

**STANDARD ARTICLE**

Evaluation of a novel monoclonal antibody-based enzyme immunoassay for detection of *Histoplasma* antigen in urine of dogs

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Abstract

Background: Commercially available, noninvasive testing options for histoplasmosis are limited outside of the United States.

Objectives: To describe the diagnostic performance of a novel *Histoplasma* antigen enzyme immunoassay (IM EIA) for the diagnosis of histoplasmosis in dogs.

Animals: Twenty dogs with histoplasmosis, 79 dogs without histoplasmosis, and 11 unclassified dogs providing 202 urine samples.

Methods: This a prospective study using stored urine samples. Samples were analyzed with the IM EIA and with the commercially available *Histoplasma* antigen EIA (MV EIA). Dogs were classified based on final proven diagnosis and performance of the IM EIA was described and compared with the MiraVista enzyme immunoassay (MV EIA).

Results: The diagnostic sensitivity (DSe), specificity (DSp), and accuracy (DAc) of the IM EIA were 70% (51%-89%), 99% (97%-100%), and 93% (81%-100%), respectively. The DSe, DSp, and DAc for the MV EIA were 95% (85%-100%), 99% (97%-100%), and 98% (95%-100%), respectively. The area under the receiver operator characteristic curve was significantly smaller for IM EIA (0.87) as compared with MV EIA (0.97, $P = .03$). This was primarily due to 6 false negative IM EIA results, 4 from dogs with disease localized to the gastrointestinal tract. The MV EIA was positive in 5/6 of these dogs.

Conclusions and Clinical Importance: The IM EIA might be useful for the diagnosis of disseminated histoplasmosis in dogs, but clinical usefulness will be limited in dogs with histoplasmosis localized to the GI tract.

KEYWORDS

canine, fungal, invasive fungal infection, mycology, mycoses, mycosis

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 dogs; HP, histoplasmosis positive dogs; IFI, invasive fungal infection; IM EIA, IMMY *Histoplasma* urine antigen enzyme immunoassay; MV EIA, MiraVista Diagnostics *Histoplasma* urine antigen enzyme immunoassay; OD, optical density; ROC, receiver operating characteristic curve; UD, undiagnosed dogs.

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1 | INTRODUCTION

Histoplasmosis is 1 of the most common enzootic invasive fungal infections (IFIs) of dogs around the world, including in the Eastern half of the United States.^{1,2} Spores of the causative agent, *Histoplasma capsulatum*, are inhaled when contaminated soil is aerosolized.³ Disease might be confined to respiratory tract or disseminate throughout the body. Although likely a manifestation of dissemination, disease localized to the gastrointestinal (GI) tract occurs in dogs.²

Clinically histoplasmosis is often initially overlooked because of vague clinical signs that overlap with other common inflammatory diseases such as bacterial infection, cancer, or immune-mediated disease. Fungal culture, once considered the gold-standard, is rarely used clinically because of poor sensitivity and delayed results (up to 4 weeks).^{2,4} Currently 1 of the most common means of confirming histoplasmosis in dogs is by identifying *H capsulatum* yeasts in fluid or tissue samples.² Collecting these samples can be invasive, potentially requiring multiple tissue aspirates or biopsies. For these reasons, the diagnosis of histoplasmosis is often challenging and delayed, which likely contributes to the relatively low 6-month survival rate of 71%.² Moreover, survival is even lower when dogs not treated are considered, which might occur due to severe clinical signs or anticipated prolonged and expensive antifungal treatment. Finally, even with long-term survival, disease relapse remains possible, in part due to challenges of treatment monitoring and determining when to discontinue antifungal treatment.²

More recently a commercially available, noninvasive *Histoplasma* antigen enzyme immunoassay (MiraVista Diagnostics, Indianapolis, Indiana; MV EIA) supports the diagnosis of histoplasmosis in dogs.⁵ The antigen detected, galactomannan, is a component of the *Histoplasma* cell wall. When urine is tested, the MV EIA is an accurate test for the diagnosis of histoplasmosis in dogs, with sensitivity and specificity of 89.5% and 100%, respectively, although the specificity is expected to be dependent upon the geographic location.⁵ For example, in an area with enzootic blastomycosis, the specificity will be lower due to cross-reactivity.⁶ In addition to diagnosis, the MV EIA is also commonly used clinically to monitor antifungal treatment and detect relapse of disease.⁷⁻¹¹

A second *Histoplasma* antigen EIA (IMMY, Norman, Oklahoma; IM EIA) is used in humans and cats.¹²⁻¹⁴ This test is not available from a service laboratory, but reagents are commercially available for an in-house test. Like the MV EIA, the IM EIA detects galactomannan, with 1 difference from the MV EIA being that it uses a monoclonal antibody for antigen capture and detection. Overall results from humans and cats have shown that the IM EIA might be useful for the diagnosis of histoplasmosis, but in general a single diagnostic cut-off cannot be used that provides a performance equal to that of the MV EIA.¹²⁻¹⁴ In attempt to improve the diagnostic performance of the IM EIA, multiple diagnostic cut-offs are used in humans and a preanalytical heat fixation step is used in cats.^{12,13}

A noninvasive diagnostic test for histoplasmosis in dogs, such as an antigen EIA, might be useful in parts of the world where shipping samples to a service laboratory in the United States is not affordable

or feasible due to other logistical reasons. The objectives of our study are to describe the diagnostic performance of the IM EIA in dogs and to compare it with the currently clinically available *Histoplasma* antigen test, MV EIA.

2 | MATERIALS AND METHODS

This study complied with the Standards of Reporting Diagnostic Accuracy Studies (STARD) guidelines published in 2015.¹⁵ Urine samples stored from dogs treated at the Veterinary Teaching Hospital at Oklahoma State University and enrolled in other clinical studies were used. All samples were collected in accordance with study protocols approved by the Institutional Care and Use Committee at Oklahoma State University. Pet-owner signed consent was obtained before inclusion into any study. Urine samples used were from dogs with histoplasmosis at the time of diagnosis and during treatment, dogs with an alternative diagnosis, and healthy control dogs. Pertinent clinical information was retrieved from medical record review and included signalment, diagnostic test results (CBC, blood biochemistry panel, urinalysis, imaging studies, cytopathology, histopathology, and hormone and infectious disease testing), definitive diagnosis, and form of histoplasmosis (disseminated, pulmonary, or GI).

2.1 | Diagnosis and classification of histoplasmosis

The diagnosis of histoplasmosis (HP) required finding *Histoplasma* yeasts on cytopathology or histopathology by a board certified veterinary clinical pathologist or board certified veterinary anatomic pathologist, respectively. Disseminated disease was defined as clinical evidence (CBC, blood biochemistry panel, urinalysis, cytopathology, imaging study results) of disease in any body system other than the lung, excluding disease apparently localized to the GI tract, which was defined as the GI form. The pulmonary form was defined as disease apparently localized to the lung. Only urine samples collected from dogs at the time of diagnosis (not during treatment) were used to determine the diagnostic performance of IM EIA and MV EIA. Urine samples from HP dogs during treatment were included because *Histoplasma* antigen testing is commonly used for treatment monitoring. These samples were only used to describe agreement between IM EIA with MV EIA and were not used to determine diagnostic performance.

2.2 | Diagnosis and classification of other disease

Conservative criteria were used in attempt to ensure dogs classified as being histoplasmosis negative (HN) did not have histoplasmosis. Exclusion criteria included any of the following unexplained findings—lymphadenopathy, lameness or joint effusion, nonregenerative anemia, parenchymal lung disease, or vomiting or diarrhea. The definitive diagnosis of an alternative disease required supporting evidence of

CBC, blood biochemistry panel, urinalysis, imaging studies, cytopathology or histopathology, or other diagnostic tests. More specifically, a dog with respiratory disease was required to have cytopathology (lung lavage or lung aspirate) or histopathology (nasal biopsy) supportive of the diagnosis. Dogs with GI disease were required to have GI biopsy and histopathology. If nonregenerative anemia was present, a bone marrow aspirate or biopsy was required. For the diagnosis of primary liver disease, an aspirate and cytopathology or liver biopsy and histopathology was required. If joint effusion or lameness was present, synovial fluid aspiration and cytopathology was required. The diagnosis of cancer required cytopathology or histopathology. Definitive diagnosis of an infectious disease required cytopathology, histopathology, microbial culture, or other supportive infectious disease testing, as appropriate. In addition to other supportive evidence, the diagnosis of immune mediated disease required a positive clinical response to immunomodulatory treatment. Finally, the diagnosis of endocrine disease required supportive CBC, blood biochemistry panel, urinalysis, and hormone testing. Multiple alternative diagnoses could be present in the same dog, so long as all of the above criteria were met for each diagnosis. Urine samples from dogs without a proven diagnosis (UD) were only used to compare results between IM EIA and MV EIA and were not used to describe the diagnostic performance of 2 assays.

2.3 | Healthy control dogs

Inclusion criteria for healthy dogs included a normal full physical examination and normal or clinically irrelevant changes on blood biochemistry panel and CBC. Exclusion criteria for healthy dogs included a positive MV EIA or reported clinical signs by the pet-owner. Clinically irrelevant changes were defined as mild (<10% above the upper reference interval) increases of cholesterol or triglyceride concentrations or mild (<10% above or below the reference intervals) changes of serum phosphorous concentration. In addition, mild mature neutrophilia (<50% above reference interval) or lymphopenia were also considered clinically irrelevant.

2.4 | Urine samples

Urine samples were collected from dogs via prepubic cystocentesis or free-catch. Urine samples were immediately refrigerated and frozen at -80°C within 8 hours. Sample storage time was up to 6 years. For IM EIA, urine samples were not exposed to a free-thaw cycle before analysis. For the MiraVista enzyme immunoassay (MV EIA), samples were exposed to 1 freeze-thaw cycle.

2.5 | IMMY *Histoplasma* antigen enzyme immunoassay

Testing was performed with commercially available monoclonal-antibody agent specific reagents (IMMY) with slight modification from that previously described in humans and cats.^{12,13} In short, an

automated plate washer (Wellwash Versa, Thermo Scientific, Waltham, Massachusetts) and spectrophotometer (Varioskan Flash, Thermo Scientific) were used for all analyses. All samples were run in duplicate. Urine samples were 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 antibody (IMMY). The plate was incubated for 55 minutes at 37°C . Wells were washed 3 times with 300 μL wash buffer. Afterward, 100 μL of horseradish peroxidase (HRP)-conjugated anti-galactomannan monoclonal antibody (IMMY) was added to each well. The plate was again 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) was then 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) was then added to all wells. Within 1 minute, dual spectroscopy was used to determine an optical density (OD) at 450 nm with a reference wavelength of 620 nm.

Each plate included negative and positive controls (IMMY) and samples of wash buffer as blanks. Standards were made by serial dilutions of a known concentration of purified antigen (IMMY) in wash buffer. Dilutions of 0.0, 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, and 25 ng/mL were used to generate a standard curve with a 4-parameter logistic curve fit and blank subtraction. *Histoplasma* antigen concentrations were calculated by mapping the unknown sample OD against the standard curve. For statistical analysis, concentrations above 25 ng/mL were reported as 25 ng/mL due to the likelihood of nonlinearity above this concentration.

2.6 | Heat fixation

After initial IM EIA analysis, a preanalytical heat fixation step was performed, and samples were reanalyzed. Urine that was reanalyzed with the heat fixation step was kept at room temperature for less than 6 hours until analyses. Fixation included heating 150 μL of urine at 120°C for 3 minutes with a heat block (Isotemp Dry Bath, Thermo Scientific) then centrifugation at 10 000g for 10 minutes. The supernatant was analyzed with IM EIA within 1 hour, as described above.

2.7 | MiraVista *Histoplasma* enzyme immunoassay

After the IM EIA was performed, samples were immediately refrozen at -80°C and shipped overnight on dry ice to the service laboratory for MV EIA (MiraVista Diagnostics). Assays were performed in a single batch, as previously described.¹⁶ The MV EIA has a lower limit of quantification (LLOQ) of 0.4 ng/mL. Positive EIA results below 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 values were reported as 0.4 ng/mL or 19.0 ng/mL, respectively.

2.8 | Validation of the IMMY *Histoplasma* antigen enzyme immunoassay

Lower limits of quantification, precision or measurement uncertainty, spiked recovery, and diagnostic accuracy were used to partially validate the IM EIA. The LLOQ was determined by adding 10 SDs to the mean of 68 blank samples.¹⁷ All plates included between 2 and 8 blank samples.

The assay precision or measurement uncertainty was demonstrated using intra-assay and interassay coefficients of variation (% CV). Intra-assay %CV was calculated using all of the samples, except for sample pairs with 1 sample having no measurable antigen and the other having very low calculated antigen concentration (<0.1 ng/mL). For intra-assay %CV, all factors except the “well” were held equal. Interassay %CV was calculated using all samples, except sample pairs with 1 sample having no measurable antigen and the other having very low calculated antigen concentration (<0.1 ng/mL). For interassay %CV, initial testing and repeated testing was performed on the same day with all factors except the “plate” being held equal.

Spiked recovery was performed to determine if there were significant interactions between dog urine and the *Histoplasma* galactomannan antigen. Urine samples from 7 HN dogs 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, and 12.5, and 25 ng/mL. Percent recovery was calculated according to the following formula: $(\text{measured concentration}_{\text{spiked sample}} - \text{measured concentration}_{\text{heat sample}}) / \text{theoretical concentration}_{\text{spiked}} \times 100$.¹⁷

2.9 | Statistical analysis

Statistical analysis was performed with commercial software (SAS 9.4, SAS, Cary, North Carolina). Statistical methods complied with guidelines for assay validation adopted by the World Assembly of Delegates of the World Organization for Animal Health (OIE).¹⁸ Mean IM EIA antigen concentrations (duplicate samples) were used for statistical analysis. The Kolmogorov-Smirnov test was used to test for normality and antigen concentrations were nonparametric. Unless specifically stated otherwise, descriptive statistics were reported as median and range for continuous variables and frequency, percentage, or both for nominal variables. Antigen concentrations for the IM EIA were compared with the clinical diagnosis for HP and HN dogs at the time of diagnosis. Youden's index was used to determine the ideal diagnostic cut-off¹⁹, then IM EIA results were dichotomized as positive or negative. For samples obtained at diagnosis, DSe (true positive (TP)/[true positive (TP) + false negative (FN)]), DSp (true negative (TN)/[TN + FP]), and DAC (TP + TN/[TP + FP + FN + TN]) were determined for the IM EIA and MV EIA. These were provided with the associated 95% confidence intervals. The McNemar change test was used to compare the DSe and DSp of the IM EIA with MV EIA. The Wilcoxon signed-rank test was used to compare IM EIA results before and after heat treatment. The Mann-Whitney *U* test was used compare MV EIA antigen concentrations between disseminated and localized histoplasmosis. Cohen's kappa statistic was used to describe the

level of agreement between IM EIA and MV EIA after samples were stratified based on MV EIA antigen concentrations (<1.0, 1.0-5.0, and >5.0 ng/mL). Agreement was considered slight, fair, moderate, and substantial for values <0.2, 0.21 to 0.40, 0.41 to 0.60, and >0.60, respectively, as previously described by Landis et al.²⁰ Receiver operating characteristic curves (ROCs) and the area under the curves (AUCs) were compared between MV EIA and IM EIA with a nonparametric approach previously described by DeLong et al.²¹ Statistical significance was set as $P \leq .05$.

3 | RESULTS

3.1 | Dogs—At time of diagnosis

A single urine sample from 110 dogs at the time of diagnosis was included in the study. This included HP dogs ($n = 20$), HN dogs with an alternative diagnosis (55), HN dogs that were apparently healthy (24), and 11 UD dogs that did not have a proven diagnosis. The median age of HP dogs was 6.1 years (1.0-13.4) and the median body weight was 9.7 kg (2.9-53.5). The median age of HN dogs was 7.7 years (0.3-17.5) and the median body weight was 20.5 kg (1.8-68.3). Breeds represented in HP dogs included mixed breeding ($n = 4$), miniature schnauzer (4), Siberian husky (2), Labrador retriever (2), and 8 breeds with 1 dog each. This included spayed females ($n = 11$), neutered males (6), intact males (2), and 1 intact female. Breeds represented in HN dogs included mixed breeding ($n = 13$), Labrador retriever (6), German shepherd dog (5), boxer (3), dachshund (3), Great Dane (3), French bulldog (3), miniature schnauzer (3), Siberian husky (3), Boston terrier (2), Australian heeler (2), Chihuahua (2), West Highland white terrier (2), Yorkshire terrier (2), and 27 breeds with 1 dog each. This included spayed females ($n = 43$), neutered males (32), intact males (3), and 1 intact female.

Histoplasma capsulatum yeast were found in a single sample in 17 dogs, including rectal scrape ($n = 9$), blood smear (5), abdominal fluid (2), liver aspirate (1), and synovial fluid (1). Organisms were found in multiple samples in 2 dogs including liver and popliteal lymph node aspirates in 1 dog and in liver, spleen and mesenteric lymph node aspirates in 1 dog. Forms of disease included disseminated ($n = 13$) and GI (7). No dog had disease localized to the lung. One dog had disease localized to the bones and joints and for the purposes of our study was classified as disseminated disease.

Thirty HN dogs had a single alternative diagnosis and 25 dogs had multiple diagnoses. Alternative diagnoses included endocrine disease ($n = 14$), urologic disease (14), cancer (13), GI/liver/pancreatic disease (8), hematologic/immune mediated disease (6), cardiovascular disease (5), neurologic disease (4), ophthalmic disease (4), dermatologic disease (3), musculoskeletal disease (3), behavioral disease (2), and respiratory disease (1). (Table 1) An additional dog had disseminated aspergillosis. Disease in UD dogs included hepatopathy ($n = 2$), parenchymal lung disease (2), lung mass (1), hepatic mass (1), hepatomegaly and intraabdominal lymphadenopathy (1), intramedullary spinal mass (1), large bowel diarrhea (1), peripheral nerve disease (1), and adrenal mass (1).

TABLE 1 Alternative diagnoses and diagnostics tests used to obtain diagnoses in dogs (n = 79) without histoplasmosis

| Disease category | Specific disease (number) | Diagnosis proven ^a |
|-------------------------------------|-----------------------------------|---|
| Endocrine (14) | Diabetes mellitus (9) | Routine lab work + UA (9) |
| | Hypothyroidism (3) | Thyroid hormone panel (3) |
| | Hyperadrenocorticism (2) | Low dose dex suppression test (2) |
| Urinary (14) | Lower UTI, bacterial (3) | Urinalysis + bacterial culture (3) |
| | USMI (3) | Routine lab work, UA, AUS (3) |
| | Pyelonephritis (2) | Routine lab work, UA, bacterial culture, AUS (2) |
| | Cystolithiasis | AUS |
| | Ureterolithiasis | AUS |
| | Protein losing nephropathy | AUS |
| | Nephrolithiasis | Urinalysis, UPC, AUS, infectious disease testing |
| | Polycystic kidney disease | AUS |
| | Chronic kidney disease | AUS Routine lab work, UA, AUS |
| Cancer (13) | Lymphoma (4) | FNA + cytology |
| | Pulmonary carcinoma (2) | FNA + cytology |
| | Hepatocellular carcinoma (2) | FNA + cytology |
| | Rectal carcinoma, metastatic | FNA + cytology |
| | Apocrine gland adenocarcinoma | FNA + cytology |
| | Transitional cell carcinoma | Traumatic catheterization, cytology |
| | Histiocytic sarcoma | FNA + cytopathology |
| | Mast cell tumor | FNA + cytopathology |
| Gastrointestinal/pancreas/liver (8) | Pancreatitis (3) | Abdominal US (3) |
| | Gall bladder mucocele (2) | Abdominal US (2) |
| | Esophageal dysmotility | Esophogram |
| | Portosystemic shunt, extrahepatic | CT portal angiogram |
| | Portosystemic shunt, intrahepatic | CT portal angiogram |
| | Ancylostomiasis | Fecal flotation |
| Hematologic/immune mediated (6) | IMHA (5) | Serum biochemistry, blood smear, infectious disease testing |
| | Immune mediated polyarthritis | Radiographs, synovial fluid cytology |
| Cardiovascular (5) | Pulmonic stenosis | Echocardiogram |
| | Dilated cardiomyopathy | Echocardiogram |
| | ARVC | Echocardiogram, ECG |
| | Systemic hypertension | Indirect measurement |
| | Endocarditis, bacterial | Echocardiogram, blood culture, <i>Bartonella</i> PCR + serology |
| Neurologic (4) | Idiopathic epilepsy (2) | Brain MRI, CSF analysis (2) |
| | MUE (2) | Brain MR, CSF analysis, infectious disease testing |
| Ophthalmic (4) | Cataracts (3) | Ophthalmic exam |
| | Retrobulbar abscess, bacterial | Head CT, FNA + cytology, bacterial culture |
| Dermatologic (3) | Atopy (3) | Skin scrape cytology, skin impression smear cytology |
| | Cutaneous keratin cyst | FNA + cytology |
| Musculoskeletal (3) | Osteoarthritis (2) | Joint radiographs, synovial fluid cytology |
| | Patellar luxation | Orthopedic exam |
| Behavioral (2) | Anxiety | Clinical diagnosis |
| | Polydipsia, psychogenic | Routine lab work, UA, urine culture, modified water deprivation test |
| Respiratory (1) | Lymphoplasmacytic rhinitis | Nasal CT, nasal tissue histopathology |
| Infectious/parasitic (1) | Aspergillosis, disseminated | Routine lab work, UA, urine sediment exam, AUS, Brain MRI, FNA + cytology |

Abbreviations: AUS, abdominal ultrasonography; CSF, cerebrospinal fluid; CT, computed tomography; dex, dexamethasone; FNA, fine needle aspirate; IMHA, immune mediated hemolytic anemia; ARVC, arrhythmogenic right ventricular cardiomyopathy; MRI, magnetic resonance imaging; MUE, meningoencephalitis of unknown etiology; PCR, polymerase chain reaction; UA, urinalysis; UPC, urine protein to creatinine ratio; US, ultrasonography; USMI, urethral sphincter mechanism incompetence; UTI, urinary tract infection.

^aThe diagnostic tests listed are not a comprehensive list of all diagnostics performed but rather those instrumental in determining the definitive alternative diagnosis.

3.2 | Dogs—During antifungal treatment

Ninety-two urine samples from 16 dogs during treatment were included in the study. These 16 dogs also had a urine sample included at the time of diagnosis. Antigen concentrations during antifungal treatment were only used to describe agreement between IM EIA and MV EIA and were not used to describe diagnostic performance (Table 2).

3.3 | Partial validation—IMMY *Histoplasma* antigen enzyme immunoassay

Intra-assay CV% was 6.7%, calculated from 191 urine sample pairs. Interassay CV% was 10.2%, calculated from 77 urine sample pairs. Spiked recovery (mean \pm SD) for concentrations of 0.4 ng/mL, 0.8 ng/mL, 1.6 ng/mL, 3.2 ng/mL, 6.3 ng/mL, 12.5 ng/mL, and 25 ng/mL were 89.7% (41.2), 69.4% (21.4), 71.1% (17.9), 71.0% (12.9), 70.4% (9.0), 68.4% (9.4), and 68.9% (9.9), respectively. Sixty-eight blank wells were used to calculate the LLOQ, which was 0.35 ng/mL.

3.4 | Diagnostic performance—MiraVista Diagnostics *Histoplasma* antigen enzyme immunoassay

Based on samples from dogs at the time of diagnosis, the MV EIA provided 19 true positives, 78 true negatives, 1 false positive, and 1 false negative. The DSe, DS_p, and DAc (and associated 95% confidence intervals) was 95% (85-100), 99% (97-100), and 98% (95-100), respectively. (Figure 1) The median antigen concentration for HP dogs at diagnosis and during treatment was 8.02 ng/mL (0-19) and 1.17 ng/mL (0-19), respectively. At the time of diagnosis, the median antigen concentration from dogs with GI histoplasmosis was 1.18 ng/mL (0-8.32) which was significantly lower than dogs with disseminated disease (12.61 ng/mL [0.4-19.0]; $P = .003$). The median antigen concentration for HN dogs was 0 (0-2.45).

3.5 | Diagnostic performance—IMMY *Histoplasma* antigen enzyme immunoassay

The median IM EIA concentration in HP dogs at the time of diagnosis was 3.64 ng/mL (0-25.0) and during treatment was 0.15 (0-25.0). The

TABLE 2 Agreement of results for MV EIA and IM EIA in dogs with ($n = 20$) histoplasmosis at the time of diagnosis (20) and during antifungal treatment (16) and dogs without histoplasmosis (79)

| IMMY <i>Histoplasma</i> antigen EIA | MiraVista <i>Histoplasma</i> antigen EIA | |
|-------------------------------------|--|----------|
| | Positive | Negative |
| Positive | 54 | 2 |
| Negative | 42 | 104 |

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

median IM EIA concentration in HN dogs was 0 (0-1.95). The ideal diagnostic cut-off was determined to be 0.5 ng/mL, with a Youden's index of 0.69 (Figure 2). Based on samples from dogs at the time of diagnosis, the IM EIA provided 14 true positives, 78 true negatives, 1 false positive, and 6 false negatives. The DSe, DS_p, and DAc was 70% (51-89), 99% (97-100), and 93% (81-100), respectively (Figure 1). Five of the 6 (83%) false negative samples tested positive on MV EIA. Four of 6 (67%) of the false negative samples came from dogs with disease localized to the GI tract. A fifth dog had disease localized to the bones and joints. The 1 false positive tested negative on the MV EIA. The DSe ($P = .06$) and DS_p ($P = 1.0$) for IM EIA were not significantly different from the MV EIA.

3.6 | Agreement—MiraVista Diagnostics and IMMY *Histoplasma* antigen enzyme immunoassays

The area of under the receiver operator characteristic curve was significantly smaller for the IM EIA (0.87) as compared with the MV EIA (0.97; $P = .03$) (Figure 3). For samples with high MV EIA antigen concentrations (>5.0 ng/mL), there was complete agreement (43/43) between the 2 assays. For samples with moderate concentrations (1.0-5.0 ng/mL), there was slight agreement ($\kappa = 0.09$ [0.16-0.32], $P = .33$) with discordant results for 10/21 (48%) samples. For samples with low antigen concentrations (<1.0 ng/mL), there was slight agreement ($\kappa = -0.03$ [-0.07 to 0.01], $P = .43$) with discordant results for 34/138 (25%) samples. Considering all 44 discordant results, 42/44 (95%) were negative on the IM EIA but positive on the MV EIA and 37/42 (88%) were from HP dogs during antifungal treatment, while the other 5/42 (12%) were at the time of diagnosis (Table 2).

3.7 | Heat fixation IMMY *Histoplasma* antigen enzyme immunoassay

Preanalytical heat fixation was performed on 198/202 (98%) of samples. The median antigen concentrations for HP dogs at the time of diagnosis was 3.2 ng/mL (0-25) and during treatment was 0.12 ng/mL (0-25). The median antigen concentration for HN dogs was 0 (0-4.87). For all samples, the median antigen concentration was significantly lower ($P = .01$) after as compared with before heat fixation. Heat fixation changed the IM EIA test result in 7/198 (3.5%) of samples. It changed 1 true positive to a false negative, and 2 true negatives to false positives. In addition, 4 samples in HP dogs during treatment changed including 1 sample that initially agreed with the MV EIA and did not agree after heat fixation and 3 samples that initially did not agree with the MV EIA and agreed after heat fixation.

4 | DISCUSSION

Our study partially validated and described the diagnostic performance of the IM EIA in dogs. Findings suggest that the IM EIA might

FIGURE 1 Flow diagram demonstrating MV EIA and IM EIA results for dogs with and without histoplasmosis at the time of diagnosis. IM EIA, IMMY *Histoplasma* antigen enzyme immunoassay; MV EIA, MiraVista Diagnostics *Histoplasma* antigen enzyme immunoassay

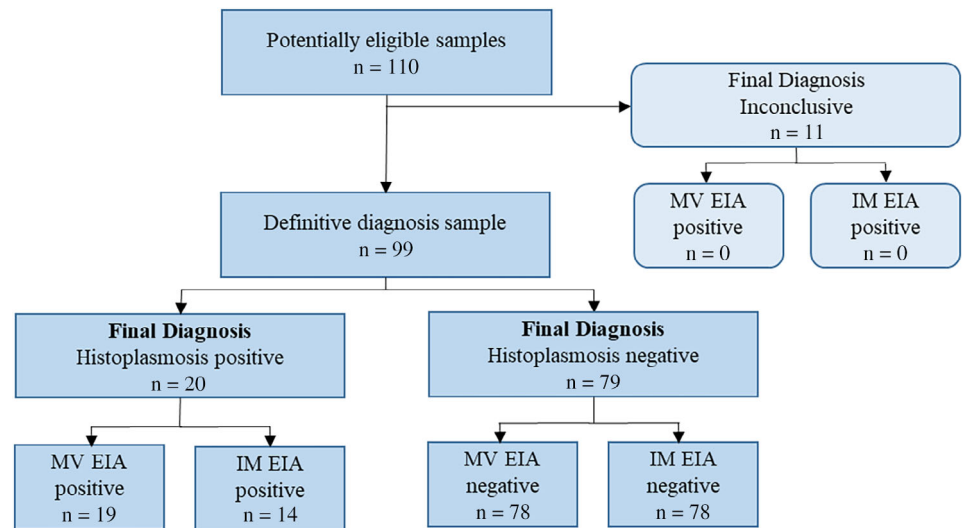
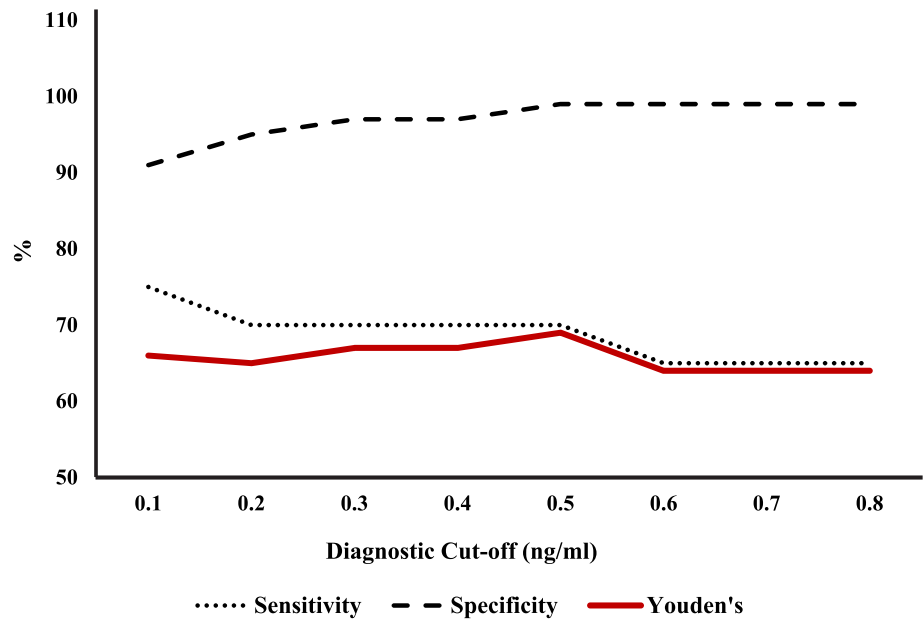


FIGURE 2 Youden's index, diagnostic sensitivity, and diagnostic specificity of the IM EIA for different diagnostic cut-offs in dogs with (n = 20) and without (79) histoplasmosis. IM EIA, IMMY *Histoplasma* antigen enzyme immunoassay



be useful for the diagnosis of disseminated histoplasmosis in dogs, but the test will be less useful for disease apparently localized to the GI tract. Disease localized to the GI tract was relatively common in our study being found in >1/3 of HP dogs. Similar findings were reported in a larger group of dogs with histoplasmosis, where 34% had disease apparently localized to the GI tract.² Although not well described in the published literature, the authors have observed histoplasmosis apparently localized to other body systems, including the eyes, skin, and skeletal system. One dog in our study had disease apparently localized to the bones/joints. If this dog was considered to have localized disease, 5/8 (63%) dogs with localized histoplasmosis had a false negative IM EIA result. The MV EIA was positive in 7/8 (88%) of these dogs, suggesting that it is the noninvasive test of choice for this subgroup of HP dogs. Interestingly the performance of the MV EIA is likely not completely unaffected by localized disease, as the majority of the few false negative MV EIA test results reported in the literature

have included dogs with disease apparently localized to the GI tract or cats with disease apparently localized to the GI tract, eyes, or skeletal system.^{5,22-24}

The relatively high number of false negative results of the IM EIA with GI histoplasmosis was likely due to decreased antigen capture by the IM EIA. This was demonstrated, in part, by the lower than expected spiked recovery. For all antigen concentrations except the lowest, recovery was approximately 70%, which is lower than a previously recommended acceptable range of 80% to 120%.¹⁷ This suggests that there is a matrix (dog urine) and analyte (galactomannan) interaction that affects the IM EIA. While urine is a convenient biological sample as it is plentiful and relatively easy to obtain, certain properties such as urea, salts, and acid can present challenges. It remains unknown what specifically affected antigen capture for IM EIA in our study. In addition to the lower antigen capture, the IM EIA in dogs with GI histoplasmosis was compounded by the significantly lower

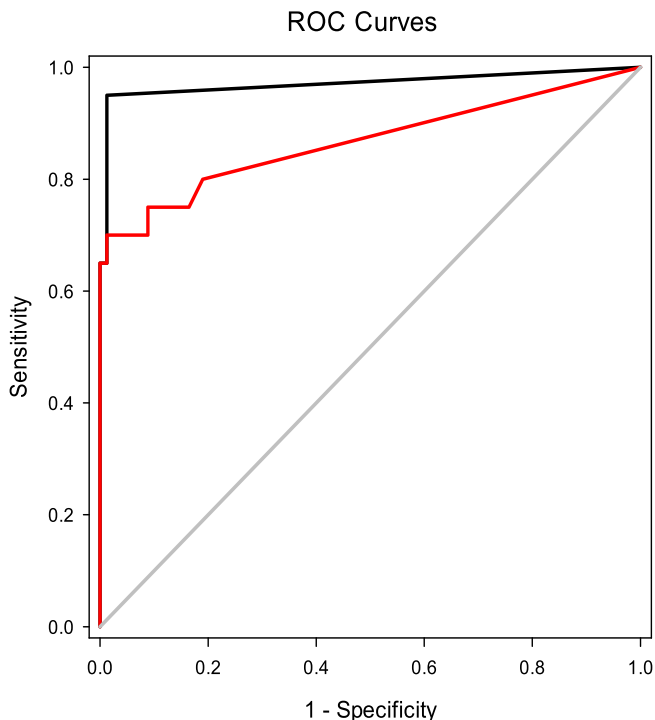


FIGURE 3 Receiving operating characteristic curves for the MV EIA and IM EIA at the time of diagnosis in dogs with ($n = 20$) and without (79) histoplasmosis. Area under the curve was significantly smaller for IM EIA (0.87) as compared with MV EIA (0.97; $P = .03$). (□ MV EIA; □ IM EIA). IM EIA, IMMY *Histoplasma* antigen enzyme immunoassay; MV EIA, MiraVista Diagnostics *Histoplasma* antigen enzyme immunoassay

antigen concentrations found in these samples as compared with those from dogs with disseminated disease. The other false negative with the IM EIA was from a dog with disseminated disease that was positive with the MV EIA, but below the limit of quantification. This was the lowest MV EIA concentration from any dog with disseminated disease. These same factors likely led to a relatively large number of negative IM EIA test results in dogs during antifungal treatment, which is a clinical scenario where lower antigen concentrations are expected. The poor agreement between the IM EIA and MV EIA in HP dogs during treatment, with the large majority of discordance being IM EIA negative and MV EIA positive, suggests that the IM EIA might not be useful clinically for treatment monitoring.

The comparison of 2 different assays is commonly based on the diagnostic cut-off used and the ultimate test result. The ideal cut-off of 0.5 ng/mL in our study is similar to the 0.4 ng/mL cut-off described in cats and humans.^{12,13} A different cut-off could be used pending the intended clinical purpose. The high specificity demonstrated in our study suggests that the IM EIA, with the 0.5 ng/mL cut-off, is good at confirming but not necessarily screening for histoplasmosis. In this study group, it was not possible to decrease the diagnostic cut-off low enough to significantly improve DSe as false negative test results were due to very low or unmeasurable antigen concentrations (<0.12 ng/mL). Comparing the AUC of ROCs is 1 way of comparing 2 different assays

without consideration to a single cut-off. The significantly lower AUC for the IM EIA suggests that overall, it was inferior to the MV EIA for the diagnosis of histoplasmosis in this group of dogs.

In attempt to improve diagnostic performance, a preanalytical heat fixation step was added, and the samples were reanalyzed. In cats, this appeared to improve the diagnostic performance, although it was not performed in enough samples to draw definitive conclusions.¹² In the current study, heat fixation led to equivocal changes and the findings do not support the addition of this preanalytical step. This finding might be due, in part, to the fact that heat fixation appeared to improve DSp in cats and the current study demonstrated a high DSp of the IM EIA before heat fixation in dogs.

In addition to diagnostic performance and spiked recovery, our study partially validated the IM EIA in dogs by describing the repeatability and LLOQ. Repeatability was adequate as the intra-assay %CV and interassay %CV was below the OIE recommended maximum of 15%.²⁵ The LLOQ of 0.35 ng/mL was similar, but slightly lower than the 0.5 ng/mL LLOQ reported in humans and cats.^{12,26} Due to how it was calculated, it is possible our study underestimated the LLOQ for the IM EIA.

Our study has multiple notable limitations. The first is inclusion of a relatively small number of HP dogs, especially when considering the different forms of disease. Based on the data in our study and clinical experience, apparently localized histoplasmosis should likely be differentiated from disseminated disease when investigating diagnostic tests. This differentiation is incorporated in guidelines for the diagnosis and treatment of histoplasmosis in humans, where even more forms are recognized.¹¹ A second limitation includes the free-thaw cycle before MV EIA. In general, freeze-thaw cycles should be avoided, or at least limited, due to the chance for degradation of the analyte. In order to perform the 2 different assays on the same urine sample, shipping the sample to the service laboratory for MV EIA required that the samples be refrozen and thawed once. This was not expected to affect the results of our study as the MV EIA is unaffected by at least 10 freeze-thaw cycles (J. Witt, personal communication, July 2020). A third limitation was the long storage time of some urine samples. In order to collect enough samples for a meaningful study, and to perform assays in batches, storage was required. Studies in humans and cats also utilized samples stored for years in some cases.^{12,14,26} Freezing, even for long periods of time, does not affect the MV EIA, but it might affect the IM EIA.²⁷ Robustness of the IM EIA, as it relates to sample storage, was not investigated in the current study. The fourth limitation was the lack of dogs with other IFI included in the study. In the authors' hospital population, IFIs other than histoplasmosis are uncommon. The 1 dog with disseminated aspergillosis included in the study tested negative on the both the IM EIA and MV EIA. Including a larger number of dogs with other IFIs would be expected to lower the specificity of both assays investigated. With that being said, many of the dogs with alternative disease had clinical signs that overlap with histoplasmosis and provided a meaningful negative control group in order to describe diagnostic performance. A final limitation includes the impossibility of proving that all HN dogs did not have histoplasmosis with 100% certainty. The study utilized conservative criteria in attempt to ensure that all HN

dogs were very unlikely to have histoplasmosis. These strict criteria led to 11/66 (17%) dogs with illness not believed to be histoplasmosis, to be classified as undiagnosed. All of these dogs tested negative on both assays. In addition to the strict diagnostic requirements, the fact that only 1 HN dog tested positive on the MV EIA, which has previously been shown to be highly specific, also supports the correct disease classification of HN dogs in our study.

5 | CONCLUSION

The IM EIA might be useful for the diagnosis of disseminated histoplasmosis in dogs, but due to unacceptably high number of false negative results, especially in dogs with histoplasmosis apparently localized to the GI tract, the clinical usefulness of the IM EIA will be limited.

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CONFLICT OF INTEREST DECLARATION

Dr. Andrew Hanzlicek became an employee of MiraVista Diagnostics after manuscript submission but before publication.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Samples were residual urine samples from other approved studies. All samples were collected in accordance with study protocols approved by the IACUC of Oklahoma State University.

HUMAN ETHICS APPROVAL DECLARATION

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

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