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STANDARD ARTICLE

A monoclonal antibody-based urine *Histoplasma* antigen enzyme immunoassay (IMMY®) for the diagnosis of histoplasmosis in cats

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Background: An in-house *Histoplasma* urine antigen test for cats might be desirable in certain situations.

Objective: To validate and compare the diagnostic performance of a monoclonal antibody-based IMMY urine *Histoplasma* antigen enzyme immunoassay (IMMY EIA) to the commercially available urine *Histoplasma* antigen enzyme immunoassay (MiraVista Diagnostics, MV EIA).

Animals: One hundred ninety-three urine samples from 105 client-owned and purpose-bred research cats.

Methods: Cats were classified as *Histoplasma* positive or negative based on diagnostic investigation. The IMMY EIA and MV EIA were performed on all urine samples. Correlation and agreement between the assays were determined. Diagnostic performance was determined and compared between assays.

Results: The IMMY EIA, with a 0.25 ng/mL diagnostic cutoff, provided a diagnostic sensitivity (DSe), diagnostic specificity (DSp), and diagnostic accuracy (DAc) of 89% (95% confidence interval [CI]; 73%-97%), 80% (67%-89%), and 83% (74%-90%), respectively. The IMMY EIA, with a 1.1 ng/mL diagnostic cutoff, provided a DSe, DSp, and DAc of 77% (95% CI 60%-90%), 97% (88%-100%), and 89% (81%-95%), respectively. The MV EIA provided a DSe, DSp, and DAc of 94% (95% CI 81%-99%), 97% (89%-100%), and 96% (90%-99%), respectively. Moderate overall agreement was found between MV EIA and IMMY EIA using the 0.25 ng/mL cut-off (κ = 0.44; 95% CI 0.31-0.57) and the 1.1 ng/mL cut-off (κ = 0.43, 95% CI, 0.31-0.56).

Conclusions and Clinical Importance: The IMMY EIA might be useful as a diagnostic test for histoplasmosis in cats. Further modifications of the IMMY EIA are required to achieve the diagnostic performance of the MV EIA.

KEYWORDS

feline, fungal, histoplasmosis, mycosis

1 | INTRODUCTION

Histoplasmosis, caused by *Histoplasma capsulatum*, occurs in temperate and subtropical climates around the world and is the second most common systemic mycosis among cats in the United States.[1] Inhalation of spores (microconidia) found in dust and soil is the most common means of infection. Organs that are commonly affected include the lung, liver, spleen, lymph nodes, bone marrow, bones, and joints.[2–4]

Abbreviations: %CV, coefficient of variation; AUC, area under the curve; BLQ, below the limit of quantification; DAc, diagnostic accuracy; DSe, diagnostic sensitivity; DSp, diagnostic specificity; HN, histoplasmosis-negative cat; HP, histoplasmosis-positive cat; IMMY EIA, IMMY urine *Histoplasma* antigen enzyme immunoassay; LLOQ, lower limit of quantification; MV EIA, MiraVista urine *Histoplasma* antigen enzyme immunoassay; NPV, negative predictive values; OD, optical density; OIE, World Health Organization; PPV, positive predictive values; ROC, receiver operating characteristic; UD, undiagnosed cat.

Affected cats often display vague clinical signs such as weight loss, anorexia, and lethargy or weakness.[2–4] Even in endemic areas, diagnosis is commonly delayed.[2] Diagnosis most commonly is confirmed by finding *H. capsulatum* organisms in cytopathologic or histopathologic samples.[2–4] Treatment includes prolonged administration of antifungal drugs.[2,3,5] Despite antifungal treatment, histoplasmosis is commonly fatal with 6-month survival only being 67%.[2]

Even with extensive searches, cytopathology or histopathology do not always reveal *H. capsulatum* organisms and in some cases tissue sampling is not feasible. A commercially available *Histoplasma* antigen enzyme immunoassay (MiraVista Diagnostics, Indianapolis, IN) is useful for supporting the diagnosis of disseminated histoplasmosis in cats, with a diagnostic sensitivity (DSe) of 94% when urine is tested.[6] It is also useful for monitoring as an indicator of remission and relapse during and after treatment, respectively.[5]

Currently, the MiraVista *Histoplasma* enzyme immunoassay (MV EIA) is the only commercially available *Histoplasma* antigen test. As such, all samples must be shipped to the service laboratory in Indianapolis, IN. Doing so might not be practical in some parts of the world. Moreover, antigen testing when used repeatedly for treatment monitoring might be cost prohibitive for some pet owners. A *Histoplasma* antigen test that can be performed in-house might be desirable in certain situations. The IMMY (Norman, Oklahoma) offers agent-specific reagents for in-house diagnostic testing. Both the MV EIA and IMMY urine *Histoplasma* antigen enzyme immunoassay (IMMY EIA) are sandwich ELISAs with proprietary capture and biotinylated detection antibodies targeting antigens on the fungal cell wall. The capture antibodies differ between the 2 tests, with the IMMY EIA using a monoclonal antibody targeting *Histoplasma* galactomannan on the fungal cell wall and the MV EIA using a polyclonal antibody targeting *Histoplasma* polysaccharide antigen on the fungal cell wall.[7–9]

The diagnostic performances of the MV and IMMY EIAs have been investigated in humans with suspected histoplasmosis.[7,9,10] Initially, the positive agreement of the IMMY EIA with the MV EIA was found to be moderate at 64.5%.[7] However, it improves to 82.3% by making a minor modification to the analytical protocol (use of a wash buffer 0.0 ng/mL calibrator, in addition to the 7 manufacturer-recommended calibrators) and adding an "indeterminate" range of test results to the manufacturer's interpretive criteria. By creating an indeterminate range of test results from 0.11 to 0.49 ng/mL, overall agreement of the IMMY EIA with the MV EIA improves to 90%, but 8% of humans fall within the indeterminate range and required further testing.[9]

To our knowledge no published studies describe the use of IMMY EIA in veterinary species. Our aims were (1) to partially validate the IMMY EIA for use in cats and (2) to describe the diagnostic performance of the IMMY EIA in cats and compare its diagnostic performance to that of the current gold standard (MV EIA).

2 | MATERIALS AND METHODS

2.1 | Cats

Surplus urine samples from client-owned and purpose-bred research cats enrolled in several clinical studies approved by the Institutional Animal Care and Use Committee of Oklahoma State University were used. Cats were confirmed histoplasmosis-positive (HP) if they had compatible clinical signs (some combination of lethargy, inappetence, weight loss, fever, lymphadenopathy, splenomegaly, joint effusion, lameness, tachypnea, or dyspnea) and *H. capsulatum* organisms were found on cytological or histopathological evaluation by a board-certified pathologist. Alternatively, cats were classified as HP based on appropriate clinical signs (listed above), positive urine MV EIA, and positive response to antifungal treatment. Histoplasmosis-negative (HN) cats included (1) ill client-owned cats with a definitive alternative diagnosis, (2) apparently healthy client-owned cats, and (3) specific pathogen-free (SPF) purpose-bred research cats, not exposed to *H. capsulatum*.

Exclusion criteria for HN cats included unexplained lymphadenopathy, splenomegaly, joint effusion, lameness, respiratory disease, or positive MV EIA. Cats that had an alternative diagnosis involving the respiratory system, liver, spleen, lymph nodes, gastrointestinal (GI) tract, bone(s), or joint(s) were required to have supportive evidence from cytology or histopathology that did not reveal *H. capsulatum* organisms. Additionally, the diagnosis of cancer required cytopathology or histopathology.

Cats that were classified as HP based on MV EIA results, but without finding organisms, were excluded from determination of the diagnostic performance of MV EIA. Additionally, cats that were classified as HN based solely on positive MV EIA, and had an alternative diagnosis, were included only in the determination of the diagnostic performance of MV EIA. Cats that did not have a definitive diagnosis were classified as undiagnosed (UD) and only were used for comparison of IMMY EIA to MV EIA results.

2.2 | Urine collection and handling

Urine samples were collected by pre-pubic cystocentesis using a 1.5-inch, 22-gauge needle and 5 or 10 mL syringe. Urine was refrigerated for up to 8 hours and then frozen at −80°C for up to 5 years until analyzed. Because of the study design, all urine samples used for MV EIA underwent 2 freeze-thaw cycles. All urine samples used for IMMY EIA underwent 1 freeze-thaw cycle, except those repeated on a different day for determination of inter-assay coefficient of variation (%CV), which underwent 2 freeze-thaw cycles.

2.3 | IMMY urine *Histoplasma* enzyme immunoassay

Testing was performed using commercially available monoclonal-antibody agent specific reagents (IMMY) with modification from that previously described.[9] An automated plate washer (Wellwash Versa; Thermo Scientific, Waltham, MA) and spectrophotometer (Varioskan Flash; Thermo Scientific) were used for all analyses. All samples were run in duplicate. Urine samples were kept frozen and analyzed within 1 hour of being thawed at room temperature. One-hundred microliters of undiluted urine was added to individual microwells in a 96-well plate coated with *Histoplasma* galactomannan monoclonal capture antibody (IMMY). The plate was incubated for 55 minutes at 37°C. Wells were washed 3 times with 300 μL wash buffer. After washing, 100 μL of horseradish peroxidase (HRP)-conjugated

anti-galactomannan monoclonal antibody (IMMY) was added to each well. The plate again was incubated for 40 minutes at 25°C. A second identical wash step was performed. One-hundred microliters of HRP enzyme substrate (3,3′,5,5′-tetramethylbenzidine) then was added to each well, followed by a final incubation of 25 minutes at 25°C. One-hundred microliters of stop solution (2-N-sulfuric acid) then was added to all wells. Immediately after, optical density (OD) was measured at 450 nm.

Each plate included samples of wash buffer (IMMY) as blanks and manufacturer-provided positive and negative controls. Standards included serial dilutions in wash buffer of a known concentration of purified antigen (IMMY): 0.0, 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, and 25 ng/mL. The standard curve was generated using a 4-variable logistic curve fit and blank subtraction. *Histoplasma* antigen concentrations were calculated by mapping the unknown sample OD against the standard curve.

2.4 | Heat fixation

In attempt to improve diagnostic performance, a pre-analytical heat fixation step was added to a subset of samples. All samples for which an adequate volume of urine remained after initial analysis, starting on the 4th day of testing, were reanalyzed after heat fixation. Heat fixation was performed on the same day that samples were analyzed using the IMMY EIA as described above. Urine that was reanalyzed using the heat fixation step was kept at room temperature for <6 hours before analyses. Fixation included heating 200 μL of undiluted urine to 120°C for 3 minutes with a heat block (Isotemp Dry Bath; Thermo Scientific) immediately followed by centrifugation at 10000*g* for 10 minutes. The sample supernatant was separated immediately and analyzed within 1 hour by IMMY EIA, which was performed as described above.

2.5 | MiraVista urine *Histoplasma* enzyme immunoassay

After the IMMY EIA was performed, samples immediately were refrozen at −80°C and shipped overnight on ice for MV EIA. Urine was kept frozen until being thawed at room temperature for analysis. Antigen tests were performed in 2 batches, as previously described.[8] The MV EIA has a lower limit of quantification (LLOQ) of 0.4 ng/mL. Positive EIA results <0.4 ng/mL were reported as positive but below the limit of quantification (BLQ). The upper limit of quantification of the assay is 19.0 ng/mL. Enzyme immunoassay results above 19.0 ng/mL were reported as positive but above the limit of quantification. For statistical analyses, these unquantifiable results were reported as 0.4 or 19.0 ng/mL, respectively.

2.6 | Validation—IMMY EIA

Lower limits of quantification, precision or measurement uncertainty, spiked recovery, and diagnostic accuracy (DAc) were used to partially validate the IMMY EIA in cats. The LLOQ was determined by adding 10 SDs to the mean of 40 blank samples.[11] All plates included between 2 and 8 blank samples.

Assay precision or measurement uncertainty was quantified using intra- and inter-assay coefficients of variation (%CV). Intra-assay %CV was calculated using all the samples with results above the LLOQ, with all factors except "well" being held equal. Inter-assay %CV was calculated using samples with results above the LLOQ on initial testing and the same sample repeated on at least 1 additional plate with all factors except "plate," and in some cases "day", being held equal.

Spiked recovery was performed to determine if significant interactions occurred between feline urine and the *Histoplasma* galactomannan antigen. Urine samples from 5 SPF, purpose-bred research cats were divided into 8 aliquots, and purified antigen solution (IMMY) was added to 7 of the aliquots at 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, and 25 ng/mL. Percent recovery was calculated according to the following formula: ([measured concentration$_{\text{spiked sample}}$ − measured concentration$_{\text{neat sample}}$]/theoretical concentration$_{\text{spiked}}$ × 100).[11]

2.7 | Statistical analysis

Statistical analysis was performed using commercial software (SAS 9.4; SAS, Cary, North Carolina). Statistical methods followed guidelines for assay validation adopted by the World Assembly of Delegates of the World Organization for Animal Health (OIE).[12] Unless otherwise stated, mean IMMY EIA antigen concentrations (performed in duplicate) were used for statistical analysis. Antigen concentrations for the IMMY EIA were compared with the clinical diagnosis for HP and HN cats. Youden's index was used to determine an ideal diagnostic cutoff with adjustment based on expected clinical use.[13] After determination of a diagnostic cutoff, IMMY EIA results were dichotomized as positive or negative. For samples obtained at diagnosis from HP and HN cats, DSe, diagnostic specificity (DSp), and DAc were determined for the IMMY EIA and MV EIA. Cohen's kappa statistic was used to compare the DSp and DSe of the IMMY EIA for each diagnostic cutoff to MV EIA. Chi-square tests were used to compare IMMY EIA results before and after heat treatment. Pearson correlation coefficients were used to describe the correlation between IMMY EIA and MV EIA. Cohen's kappa statistic was used to evaluate agreement between IMMY EIA and MV EIA before and after samples were stratified based on MV EIA antigen concentrations (<1.0 ng/mL, 1.0-5.0 ng/mL, 5.01-10.0 ng/mL, and > 10.0 ng/mL). Agreement was considered slight, fair, moderate, and substantial for values 0-0.2, 0.21-0.40, 0.41-0.60, and 0.61-0.80, respectively, as previously described.[14] Dichotomized test results (positive or negative) of IMMY EIA and MV EIA as compared using the ultimate clinical diagnosis (HP or HN) were used to generate receiver operating characteristic (ROC) curves and area under the curve (AUC) was compared between MV EIA and IMMY EIA using a nonparametric approach as previously described.[15] Statistical significance was set at $P \leq .05$.

3 | RESULTS

3.1 | Cats

One-hundred and ninety-three urine samples from 105 cats were included in the study. Cats included 40 HP cats, 59 HN cats, and

6 UD cats. For HP cats, 35 urine samples were obtained from 35 cats at the time of diagnosis and 93 urine samples were obtained from 31 cats during treatment. Except for the calculation of diagnostic performance of the MV EIA, 2 cats were excluded from statistical analysis because they were not believed to have histoplasmosis but were MV EIA positive. Of the HP cats, 32 of 35 (91%) were diagnosed based on finding *H. capsulatum* organisms. The remaining 3 cats had compatible clinical signs, positive MV EIA results, and clinical improvement after antifungal treatment. Clinical presentations of these 3 cats included anorexia, weight loss, dyspnea, interstitial lung disease, and fever (n = 1); anorexia, weight loss, tachypnea, fever, and nonregenerative anemia (1); and, anorexia, weight loss, diarrhea, dyspnea, structured interstitial lung disease, and fever (1). Of the HN cats, 40 of 59 (68%) had an alternative definitive diagnosis and 19 of 59 (32%) were considered healthy. Alternative diagnoses included hyperthyroidism (n = 9), neoplasia (6), hypertrophic cardiomyopathy (4), feline bronchial disease (2), FeLV infection (2), chronic kidney disease (2), obesity (2), and 1 each of bacterial osteomyelitis, neutrophilic cholangitis, herpes virus conjunctivitis, cytauxzoonosis, diabetes mellitus, hyperaldosteronism, inflammatory bowel disease, idiopathic cystitis, mammary hyperplasia, osteoarthritis, toxoplasmosis, tracheal hypoplasia, and immune-mediated vasculitis. Neoplasms, all diagnosed in 1 cat each, included multicentric lymphoma, cutaneous mast cell tumor, nasal carcinoma, nasal sarcoma, pulmonary carcinoma, and intestinal lymphoma. The apparently healthy cats included SPF, purpose bred-research cats (n = 11) and client-owned cats (8). In the cats classified as UD (n = 6), clinical signs included chronic GI disease (3), and 1 each of hepatobiliary disease, anemia and fever of unknown origin, and chronic upper respiratory disease.

3.2 | Validation IMMY enzyme immunoassay

Positive and negative controls were within the manufacturer's acceptable concentrations for all plates. The mean (SD) calculated antigen concentration for blank samples was 0.017 ng/mL (0.048) with a calculated LLOQ of 0.50 ng/mL. Intra-assay %CV was 9.9% and inter-assay %CV was 22.9%. Thirty-five urine samples were analyzed on at least 2 plates and were used to calculate inter-assay %CV. The mean (SD) recovery for urine samples spiked with 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, and 25 ng/mL was 95.5% (47.8), 124.9% (24.6), 127.7% (16.0), 115.4% (11.0), 110.7% (6.2), 107.7% (7.7), and 102.6% (7.2), respectively.

3.3 | IMMY enzyme immunoassay results and diagnostic performance

Mean (SD) antigen concentrations were 5.35 ng/mL (5.80), 0.2 ng/mL (0.43), and 0.14 ng/mL (0.28) for HP, HN with alternative diagnosis, and apparently healthy HN cats, respectively. (Figure 1) The ideal single diagnostic cutoff based on the Youden's index was 1.1 ng/mL, which provided DSe, DSp, and DAc of 77% (95% confidence interval [CI] 60%-90%), 97% (88%-100%), and 89% (81%-95%), respectively. (Figure 2) This cutoff was considered to have an unacceptably low sensitivity for many clinical applications. As such, a diagnostic cutoff of 0.25 ng/mL also was investigated to improve the sensitivity of

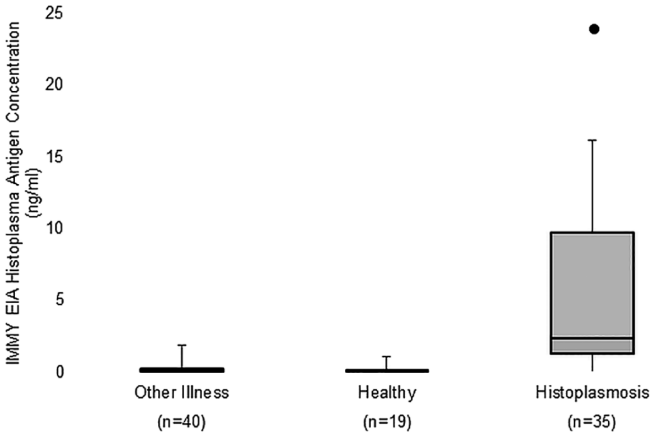

FIGURE 1 Box and Whisker plots demonstrating the antigen concentrations as measured by the IMMY urine *Histoplasma* antigen enzyme immunoassay in 35 cats with histoplasmosis at the time of diagnosis, 40 cats with alternative diagnosis at the time of diagnosis, and 19 apparently healthy cats

the test. This cutoff was chosen by visual inspection of the ROC curve (Figure 3) and the graph comparing DSe, DSp, and DAc at various diagnostic cutoffs (Figure 1).

When use of the MV EIA was removed as an inclusion or exclusion criteria, DSe, DSp, and DAc of the MV EIA were 94% (95% CI 81%-99%), 97% (89%-100%), and 96% (90%-99%), respectively. When a diagnostic cutoff of 0.25 ng/mL was used, the IMMY EIA had significantly lower DSp (P = .002) as compared to the MV EIA. The DSe was not significantly different (P = .16). When a diagnostic cutoff of 1.1 ng/mL was used, the IMMY EIA had a significantly lower DSe (P = .01). The DSp was not significantly different (P = .65). The AUC for the ROC curve was 0.93 (95% CI 0.86-0.97) and 0.97 (0.91-1.00) for the IMMY EIA and MV EIA, respectively. These were not significantly different (P = .13; Figure 3).

3.4 | Relationship between IMMY enzyme immunoassay and MiraVista enzyme immunoassay before heat fixation

A significant positive correlation was found between IMMY EIA and MV EIA (R = 0.92; P < .0001). Moderate overall agreement was found between MV EIA and IMMY EIA using the 0.25 ng/mL cutoff (κ = 0.44; 95% CI 0.31-0.57) and the 1.1 ng/mL cut-off (κ = 0.43; 0.31-0.56). When the MV EIA was <1.0 ng/mL, poor agreement was found between MV EIA and IMMY EIA using the 0.25 ng/mL cutoff (κ = 0.07; 95% CI −0.10 to 0.25) and 1.1 ng/mL cut-off (κ = −0.2; −0.14 to 0.09). All discordant results between IMMY EIA and MV EIA occurred on samples for which MV EIA was <1.0 ng/mL.

3.5 | Heat fixation

Heat fixation was performed on 66 urine samples including those from HP cats during treatment (n = 32) and at the time of diagnosis (11) and those from HN client-owned cats (23). These assays were performed after the samples were tested without heat fixation. The mean (SD) heat fix IMMY EIA, standard IMMY EIA, and MV EIA were

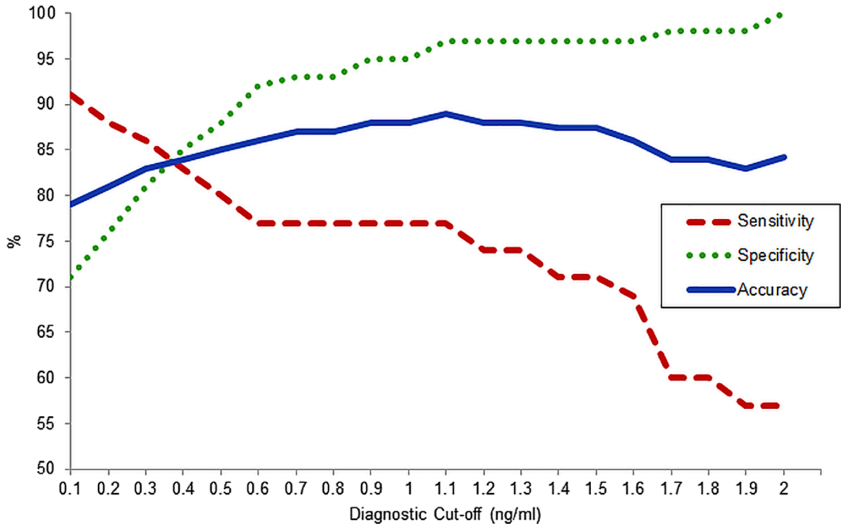

FIGURE 2 The diagnostic sensitivity, specificity, and accuracy of the IMMY urine *Histoplasma* antigen enzyme immunoassay, at different diagnostic cutoffs, for the diagnosis of histoplasmosis in 35 cats with histoplasmosis, 40 cats with an alternative diagnosis, and 19 apparently healthy cats

0.5 ng/mL (1.36), 0.61 ng/mL (1.48), and 0.86 ng/mL (2.13), respectively. When a diagnostic cutoff of 0.25 ng/mL was used, agreement of the IMMY EIA with MV EIA was not significantly different before and after heat fixation (P = .08). With a diagnostic cutoff of 0.25 ng/mL, IMMY EIA agreed with MV EIA in 44 of 66 (67%) samples before heat treatment and in 53 of 66 (80%) samples after heat treatment. Heat fixation changed the test result in 13 of 66 cases (20%; Table 1). In effect, heat fixation led to 5 false positives becoming true negatives and 1 true negative becoming a false positive. Agreement with MV EIA in these cats before and after heat fixation was 1 of 6 (17%) and 5 of 6 (83%), respectively. Heat fixation did not change the test result of 4 additional samples that were classified as false negatives (n = 3) or false positive (1) at the time of diagnosis. When a 1.1 ng/mL diagnostic cutoff was used, heat fixation changed the test result in 5 of 66 (8%) cases. This number includes HP cats at the time of diagnosis (n = 2) and during treatment (2) and HN client-owned cats (1). Agreement between the IMMY EIA and the MV EIA before and after heat treatment was 3 of 5 (60%) and 2 of 5 (40%), respectively. In effect, heat fixation caused 1 false positive to become a true negative and 2 true positives to become false negatives (Table 1).

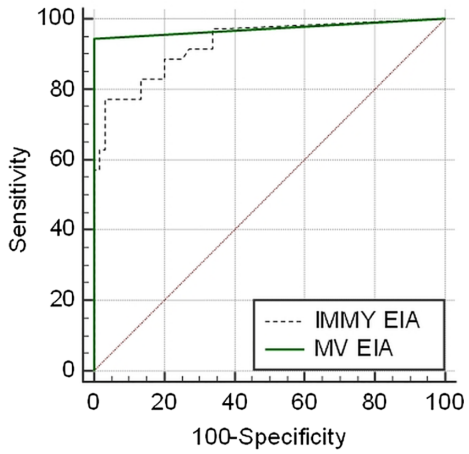

FIGURE 3 Receiver operating curves for the IMMY urine *Histoplasma* antigen enzyme immunoassay and the MiraVista urine *Histoplasma* antigen enzyme immunoassay for the diagnosis of histoplasmosis in 35 cats with histoplasmosis and 59 cats without histoplasmosis

4 | DISCUSSION

Ours is the first study to partially describe and validate the diagnostic performance of the IMMY EIA in cats. Our findings suggest that the IMMY EIA might be useful as a diagnostic test for histoplasmosis in cats. Further modifications of the IMMY EIA are required to achieve the diagnostic performance of the MV EIA.

There are no universally accepted validation methods, and development of an antigen assay is dependent upon the intended use.[16] Based on the World Organization for Animal Health (OIE), "fitness for purpose" categories, the intended purpose for the IMMY EIA are categories 1 and 4 (ie, demonstration of freedom from infection and confirmatory diagnosis of clinical cases, respectively).[16] For these purposes, the OIE considers DSe, DSp, negative and positive predictive values

TABLE 1 Agreement between IMMY EIA and MV EIA before and after heat fixation with two diagnostic cutoffs (0.25 and 1.1 ng/mL) in cats with and without histoplasmosis

Clinical diagnosis	Agreement before heat fixation (%)	Agreement after heat fixation (%)
Diagnostic cutoff (0.25 ng/mL)		
Histoplasmosis positive (n = 7)	1/7 (14)	6/7 (86)
Histoplasmosis negative (n = 6)	1/6 (17)	5/6 (83)
All (n = 13)	2/13 (15)	11/13 (85)
Diagnostic cutoff (1.1 ng/mL)		
Histoplasmosis positive (n = 4)	3/4 (75)	1/4 (50)
Histoplasmosis negative (n = 1)	0/1 (0)	1/1 (100)
All (n = 5)	3/5 (60)	2/5 (40)

Abbreviations: IMMY EIA, IMMY urine *Histoplasma* antigen enzyme immunoassay; MV EIA, MiraVista urine *Histoplasma* antigen enzyme immunoassay.

(NPV and PPV), turn-around time, quality assurance capability, and reproducibility and repeatability as essential factors to consider.[16] Many of these factors are described herein.

Considering the intended purpose of the IMMY EIA, our study partially validated the IMMY EIA for the diagnosis of histoplasmosis in cats, because it demonstrated acceptable repeatability, lower limits of quantification, and detection of the analyte of interest across a wide range of known concentrations. Although there is no universally acceptable %CV, based on OIE recommendations, intra-assay %CV should be <15%, except for very low concentrations.[17] The intra-assay %CV of 9.9% reported herein suggests adequate repeatability. Urine is a convenient biological specimen because it is easy to obtain and usually is available in abundance. Potential challenges to using urine include variability in pH and high concentrations of urea and salts. For these reasons, investigating matrix/analyte interactions is important. Universally acceptable spiked sample recovery concentrations are not available, but a suggested range is 80%-120%.[11] All but 2 of the known concentrations had recovery within this range, and the 2 concentrations outside this range were very close to it. This finding suggests that, in the short term, significant interactions do not occur between feline urine and *Histoplasma* galactomannan antigen that negatively affect the IMMY EIA. Because of a small number of very high antigen concentrations, the upper limit of quantification was not determined. All IMMY EIA results reported herein fall within the previously reported linear range in humans (0.5-50.0 ng/mL).[7] The LLOQ determined in our study was identical to that previously described in humans (0.5 ng/mL).[7] Pending the intended use of the assay, the LLOQ might be higher than the preferred diagnostic cutoff. If so, the range between the OD diagnostic cutoff and LLOQ would be interpreted as positive but BLQ, as is done with the MV EIA. Additional validation methods that should be considered in future studies include investigation of the upper limit of quantification, robustness, sample stability, and dilutional linearity or parallelism, among others.

The diagnostic cutoff is determined based on the intended use of the assay. If used as a screening test, a high DSe is desired. If used as a confirmatory test, a high DSp is desired. When adjusting the diagnostic cutoff, increasing DSe is done at the expense of Dsp and vice versa. In our study, the most accurate diagnostic cutoff of 1.1 ng/mL would likely provide an unacceptably low DSe if the intended purpose is to help support the diagnosis of suspected histoplasmosis. For that reason, a second diagnostic cutoff of 0.25 ng/mL also was investigated. The lower cutoff provided a more acceptable DSe at the expense of DSp. In humans, 2 diagnostic cutoffs have been used concurrently. In doing so, a range of indeterminate test results is produced. For indeterminate results, the IMMY EIA alone cannot be used to make diagnostic and therapeutic decisions but does suggest the need for further testing. A similar approach could be taken with the IMMY EIA in cats. For example, an indeterminate range of 0.25-1.90 ng/mL could be used. In the group of cats reported herein, doing so would have provided a DSe of 89%, DSp of 100%, and a DAc of 96%. As a trade-off, 23 of 94 (24%) cats would have fallen in the indeterminate range and required additional testing. Of those cats, 22 of 23 (96%) had MV EIA results that agreed with the clinical diagnosis. In this scenario, the IMMY EIA could be used as an initial in-house test with indeterminate results requiring that samples be sent for MV EIA.

When compared with the MV EIA, the IMMY EIA was diagnostically inferior. No single diagnostic cutoff for the IMMY EIA could achieve both the DSe and DSp of the MV EIA, which was evidenced by the significantly lower DSe (1.1 ng/mL cutoff) and DSp (0.25 ng/mL cutoff). Although the MV EIA was used as an exclusion criterion for HN cats, it only led to the exclusion of 2 cats. In other words, the MV EIA was used to help ensure HN cats did not have histoplasmosis, but in our study a MV EIA-positive test result was very uncommon in cats not believed to have histoplasmosis. The 2 cats excluded because of being MV EIA positive were believed to be HN. They were considered false positives for the calculation of the diagnostic performance of the MV EIA. In addition, the 3 cats that were classified as HP based on the MV EIA, without cytologic or histologic confirmation, were removed from the calculation of diagnostic performance for the MV EIA. Removing the MV EIA results as inclusion or exclusion criteria was important to prevent selection bias when describing the diagnostic performance of the MV EIA in this group of cats.

When all samples (at diagnosis and during treatment) were considered, the IMMY EIA had moderate overall agreement with the MV EIA. Discordant test results only were found with lower antigen concentrations (<1.0 ng/mL). This phenomenon has been described, albeit to a lesser extent, in humans with discordant results, often falling between 0.11 and 0.49 ng/mL.[9] In fact, low-level antigenuria presents a clinical conundrum in humans.[18] In an attempt to improve differentiation between HN cats and HP cats with low-level antigenuria, an additional heat fixation step was added to the pre-analytical protocol and approximately 1/3 of the samples were reanalyzed. Although heat fixation did not significantly increase agreement with MV EIA, it did appear to improve DSp, but only when a lower diagnostic cutoff (0.25 ng/mL) was used. In fact, 5 of 6 IMMY EIA results that were considered false positives became true negatives and only 1 true negative became a false positive. Heat fixation did not appear to improve the DSe of low-level antigenuria in the small number of heat-fixed samples analyzed, as none of the false negatives changed to true positives. Although the reason heat fixation improved DSp is unknown, these findings collectively suggest that heating, centrifugation, or both decrease the presence of an interfering substance such as cellular debris or protein that cross-reacts with the assay. Further research regarding the effects of heat fixation on the IMMY EIA is warranted.

Negative predictive values and PPV are dependent upon the diagnostic performance of the test and the pretest probability of disease. If the IMMY EIA (cutoff 0.25 ng/mL) was used as a screening test in all cats in our hospital (prevalence = 4.5%), the NPV and PPV would be approximately 99% and 17%, respectively. A negative test would rule out histoplasmosis but a positive test would mean very little. If the test were used in cats with suspect histoplasmosis where the pretest probability was, for example, 80%, the NPV and PPV would be approximately 64% and 95%, respectively. In this scenario, a cat that tests positive with the IMMY EIA very likely has histoplasmosis. In other words, interpretation of IMMY EIA test results, as with any diagnostic test, should account for the pretest probability of disease.

Our study had some limitations. The first is the fact that the clinical diagnosis was used as the standard by which both assays were

compared. Clinical diagnoses are not perfect and every organ system in every cat was not sampled. Much effort was used to ensure that HP cats truly had histoplasmosis and that HN cats truly did not. All but 3 HP cats would be considered unequivocal reference standards by the OIE, the highest level.[16] Clinically determining HN cats is considerably more challenging. All HN cats would be considered relative reference standards by the OIE.[16] As such, guidelines to use other well defined test methods were followed.[16] Cytology or histopathology was required when making an alternative diagnosis involving organ systems commonly affected by histoplasmosis. This, in addition to requiring the highly sensitive MV EIA to be negative, was considered adequate for HN cats to serve as negative controls.

A second limitation is the lack of cats with other systemic mycoses to serve as negative controls. In our hospital, other systemic mycoses in cats are very uncommon. Although cross-reactivity of other fungal organisms with the IMMY EIA has not been extensively studied, in 1 study of humans, 6 of 10 blastomycosis controls and 0 of 13 aspergillosis controls tested positive. As such, the DSe of the IMMY EIA reported herein might be lower in locations where other systemic mycoses are more common.

A third limitation was the difference in freeze-thaw cycles between the IMMY EIA and the MV EIA. Additionally, some of the repeat samples used to determine the IMMY EIA inter-assay %CV were subjected to an additional freeze-thaw cycle. In general, freeze-thaw cycles should be avoided because they can cause degradation of the analyte. Because the MV EIA is only performed in the service laboratory in Indianapolis, Indiana, the samples were refrozen before shipment. Doing so allowed the MV EIA to be performed in batches, and this advantage was considered to outweigh the disadvantages of an additional freeze-thaw cycle.

A fourth limitation was that all samples were stored, some for a prolonged period of time. This did not affect the comparisons between the 2 assays because both were subjected to the essentially the same storage conditions. Studies describing the IMMY EIA in humans also used stored samples; in some instances, samples were stored for several years.[7,10] We are unaware of published information investigating the effects of storage time on the IMMY EIA, and thus how storage affected the diagnostic performance reported herein is unknown. Future study of the stability of *Histoplasma* galactomannan in feline urine and the potential effects of storage on the IMMY EIA is warranted.

A final limitation is that heat fixation was not used for all analyses, because it appears to improve specificity. Heat fixation has not been included in published reports of the IMMY EIA in humans and thus was not included in initial testing. Additionally, not all sample volumes were large enough to be tested using the IMMY EIA with and without heat fixation and with the MV EIA. Further research regarding the effect of heat fixation on the IMMY EIA is needed.

In conclusion, the IMMY EIA might be useful to support the diagnosis of histoplasmosis in cats. With the analytical protocol reported herein, the diagnostic performance of the IMMY EIA is inferior to the commercially available MV EIA. Further research investigating preanalytical and analytical modifications to improve diagnostic performance of the IMMY EIA is warranted.

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CONFLICT OF INTEREST DECLARATION

Dr. Andrew Hanzlicek has in the past, and is currently, collaborating with MiraVista Diagnostics on multiple studies relating to veterinary clinical mycology.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

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

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