mild in 1, mild in 6, and mild to moderate in 7), followed by fibrosis in 4, and lacteal dilatation in 4. One cat was reported to have occasional crypt abscesses.

3.3 | Immunohistochemistry

Lymphocytes infiltrating the lamina propria stained positive for CD3 in all cats. In 12 cats, a CD3+ epitheliotropic lymphocyte population was reported. In 5 cats, a mixed epitheliotropic and lamina proprial lymphocytic infiltrate staining positive for CD3 was identified. In 3 cases, the pathologist reported a CD3+ lymphocytic infiltrate without further comments on localization. In no case did the pathologist suggest that any additional stains were needed for a final diagnosis.

3.4 | PCR for Antigen Receptor Rearrangements

Molecular clonality testing of T cell receptor genes (TRG) was performed in all cats. In addition, clonality analysis using B-cell primers including IgH2, IgH3, and κ-deleting element was performed on sections of duodenal biopsy specimens from 1 cat.

Molecular clonality testing of the TRG identified clonal rearrangements in duodenal biopsy specimens of 8 cats. In 5 cats, clonal rearrangements in a polyclonal background were reported. In 6 cats, including the single case in which T- and B-cell primers were used, rearrangements were determined to be polyclonal. In 1 cat, TRG clonality analysis was interpreted as pseudoclonal, likely because of insufficient DNA retrieval.

3.5 | Integrated interpretation based on H&E staining, immunohistochemistry, and PARR

Results from H&E stains, immunohistochemistry, and PARR were integrated by the external pathologists and reported as case interpretations. Results were interpreted as consistent with duodenal SCL in 12 cats and emerging SCL in 1 cat. Six cats were reported as having lymphocytic enteritis. In 1 cat, the findings were deemed uninterpretable because of pseudoclonality.

3.6 | Correlation among laboratory findings, histopathology, and results of clonality testing

No association was found between laboratory findings and histopathology or results of clonality testing (P = .28 and .18, respectively). Similarly, no association was identified between histopathology and results of clonality testing (P = .38).

3.7 | Follow-up data

Follow-up data were available for all 20 cats. Two cats were euthanized because of signs of gastrointestinal disease, including weight loss and vomiting, 295 and 654 days post-endoscopy, respectively (see Supporting Information Table S1, cases 10 and 19). Both cats were diagnosed previously with SCL based on the first histopathologic examination as well as laboratory results on H&E, immunohistochemistry, and PARR. The owner reported that the former cat had developed weight loss (approximately 1 kg) and frequent vomiting approximately 9 months after endoscopy. The cat underwent abdominal ultrasound examination before euthanasia during which thickened segments within the SI tract and abdominal lymphadenomegaly were identified. This cat did not receive any treatment. The second cat developed weight loss (1.3 kg), sarcopenia, vomiting, and chronic kidney disease (International Renal Interest Society stage 3, proteinuric, non-hypertensive). The cat was treated with prednisolone and budesonide before euthanasia eventually was elected. Neither cat was available for postmortem examination.

Another cat developed severe non-self-limiting vomiting approximately 513 days post-endoscopy (see Supporting Information Table S1, case 13). The owner reported that a CBC, serum biochemistry profile, and abdominal ultrasound examination had been within normal limits and that the cat's body weight had been unchanged after endoscopy. Upon treatment with a prescription hydrolyzed protein diet, signs of gastrointestinal disease ceased within days. At the time of study enrollment, this cat was the only 1 in which ileo-colonoscopy was performed in addition to gastroduodenoscopy. Histopathology at that time showed mild diffuse and moderate nodular lymphocytic-plasmacytic gastritis with mild fibrosis, mild diffuse lymphocytic-plasmacytic duodenitis with minimal diffuse hyperplasia of crypts, minimal diffuse lymphocytic-plasmacytic ileitis, and mild diffuse lymphocytic-plasmacytic colitis with multifocal nodular lymphocytic aggregates. The WSAVA scores of the stomach, duodenum, and colon were 2.5, 2.0, and 2.0, respectively. Immunohistochemistry was consistent with a mildly epitheliotropic lymphocyte population staining positive for CD3 in both the upper and lower SI tract. Clonality testing identified polyclonal rearrangements in samples from the upper and lower SI tract, with small reproducible peaks within the polyclonal background, suggestive of a decreased T cell receptor repertoire. Lesions were interpreted as consistent with

lymphocytic enteritis with mild epitheliotropism in the upper SI tract and lymphocytic enteritis within the lower SI tract.

Owners' responses to follow-up questionnaires for the remaining 17 cats indicated no signs of chronic gastrointestinal disease after a median of 709 days post-endoscopy (range, 219-869 days). Of these 17 cats, duodenal biopsy specimens previously had been interpreted as consistent with SCL in 10, emerging SCL in 1, and lymphocytic enteritis in 5. One case was deemed uninterpretable because of pseudoclonality.

4 | DISCUSSION

To the best of our knowledge, ours is the first study describing the results of histopathology, immunohistochemistry, and molecular clonality testing in endoscopically obtained upper SI biopsy specimens of clinically healthy client-owned cats with demographic characteristics similar to those of cats that present with signs of CE.

All 20 cats in our study had histopathological changes that are considered abnormal based on current WSAVA standards. Although many inflammatory changes were considered minimal to mild, most of the cats had inflammatory lesions that were rated as mild to moderate lymphocytic-plasmacytic enteritis. Two cats were diagnosed with SCL based on histopathology alone. Other histological features seen in our population of clinically healthy cats included lymphocytic nodule formation in the stomach, fibrosis, crypt hyperplasia, lacteal dilatation, and crypt abscesses.

Results of histopathologic studies of intestinal biopsy specimens from clinically healthy cats have been described before and are the basis for the WSAVA histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy specimens from both dogs and cats.[1–3,13] However, upon careful examination of the reference population for the WSAVA standards, the definition of a normal histological baseline becomes somewhat questionable. Although guidelines for establishing a normal histopathological baseline are lacking, guidelines exist for establishing reference intervals for laboratory values. Generally, a reference interval is defined as "an interval that, when applied to the population serviced by the laboratory, correctly includes most of the subjects with characteristics similar to the reference group, but excludes others."[14] In other words, a reference should be established in a cohort of healthy individuals with otherwise the same characteristics as the patient population to which it is intended to be compared. The same principle likely should apply to establishing histopathologic standards. Depending on the study and underlying diagnosis of IBD or SCL, cats with CE have a reported median age between 6 and 13.5 years.[4–9] However, the WSAVA criteria were developed based mainly on full-thickness biopsies from SPF colony cats that were relatively young.[2,3] One study used adult cats of undetermined origin from an Animal Control Center in Japan.[15] However, the authors also stated that, based on the dental status, the cats were considered young adults. In addition, this study mostly contributed to the WSAVA criteria through the description of epithelial globular leukocytes, rather than normal architecture and total mucosal leukocytes.

The intestinal tract and its gut-associated lymphoid tissue is the largest lymphoid organ in the body, with an extremely high plasticity and compensatory capacity.[16] With increasing age and chronic antigen exposure, gastrointestinal histology may change without necessarily representing a pathological condition. Therefore, most of the histopathologic lesions seen in our population of cats might in fact be normal for older cats.

On the other hand, clinical or subclinical CE is very common in geriatric cats, as well as in elderly people, with up to 40% of geriatric human patients reporting at least 1 gastrointestinal complaint during a routine physical examination.[17] Therefore, histopathological changes seen in our population also might reflect true subclinical disease. At least for the 2 cats diagnosed with SCL on histopathology, this appears likely, because those cats developed severe clinical signs of CE and eventually were euthanized approximately 10 and 22 months post-endoscopy. Another cat developed clinical signs of CE with severe vomiting approximately 17 months post-endoscopy. However, this cat had only minimal to mild histopathological changes in both the upper and lower SI tract, and the lymphocyte population was determined to be polyclonal on PARR. In addition, clinical signs subsided after treatment with a hypoallergenic diet. The long lag time between endoscopy and development of clinical signs, mild histopathological changes and polyclonality at the time of endoscopy, and response to diet imply that, in this particular cat, the clinical signs might have been caused by a new onset of CE unrelated to previous changes, and the disease might be categorized as food-responsive enteropathy. The remaining 17 of 20 cats had no clinical signs of chronic gastrointestinal disease after a median of 709 days post-endoscopy (range, 219-869 days), and thus subclinical disease appears less likely for this group of cats.

Finally, observer-dependent variability is another possible explanation for our results. Despite standardized scoring criteria, interpretation of histopathological findings may vary substantially among pathologists.[18,19] In addition, the extent of cellular infiltration in the lamina propria was judged subjectively, without using computer-assisted morphometry methods. Therefore, observer-dependent subjectivity and error cannot be ruled out and another pathologist might have assessed and scored the biopsy specimens differently.

In addition to histopathological examinations by 1 pathologist, samples were sent to an external laboratory for immunohistochemistry and molecular clonality analysis of FFPE duodenal tissue samples. Pathologists at the external laboratory performed their own H&E-staining evaluation and integrated results from H&E-based histopathology, immunohistochemistry, and PARR to formulate a diagnosis and interpretation of each case as performed routinely on samples from cats submitted to the laboratory for diagnostic purposes. Clonality analysis of the TRG revealed clonal rearrangements in 13 of 20 cats with or without a polyclonal inflammatory background and thus they were interpreted as consistent with SCL (n = 12) or emerging SCL (n = 1). Only 6 cats were found to have polyclonal T cell receptor (TCR) rearrangements. One cat was found to have results consistent with

pseudoclonality, most likely because of insufficient DNA retrieval. One possible explanation for the frequent finding of clonal rearrangements would be the presence of malignant but indolent lymphocyte clones. Again, this would appear to be a reasonable explanation for the 2 cats that were diagnosed with SCL on histopathology and much later developed clinical signs of CE. We cannot exclude the possibility that the remaining 11 cats had SCL elsewhere in the gastrointestinal tract with trafficking neoplastic lymphocytes causing positive clonality results. Lymphocyte trafficking is a well-known phenomenon, allowing naïve and memory T cells to be recruited to the lamina propria or epithelium of the intestinal tract by a process called lymphocyte homing.[20] Trafficking of neoplastic (clonal) lymphocytes might cause detection of clonal TRG rearrangements in the present lymphocyte population without apparent histopathological changes. However, the long disease-free follow-up time for these cats makes this scenario somewhat unlikely.

Another explanation would be the occurrence of benign clones. In some instances, chronic antigenic stimulation may lead to disproportional proliferation of a lymphocytic subpopulation, resulting in true but benign clonal expansion, often in a polyclonal background.[11] Benign clonal expansion has been documented in humans[21,22] and dogs with infectious and autoimmune diseases, neoplasia, and drug administration.[23–25] Other causes for the detection of clonality in the absence of neoplasia include canonical rearrangements of certain γδ T cell clones and nonspecific amplification of sequences other than rearranged TRGs.[11] Benign clonal expansion is a plausible explanation, especially for the 5 cats in which clonality analysis identified clonal rearrangements in a polyclonal background. However, we did not find an association between extent of inflammation and results of the clonality assay. Also, even if such benign clonal expansion were to be the reason for the many cats that were positive for PARR, this would severely hamper the clinical usefulness of PARR.

The presence of pseudoclonal results may be another valid explanation for our findings. Pseudoclonal profiles may result from a lack of primers covering the rearranged genes, mutation of primer binding sites (common in somatic hypermutation in B-cells), absence of rearranged T cell receptor gamma chains (NK-cell neoplasms), or insufficient target DNA.[11] In PCR-based clonality assays, the amount of input DNA is standardized and determined mainly by the size and amount of tissue available for DNA retrieval. However, the target DNA is the DNA that is amplified during the PCR (ie, DNA from T cells in TRG clonality assays). With low numbers of lesional T cells, the ratio of target DNA to total DNA decreases and preferential amplification and pseudoclonality may occur despite adequate total DNA concentration and purity.[11,26] This is an important reason that clonality assays should always be interpreted in the context of histopathology and immunohistochemistry, and thus should be performed in the same laboratory, preferably by the same pathologist. In our study, 1 cat was reported to have pseudoclonal rearrangements, most likely because of low target DNA in the sample, and thus results were deemed uninterpretable. In all other cats, the clonality assays were judged to have sufficient input DNA for the assay to be performed and interpretations of integrated results from histopathology, immunohistochemistry, and PARR were reported for these cats. Polymerase chain reaction-based analysis of Ig/TCR rearrangements is widely used in human medicine and is considered to be the gold standard for clonality testing.[26] However, both false negative and false positive results have been a problem, especially in the early years of assay use. This has led to the formation of the EuroClonality (BIOMED-2) consortium and the development of standardized multiplex PCR assays for nearly all Ig/TCR targets in humans.[26,27] This standardization made it technically feasible to bring this test into a routine diagnostic setting. However, besides the analytical phase, pre- and post-analytical aspects should be considered. Thus, interpretation algorithms have been introduced that take into account peak heights and ratios to define truly clonal rearrangements.[26–30] Such standardization is currently lacking among veterinary laboratories, and thus differences in primers, laboratory practices, and result interpretation among laboratories might explain our findings.[28]

Since the introduction of clonality assays for the diagnosis of intestinal T cell lymphoma in cats in 2005, several studies have investigated the value of PCR-based clonality assays in the diagnosis of intestinal and extraintestinal T cell lymphoma in cats.[9,12,31,32] Subsequently, clonality assays have become the gold standard for the diagnosis and differentiation of lymphoma in cats.[33] However, similar to the WSAVA criteria, PARR for the molecular diagnosis of intestinal T cell lymphoma in cats was developed based on samples obtained from healthy young (ie, 12-18 months old) SPF colony cats and thus might not be representative of the target population. Although the sensitivity of PCR-based clonality assays performed on FFPE tissue generally is considered high (>90%), a study in human patients with lymphoproliferative disease identified specificities as low as 54.3% in patients with reactive lesions, even with the use of standardized BIOMED-2 clonality assays.[34] A recent study in cats with CE reclassified cats diagnosed with IBD on the basis of histopathology as having SCL instead, based on their PARR analysis.[35] Our results, as well as results from human pathology, imply that reclassification based on clonality results alone may not be justified.

Our study had several limitations. So as not to severely prolong anesthesia time, endoscopy and collection of biopsy specimens were restricted to the upper SI tract and stomach in most of our cats. Thus, we cannot exclude the possibility that some cats had SCL elsewhere inside or outside the intestinal tract, which might explain the number of clonal results in our study. However, once again, this seems unlikely because most of the cats never developed any clinical signs of CE even after having been followed for several months to years. In addition, we cannot entirely exclude the possibility of subclinical disease being present in this population of cats. Most of the cats in our study were slightly overweight with a median body condition score of 6 (range, 5 to 9 out of 9), and thus obesity could be viewed as a clinical abnormality. However, for a number of different diseases in humans, mild obesity has been shown to be associated with longer survival compared to patients with a body condition that is considered ideal.[36] Based on our clinical experience, most healthy geriatric cats are overweight, and restricting the body condition score to an ideal score of 5 out of 9 likely would have introduced a substantial bias into the study population. Our inclusion criteria permitted cats that were

vomiting up to twice per month. In addition, some cats had laboratory abnormalities such as increased serum folate concentration or increased serum fPLI concentration without associated clinical signs. Cats that were vomiting either were long-haired cats vomiting predominately hairballs or had occasional vomiting up to twice per month without any other clinical signs. Both cats with SCL were among the 4 cats that had occasional vomiting, and thus we cannot exclude that this might have been an early sign of gastrointestinal disease. Progression of IBD to SCL over months to years has long been suspected, and inflammatory lesions frequently coexist with SCL.[9,12] Although results of histopathology and clonality testing did not correlate with clinical or laboratory abnormalities, subclinical CE remains a possible reason for the findings in our study. Finally, because of the stepwise approach the pathologists took for immunohistochemistry, tissue biopsy specimens did not routinely undergo staining for B or NK cells. Therefore, mixed infiltrates were likely missed in the tissue biopsy specimens. However, we indented not to interfere with the pathologists' approach and to assess whether this cohort of cats would be identified correctly as clinically healthy based on tests that currently are considered to be the gold standard for the diagnosis of CE in cats.

5 | CONCLUSIONS

We characterized results of histopathology, immunohistochemistry, and molecular clonality testing in endoscopically-obtained upper SI biopsy specimens from healthy client-owned cats with demographic characteristics resembling those of cats that present with signs of CE. Intestinal biopsy samples commonly had histopathologic findings considered abnormal based on current WSAVA standards. Similarly, results of clonality testing identified many cats with clonal rearrangements within this group of healthy cats. Our results suggest that histological scoring criteria may need to be revised and adapted to a more adequate reference population. Although the sensitivity of molecular clonality testing generally is considered to be high, our results imply that further assessment of the specificity of this diagnostic modality may be warranted.

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CONFLICT OF INTEREST DECLARATION

All authors are either employed by (Marsilio, Lidbury, Suchodolski, and Steiner) or affiliated with (Ackermann) the Gastrointestinal Laboratory at Texas A&M University, which offers laboratory tests, including histopathology services, on a fee-for-service basis.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study protocol was approved by the Texas A&M University Animal Care and Use Committee (IACUC 2015-0276 CA) and written owner consent was obtained for each cat prior to enrollment into the study.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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