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Use of a Granulocyte Immunofluorescence Assay Designed for Humans for Detection of Antineutrophil Cytoplasmic Antibodies in Dogs with Chronic Enteropathies

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Background: Perinuclear antineutrophil cytoplasmic antibodies (pANCA) previously have been shown to be serum markers in dogs with chronic enteropathies, with dogs that have food-responsive disease (FRD) having higher frequencies of seropositivity than dogs with steroid-responsive disease (SRD). The indirect immunofluorescence (IIF) assay used in previous publications is time-consuming to perform, with low interobserver agreement.

Hypothesis/Objectives: We hypothesized that a commercially available granulocyte IIF assay designed for humans could be used to detect perinuclear antineutrophil cytoplasmic antibodies in dogs.

Animals: Forty-four dogs with FRD, 20 dogs with SRD, 20 control dogs, and 38 soft-coated wheaten terrier (SCWT) or SCWT-cross dogs.

Methods: A granulocyte assay designed for humans was used to detect pANCA, cANCA, and antinuclear antibodies (ANA), as well as antibodies against proteinase-3 protein (PR-3) and myeloperoxidase protein (MPO) in archived serum samples.

Results: Sensitivity of the granulocyte assay to predict FRD in dogs was 0.61 (95% confidence interval (CI), 0.45, 0.75), and specificity was 1.00 (95% CI, 0.91, 1.00). A significant association was identified between positive pANCA or cANCA result and diagnosis of FRD ($P < 0.0001$). Agreement between the two assays to detect ANCA in the same serum samples from SCWT with protein-losing enteropathy/protein-losing nephropathy (PLE/PLN) was substantial (kappa, 0.77; 95% CI, 0.53, 1.00). Eight ANCA-positive cases were positive for MPO or PR-3 antibodies.

Conclusions and Clinical Importance: The granulocyte immunofluorescence assay used in our pilot study was easy and quick to perform. Agreement with the previously published method was good.

Key words: Antineutrophil cytoplasmic antibodies; Chronic enteropathy; Food-responsive disease; Indirect immunofluorescence.

Antineutrophil cytoplasmic antibodies (ANCA) are directed against neutrophil granule components.[1] The location of antigen–antibody complexes after incubation allows these antibodies to be classified as perinuclear antineutrophil cytoplasmic antibodies (pANCA) or cytoplasmic antineutrophil cytoplasmic antibodies (cANCA) when visualized using indirect immunofluorescence (IIF). Specific antigens can be further identified by testing for antibodies against myeloperoxidase protein (MPO) and proteinase-3 protein (PR-3). These proteins are enzymes that may be involved in neutrophil degranulation and generation of reactive oxygen species, among other functions.[2,3]

After increasing use in the human medical field, ANCA have been documented in diseases of dogs associated with an inappropriate immune response, including canine inflammatory bowel disease, protein-losing enteropathy/nephropathy (PLE/PLN), immune-mediated hemolytic anemia, and vasculitis-related illness.[4–7] Antineutrophil cytoplasmic antibodies have been documented in patients with neoplastic and infectious diseases.[6,8] Chronic enteropathies are well documented

Abbreviations:

ANA	antinuclear antibodies
cANCA	cytoplasmic antineutrophil cytoplasmic antibodies
FRD	food-responsive disease
IIF	indirect immunofluorescence
MPO	myeloperoxidase protein
pANCA	perinuclear antineutrophil cytoplasmic antibodies
PLE	protein-losing enteropathy
PLN	protein-losing nephropathy
PR-3	proteinase-3 protein
SCWT	soft-coated wheaten terrier
SRD	steroid-responsive disease

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in veterinary medicine and have been divided into diseases that are responsive to dietary management alone (termed "food-responsive disease," [FRD]), antibiotic therapy (termed "antibiotic-responsive disease"), or diseases that require immunosuppressive drugs for control of clinical signs (termed "steroid-responsive disease," [SRD]).[9] The presence of pANCA in dogs with chronic enteropathies has been suggested to correlate with a positive response to management with strict elimination diet implementation alone. Despite use as a potential marker for FRD, no correlation with clinical disease severity scoring was found.[10] Studies involving a cohort of soft-coated wheaten terriers (SCWT) and SCWT-beagle crossbred dogs with PLN/PLE indicated that antibodies appeared up to 2 years before development of clinically relevant disease, suggesting possible use of ANCA to predict onset of disease.[5] Titers for ANCA remained positive once identified. These various studies suggest that ANCA testing could be utilized for a number of immune-mediated disease syndromes although an assay designed for dogs is not currently widely available.

Previously reported methods for analysis of ANCA in veterinary medicine have involved the use of relatively time-consuming laboratory techniques, with a need for collection of serum from donor dogs from which neutrophils were isolated and harvested using centrifugation of blood with polymorph preparation designed to isolate polymorphonuclear leukocytes.[4,a] Granulocytes subsequently were fixed with ethanol before sample testing. Neutrophil isolation produced inconsistent results because of variability in canine granulocyte purification, and interobserver variation in immunofluorescence result interpretation was reported to be high, adding to the inherent subjectivity of the test.[4] A commercially available assay for ANCA could decrease the time needed to perform the assay while ensuring improved reliability and consistency of results. By the previously reported ANCA assay, 8–10 samples could be run per day, including a positive and negative control.

The primary aim of our pilot study was to evaluate whether a commercially available granulocyte IIF assay designed for use in humans could be used to detect ANCA in dogs. We hypothesized that pANCA would be detectable in patients with FRD. In addition, we hypothesized that agreement between the results from the canine granulocyte assay and the assay designed for humans would be high when using samples from dogs tested using both methods.

Materials and Methods

Patient Selection

Archived serum samples from 44 dogs of different breeds with FRD, 20 dogs of different breeds with SRD, and 20 healthy dogs were included in the study. Patients with FRD were defined as those that had suffered from chronic diarrhea for >4 weeks and had a diagnosis based on exclusion of other potential underlying diseases, based on results of CBC, serum biochemistry profile, fecal evaluation, and abdominal ultrasound examination. These dogs had all had gastrointestinal biopsy samples collected at the time of serum sample submission. All dogs in the FRD group had been treated using a strict exclusion diet at the discretion of the clinician in charge of the case and had responded favorably, with normalization of clinical signs within 2 weeks. These patients then were maintained on the exclusion diet for at least 8 weeks, without relapse of clinical signs.

All dogs within the SRD group had undergone comprehensive diagnostic investigations, including CBC, serum biochemistry profile, abdominal ultrasound examination, and endoscopy with gastrointestinal biopsies. Dogs with SRD were defined as those that had undergone at least 1 unsuccessful dietary and antimicrobial trial with either metronidazole or tylosin, at clinician discretion, for at least 4 weeks, and required immunosuppressive therapy to control their clinical signs. An unsuccessful dietary trial was defined as a trial that did not lead to substantial improvement or resolution of clinical signs within 2–4 weeks.[11] A number of combinations of prednisolone, cyclosporine, and azathioprine treatment were included in this group. In all dogs with FRD and SRD, endoscopic biopsy samples from some combination of stomach, duodenum, colon, and ileum had been analyzed. Biopsy samples were evaluated by an experienced pathologist in conjunction with the World Small Animal Veterinary Association (WSAVA) guidelines.[12]

All healthy control dogs had undergone complete physical examination, had no history of diarrhea, and had had an unremarkable comprehensive laboratory evaluation.

In addition, 38 serum samples from a colony of SCWT or SCWT-Beagle crossbred dogs with PLE/PLN at North Carolina State University were analyzed. These samples previously had been evaluated for the presence of ANCA using the published method for dogs[4] and had residual samples available for testing that had been stored at −80 C. Detection of ANCA is possible after several freeze–thawing cycles. Samples are stable over years in serum frozen at temperatures below −20°C (Allenspach, unpublished observations). Details regarding these 38 patients can be found in a previously reported study.[5] Agreement between the two methods was assessed by testing a subset of 10 samples by the same person (KA), using both assays.

All serum samples used in our study consisted of residual diagnostic blood samples. The archived serum and biopsy samples for this study had been taken for diagnostic purposes only. The study was approved by the Royal Veterinary College Ethics and Welfare Committee.

Sample Processing and Indirect Immunofluorescence Technique

An assay[b] not previously reported in the veterinary literature for dogs was utilized for analysis of antibodies in the serum samples. Granulocyte mosaic slides containing biochips with human granulocytes fixed in ethanol, human granulocytes fixed in formalin, myeloperoxidase protein (MPO), proteinase-3 protein (PR-3), and HEp-2 cells were used. This combination allows detection of pANCA, cANCA, and antinuclear antibodies (ANA) on the same slide as well as enabling several patient samples to be analyzed at 1 time. It also allows assessment for antibodies to specific antigens (MPO and PR-3) by provision of antigen-specific substrates. The method of performing the assay is standardized, and each biochip is identical in appearance and content. Up to 40 samples can be run in 1 day.

The novel assay was performed according to manufacturer's instructions. Briefly, patient serum was diluted 1:10 using 0.2% phosphate-buffered saline (PBS)–Tween. Biochip slides were incubated with 30 μL of diluted serum for 30 minutes at room temperature. After washing with PBS–Tween, 25 μL of fluorescein isothiocyanate (FITC)-labeled goat anti-dog immunoglobulin was added to each reaction field. After further incubation for 30 minutes and washing, the slides were embedded in glycerol medium,

covered with a cover glass and each biochip was fully evaluated with a 4-quadrant technique with a fluorescence microscope at 200–400× magnification by two independent observers who were trained in the technique by an operator with extensive previous IIF experience. Results were interpreted as positive or negative by each observer. A third experienced reviewer was used if results were ambiguous. Human serum positive and negative controls were utilized as provided by the manufacturer for each sample.

Statistical Analysis

Data analysis was performed by commercial statistical software[c] . A chi-square test was used to determine the association of a positive ANCA result with the diagnosis of FRD. Sensitivity and specificity for the correct detection of FRD or SRD with 95% CI were calculated for use of the new assay designed for humans in dogs. Agreement between the previously published granulocyte ANCA assay for dogs and the new granulocyte assay for humans, performed on the same samples and by the same person, was calculated by the unweighted kappa statistic. Significance for all statistical testing was set at $P < 0.05$. Interpretation of the kappa statistic was performed as reported previously, with <0 representing poor agreement and >0.8 representing almost perfect agreement.[13]

Results

Of the dogs diagnosed with FRD, 22 were female and 22 were male. Neuter status was not always recorded. There were 39 purebred dogs included and five crossbred dogs. Dogs were all of different individual breeds with only the French bulldog and German Shepherd dog included more than once (4 and 3 times, respectively). Their ages ranged from 8 months to 9 years (mean age, 2.6 years). All histopathology findings included evidence of inflammatory change in at least two anatomical sections of the gastrointestinal tract. The inflammatory changes were predominantly lymphoplasmacytic in nature with no infectious agents or neoplastic infiltrates reported. Variable degrees of villous atrophy and lymphoid hyperplasia were reported.

Of the 20 dogs diagnosed with SRD, 3 were crossbred dogs and 17 were purebred dogs. Their ages ranged from 2 to 13 years (mean age, 7.1 years). Histopathologic evaluation had identified lymphoplasmacytic inflammatory changes in at least 2 anatomic sections of the gastrointestinal tract with variable degrees of villous atrophy and lymphoid hyperplasia. No infectious agents or neoplastic infiltrates were reported for any dog.

Of the control dogs, 5 were crossbred dogs, 4 were beagles, and 11 were purebred dogs. Their ages ranged from 1 to 13 years (mean age, 6.1 years). Data from the SCWT/SCWT-Beagle crossbred dogs were described previously.[5]

Accuracy of the Granulocyte Assay Designed for Humans to Diagnose FRD and SRD in Dogs

By the new assay designed for humans, all 20 samples from healthy dogs were negative for ANCA and ANA. All 20 dogs with SRD also were negative for ANCA and ANA. Of the 44 dogs with FRD, 27 (61.4%) were positive for antibodies to pANCA (Fig 1) or cANCA (Fig 2) by the new method (Table 1). Sensitivity of the granulocyte assay designed for humans to predict FRD in dogs was 0.61 (95% CI, 0.45, 0.75), and specificity was 1.00 (95% CI, 0.91, 1.00). Significant association between a positive ANCA result and a diagnosis of FRD was identified ($P < 0.0001$). In 8 positive cases, either MPO or PR-3 autoantibodies were identified on the biochips with 5 samples positive for PR-3 and three samples positive for MPO.

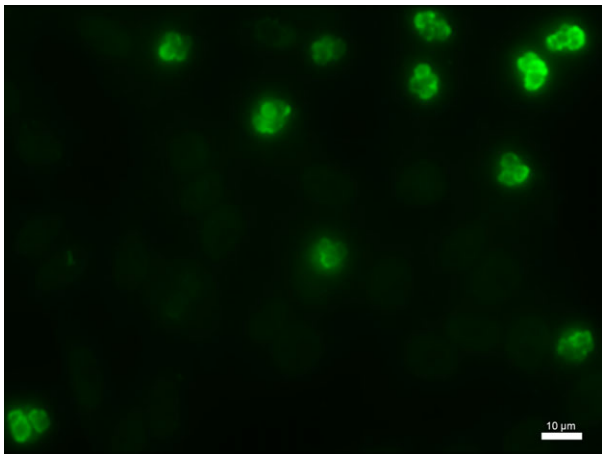

Fig 1. Positive canine pANCA IIF by the human granulocyte assay and FITC-labeled goat anti-dog immunoglobulin. Green florescence indicates a positive result. ×400 magnification.

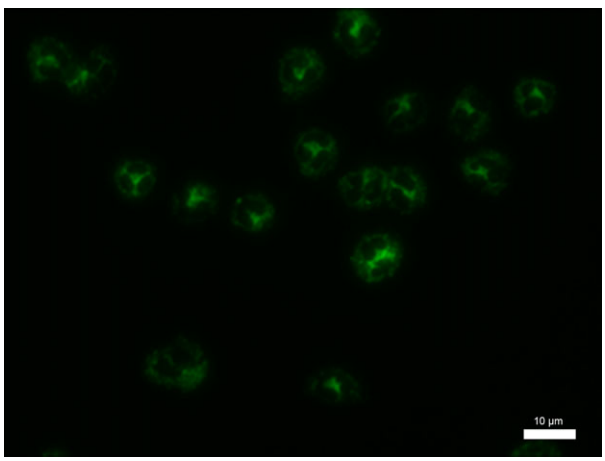

Fig 2. Positive canine cANCA IIF by the human granulocyte assay and FITC-labeled goat anti-dog immunoglobulin. Green florescence indicates a positive result. ×400 magnification.

Comparison of the Granulocyte Assay Designed for Humans with the Granulocyte Assay for Dogs using the Same Canine Serum Samples

Of the 38 SCWT and SCWT-crossbred dog serum samples, 31 tested positive for ANCA by the previously published granulocyte method for dogs. By the granulocyte assay designed for humans described here, 28 of

Table 1. ANCA results by the new human granulocyte assay.

	Human granulocyte ANCA assay	
	Positive	Negative
CTR	0	20
SRD	0	20
FRD	27	17

ANCA, antineutrophil cytoplasmic antibodies; CTR, healthy control dogs; FRD, dogs with food-responsive disease; SRD, dogs with steroid-responsive disease.

Table 2. Comparison of results between the previously published canine granulocyte ANCA assay and the new human granulocyte ANCA assay using the same samples from SCWT with PLE/PLN.

		Canine granulocyte ANCA assay		
		Positive	Negative	Total
Human granulocyte ANCA assay	Positive	28	0	28
	Negative	3	7	10
Total		31	7	38

ANCA, antineutrophil cytoplasmic antibodies; SCWT, soft-coated wheaten terrier; PLE/PLN, protein-losing enteropathy/protein-losing nephropathy.

those same dog samples tested positive (Table 2). Agreement between the 2 assays to detect ANCA in serum of the same samples was substantial (kappa = 0.77; 95% CI, 0.53, 1.00).[13]

Discussion

Results of our study indicate that pANCA and cANCA can be detected using human granulocyte mosaic biochip slides. This method of testing was helpful to differentiate patients with FRD from those with SRD or those without disease, with no positive results documented for either of the latter testing categories. Sensitivity and specificity to diagnose FRD or SRD by this method was similar to previously published results.[3,7]

Because IIF interpretation is subjective, improved procedure and ease of interpretation of results is paramount for optimal use of such a test. Use of a test with purified granulocytes adhered on the slides as well as the use of human serum positive and negative controls provided by the company made interpretation easier than with the previously published method. Purification of canine granulocytes before performing the immunofluorescence assay was not required, which shortened the time taken to perform the assay to <1 day. The design of the new assay allowed several patient samples to be analyzed at 1 time, theoretically improving reliability and throughput of patient samples.

Agreement between the 2 assay methods was substantial with a calculated kappa statistic of 0.77. Although this agreement is good, it does indicate that in approximately 20% of cases, the assays did not agree. This discrepancy is demonstrated in Table 2. The possibility of this disagreement should be taken into consideration when interpreting results using either ANCA test. Calculated sensitivity and specificity of the methodology designed for humans indicates that the test appears to be less sensitive than the method using dog granulocytes, although for the purposes of our study it was only used to assess for ANCA in samples of SCWT/SCWT-crossbred dogs with PLE/PLN.[4] Although the sensitivity of this test was only 0.61, the primary aim of our study was to report the use of a granulocyte test designed for humans in dogs, and not to conclusively document clinical utility of the test in a large cohort of patients. Additional prospective testing now should be performed to assess the ability of this test to differentiate disease etiologies.

Assuming there is a loss of sensitivity with the new assay, it is possible that by using human granulocytes instead of dog granulocytes, some antigens are not being detected, therefore decreasing sensitivity of the test. Sequence homology between canine and human MPO is 88% when the sequences are compared.[14] The amino acid sequence of canine PR-3 currently is not available and comparison to the human sequence cannot be made. Although canine and human MPO are expected to exhibit close homology, there is a possibility that some epitopes of canine MPO may not be detected by the assay designed for humans, leading to false-negative results. It is also possible that differences in preparation of slides and the drying and fixation process may have affected the sensitivity results, although every effort was made to perform the analyses by the same methodology under consistent conditions.

The possibility of false positives also cannot be conclusively excluded. There is no accepted gold standard method available to conclusively document the presence or absence of ANCA in veterinary patients. This situation hampers evaluation of agreement between true-positive and true-negative results, and this possibility should always be considered during test result interpretation. Once specific antigens for ANCA are identified, such as PR-3 and MPO, Western blot to detect antibodies against the canine proteins could be used as a gold standard.

In 8 patients, the antigen being targeted also could be identified. As a consequence of the small number of samples in which this was possible, the clinical relevance of this finding remains unclear and requires further clinical study. In human patients with ulcerative colitis, pANCA are more commonly identified in patients with a more clinically aggressive disease course.[15] The PR-3 autoantibodies may be of importance in ulcerative colitis, and their presence has been documented in this disease.[16] Due to the nature of our study, prospective follow-up on patients with ANCA was not possible. The small number of patients in which specific antigen identification was observed may further support a loss of sensitivity by

use of an assay designed for humans. Alternatively, the underlying antigen may be different in the majority of affected dogs. In humans, the main ANCA proteins that have been identified are lactoferrin, elastase and bactericidal increasing protein.[16] Further research to identify the specific proteins targeted by canine ANCA is required, although they may be similar to those documented in human patients.

In conclusion, this granulocyte ANCA assay designed for use in humans can be used with canine serum to assess for the presence of ANCA, with good agreement between the two assays when performed on the same set of serum samples. This standardized method was easy to perform and did not require donor dogs for granulocyte extraction. In a subset of these patients, specific antigens could be identified that may allow further research into a variety of immune-mediated diseases in dogs. Despite a lack of sensitivity, this assay appears to be highly specific. The availability of a commercial test allows additional large studies on ANCA to be performed, evaluating its potential role in clinical case investigation.

Footnotes

[a] Polymorphprep medium, Axon Lab AG, Oslo, Norway
[b] EUROIMMUN, Ashville House, Wimbledon, London
[c] IBM SPSS Statistics, version 22, Armonk, NY

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Conflict of Interest Declaration: The reagents utilized for this study were provided by EUROIMMUN. L Perazzotti and K Affeldt are employees of EUROIMMUN. R Wagner is an employee of Laboklin Ltd.

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

References

1. Mancho C, Sainz Á, García-Sancho M, et al. Detection of perinuclear antineutrophil cytoplasmic antibodies and antinuclear antibodies in the diagnosis of canine inflammatory bowel disease. J Vet Diagn Invest 2010;22:553–558.

2. Grigorieva DV, Gorudko IV, Sokolov AV, et al. Myeloperoxidase stimulates neutrophil degranulation. Bull Exp Biol Med. Springer US 2016;161:1–6.

3. Korkmaz B, Moreau T, Gauthier F. Neutrophil elastase, proteinase 3 and cathepsin G: Physicochemical properties, activity and physiopathological functions. Biochimie 2008;90:227–242.

4. Allenspach K, Luckschander N, Styner M, et al. Evaluation of assays for perinuclear antineutrophilic cytoplasmic antibodies and antibodies to Saccharomyces cerevisiae in dogs with inflammatory bowel disease. Am J Vet Res 2004;65:1279–1283.

5. Allenspach K, Lomas B, Wieland B, et al. Evaluation of perinuclear anti-neutrophilic cytoplasmic autoantibodies as an early marker of protein-losing enteropathy and protein-losing nephropathy in Soft Coated Wheaten Terriers. Am J Vet Res 2008;69:1301–1304.

6. Karagianni AE, Solano-Gallego L, Breitschwerdt EB, et al. Perinuclear antineutrophil cytoplasmic autoantibodies in dogs infected with various vector-borne pathogens and in dogs with immune-mediated hemolytic anemia. Am J Vet Res 2012;73:1403–1409.

7. Felsburg PJ, HogenEsch H, Somberg RL, et al. Immunologic abnormalities in canine juvenile polyarteritis syndrome: A naturally occurring animal model of Kawasaki disease. Clin Immunol Immunopathol 1992;65:110–118.

8. Mancho C, Sainz Á, García-Sancho M, et al. Evaluation of perinuclear antineutrophilic cytoplasmic antibodies in sera from dogs with inflammatory bowel disease or intestinal lymphoma. Am J Vet Res 2011;72:1333–1337.

9. Dandrieux JRS. Inflammatory bowel disease versus chronic enteropathy in dogs: Are they one and the same? J Small Anim Pract 2016;57:589–599.

10. Luckschander N, Allenspach K, Hall J. Perinuclear antineutrophilic cytoplasmic antibody and response to treatment in diarrheic dogs with food responsive disease or inflammatory bowel disease. J Vet Internal Med 2006;20:221–227.

11. Marks SL, Laflamme DP, McAloose D. Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. Vet Ther 2002;3:109–118.

12. Day MJ, Bilzer T, Mansell J, et al. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: A report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J Comp Pathol 2008;138:S1–S43.

13. Watson PF, Petrie A. Method agreement analysis: A review of correct methodology. Theriogenology 2010;73:1167–1179.

14. Available at https://www.ncbi.nlm.nih.gov/genome. Accessed April 2017.

15. Lombardi G, Annese V, Piepoli A, et al. Antineutrophil cytoplasmic antibodies in inflammatory bowel disease. Dis Colon Rectum 2000;43:999–1007.

16. Kyriakidi KS, Tsianos VE, Karvounis E, et al. Neutrophil anti-neutrophil cytoplasmic autoantibody proteins: Bactericidal increasing protein, lactoferrin, cathepsin, and elastase as serological markers of inflammatory bowel and other diseases. Ann Gastroenterol 2016;29:258–267.