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Journal

Journal of Veterinary Internal Medicine, 37(2)

ISSN

0891-6640

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Publication Date

2023-03-01

DOI

10.1111/jvim.16636

Peer reviewed

Received: 13 May 2022 | Accepted: 6 January 2023

DOI: 10.1111/jvim.16636

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Clinical utility of an immunoglobulin A-based serological panel for the diagnosis of chronic enteropathy in dogs

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Abstract

Background: A panel of IgA-based serologic assays might aid in the diagnosis of chronic enteropathy (CE) in dogs, a syndrome encompassing conditions such as food-responsive enteropathy, immunosuppressant-responsive enteropathy, and inflammatory bowel disease (also referred to as chronic inflammatory enteropathy). However, it is unclear whether these biomarkers discriminate between CE and other types of primary intestinal disorders.

Objectives: To evaluate a diagnostic panel that measures serum concentrations of IgA directed against OmpC (ACA), canine calprotectin (ACNA), and gliadin-derived peptides (AGA) in dogs with well-characterized intestinal diseases.

Animals: Fifty-five dogs with primary intestinal disease.

Methods: Serum ACA, ACNA, and AGA concentrations were measured in 30 dogs with CE and 25 dogs with other intestinal diseases (non-CE population), including histoplasmosis, parasitism, *E. coli*-associated granulomatous colitis, and lymphoma. Serum IgA concentrations were compared among populations, and sensitivities and specificities were calculated using laboratory-provided cut-points.

Results: Twenty-six of 30 (87%) CE dogs and 21 of 25 (84%) non-CE dogs had abnormal concentrations (intermediate or high) of at least 2 markers; these proportions were not significantly different ($P = .99$). A serum ACA concentration ≥15 EU/mL was 86.7% (95% confidence interval [CI], 69.3%-96.2%) sensitive and 24.0% (95% CI, 9.4%-45.1%) specific for CE diagnosis. High AGA concentrations were observed in 16 of 25 (64%) non-CE dogs.

Conclusions and Clinical Importance: The evaluated serologic markers were poorly specific for CE diagnosis, which raises concerns that their use in clinical practice might lead to misdiagnoses and delayed or even detrimental treatments in dogs with non-CE intestinal diseases.

Abbreviations: ACA, IgA antibodies against bacterial OmpC; ACNA, IgA antibodies against canine calprotectin; AGA, IgA antibodies against gliadin-derived peptides; CE, chronic enteropathy; CIE, chronic inflammatory enteropathy; FRE, food-responsive enteropathy; IBD, inflammatory bowel disease; IRE, immunosuppressant-responsive enteropathy; PLE-L, protein-losing enteropathy with lymphangiectasia.

KEYWORDS
ACA, AGA, biomarkers, food-responsive enteropathy, IgA, inflammatory bowel disease

1 | INTRODUCTION

Chronic intestinal diseases are important causes of morbidity and even mortality in the pet dog population.[1-3] Untreated or poorly controlled chronic intestinal diseases can result in myriad consequences including weight loss, protein-energy malnutrition, electrolyte abnormalities, micronutrient derangements, hormonal imbalances, and death.[2,4-9] Mucosal inflammation is present in many cases, but a specific etiology often is not identified despite a thorough diagnostic investigation. These cases are commonly termed chronic enteropathy (CE) or chronic inflammatory enteropathy (CIE).[1,10,11] The syndrome of CE encompasses several conditions that typically are classified by therapeutic response, such as food-responsive enteropathy (FRE), antibiotic- or microbiota-responsive enteropathy, and immunosuppressant-responsive enteropathy (IRE) or inflammatory bowel disease (IBD).[7,10] The terms CIE and IBD are sometimes used interchangeably, but the terminology used to describe CE syndromes is not universally agreed upon.[10] The pathophysiologic mechanisms responsible for CE are also a subject of ongoing debate and research.[7,10] Still, available evidence suggests that breakdown of immunologic tolerance to luminal antigens, alterations in gut barrier function, and an altered intestinal microbiome, all potentially on a genetic susceptible background, are involved in the pathogenesis of CE in dogs.[10,12-14]

The diagnosis of CE is complex and involves integration of historical and physical examination findings, laboratory tests, diagnostic imaging, as well as therapeutic trials.[15] In addition, histopathologic assessment of endoscopically or surgically collected intestinal biopsy samples is required in many cases to evaluate for possible neoplastic or infectious causes.[15,16] The costs and challenges associated with diagnosing CE have prompted numerous investigations of serum and fecal biomarkers.[11,17] Many of these markers have contributed to improved understanding of CE, but only a few markers are routinely used in clinical practice.[17-20] Serum folate and cobalamin concentrations are measured frequently to assess intestinal absorptive function, and they also have implications for disease management.[21,22] Serum C-reactive protein concentrations and fecal calprotectin concentrations can be used to assess disease severity and treatment responses.[20,23] These and other markers can help in the evaluation of CE cases, but they do not discriminate between CE and other forms of chronic intestinal disease.[11]

A panel of 3 serologic markers has been developed to aid in the diagnosis of CE and IBD in dogs.[24] The panel consists of measurements of serum IgA antibodies against bacterial OmpC (ACA), canine calprotectin (ACNA), and gliadin-derived peptides (AGA). This panel is now offered as a commercial test, and the "CE-IBD" assay has garnered considerable attention in the veterinary community.[25,26] Indeed, blood-based markers capable of differentiating CE from other forms of intestinal disease would be extremely valuable to practitioners when considering the current challenges and costs of definitively diagnosing CE. However, the use of the CE-IBD assay in clinical settings has raised several concerns, and a previously published study suggesting that these markers distinguish CE from other gastrointestinal disorders has been retracted.[27-29] Critical evaluations of the serologic markers in well-characterized populations of dogs are needed before routine clinical use can be recommended. Our objective was to evaluate serum ACA, ACNA, and AGA concentrations in dogs with CE and dogs with non-CE intestinal disease that could mimic CE in a clinical setting.

2 | MATERIALS AND METHODS

2.1 | Design overview

The diagnostic performance of the CE-IBD assay was investigated by measuring concentrations of the 3 serologic markers in 2 populations of dogs with well-characterized intestinal disease, which included those dogs with CE and those dogs with other forms of primary intestinal disease (non-CE population). A sample size calculation was performed using extrapolated data from a population of dogs with possible CE.[27,29] The calculation was based on potential differences in ACA concentrations because it is the primary marker used by the commercial laboratory to determine if the results are consistent with CE.[26] In order to detect an approximate 30% difference in ACA concentrations between CE and non-CE populations with alpha of 0.05 and power of 0.8, at least 14 dogs would need to be included in each population. If at least 23 dogs were included in each population, the power would be >0.95 to detect this potential difference.

Both banked serum specimens and prospectively collected specimens were used for the study. All samples were collected from dogs at the time of a veterinary evaluation for active gastrointestinal disease. Several study investigators had banked specimens that had been collected in the years 2012 to 2022 as part of diagnostic submissions or clinical research projects, and surplus serum had been stored at −80°C. Investigators reviewed these cases to identify those in which medical records contained sufficient information to allow classification into 1 of the study populations as described below, and sufficient serum volume was available for analyses. In addition, study investigators also prospectively collected samples from patients that were undergoing clinical evaluations between June 2021 and January 2022 that met the criteria outlined below. All serum samples were submitted to a commercial laboratory (Antech Diagnostics, Inc) for measurements of ACA, ACNA, and AGA concentrations. Complete analytical validation has not been published, but limited information about assay characteristics can be found elsewhere.[24,27,29]

2.2 | Case classification

The CE population consisted of 3 subsets of dogs that included FRE, IRE, and idiopathic protein-losing enteropathy associated with lymphoplasmacytic inflammation and lymphangiectasia (PLE-L). Dogs with potential antibiotic- or microbiota-responsive enteropathy were not included in our study because of the controversies surrounding the diagnosis and classification of this population.[10,30-32] All dogs within the CE population were required to have ongoing diarrhea or vomiting ≥3 weeks in duration at the time of blood collection in addition to minimum diagnostic testing, which included a CBC, serum or plasma biochemical profile, fecal flotation or empirical anthelmintic treatment, and an abdominal ultrasound examination. Dogs were eligible for inclusion in the CE population if there was an absence of historical, hematologic, biochemical, or sonographic evidence of metabolic or extra-intestinal disease that could account for the gastrointestinal signs, and either a negative fecal flotation for gastrointestinal parasites or failure to respond to empirical anthelmintic treatment. Previous or current antimicrobial or probiotic usage were not exclusion criteria, but dogs were required to have persistent gastrointestinal disease despite these treatments. Dogs were excluded if they had been treated with glucocorticoids or other immunosuppressive medications in the 4 weeks preceding sample collection.

In addition to these general criteria, CE dogs were required to meet additional inclusion criteria to permit classification of CE as either FRE, IRE, or PLE-L.[7,10,15] The classification of FRE was based on therapeutic response assuming the general CE inclusion criteria were met whereas the IRE and PLE-L dogs were required to have undergone additional laboratory testing and endoscopic examination with histologic assessment of intestinal biopsy samples. Dogs were classified as FRE if they had resolution of gastrointestinal signs without recurrence for at least 1 month after feeding either a hydrolyzed protein diet, a novel protein/limited ingredient diet, or a therapeutic gastrointestinal diet.

Dogs with IRE and PLE-L were required to have undergone additional testing for hypoadrenocorticism and exocrine pancreatic insufficiency. Hypoadrenocorticism was excluded in IRE and PLE-L dogs by documenting baseline cortisol concentrations >55 nmol/L (2 μg/dL) or post ACTH-stimulated cortisol concentrations >138 nmol/L (5 μg/dL).[33] Exocrine pancreatic insufficiency was excluded by documenting trypsin-like immunoreactivity concentrations ≥5.7 μg/L.[15,34] Dogs with IRE and PLE-L were required to undergo histopathologic assessment of intestinal biopsy samples.[7,10,15] Dogs were classified as IRE if histopathology confirmed a nonsuppurative mucosal inflammatory infiltrate in the absence of an overt infectious or neoplastic disease, clinical signs failed to respond to either a hydrolyzed protein or novel protein/limited ingredient diet, and clinical signs responded to subsequent administration of immune-modulating treatments. Dogs were classified as PLE-L if serum albumin concentration was <2.5 g/dL, no proteinuria was detected on urinalysis, serum bile acid concentrations were normal, and histopathology disclosed lacteal dilatation accompanied by a nonsuppurative mucosal inflammatory infiltrate in the absence of an overt infectious or neoplastic disease.[35,36] The PLE-L subset potentially could be a form of IRE given the mucosal inflammatory infiltrates, but they were treated as a distinct subset given the controversies surrounding the classification and treatment of intestinal lymphangiectasia.[2,4,7,10,15]

Dogs were not considered for inclusion in the CE population unless they met the general inclusion criteria for CE as well as the specific criteria for classification into the FRE, IRE, or PLE-L subpopulations. A canine inflammatory bowel disease activity index (CIBDAI) score also was calculated for each dog in the CE population when sufficient information was available in the medical record.[21]

The non-CE population consisted of dogs with diarrhea or vomiting that was caused by intestinal parasitism, histoplasmosis, lymphoma, or *E. coli*-associated granulomatous colitis. These diseases were specifically targeted because they represent primary gastrointestinal disorders that are likely to have similar or overlapping clinical presentations to CE.[6,9,15,37,38] Dogs were included in the parasitism subpopulation if a fecal flotation or smear confirmed the presence of parasitic ova or cysts and clinical signs resolved after anthelmintic treatment. Dogs were included in the histoplasmosis subpopulation if a rectal mucosal scraping or intestinal biopsy results showed the presence of organisms consistent with *Histoplasma spp*. The lymphoma subpopulation included dogs in which abdominal ultrasound examination identified structural changes consistent with infiltrative intestinal disease, and cytology or biopsy of lymph nodes or intestine identified a monomorphic population of intermediate to large neoplastic lymphocytes (e.g., lymphoblastic lymphoma). For this study, sonographic evidence of infiltrative intestinal disease was defined as single, multifocal, or diffuse areas of severe thickening (wall thickness ≥8 mm) or loss of normal wall layering. The granulomatous colitis subpopulation included dogs in which intestinal histopathology identified granulomatous mucosal infiltrates with periodic acid-Shiff positive macrophages, and either fluorescence in situ hybridization or immunohistochemistry confirmed *E. coli* within mucosal macrophages.

2.3 | Data and statistical analysis

Data distributions were assessed using Shapiro-Wilk testing and boxplot analysis; normally distributed data were reported as mean ± SD whereas data that were not normally distributed were reported as median and interquartile range (IQR). Baseline characteristics of CE and non-CE populations were compared using Mann-Whitney U tests, unpaired Student's *t*-tests, or Fisher exact tests as appropriate. Spearman rank correlation coefficients (ρ) were calculated to evaluate potential associations of CIBDAI scores and biomarker concentrations within the CE population. The serologic markers were compared between CE and non-CE populations using Mann-Whitney U tests. Fisher exact testing was used to compare the proportion of dogs in each population that had abnormally high concentrations of 2 and 3 markers. The sensitivity, specificity, and diagnostic accuracy of various biomarker cut-points for the diagnosis of CE were calculated. The evaluated cut-points were based on values provided by the commercial laboratory offering these assays for clinical use.[26] The laboratory

also provides a summarizing interpretive statement as to whether the panel is consistent with CE.[26] The sensitivity and specificity of the collective CE-IBD assay also were calculated based on whether the panel was consistent with, or not consistent with, a diagnosis of CE.

Multivariable linear regression analyses also were performed to investigate potential associations of age, sex, neuter status, diagnosis (CE or non-CE), and duration of sample storage with the primary outcome variables of ACA, ACNA, and AGA concentrations. For regression analyses, biomarker results were log-transformed to ensure normal distribution of residuals, which were assessed by histograms and normal probability plots. Multicollinearity was assessed by variance inflations factors, which were <2.5 for all variables included in the models. Variables with $P > .20$ were removed from the models in step-wise fashion. Coefficients of determination (R^2) were calculated for any continuous variables that were significantly associated with ACA, ACNA, or AGA concentrations in the regression modeling. Statistical analyses were performed using commercially available software packages (GraphPad Prism Version 6.0; GraphPad Software Inc, La Jolla, California or NCSS 2019 Statistical Software, Version 19, Kaysville, Utah), and for all analyses, P values ≤.05 were considered significant.

3 | RESULTS

3.1 | Dogs

A total of 55 dogs, including 30 in the CE population and 25 in the non-CE population, were included in the study. Baseline characteristics of the CE and non-CE populations are summarized in Table 1. The subpopulations comprising the CE population included 18 dogs with FRE, 6 dogs with IRE, and 6 dogs with PLE-L. The median CIBDAI score for the CE population was 6 (range, 3-16). The CIBDAI scores were not significantly correlated with serum ACA ($\rho = 0.073$, $P = .7$), ACNA ($\rho = -0.008$, $P = .97$), or AGA ($\rho = -0.031$, $P = .87$) concentrations. Twenty of 30 dogs in the CE population had failed previous antimicrobial trials with metronidazole (n = 13), tylosin (n = 1), doxycycline (n = 1), or both metronidazole and tylosin (n = 5). Two dogs were still receiving antibiotics at the time of sample collection despite the lack of clinical resolution. Eight CE dogs had failed treatment with probiotics, including 2 dogs that were still receiving probiotics at the time of sample collection despite the lack of clinical resolution.

Although not a requirement for study inclusion, 14 FRE dogs had assessments of either baseline or ACTH-stimulated cortisol concentrations and 13 FRE dogs had assessments of trypsin-like immunoreactivity concentrations that excluded hypoadrenocorticism and exocrine pancreatic insufficiency, respectively. Ten FRE dogs had endoscopically collected intestinal biopsy samples that identified non-suppurative mucosal inflammatory infiltrates. Resolution of gastrointestinal signs was achieved in all 18 FRE dogs with feeding of a prescription hydrolyzed protein diet.

The 6 dogs with IRE had all failed to respond to a hydrolyzed protein diet and had variable degrees of lymphoplasmacytic mucosal inflammatory infiltrates observed in endoscopically collected intestinal biopsy specimens. Improvement or resolution of gastrointestinal signs was achieved with glucocorticoids alone (n = 3), cyclosporine alone (n = 2), or glucocorticoids and cyclosporine (n = 1). The 6 dogs with PLE-L had moderate to severe lacteal dilatation and variable degrees of lymphoplasmacytic mucosal inflammatory infiltrates observed in endoscopically collected intestinal biopsy specimens. Improvement or resolution of gastrointestinal signs was achieved using a fat-restricted diet and glucocorticoids (n = 4) or a hydrolyzed protein diet and cyclosporine (n = 1) in 5 PLE-L dogs. Follow-up information was unknown for 1 PLE-L dog.

The subpopulations included in the non-CE population included 8 dogs with intestinal histoplasmosis, 10 dogs with intestinal parasitism, 4 dogs with intestinal lymphoma, and 3 dogs with granulomatous colitis. The diagnosis of histoplasmosis was based on cytologic analysis of rectal mucosal scrapings in all 8 dogs, 7 of which also had *Histoplasma spp*. antigen detected in a urine specimen.[39] Six dogs with histoplasmosis were successfully treated with either itraconazole (n = 4) or fluconazole (n = 2) and had complete resolution of gastrointestinal disease. Tapering courses of glucocorticoids were used in conjunction with antifungal treatment in 5 of these 6 dogs. Two dogs with histoplasmosis, including 1 with suspected neurologic involvement, died despite treatment with antifungal drugs and prednisone. All 10 dogs with parasites had ova identified on fecal flotations; 4 had ancylostomiasis (hookworms), 3 had trichuriasis (whipworms), and 3 had toxocariasis (roundworms). All dogs with parasitism had resolution of gastrointestinal signs after anthelmintic treatment, which included administration of pyrantel pamoate (n = 4) or fenbendazole (n = 6). The diagnosis of lymphoblastic lymphoma was based on intestinal histopathology in 3 dogs and cytologic analysis of intra-abdominal lymph nodes in 1 dog in which ultrasound identified diffuse intestinal thickening with multifocal areas of loss of wall-layering in addition to

TABLE 1 Baseline characteristics of the dogs included in the study of the CE-IBD assay

Variable	CE Population	Non-CE Population	*P* value
Age (years)	5.2 (3.5-8.9)	3.0 (0.8-6.3)	.01
Sex (male/female)	18/12	11/14	.29
Status (altered/intact)	29/1	12/13	<.001
Weight (kg)	25.1 ± 12.4	15.7 ± 9.2	.005

Note: Data are presented as median (interquartile range) for age and mean ± SD for body weight. Sex and sex status are presented as absolute numbers. The chronic enteropathy population (CE) consisted of 30 dogs and the non-CE population consisted of 25 dogs.

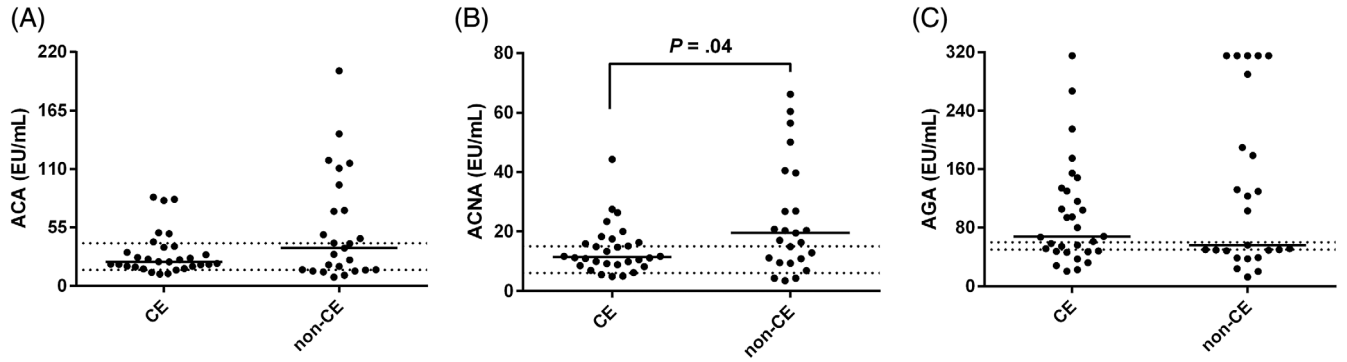

FIGURE 1 Scatterplots depicting serum concentrations of (A) ACA, (B) ACNA, and (C) AGA in 30 dogs with chronic enteropathy (CE) and 25 dogs with other forms of intestinal disease (non-CE). The central line within each scatter plot represents the median, and the dashed lines across each panel represent the laboratory-provided cut-points for intermediate and high values. Note, 1 dog in the non-CE population had an ACNA concentration of 126.5 EU/mL, which is not depicted in the figure. Six dogs had AGA concentrations reported as >315 EU/mL, and these values are depicted as 315.1 EU/mL in the figure. ACA, IgA antibodies against bacterial OmpC; ACNA, IgA antibodies against canine calprotectin; AGA, IgA antibodies against gliadin-derived peptides

multiple enlarged abdominal lymph nodes. Granulomatous colitis was diagnosed based on histopathologic assessments in all 3 dogs that was accompanied by fluorescence in situ hybridization for intraepithelial *E. coli* in 2 dogs and immunohistochemistry for *E. coli* 1 dog. Fluoroquinolone antibiotic treatment resulted in disease resolution in 1 granulomatous colitis dog whereas 1 granulomatous colitis dog was reported to fail initial antibiotic treatment. Outcome was unknown for the remaining dog.

3.2 | Assay results and test performance

Serum ACA ($P = .36$) and AGA ($P = .70$) concentrations were not significantly different between the CE and non-CE populations (Figure 1). However, serum ACNA concentrations (median, IQR) in the CE population (11.4 EU/mL, 8.7-16.6 EU/mL) were lower than in the non-CE population (19.5 EU/mL, 10.2-40.1 EU/mL; $P = .04$). Twenty-six of 30 (87%) CE dogs and 21 of 25 (84%) non-CE dogs had increased concentrations of at least 2 markers, and 18 of 30 (60%) CE dogs and 13 of 25 (52%) non-CE dogs had increased concentrations of all 3 markers. The proportions of dogs in each population having increased concentrations of 2 markers ($P = .99$) and 3 markers ($P = .59$), respectively, were not significantly different. A serum ACA concentration ≥15 EU/L, which is the upper limit of the laboratory-provided reference interval, had a sensitivity of 86.7% (95% CI, 69.3%-96.2%) and a specificity of 24.0% (95% CI, 9.4%-45.1%). The sensitivities, specificities, and accuracies of laboratory-provided cut-points for the 3 markers are shown in Table 2. No evaluated cut-point for any of the 3 markers had concurrent sensitivity and specificity >60%. The summative interpretation of the panel provided by the diagnostic laboratory was "consistent with CE-IBD" in 26 of 30 (87%) CE dogs and 19 of 25 (76%) non-CE dogs, and these proportions were not significantly different ($P = .48$). Biomarker concentrations in each of the CE and non-CE subpopulations as well as additional statistical comparisons are available online (Tables S1 and S2).

All 8 dogs with histoplasmosis had abnormally high concentrations of all 3 markers. Six of 10 dogs with intestinal parasitism had high ACA concentrations, 10 of 10 had high ACNA concentrations, and 5 of 10 had high AGA concentrations (File S1). All 4 dogs with lymphoma had high ACA concentrations, 3 had high ACNA concentrations, and 2 had high AGA concentrations. One dog with granulomatous colitis had high concentrations of all 3 markers whereas the other 2 dogs with granulomatous colitis had normal concentrations of all 3 markers.

3.3 | Covariate associations with biomarker concentrations

Sex, neuter status, and weight were not associated with ACA, ACNA, or AGA concentrations ($P > .2$ for all comparisons). Age was weakly and positively associated with ACA concentrations ($P = .04$, $R^2 = 0.032$), but not ACNA ($P = .14$) or AGA concentrations ($P = .53$). Duration of sample storage was weakly and positively associated with ACNA concentrations ($P = .04$, $R^2 = 0.069$), but not ACA ($P = .5$) or AGA concentrations ($P = .22$). A diagnosis of non-CE was associated with higher ACA ($P = .03$) and ACNA concentrations ($P = .02$) in the multivariable linear regressions. The final models for these relationships were:

$$\log(\text{ACA}) = 1.238 + 0.212^{*}(\text{Diagnosis}) + 0.029^{*}(\text{age in years}), \text{and}$$

$$\log(\text{ACNA}) = 1.009 + 0.197^{*}(\text{Diagnosis}) + 0.002^{*}(\text{sample storage in months}),$$

where CE = 0 and non-CE = 1. One dog with histoplasmosis had a sample storage duration of 109 months, which was nearly 2-fold

TABLE 2 Sensitivities, specificities, and accuracies of ACA, ACNA, and AGA for the diagnosis of chronic enteropathy in dogs

Marker	Cut-point	% Sensitivity (95% CI)	% Specificity (95% CI)	% Accuracy (95% CI)
ACA	≥15 EU/mL	86.7 (69.3-96.2)	24.0 (9.4-45.1)	58.2 (44.1-71.4)
	>40 EU/mL	20.0 (7.7-38.6)	56.0 (34.9-75.6)	36.4 (23.8-50.4)
ACNA	≥6 EU/mL	90.0 (73.5-97.9)	12.0 (2.6-31.2)	54.6 (40.6-68.0)
	>15 EU/mL	33.3 (17.3-52.8)	40.0 (21.1-61.3)	36.4 (23.8-50.4)
AGA	≥50 EU/mL	70.0 (50.6-85.3)	36.0 (18.0-57.5)	54.6 (40.6-68.0)
	>60 EU/mL	56.7 (37.4-74.5)	52.0 (31.3-72.2)	54.6 (40.6-68.0)

Note: The sensitivities, specificities, and accuracies (and their respective 95% confidence intervals [CI]) of selected ACA, ACNA, and AGA cut-points for the diagnosis of chronic enteropathy (CE) are shown in the table. The cut-points were chosen based on values utilized by the commercial laboratory to classify concentrations as normal, intermediate, or high for each of the 3 markers. The performance metrics were calculated using data from 30 dogs with CE and 25 dogs with non-CE intestinal diseases.
Abbreviations: ACA, IgA antibodies against bacterial OmpC; ACNA, IgA antibodies against canine calprotectin (ACNA); AGA, IgA antibodies against gliadin-derived peptides.

longer than any other dog. When this single dog was excluded from regression analysis, the duration of sample storage was not significantly associated with ACNA concentrations ($P = .28$).

4 | DISCUSSION

We documented that increased serum ACA, ACNA, and AGA concentrations are common in dogs with CE as well as in dogs with various parasitic, infectious, and neoplastic enteropathies. High concentrations of ACA, ACNA, and AGA have been suggested to indicate breakdown of the gut mucosal barrier resulting in an immune response to bacterial antigens, nonspecific intestinal inflammation, and susceptibility to gliadin sensitization, respectively.[24,27,29] The 3 individual markers as well as the laboratory provided assessment of the panel (eg, consistent with, or not consistent with, CE-IBD) were moderately sensitive for CE diagnosis, which is similar to previous observations.[24,27,29] Conversely, the specificities of these markers for CE diagnosis were low in our study, which is notably discordant from specificities of approximately 80% to 100% that were reported in a previous study that has since been retracted.[27-29] We speculate that these discrepancies are because of the composition of study populations. The non-CE population in our study consisted of dogs with primary intestinal diseases that have similar clinical features as CE whereas the previous study utilized non-CE populations consisting of dogs with non-intestinal or systemic disease processes.[27,29] Another study describing these markers for the first time contrasted dogs with CE and healthy dogs and dogs with acute enteropathies.[24] The challenges in diagnosing CE are seldom differentiating dogs with CE from dogs with acute gastroenteritis or differentiating dogs with CE from dogs with secondary gastrointestinal disorders. These distinctions are usually readily achieved by integrating history, clinical findings, and routine laboratory assessments.[15] Biomarkers that are proposed to aid in the initial diagnosis of CE-IBD would need to reliably discriminate between CE and other forms of primary intestinal disease, and none of the markers evaluated in our study accurately discriminated between CE and non-CE populations.[7,11,17]

Serum ACNA concentrations were higher in non-CE dogs as compared to the CE population. Perhaps this finding indicated a higher degree of intestinal inflammation, but systematic intestinal histopathologic assessments were not performed in many non-CE dogs. Despite the statistical difference between CE and non-CE populations, substantial overlap still was found in results that would limit diagnostic utility. Serum ACA concentrations, which are used as the primary indicator of the presence or absence of CE based on the laboratory's algorithm for interpreting the CE-IBD panel, were high in a similar proportion of CE and non-CE dogs, although the results might be higher in non-CE dogs depending on how the data are analyzed. Even within the CE population, increased ACA concentrations did not appear to be associated with any specific treatment response because some dogs with high ACA concentrations responded to dietary manipulation alone whereas others failed dietary therapy and required immunosuppressant medications. In addition to the poor diagnostic specificity, approximately 20% to 25% of dogs with CE have been reported to have normal ACA concentrations, which is slightly higher than the 13% observed in our CE population.[24] These findings and observations suggest that utilizing the CE-IBD panel to guide diagnostic or treatment decisions would seemingly be of minimal value and could even lead to misdiagnoses and inappropriate treatments being administered. Some clinical utility might be possible if these biomarkers provided prognostic information, or if serial monitoring provided ancillary support of disease status, but exploring these possibilities was beyond the scope of our study.

High AGA concentrations are interpreted by the laboratory to indicate sensitization to gliadin, which is a component of gluten.[26] The interpretative statement of the CE-IBD panel in dogs with high AGA concentrations also suggests that a feeding trial with a gliadin-free diet should be considered in stable dogs (File S1). The inclusion of AGA in the panel as well as the suggestion of a gliadin-free diet are surprising. A gluten-sensitive enteropathy in Irish Setters and a gluten-sensitive dyskinesia in Border Terriers have been well-

characterized.[40,41] Beyond these conditions, gluten hypersensitivity appears to be an uncommon entity in dogs, and adverse reactions to animal protein sources such as beef, dairy, and chicken are far more common causes of chronic gastrointestinal disease in dogs.[42] Furthermore, blood-based testing is often inaccurate for identifying the offending dietary component in dogs with adverse food reactions.[43] These observations suggest that AGA measurements are unlikely to be clinically useful in dogs with chronic intestinal disease. In support of this conclusion, high AGA concentrations were found in a similar proportion of CE and non-CE dogs. All dogs in our study with histoplasmosis had abnormal AGA concentrations, and there is no evidence that dogs recovering from histoplasmosis develop gliadin sensitization.[38] Half of the dogs with intestinal parasitism in our study had high AGA concentrations, and clinical signs resolved in these dogs after anthelmintic treatment alone. Lastly, even some apparently healthy dogs have increased AGA concentrations.[24]

Our study had several limitations, including potential confounding factors. Many samples used in this study had been stored at -80^0 C for variable periods of time, and the stability of the measured IgA antibodies is not known. Some baseline characteristics of the 2 study populations, including age, neuter status, and weight, were different. Antibodies often are stable for several years or longer even after multiple freeze-thaw cycles.[44,45] Substantial overlap also occurred between the ages and weights of the CE and non-CE dogs, and CE is diagnosed in dogs with wide ranges in age, weight, and breed genetics.[15] Furthermore, our regression analyses did not identify any associations of sex, neuter status, or weight with biomarker concentrations, and the weak associations of age with ACA concentrations ($R^2 = 0.032$) and duration of sample storage with ACNA concentrations ($R^2 = 0.069$) are unlikely to be clinically relevant. The lack of intestinal histopathology and extensive biochemical testing for some of the FRE dogs could be considered another limitation. The FRE dogs had negative fecal flotation results or failed empirical deworming or both, as well as a CBC, chemistry profile, and abdominal ultrasound examination that did not indicate extra-intestinal disease, and clinical signs that remained resolved for at least 1 month upon feeding of a hydrolyzed protein diet. Still, we cannot definitively exclude the possibility that concurrent or extraintestinal illnesses were present in some of these dogs. Finally, the study was well-powered to detect potential differences in biomarker concentrations between the CE and non-CE populations, but it was not well-powered to detect potential differences in biomarker concentrations among the various subpopulations within the CE and non-CE populations (Table S1 and S2). The clinical relevance of any potential differences is uncertain. Although IRE dogs have higher ACA concentrations than FRE dogs, overlap still was found among these groups. More importantly, there is substantial overlap of ACA concentrations of both of these CE groups with the non-CE subpopulations that require different approaches for diagnostic testing and management.

In summary, we documented that ACA, ACNA, and AGA have poor accuracy for diagnosing CE in dogs. Dogs with intestinal histoplasmosis, parasitism, granulomatous colitis, and lymphoma can have similarly high or even higher concentrations of the 3 markers. Nearly 15% of dogs with CE did not have results interpreted to be consistent with CE, which also raises concerns about using the serologic panel as a screening test for CE. Finally, high AGA concentrations frequently were identified in dogs with diseases other than FRE. Veterinarians should be mindful of these findings when considering the use of these 3 serologic markers in practice, and any results must be interpreted cautiously because of the variability of ACA, ACNA, and AGA concentrations in dogs with and without CE.

ACKNOWLEDGMENT

No funding was received for this study. Portions of this work were presented in abstract form at the 2022 American College of Veterinary Internal Medicine Forum, Austin, Texas. The authors thank Dr Jamie Morrison and her team at Capital Area Humane Society in Lansing, MI for assisting with case identification, Dr Leslie Mitchell and Dr Patricia Ishii for sample contribution, and Dr. Joe Hauptman for statistical support.

CONFLICT OF INTEREST DECLARATION

Dr. Steiner and Dr. Tolbert are employees of the Texas A&M Gastrointestinal Laboratory, which offers diagnostic tests for gastrointestinal disorders in dogs and cats on a fee-for-service basis. Dr. Jablonski previously served on an advisory board for IDEXX Laboratories, Inc., Dr. Steiner is a consultant for IDEXX Laboratories, Inc., and Dr. Jaffey is a part-time case consultant for IDEXX Laboratories, Inc. Neither of these laboratories offers the diagnostic tests evaluated in this study. The authors declare no additional potential conflicts of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

The authors declare no off-label antimicrobial use.

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

This study was approved by the IACUC at Michigan State University (PROTO201900056).

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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

How to cite this article: Langlois DK, Pritchard JC, Tolbert MK, et al. Clinical utility of an immunoglobulin A-based serological panel for the diagnosis of chronic enteropathy in dogs. *J Vet Intern Med*. 2023;37(2):446-454. doi:10.1111/jvim.16636