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Development and Evaluation of an ELISA for the Quantitation of Anti-*Lagenidium giganteum* forma *caninum* Antibodies in Dogs

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Background: *Lagenidium giganteum* forma *caninum* infection causes severe cutaneous and disseminated disease in dogs. Currently, diagnosis requires culture and rRNA gene sequencing.

Objective: To develop and evaluate an ELISA for quantitation of anti-*L. giganteum* f. *caninum* IgG in canine serum.

Animals: Sera were evaluated from 22 dogs infected with *L. giganteum* f. *caninum*, 12 dogs infected with *Paralagenidium karlingii*, 18 dogs infected with *Pythium insidiosum*, 26 dogs with nonoomycotic fungal infections or other cutaneous or systemic diseases, and 10 healthy dogs.

Methods: Antigen was prepared from a soluble mycelial extract of *L. giganteum* f. *caninum*. Optimal antigen and antibody concentrations were determined by checkerboard titration. Results were expressed as percent positivity (PP) relative to a strongly positive control serum.

Results: Medians and ranges for PP for each group were: *L. giganteum* f. *caninum* (73.9%, 27.9–108.9%), *P. karlingii* (55.0%, 21.0–90.6%), *P. insidiosum* (31.3%, 15.8–87.5%), nonoomycotic fungal infection or other cutaneous or systemic diseases (19.2%, 3.2–61.0%), and healthy dogs (9.9%, 7.6–24.6%). Using a PP cutoff value of 40%, sensitivity and specificity (with 95% CI) of the ELISA for detecting *L. giganteum* f. *caninum* infection in clinically affected dogs were 90.9% (72.2–97.5%) and 73.2% (60.4–83.0%), respectively. Specificity in dogs infected with *P. karlingii* was 41.7% (19.3–68.1%) and with *P. insidiosum* was 66.7% (43.8–83.7%).

Conclusions and Clinical Importance: Quantitation of anti-*L. giganteum* f. *caninum* antibodies for detection of this infection in dogs has moderately high sensitivity but poor specificity, the latter because of substantial cross-reactivity with anti-*P. karlingii* and anti-*P. insidiosum* antibodies.

Key words: Lagenidiosis; *Paralagenidium*; Pythiosis; Serology.

Over the past 15 years, 2 novel oomycete pathogens that appeared to be members of the genus *Lagenidium* have become increasingly recognized in dogs as causes of cutaneous lesions that resemble those associated with pythiosis.[1] Recently, multigene phylogenetic analyses have allowed formal names to be published for these pathogens.[2] The first pathogen, which causes severe, progressive cutaneous disease, lymphadenopathy, pulmonary nodules, and great vessel invasion,[3] has been formally described as *Lagenidium giganteum* forma *caninum* because of its close phylogenetic relationship with the mosquito larval pathogen *L. giganteum*. Dogs infected with *L. giganteum* f. *caninum* typically have lesions that disseminate beyond the skin and regional lymph nodes to involve the thoracic or abdominal cavities, eventually resulting in death in the majority of cases despite surgical or medical treatment. The second pathogen causes more slowly progressive disease that is limited to cutaneous and subcutaneous tissues, and often can be cured when complete surgical resection with wide margins can be achieved, or when surgical resection is combined with long term antifungal treatment.[a] Although this second pathogen shares many antigenic and morphologic similarities with *L. giganteum* f. *caninum* and other *Lagenidium* species,[a] recent phylogenetic analyses based on rRNA and *cytochrome c oxidase I* gene sequences support its placement in the new genus *Paralagenidium,* with the species name *Paralagenidium karlingii*.[2]

Abbreviations:

BSA	bovine serum albumin
CI	confidence interval
CV	coefficient of variation
IgG	immunoglobulin G
OD	optical density
PBS	phosphate-buffered saline
PP	percent positivity
ROC	receiver operating characteristics
rRNA	ribosomal ribonucleic acid

Because they share similar clinical and histologic features (all cause deep, nodular, or ulcerative dermatitis characterized by pyogranulomatous and eosinophilic inflammation associated with broad, poorly septate hyphae), infections caused by the oomycetes *L. giganteum* f. *caninum*, *P. karlingii*, and *Pythium insidiosum*, and the zygomycetes *Basidiobolus ranarum* and *Conidiobolus* spp often are difficult to distinguish from one another. However, differentiating among them is clinically important because prognosis and response to medical treatment differ.[1] Currently, the definitive diagnosis of *L. giganteum* f. *caninum* and *P. karlingii* infection requires amplification and sequencing of ribosomal

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DNA extracted from either a cultured isolate or directly from tissue. This can be challenging as oomycetes often do not grow on the media used for routine fungal culture, and microbiology personnel in veterinary diagnostic laboratories are not typically trained to isolate or identify these organisms. Previous immunoblot analyses showed that serum from dogs infected with either *L. giganteum* f. *caninum*[3] or *P. karlingii* [a] contains antibodies that bind to a large number of antigens of *L. giganteum* f. *caninum,* suggesting that a serologic assay for detection of anti-*L. giganteum* f. *caninum* antibodies might be of diagnostic value. In addition, our previous development and subsequent routine use of a highly sensitive and specific ELISA for the detection of anti-*P. insidiosum* antibodies in dogs prompted us to consider use of the ELISA format to detect anti-*Lagenidium* antibodies. Therefore, the purpose of this study was to develop and optimize an ELISA for the quantitation of anti-*L. giganteum* f. *caninum* antibodies in dogs, and to assess its diagnostic utility.

Methods

Antigen Preparation

An isolate of *L. giganteum* f. *caninum* (C02-SW136, CBS[b] 135280) originally recovered from a canine mammary mass was used for antigen production. Soluble mycelial antigen extraction was performed using a modification of a technique previously described for *P. insidiosum*.[4] Briefly, several 2 mm-diameter plugs of agar with attached hyphae taken from the edges of a 3–4-day-old colony were used to inoculate 500 mL of peptone-yeast extract-glucose broth. After 5 days of stationary growth at 30°C, the culture was killed by addition of thimerosal (2% wt/vol). The mycelial mat was collected by vacuum filtration, washed 3 times with cold phosphate-buffered saline (PBS), and ground in the presence of liquid nitrogen. Approximately 200 mg of ground mycelium was suspended in 1 mL of PBS and vortexed at high speed for 1 minute. The suspension was kept on ice overnight, vortexed again, and centrifuged at 10,000 × *g* for 30 minutes at 4°C. The supernatant was collected, and the protein content was determined by means of a colorimetric assay.[c] The supernatant then was stored at −20°C until use.

ELISA

Optimal concentrations of the coating antigen and conjugated antibody were determined by checkerboard titration.[5] Flat bottom microtiter plates[d] were coated overnight at 4°C with 50 μL/well of antigen solution diluted in carbonate coating buffer (pH 9.6) to a protein concentration of 7.5 μg/mL. After coating, the plates were washed 4 times with PBS + 0.05% Tween (PBST) with a semiautomated plate washer. The wells were blocked with 100 μL of 1% bovine serum albumin (BSA)[e] in PBST for 1 hour at 37°C. After 4 washes, test serum diluted in 1% BSA-PBST (50 μL/well) was added and incubated at 37°C for 1 hour. After 4 additional washes, 50 μL of secondary antibody (horseradish peroxidase-conjugated anti-canine IgG made in rabbit)[f] diluted 1 : 7,500 in 1% BSA-PBST was added to each well and incubated at 37°C for 1 hour. After 4 washes, 100 μL per well of TMB substrate[g] was added and color was allowed to develop at room temperature for 10 minutes. The reaction was stopped with the addition of 100 μL per well of 0.1 M H_2SO_4. Absorbance was read at 450 nm, and the median optical density (OD) of quadruplicate samples was recorded for each test sample and control.

Determination of Screening Dilution

To determine the optimal screening dilution for test samples in the ELISA, sera from 2 *L. giganteum* f. *caninum*-infected dogs (1 strongly positive and 1 weakly positive serum as previously determined by immunoblot analysis),[3] 1 *P. insidiosum*-infected dog, and 1 healthy dog were evaluated using serial dilutions from 1 : 250 through 1 : 16,000. The median optical densities from 3 replicates of each dilution were plotted against the reciprocal dilution. A screening dilution of 1 : 2,000 was chosen for use in the ELISA because it provided good separation between the weak positive *L. giganteum* f. *caninum* serum and the *P. insidiosum* serum, and because optical densities for the majority of sera to be tested were expected to fall on the straight parts of their respective curves at this dilution.

Test Sera

Sera evaluated in the ELISA were archived samples that had been submitted to the *Pythium* Laboratory, Louisiana State University, for diagnostic or research purposes, and had been stored for up to 15 years at −70°C. These included samples from: 22 dogs infected with *L. giganteum* f. *caninum*; 12 dogs infected with *P. karlingii*; 18 dogs infected with *P. insidiosum* (cutaneous in 14, gastrointestinal in 4); 26 dogs with nonoomycotic fungal or algal infection (18), or nonfungal disease that resembled lagenidiosis (8); and, 10 healthy dogs. The diagnosis of *L. giganteum* f. *caninum* infection, *P. karlingii* infection, and *P. insidiosum* infection in each case was suspected based on clinical and histologic findings and confirmed by morphologic[2,6] and molecular[2,7] identification of a cultured isolate. For the *Lagenidium* and *Paralagenidium* groups, all stored sera that were available from dogs with a culture-confirmed diagnosis were included. For the *P. insidiosum* group, random samples of stored sera from culture- and PCR-confirmed cases were included. The diagnoses in the 18 dogs with nonoomycotic fungal or algal infections included blastomycosis (5), cryptococcosis (4), zygomycosis (3), aspergillosis (3), and 1 dog each with sporotrichosis, protothecosis, and abdominal mycetoma. Diagnoses in these dogs were based on histologic, cytologic, or culture findings. The 8 dogs with nonfungal disease had clinical signs consistent with inflammatory dermatopathy, pulmonary masses, or lymphadenopathy. Their final diagnoses, based on evaluations performed at the Louisiana State University Veterinary Teaching Hospital, included flea allergy dermatitis (2); and 1 dog each with acral lick dermatitis and bacterial furunculosis; bacterial folliculitis and furunculosis; idiopathic sterile nodular panniculitis; pemphigus vulgaris; eosinophilic pulmonary granulomatosis; and lymphoma. Histopathology was performed in each of these dogs except the 2 with flea allergy dermatitis. The 10 healthy dogs were owned by veterinary students and were living in southeastern Louisiana at the time of serum sampling.

Quadruplicate samples of test serum diluted at 1 : 2,000 were evaluated in the ELISA. A strongly positive control serum (as previously determined by immunoblot analysis)[3] and a PBS negative control were included on each plate. The order of placement of the test sera on the plates was randomized using a computer-based random number generator.[h] The median OD of 4 replicates for each control and test sample was recorded.

Data Analysis

Results of the ELISA were expressed as percent positivity (PP) relative to the strong positive control serum from the same plate,[8] calculated as: (median OD for test serum/median OD for strong positive control) × 100. Sensitivity and specificity of the ELISA for detecting dogs with *L. giganteum* f. *caninum* infection

(n = 22) and distinguishing them from clinically affected dogs without *L. giganteum* f. *caninum* infection (n = 56) were calculated, with the cutoff value determined by receiver operating characteristics (ROC) curve analysis. Because false positive results could prompt unnecessary surgery or euthanasia, our goal in choosing a cutoff value was to either eliminate false positive results completely, or, alternatively, to maximize sensitivity while still maintaining reasonable specificity.

In addition, because of the expectation that sera from *P. karlingii*-infected dogs would show substantial cross-reactivity in the anti-*L. giganteum* f. *caninum* IgG ELISA, these same calculations were performed using the assay to detect either *L. giganteum* f. *caninum* or *P. karlingii* infection. Plots and ROC curve analyses were generated by using a statistical software package.[i] Confidence intervals for sensitivity and specificity were calculated by using the Newcombe-Wilson method without continuity correction[9] and an online calculator.[j]

The precision of the assay was evaluated by calculating the coefficient of variation (CV) for replicates within a plate, between plates, and between runs of the assay. For these analyses, 22 randomly selected sera that represented the full range of PP values were evaluated. Each serum sample was evaluated in quadruplicate on 3 plates run concurrently on the same day and on 3 plates each run on different days. Calculation of within-plate CV was based on OD values, whereas calculation of plate-to-plate and day-to-day CV was based on PP values.

Results

Results of the ELISA are shown in Figure 1. Medians and ranges for PP in each group were: *L. giganteum* f. *caninum* (73.9%, 27.9–108.9%), *P. karlingii* (55.0%, 21.0–90.6%), *P. insidiosum* (31.3%, 15.8–87.5%), nonoomycotic fungal infection or other cutaneous or systemic diseases (19.2%, 3.2–61.0%), and healthy dogs (9.9%, 7.6–24.6%). When using the assay to detect dogs with *L. giganteum* f. *caninum* infection, the area under the ROC curve (with 95% CI) was 0.867 (0.787–0.946; Fig 2). Using a cutoff value of 40%, sensitivity and specificity (with 95% CI) of the ELISA for detecting *L. giganteum* f. *caninum* infection in clinically affected dogs were 90.9% (72.2–97.5%) and 73.2% (60.4–83.0%), respectively. Specificity in dogs infected with *P. karlingii* was 41.7% (19.3–68.1%) and with *P. insidiosum* was 66.7% (43.8–83.7%). There were 2/26 dogs without oomycosis that had PP values >40%. These included 1 dog with gastrointestinal cryptococcosis (PP = 61.0%) and 1 dog with flea allergy dermatitis (PP = 53.7%). When the cutoff was decreased to a PP of 25%, sensitivity for detecting *L. giganteum* f. *caninum* infection was 100% (85.1–100%), but specificity was only 44.6% (32.4–57.6%).

When using the assay to detect dogs with either *L. giganteum* f. *caninum* or *P. karlingii* infection, the area under the ROC curve (with 95% CI) was 0.869 (0.790–0.948). Using a cutoff value of 40%, the sensitivity and specificity (with 95% CI) of the ELISA to detect either *L. giganteum* f. *caninum* or *P. karlingii* infection were 79.4% (63.2–89.7%) and 81.8% (68.0–90.5%), respectively. When dogs with pythiosis were removed from the analysis, the specificity (with 95% CI) increased to 92.3% (75.9–97.9%).

The mean within-plate (well-to-well) CV for OD for all the plates in the study was 4.5%. The mean plate-to-plate CV for PP calculated from 3 plates run on the same day was 3.8%. The mean day-to-day CV for PP calculated from 3 plates run on different days was 13.1%.

Discussion

Results of our study suggest that ELISA-based quantitation of anti-*L. giganteum* f. *caninum* IgG has fairly high sensitivity for the detection of *L. giganteum* f. *caninum* infection in dogs (91% with a 40% cutoff; 100% with a 25% cutoff), but limited to poor specificity. The latter is because of extensive cross-seroreactivity observed in dogs infected with either of the related

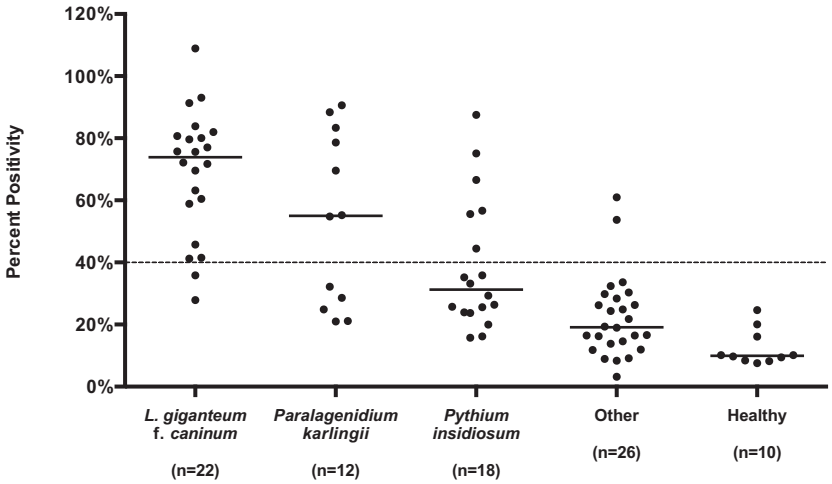

Fig 1. Scatterplot showing anti-*Lagenidium giganteum* forma *caninum* ELISA results in dogs with *L. giganteum* f. *caninum* infection, *Paralagenidium karlingii* infection, *Pythium insidiosum* infection, nonoomycotic fungal infections or other cutaneous/systemic diseases (labeled "Other"), and healthy dogs. Results are expressed as percent positivity in relation to a strongly positive control serum. The dashed line at 40% represents the proposed cutoff value. The 2 data points in the "Other" group that are above the cutoff line represent 1 dog with gastrointestinal cryptococcosis (PP = 61.0%) and 1 dog with flea allergy dermatitis (PP = 53.7%).

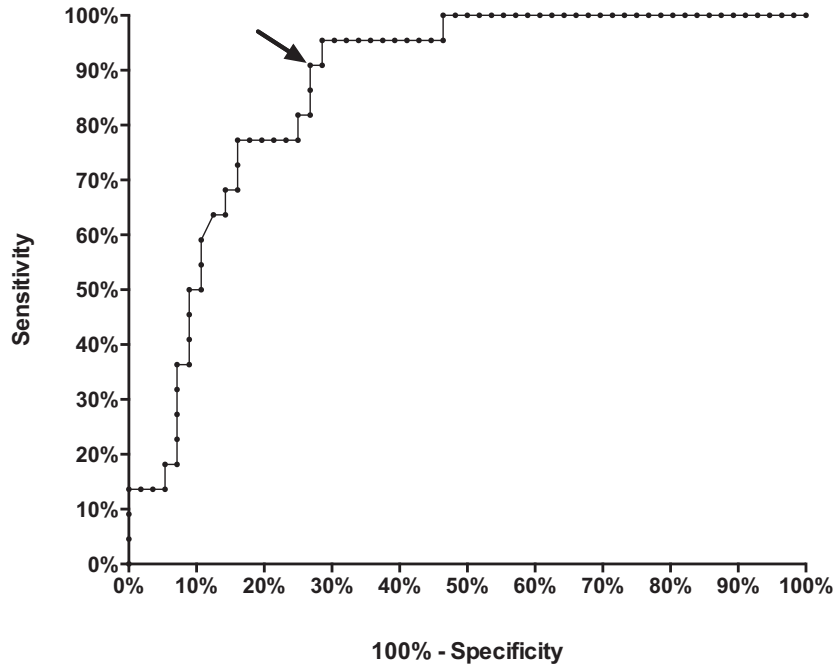

Fig 2. Results of ROC curve analysis showing performance of the assay when used to differentiate dogs with *Lagenidium giganteum* forma *caninum* infection (n = 22) from clinically affected dogs without *L. giganteum* f. *caninum* infection (n = 56). The area under the curve (with 95% CI) is 0.867 (0.787–0.946); the proposed PP cutoff value of 40% is labeled with an arrow.

oomycetes *P. karlingii* and *P. insidiosum*, and to a lesser degree to seroreactivity in dogs without oomycosis (as observed in 2 of 26 dogs in this study). Because of the generally poor prognosis and expensive, invasive therapies associated with *L. giganteum* f. *caninum* infection, false positive results could have a devastating effect on clinical decision making by prompting unnecessary amputation or euthanasia. Therefore, anti-*Lagenidium* antibody results should never be used alone as the basis for a diagnosis of *L. giganteum* f. *caninum* infection in dogs.

On the other hand, the relatively high sensitivity of the assay for detecting *L. giganteum* f. *caninum* infection suggests that it could be used to rule out the disease, or at least to suggest that it is unlikely. This might be important when owners of dogs that have clinical signs supportive of cutaneous oomycosis or zygomycosis are weighing the cost of a diagnostic evaluation (which may include imaging of the chest and abdomen as well as biopsy, culture, and molecular diagnostic testing) against the possibility of a poor outcome. In addition, given that several dogs in the same household may become infected,[3] a serologic *Lagenidium* assay with high sensitivity could be useful in screening housemates when an infected dog is identified. In these situations, a negative test would make *L. giganteum* f. *caninum* infection unlikely (especially when PP is <25%), but a positive test would have to be followed by other diagnostic tests such as histopathology and culture. Future evaluation of sera from a larger number of dogs infected with *L. giganteum* f. *caninum*, especially those that are early in the course of disease, will be important for more accurately determining the frequency of false negative results. Likewise, further evaluation of sera from a large number of dogs exposed to but not infected by *L. giganteum* f. *caninum* or *P. karlingii* (such as clinically healthy housemates of infected dogs) will be needed to more accurately determine the frequency of false positive results.

Another potential application of the ELISA that would be supported by its relatively high sensitivity would be to monitor response to treatment in dogs treated either surgically or medically for *L. giganteum* f. *caninum* infection. Although assessing the ELISA as a monitoring tool was not an objective of this study, the authors have had the opportunity to evaluate ELISA results before and after treatment in a single dog with *L. giganteum* f. *caninum* lesions that were limited to a distal limb. Thirteen months after forelimb amputation followed by long-term terbinafine administration, anti-*L. giganteum* f. *caninum* ELISA results had decreased from 83% (presurgery) to 17%, and the dog was clinically normal (A.M. Grooters, unpublished data). This result suggests that the anti-*Lagenidium* antibody ELISA may be a useful tool for monitoring response to treatment in dogs infected with *L. giganteum* f. *caninum*, but because the majority of infected dogs are not successfully treated, opportunities to use the ELISA in this context are expected to be rare.

Because *P. karlingii* isolates are slow growing, produce relatively small amounts of mycelium, and are difficult to maintain in the laboratory, it would be challenging to develop and maintain an assay that utilizes mycelium-derived antigen for detection of

anti-*Paralagenidium* antibodies in dogs. For this reason, we also assessed the ability of the anti-*L. giganteum* f. *caninum* antibody ELISA to detect dogs infected with either *L. giganteum* f. *caninum* or with *P. karlingii*. In this context, using a cutoff PP value of 40%, both the sensitivity and specificity of the assay were approximately 80%. However, when dogs with pythiosis were excluded, the specificity increased to approximately 92%, suggesting that the assay could actually be quite good at correctly identifying dogs infected with either *L. giganteum* f. *caninum* or with *P. karlingii* if used after or in conjunction with anti-*P. insidiosum* serology to identify dogs with pythiosis.

In comparison to the similarly developed ELISA for detection of anti-*P. insidiosum* antibodies (for which sensitivity and specificity both approach 100%),[4] the overall diagnostic performance of the *Lagenidium* antibody ELISA is clearly inferior. One reason for this result is the antigenic similarity between *L. giganteum* f. *caninum* and *P. karlingii*, which decreases the specificity of the assay. Another likely reason is that dogs with pythiosis appear to have higher levels of seroreactivity in general than those infected with *L. giganteum* f. *caninum* or *P. karlingii*. In previous immunoblot analyses in which sera from dogs with pythiosis and those with *L. giganteum* f. *caninum* infection were diluted as needed to produce similar signal levels when probing mycelial antigens extracted from their respective pathogens, samples from dogs with pythiosis were diluted in the range of 1 : 1,000 to 1 : 10,000, whereas samples from dogs with *L. giganteum* f. *caninum* infection were diluted in the range of 1 : 250 to 1 : 1,000.[k] The reasons for this difference are unknown, but potential explanations might be that *Lagenidium* and *Paralagenidium* organisms are less antigenically stimulating than *P. insidiosum*, that non-IgG isotypes are more important in the humoral response to *Lagenidium* and *Paralagenidium* than to *P. insidiosum*, or that the disseminated nature of *L. giganteum* f. *caninum* infection compromises the canine's immune response.

In general, ELISA results can be reported in a number of ways, with some of the more common methods being as OD values (usually corrected for background signal from nonspecific binding), as end-point titers determined by serial dilution of test sera, or by comparison to a standard curve.[8] We elected to report results as PP, which is calculated as the ratio of the median OD for the test sample to the median OD for a high positive reference standard included in the same run of the assay. The advantages of utilizing PP are that it requires only a single serum dilution, it is expressed on a continuous scale between 0 and 100, and it does not assume uniform background activity, the latter of which helps to minimize interassay variability.[8] Assay precision as assessed by intra- and interassay CV generally is considered excellent at levels below 5 and 10%, respectively, with 7 and 15% being typical for most assays.[10] Values above 10% (for intra-assay CV) and 20% (for interassay CV) are considered unacceptably high.[10] Using these guidelines, the precision of the assay described here would be considered excellent for intra-assay CV (3.8%) and acceptable for interassay CV (13.1%). Because of this interassay variability, when using the assay to monitor antibody levels in dogs being treated for *L. giganteum* f. *caninum* infection, it would be important to include both pretreatment and posttreatment serum samples on the sample plate when assessing a potential change in antibody levels.

Some limitations of our study should be noted. The first is the relatively small number of samples from dogs without oomycosis that were evaluated. Given that 2 of the 26 sera in this category produced results well above the proposed 40% PP cutoff, and that the reasons for these false-positive results (especially in the dog with allergic dermatitis) are unknown, it will be important to obtain a more precise estimate of the specificity of the assay in this group of dogs by evaluating a larger number of samples. This is especially true considering the very low prevalence of *L. giganteum* f. *caninum* and *P. karlingii* infections in the overall population of dogs with chronic dermatopathies that might be tested for these diseases. A second limitation of the study is that samples were drawn from a pool of archived sera for which a diagnosis was already known, rather than being randomly selected from a population of dogs with clinical signs suggestive of *L. giganteum* f. *caninum* infection. For this reason, we elected not to calculate predictive values for the ELISA.

In conclusion, the assay described here appears to be a sensitive but nonspecific serologic test for the identification of *L. giganteum* f. *caninum*-infected, and to a lesser degree, *P. karlingii*-infected dogs. Our recommendation would be that the assay only be used in conjunction with the more sensitive and specific *Pythium* antibody ELISA. In this context, a dog that has a positive *Pythium* serology result would be extremely likely to have pythiosis, regardless of the *Lagenidium* serology result. A dog that is negative on both assays would be unlikely to have either disease, and if the *Lagenidium* ELISA PP value is <20%, diagnostic tests should focus on other potential causes of the canine's clinical signs. Dogs in which *Pythium* ELISA results are negative, but *Lagenidium* ELISA results are >40% should have additional diagnostic procedures that include biopsy for histopathology and oomycete culture.

Footnotes

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[b] Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands

[c] BCA Protein Assay; Pierce Chemical Company, Rockford, IL

[d] Immulon 2 Microtiter Plates; Dynex Technologies, Inc, Chantilly, VA

[e] Bovine serum albumin; fraction V powder, minimum 95%; Amresco Co, Solon, OH

[f] Peroxidase Conjugated Affinity Purified Anti-Dog IgG; Rockland Immunochemicals, Gilbertsville, PA

[g] TMB 2-component substrate system; Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MA

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Conflict of Interest Declaration: Dr. Amy Grooters is an associate editor at the Journal of Veterinary Internal Medicine.

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